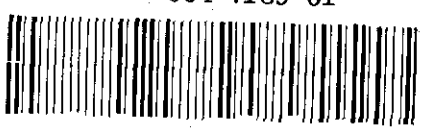

SOME STUDIES OF POSITIONAL SPECIFICITIES OF ENZYMIC DESATURATION OF LONG CHAIN FATTY ACIDS

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A Thesis

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To Pat

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SUMMARY

Aerobic desaturation of long chain fatty acids is virtually a universal process and it has been noted that under identical conditions the same organism or enzyme system will introduce the double bond into the same position in the fatty acid chain. The biosynthesis of long chain fatty acids is therefore a highly specific process. To date the specific factors which govern the position of the double bond in the fatty acid molecule have not been investigated. This thesis deals with work done to demonstrate the existence of aerobic desaturation in five systems typical of the whole spectrum of life viz. the yeast Torulopsis apicola the green alga Chlorella vulgaris, the embryo and endosperm of the castor plant Ricinis communis, a microsomal fraction of hen liver and a microsomal fraction from the mammary gland of a goat. When aerobic desaturation had been demonstrated to be operative in the system the positional specificity of enzymic desaturation was investigated by incubation of a homologous series of radiolabelled saturated fatty acids and radiolabelled homologous and positionally isomeric monoenoic fatty acids, synthesised as part of the work, with the above systems. Conclusions were drawn concerning the number of enzymes responsible for the desaturations and also concerning the control of the positional specificity of enzymic desaturation in these systems.

Some indirect evidence of the shape of the enzyme in the vicinity of the substrate in the enzyme substrate complex was also obtained. The incorporation of the substrate fatty acids and the products of aerobic desaturation into various lipid classes was studied in some of the systems and investigations were also made into the positional specificity of hydroxylation of homologous and positionally isomeric monoenoic fatty acids in the embryos and endosperms of the castor plant Ricinis communis.

CONTENTS

Abbreviations used in this thesis:-

NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
TLC	Thin layer chromatography
RTLC	Radiochemical thin layer chromatography
GLC	Gas liquid chromatography
RGLC	Radiochemical gas liquid chromatography
ATP	Adenosine 5' triphosphate
CoA	Coenzyme A
ACP	Acyl carrier protein
DMSO	Dimethyl sulphoxide
FFAP	Free fatty acid phase
PEGA	Polyethylene glycol adipate
PFS	Particle free supernatent
BSA	Bovine Serum Albumin
PPO	2,5 diphenyloxazole

GENERAL INTRODUCTION

This thesis concerns some aspects of the formation of long chain fatty acids in natural systems. Fatty acids are naturally occurring materials seldom found in the free acid form but rather as esters combined with the trihydric alcohol glycerol to form lipids. This having been said, in the description of fatty acids which follows they will be discussed in their free acid form. Long chain fatty acids, for the sake of this thesis, have an aliphatic carbon chain having at least 10 carbon atoms and also having a terminal carboxyl group. By virtue of their mode of synthesis from acetate[1] the majority of naturally occurring fatty acids have an even number of carbon atoms. Odd chain acids arising from an initial proprionic acid molecule[2] are very much less common.

Naturally occurring fatty acids may be conveniently divided into three groups - the saturated fatty acids, the unsaturated fatty acids and the substituted fatty acids. Some examples of each of these groups along with the trivial name, where commonly used, is contained in the tables below.

Table I Saturated fatty acids

Systematic Name	Trivial name	structure
n-decanoic acid	capric	$CH_3(CH_2)_8$ COOH
n-dodecanoic	lauric	$CH_3(CH_2)_{10}$ COOH
n-tetradecanoic	myristic	$CH_3(CH_2)_{12}$ COOH
n-hexadecanoic	palmitic	$CH_3(CH_2)_{14}$ COOH
n-heptadecanoic	margaric	$CH_3(CH_2)_{15}$ COOH
n-octadecanoic	stearic	$CH_3(CH_2)_{16}$ COOH
n-eicosanoic	arachidic	$CH_3(CH_2)_{18}$ COOH
n-docosanoic	behenic	$CH_3(CH_2)_{20}$ COOH
n-tetracosanoic	lignoceric	$CH_3(CH_2)_{22}$ COOH

Table II Common unsaturated fatty acids

Systematic name	Trivial Name	Structure
cis 9 hexadecenoic	Palmitoleic	$CH_3(CH_2)_5CH = CH\ (CH_2)_7\ COOH$
cis 9 octadecenoic	Oleic	$CH_3(CH_2)_7CH = CH\ (CH_2)_7\ COOH$
cis 9, cis 12 octadecadienoic	linoleic	$CH_3(CH_2)_4CH = CH\ CH_2\ CH = CH(CH_2)_7\ COOH$
cis 9, cis 12, cis 15 octadecatrienoic	linolenic	$CH_3(CH_2\ CH = CH)_3(CH_2)_7\ COOH$
cis 5, cis 8, cis 11, cis 14 eicosatetrenoic	arachidonic	$CH_3(CH_2)_4(CH = CH\ CH_2)_4(CH_2)_2COOH$
cis 11, octadecenoic	vaccenic	$CH_3(CH_2)_5\ CH = CH(CH_2)_9\ COOH$
cis 13, eicosenoic	erucic	$CH_3(CH_2)_7\ CH = CH(CH_2)_9\ COOH$
trans 9 octadecenoic	elaidic	$CH_3(CH_2)_7\ CH = CH(CH_2)_7\ COOH$
9 octadecynoic	stearolic	$CH_3(CH_2)_7\ C \equiv C(CH_2)_7\ COOH$

Unsaturated fatty acids usually have cis double bonds and where there are two or more unsaturated centres they are arranged in the methylene interrupted or divinyl methane pattern see below :

$$R - CH = CH - CH_2 - CH = CH - R'$$

A methylene interrupted sequence of double bonds

trans Double bonds acetylenic bonds and conjugated double bonds occur more rarely and are discussed later.

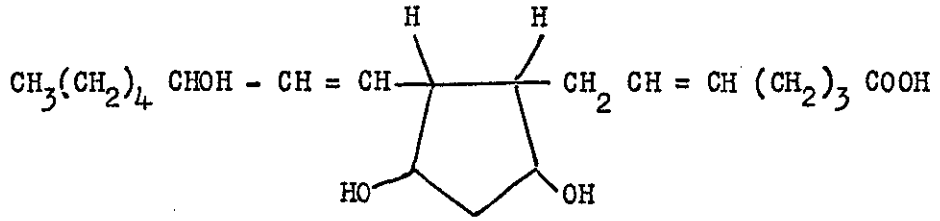
Table III Some substituted fatty acids

Systematic name	Trivial name	Structure
18 fluoro 9 cis octadecenoic	-	$FCH_2(CH_2)_7CH = CH\ (CH_2)_7\ COOH$
9,10 epoxyoctadecanoic	9,10 epoxystearic	$CH_3(CH_2)_7\ CH\text{-}O\text{-}CH(CH_2)_7\ COOH$
10 hydroxyoctadecanoic	-	$CH_3CHOH\ (CH_2)_{15}\ COOH$
d-10 methyl octadecanoic	tuberculostearic	$d\text{-}CH_3(CH_2)_7\ \overset{CH_3}{CH}(CH_2)_8\ COOH$
w-(2-n-octyl cyclo propyl)-octanoic	lactobacillic	$CH_3(CH_2)_5\ CH\text{-}CH(CH_2)_9\ COOH$ (CH$_2$ bridge)

Some substituted acids also have unsaturated centres e.g. ricinoleic acid, 12 hydroxy cis 9 octadecenoic acid and hence can be included in either classification.

The importance of certain unsaturated fatty acids as essential constituents in the diet of animals has been realised for a long time[3]. Animals deficient in these so called essential fatty acids do not maintain normal growth, or reproduction, develop skin lesions and eventually die. The acids which have been found to alleviate the above symptoms are the linoleic acid series e.g. linoleic, γ linolenic and arachidonic acids[4]. These all have the same structure at the methyl end of the molecule and linoleic acid can be converted to arachidonic by sequential elongations and desaturations. Recently the W3 linolenic acid series has been found to have some essential properties[5].

Recently a relationship between essential fatty acids and a group of naturally occurring substances called prostaglandins which have marked physiological properties such as vasto depressor activity[6] has been discovered. For example arachidonic acid may be converted to the prostaglandin (PGE_2) as shown below.



Work is still continuing, but it may well be that the ability to form these compounds is the sole reason for the special essential properties of certain polyenoic fatty acids.

Although the essential fatty acids linoleic and linolenic are produced exclusively by plant systems the biosynthesis of unsaturated fatty acids is carried out by the whole spectrum of living systems. It is the object of this thesis to investigate the biosynthesis of unsaturated fatty acids in

selected systems representative of this spectrum and to add to the existing knowledge of this important process.

The synthesis of saturated fatty acids is now well understood. The studies of Wakil[7,8], Vagelos[9], Gurin[10-12], and Porter[13] with animal systems, Vagelos[9,14-23], Yamamura[24], Lynen[1], Bloch[25], Klein[26] and Wakil[27-30] with bacteria and Stumpf[31-33], James[34] and Cheniae[35] with plants have shown that the synthesis of saturated fatty acids in all these systems is essentially the same. This pathway, the so called malonyl CoA pathway, proceeds in the following steps and is repeated until the required chain length is obtained.

Acetyl CoA + enzyme-SH ⟶ acetyl enzyme + CoASH

Malonyl CoA + acetyl-enzyme ⟶ acetoacetyl-enzyme + CoASH + CO_2

Acetoacetyl-enzyme + NADPH + H^+ ⟶ β-hydroxy butyryl-enzyme + $NADP^+$

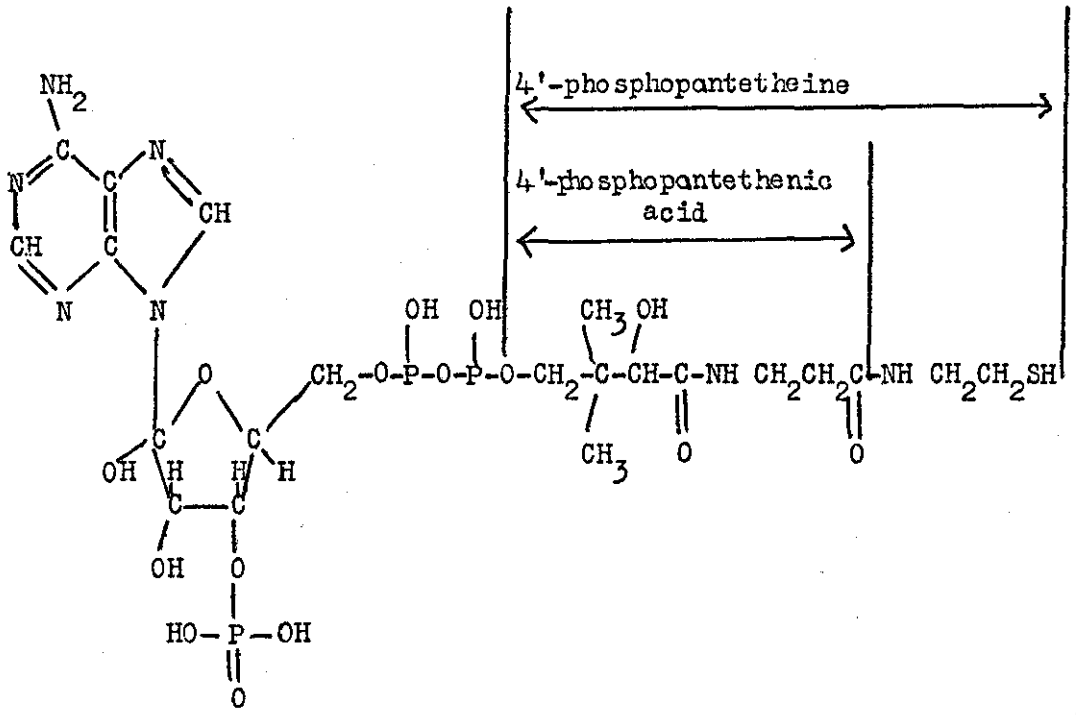
β-hydroxybutyryl-enzyme ⟶ crotonyl-enzyme + H_2O

Crotonyl-enzyme + NADPH + H^+ ⟶ butyryl-enzyme + $NADP^+$

butyryl-enzyme + malonyl CoA ⟶ Repeat cycle.

This cycle then repeats to give the required saturated fatty acid.

The enzyme which carries the functional groups during the synthetic cycle has as an integral part of it an acyl carrier protein. In some systems e.g. yeast and pigeon liver,[13] it is not separable from the enzyme whilst in other systems e.g. E.Coli[14,25] it is easily separable and has been fractionated from it. The heat stable protein which carries the acyl residues during the above sequence of reactions was first isolated by Vagelos from C.Kluyveri[19,36] and was called acyl carrier protein A.C.P. This protein which has since been studied in extracts of E.Coli[37,38,39] and avocado[31] has a molecular weight of 9,500. It has the same active prosthetic grouping as Coenzyme A (see below) viz the 4' phosphopantotheine and in the protein this is bound to a Serine residue[36a,36b] in the peptide chain.



Stucture of Coenzyme A

The biosynthesis of unsaturated fatty acids has been shown to proceed via two distinct pathways. These pathways are not competitive in any respect, indeed they are believed to be mutually exclusive since no living system has been discovered that is capable of performing both.

These two pathways are referred to as either the aerobic or the anaerobic depending on the requirement of the system for molecular oxygen as an essential co-factor.

The anaerobic pathway was first postulated by Bloch[40] who demonstrated the biosynthesis of unsaturated fatty acids in Clostridium butyricum, an obligate anaerobe. This pathway appears to be restricted to bacteria of the orders Eubacteriales and Pseudomonadales.

In C.butyricum and C.kluyvai Bloch[40,41,42] showed that lauric and myristic acids were converted only to long chain saturated acids whilst octanoic and decanoic acids were converted to 9-hexadecenoic acid and 11-octadecenoic acid and to 7-hexadecenoic acid and 9-octadecenoic acid respectively.

To account for this the following pathway was postulated[43].

acetate	Malonyl CoA (3 elongations) →	octanoate	(1)
octanoate	+ C_2 Malonyl CoA →	3 ketodecanoate	(2)
3 ketodecanoate	Reductase →	3 hydroxydecanoate	(3)
3 hydroxydecanoate	Dehydrase →	cis 3 decenoate	(4)
cis 3 decenoate	+ $3C_2$ Malonyl CoA →	cis 9 hexadecenoate	(5)
cis 9 hexadecenoate	+ C_2 Malonyl CoA →	cis 11 octadecenoate	(6)

This demands that the pathway for the synthesis of saturated fatty acids may branch at the hydroxy stage of the C_8 - C_{10} levels but no higher and that the division occurs at the dehydration step with the formation of the cis 3 acid yielding a final unsaturated product, instead of the trans 2 acid which is the intermediate for the saturated fatty acid pathway.

Finally the scheme demands that the subsequent elongation of the unsaturated acid thus formed occurs by the addition of C_2 units at the carboxyl end without reduction or isomerisation of the double bond.

Amongst evidence which established this as the correct sequence was the identification by O'Leary[44] of small amounts (1% or less) of cis 3 decenoic, cis 5 dodecenoic and cis 7 tetradecenoic acids in several lactobacilli and streptococci. Similar evidence of intermediates in the identical sequence beginning at decanoic acid has also been obtained[44].

Evidence for the retention of the cis double bond during the extension of the chain by C_2 units at the carboxyl end was obtained by Baronowski et al[45] who demonstrated the conversion of ($1^{14}C$)-cis 3-decenoic acid into cis 9-hexadecenoic acid and cis 11-octadecenoic acid with no evidence of any saturated acids being formed at all.

The key reaction of the sequence, viz the $\beta\gamma$ dehydration of the 3 hydroxy acid to yield the cis 3 monoenoic acid (reaction (4) above), was provided by Lennarz et al[25] who showed that a partially purified fatty acid synthetase

from E coli, which produces a large amount of cis 11-octadecenoic acid, would furnish the cis 3 acid from 3 hydroxydecanoyl CoA. Norris et al[46] on further purification of this enzyme showed that it was specific for the D-β-hydroxy-decanoyl thioester.

The mechanism of the anaerobic pathway has thus been largely elucidated and the factors which govern the position of the double bond in the final product can easily be seen.

The anaerobic pathway however is confined to some bacteria and is not of major importance in nature generally. By far the more important pathway in the production of unsaturated fatty acids is the direct desaturation or aerobic pathway. This pathway occurs across the whole range of living systems from the protozoa and bacteria to the higher animals.

Early studies[40] indicated that monounsaturated fatty acids could be formed by direct desaturation of a long chain precursor. This was proved by Bloomfield and Bloch[47] who showed that cell free extracts of the yeast Saccharomyces cerevisiae could convert palmitate to palmitoleate via its co-enzyme A thiolester. Direct desaturation of stearate to oleate in yeast has been demonstrated in the same way by Bloch[47].

Direct aerobic desaturations have been observed in animals[48,49,50], bacteria[47,51-54], fungi[53,55] and algae[53,56]. Although some of the more primative photosynthetic algae e.g. chlorella vulgaris[56] are able to produce oleate from directly added stearate, the higher plants are not. James[57] showed that even chain length acids of 14 carbon atoms or less would yield aerobically but not anaerobically oleic acid and linoleic acids in the isolated leaves of the castor plant Ricinis communis, whereas neither palmitate nor stearatewere converted at all though they were incorporated into lipids showing that the CoA thiolester had been formed. In the same work he showed that the formation of oleate was an aerobic process and that oleate could also be converted directly to linoleate in these leaf systems. On the basis of these

and other corroborative results in barley seedlings[58] and in the ice plant Carpobrotus chilense[59] a separate plant pathway was postulated. James and Harris[60] showed the conversion of stearic acid to oleic acid in plant leaves. If the stearic acid was allowed to be built up first from acetate in a non-photosynthetic environment i.e. anaerobically in the dark, then on allowing photosynthesis to begin, the conversion to oleic acid was observed. Thus it was shown indirectly that the higher plants had the ability to desaturate stearate formed from acetate but were not able to desaturate added stearic acid. This desaturation pathway in higher plants was proved to be the same as the aerobic pathway by Nagai and Bloch[61] who demonstrated the conversion of stearoyl-S-ACP to oleate in spinach leaf chloroplasts. Thus it would seem that the inability to desaturate palmitate and stearate or their coenzyme A derivatives is simply due to the fact that these systems lack the acyl transferase enzymes capable of converting stearoyl-S-CoA to stearoyl-S-ACP.

When the green alga Chlorella vulgaris was incubated with stearic acid it readily desaturated it to oleic and linoleic acid[62]. This is also a difference between plant systems and other living systems. Plants tend to undergo a sequence of desaturations to yield trienes by direct desaturation. The formation of polyenoic acids will be discussed later in more detail. Therefore, from the above result, it was clear that Chlorella vulgaris does possess the acyl transferases necessary to convert the acyl-S-CoA to the corresponding acyl-S-ACP. However, when James, Harris and Bezard[63] carried out the incubations in the presence of sterculic acid 8-(2-octyl-1-cyclopropenyl) octanoic acid, the system behaved as if it was following the plant pathway i.e. stearate was incorporated into lipids but was not desaturated although the build up of oleate from acetate was unaffected. Clearly the sterculic acid inhibition was acting on the acyl transferase enzymes, which convert the stearoyl-coenzyme A thiolester to the stearoyl ACP thiolester. This work is further evidence that the true substrate for desaturation in the plant kingdom is the ACP thiolester.

Stumpf[31] and Bloch[61] have shown the presence of similar proteins in higher plants and leaf chloroplasts.

The aerobic desaturation process was found to require either the reduced form of nicotinamide adenine dinucleotide (NADH) or the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) and has an absolute requirement for molecular oxygen[47].

The precise role of the oxygen and the reduced pyridine nucleotides is not clear at the moment and further investigation awaits the fractionation and purification of the enzyme system.

With molecular oxygen being an essential cofactor and the fact that the artificial electron acceptors methylene blue or phenazine methosulphate do not substitute for oxygen[47] makes a flavoprotein linked dehydrogenase mechanism unlikely.

The requirement for NADPH and oxygen along with the insensitivity of the reaction to cyanide[47] would seem to favour an oxygenase reaction. This would involve the formation of a hydroxy intermediate which, on subsequent dehydration, would give the required monoene.

This theory has the advantage that fatty acids possessing an oxygenated function are quite common. Some examples are shown below:-

Trivial name	Structure
9(10) hydroxy stearic	$CH_3 (CH_2)_8 CHOH(CH_2)_7 COOH$
ricinoleic	$CH_3 (CH_2) CHOH CH_2 CH = CH -(CH_2)_7 COOH$
densipolic	$CH_3CH_2CH = CH (CH_2)_2 CHOH CH_2 CH = CH - (CH_2)_7 COOH$
9,10 epoxystearic	$CH_3 (CH_2)_7 \overset{O}{\overset{/\backslash}{CH - CH}} (CH_2)_7 COOH$
vernolic	$CH_3 (CH_2)_4 \overset{O}{\overset{/\backslash}{CH - CH}} CH_2 CH = CH - (CH_2)_7 COOH$
coronaric	$CH_3 (CH_2)_4 CH = CH CH_2 \overset{O}{\overset{/\backslash}{CH - CH}}(CH_2)_7 COOH$

Trivial name	Structure
15,16 epoxylinoleic	CH_3CH_2 $CH\ CH\ CH_2\ CH = CH - CH_2\ CH = CH - (CH_2)_7\ COOH$
9-ketostearic	$CH_3(CH_2)_8\ C\ (CH_2)_7\ COOH$

Studying the structures of the above naturally occurring oxygenated acids the dehydrase theory is very attractive.

James et al[64] having discovered large amounts of 10 hydroxy stearic acid in faecal lipids of persons who suffered from steatorrhea and showed that faecal lipids could convert stearate to 10 hydroxystearate suggested that this might be an intermediate in the formation of oleic acid.

Marsh and James[49] demonstrated that no significant amount of oleate was produced from 9 or 10 hydroxystearate substrate by either fractionated rat liver preparations or cell free yeast systems. Similar results were obtained when the CoA thiolesters of the hydroxy acids were used showing that it was not an activation barrier that was operating in the case of the hydroxy acids.

Thus they concluded that the hypothesis of an hydroxyintermediate in the biosynthesis of oleate from stearate was only feasible if it was assumed that this intermediate was irreversibly bound to the enzyme. This reservation is the same as has been postulated in the case of the biosynthesis of long chain fatty acids from malonyl CoA[65].

Keto acids have been suggested as possible intermediates in the biosynthesis of unsaturated fatty acids[66], but 9 ketostearate was not converted to oleate either in a yeast or a liver system[49,67].

Other oxygenated species that were found to be incapable of conversion to oleate were the phosphate esto of 10 hydroxystearate, 9,10 dihydroxystearate, 9,10 cis epoxystearate[49,67] and stearolic acid[68].

In the face of this evidence Light et al[52] suggested that the desaturation of saturated fatty acids occurs by the direct abstraction of hydrogen

atoms from the saturated chain by oxygen acting as an electron acceptor without at any time entering into covalent linkage with the carbon atom of the acid.

Shroepfer and Bloch[69] produced evidence as to the stereospecificity of the desaturation when they incubated all four stereospecifically labelled monotritiated stearic acids prepared from the enantiomorphic 9-hydroxystearates and 10-hydroxystearates with growing cultures of Corynebacterium diphtheriae.

The oleic acid formed in each case was isolated and there was found to be complete loss of tritium in the 9D and 10D tritiostearic acids and retention in the case of the 9-L and 10-L tritiostearic acids. Thus it appears that the removal of the hydrogens at the 9 and 10 carbon atoms is stereospecific in the formation of oleate from stearate, the D hydrogens being removed at each position.

Furthermore the presence of a substantial kinetic isotope effect on the removal of the hydrogen atom at the C9 position and not at C10 suggested that the removal was not synchronous but stepwise with the initial removal of the hydrogen at C9 being the rate limiting step.

Although this work established the absolute configuration of the hydrogens removed in the desaturation, it did not solve the mechanism by which they were removed or the conformation of the substrate molecule at the active site. The results may be interpreted by suggesting a cis abstraction, a substitution with retention of configuration followed by a cis abstraction or a substitution with inversion followed by a trans elimination. The substitutions of, for example, hydroxy groups would have to occur with the substrate irreversibly bound to the enzyme but these results do not preclude this as a possibility, indeed the stepwise mechanism suggested by the kinetic isotope studies is in favour of this as the mechanism in this system.

The mechanism and stereochemistry of desaturation was also investigated by Morris et al[70] in the green alga Chlorella vulgaris.

This organism forms not only monoene by direct desaturation of the saturated precursor but also by a series of sequential desaturations forms a diene

and also a triene. In the case of stearic acid the desaturation products are oleic acid, linoleic acid and α linolenic acid respectively.

By the synthesis of racemic erythro and threo - 9,10 dideutero stearate, erythro and threo 12,13, dideutero oleate and erythro and threo 15,16 dideutero oleate, Morris et al[70] were able to show in all three cases that the hydrogen atoms removed were of the cis relative configuration since the erythro substrate lost both deuterium atoms from one enantiomer and none from the other. The threo substrate lost half the deuterium from each enantiomer. Removal of trans rather than cis hydrogens would have given the opposite results.

By synthesis of D- and L-9 tritio stearic acid and D- and L-12 tritio stearic acid these workers were also able to show that the D hydrogens are the ones removed in desaturation at these centres.

It may be reasonably assumed that the hydrogens removed in formation of the 15,16 double bond of linolenic acid are also of the D configuration although this has not yet been actually proved. All three desaturations were completely stereospecific and all the same as the desaturation of stearate to oleate in bacteria[69], goat mammary gland[71], hen liver[72] and fish liver[73].

The results of Morris et al with the deuterated substrates indicated a substantial kinetic isotope effect against deuterium in the formation of all three double bonds and for the stearate to oleate desaturation at least this effect had to apply at both positions of the putative double bond. This result which was observed also in animal systems[71] suggests a simultaneous concerted removal of the hydrogen atoms rather than the stepwise process as suggested by Shroepfer and Bloch as a result of their work in the bacterial system.

Aerobic desaturation is not confined to the formation of one double bond per chain. All animals, plants and micro organisms with the exception of the Pseudomonadales, Eubacteriales and Actinomycetales are able to form polyunsaturated fatty acids e.g. 74, 75, 76.

These polyunsaturated fatty acids are different in animal and plant

systems. Although the position of the first double bond in both kingdoms is almost exclusively in the 9-10 position, the position of the other double bonds differs according to the system.

In the plant kingdom successive desaturations are carried out towards the methyl end of the molecule except in a few isolated cases. Animal systems, on the other hand, can only introduce bonds between an existing one and the carboxyl end of the molecule. It is because of this that animals require dietary linoleic and linolenic acids, particularly the former, since only from these can they produce the essential polyenoic acids they require for ideal membrane function and prostaglandin synthesis e.g. arachidonic acid by elongation coupled with successive desaturations towards the carboxyl end of the molecule.

In animal systems there are essentially three series of polyenoic acids. The W9 series, which is the only one they can synthesise de novo (e.g. 5, 8, 11-20 : 3) and the W6 series (e.g. 5, 8, 11, 14-20 : 4) and the W3 series (e.g. 4, 7, 10, 13, 16, 19-22 : 6). For the W3 and W6 series animals require dietary linoleate or linolenate as starters. Plants generally produce oleate, linoleate and α linolenate as their characteristic unsaturated acids although some animal type products have been isolated from plants. Arachidonic acid has been demonstrated in mosses and ferns[77], γ linolenic in seeds of the Onagraceae[78] and Boraginaceae[79] and stearidonic in the seeds[80] and fruit[79] of various genera of the Boraginaceae.

Free fatty acids are not usually found in living systems in any large amount, indeed they bind readily to many proteins and are potent enzyme inhibitors. They exist, usually bound as esters, to the trihydric alcohol glycerol.

When fatty acids are stored as energy reserves in depot fats or as insulation, they are bound up as triglycerides I.

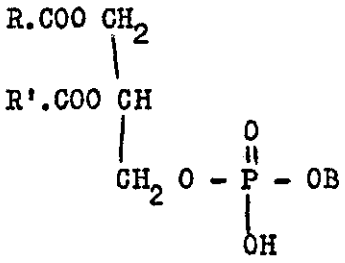
$$\begin{array}{l} CH_2\ OCOR \\ | \\ CH\ \ OCOR' \\ | \\ CH_2\ OCOR'' \end{array}$$

I

R, R' R" are fatty acyl groups and may be similar or different.

Where the lipids have a more physiological function their structure is more complex. Examples and structures of these complex lipids are as follows:

I Phospholipids

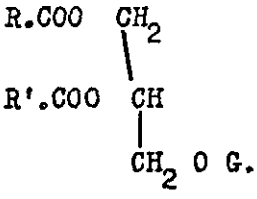


R and R' are fatty acyl groups.

B is an organic base or polyhydroxy compound and depending on this group then the phospholipids are named as below.

B = $-CH_2\ CH_2\ \overset{+}{N}\ (CH_3)_3\ \overset{-}{O}H$	3 - phosphatidyl choline (P.C.)
B = $CH_2\ CH_2\ NH_2$	3 - phosphatidyl ethanolamine (P.E.)
B = serine	3 - phosphatidyl serine (P.S.)
B = inositol	3 - phosphatidyl inositol (P.I.)
B = glycerol	3 - phosphatidyl glycerol (P.G.)

Glycolipids



G is a carbohydrate unit, which is either galactose, or a dimer of galactose usually, though other residues are possible. The lipids are denoted as MGDG monogalactosyl diglyceride or DGDG digalactosyl diglyceride.

It is in these more complex lipids that the fatty acids that have been freshly synthesised or desaturated are first found to accumulate. In animal systems they are found in the phospholipids especially in lecithin (PC). In some plant systems actively synthesised fatty acids are found first in PC,

PG and MGDG. Nichols and James[81] studied the time sequence of uptake of label from ^{14}C-acetate into the individual fatty acids of the separate lipid classes of Chlorella vulgaris and showed that uptake and turnover of label was most rapid in PC, PG and MGDG. From their results they concluded that all these three lipid classes were "carriers" of stearic acid while PG and MGDG function for myristic, palmitic and palmitoleic acids. Both oleic and linoleic acid appeared to pass through PC, PG and MGDG and the high turnover rates suggested that the acyl lipid is behaving in a manner similar to that expected for a true intermediate in the fatty acid synthetic sequence. This could be achieved in one of three ways, either with the acyl lipid acting as a transport agent moving the acid to the enzyme or the lipid could act as the true substrate for desaturation or that the pools of acid in the synthetic sequence are only connected via the acyl lipid.

The close involvement of lipid in the desaturation steps of fatty acid biosynthesis is further indicated by the observed tendency[82] for the freshly desaturated acyl groups to appear at the second position of the lipid. Gurr James and Robinson[83] demonstrated in Chlorella vulgaris chloroplast preparations that 2-oleoyl phosphatidyl choline was as good a precursor for desaturation as oleyoyl CoA thioester and that the resulting linoleate was bound exclusively to the 2 position of the same lipid class (i.e. PC). As a result of this they concluded that either the acyl group was transferred directly from the lipid to the desaturase and immediately returned in a desaturation-acylation cycle or that the lipid itself is the actual substrate for desaturation. They intend to distinguish between these two possibilities by the use of the oleyl ether analogue of lecithin which is isosteric with the natural ester linked lipid but which will not be affected by acyl transferases.

Although the vast majority of double bonds that occur in natural fatty acids have the cis configuration, some fatty acids having trans bonds have been found. Little is known of the biosynthesis of trans acids except for the work of Nichols et al[84] on trans 3-hexadecenoic acid.

These workers found that this acid was derived from palmitic acid by a direct oxygen requiring dehydrogenation. However unlike the usual cis 9-10 acid formation this process also required light. Although the formation of some cis acids in photosynthetic systems is increased by the presence of light e.g. α linolenic acid, they are in fact synthesised to a marked degree in the absence of light. Thus the absolute requirement of trans 3 hexadecenoic acid formation for light is unique in these systems.

There also appears to be a close lipid involvement in the formation of this acid since Nichols et al[84] showed that it was present only in phosphatidyl glycerol. Furthermore Haverkate and Van Deenen[85] showed in spinach that this acid was present only in the 2 position of PG with usually α linolenic acid in the 1 position.

As mentioned earlier, a main feature of the common polyunsaturated acids in both plants and animals is their methylene interrupted sequence of double bonds. The biosynthetic control giving rise to this structure is as yet unknown and although the vast majority of polyunsaturated acids have this arrangement of bonds, some 40 acids are known which exhibit conjugation[86].

All these acids are found in the plant kingdom and have eighteen carbon atom chains. The majority of these acids have two or three unsaturated centres which may be either cis or trans. A table of these acids is shown below.

Table IV. Naturally occurring conjugated fatty acids

Carbon chain length	Position and conf.of bonds	Trivial name	Source (Seed)	Ref.
18	8c, 10t, 12c	jacaric	Jacaranda mimosifolia	87
18	8t, 10t, 12c	calendic	Calendula officinalis	88
18	9c, 11t, 13c	punicic	Punicum granatum	89
18	9c, 11t, 13t	α-eleostearic	Aleurites fordii	89
18	9t, 11t, 13c	catalpic	Catalpa ovata	90
18	9a, 11a, 13c	bolekic	Onguekoa gore	91
18	9c, 12a, 14c	dehydrocrepenynic	Afzelia cuanzensis	92,97

a = acetylenic, c = cis, t = trans

Although the biosynthesis of these acids is unknown, there have been several speculative pathways put forward[e.g. 93,94]. One of the most interesting is due to Gunstone[94] who suggested that the precursor of these acids is linoleic acid and that conjugation occurs via hydroxylation or epoxidation and rearrangement followed by dehydration. This is illustrated below for the case of α-eleostearic acid (fig.I). This hypothesis has the advantage that its intermediates are known naturally occurring materials and that oxygenated functions are common amongst this class of material.

Fig.I Proposed biosynthetic route to α-eleostearic acid

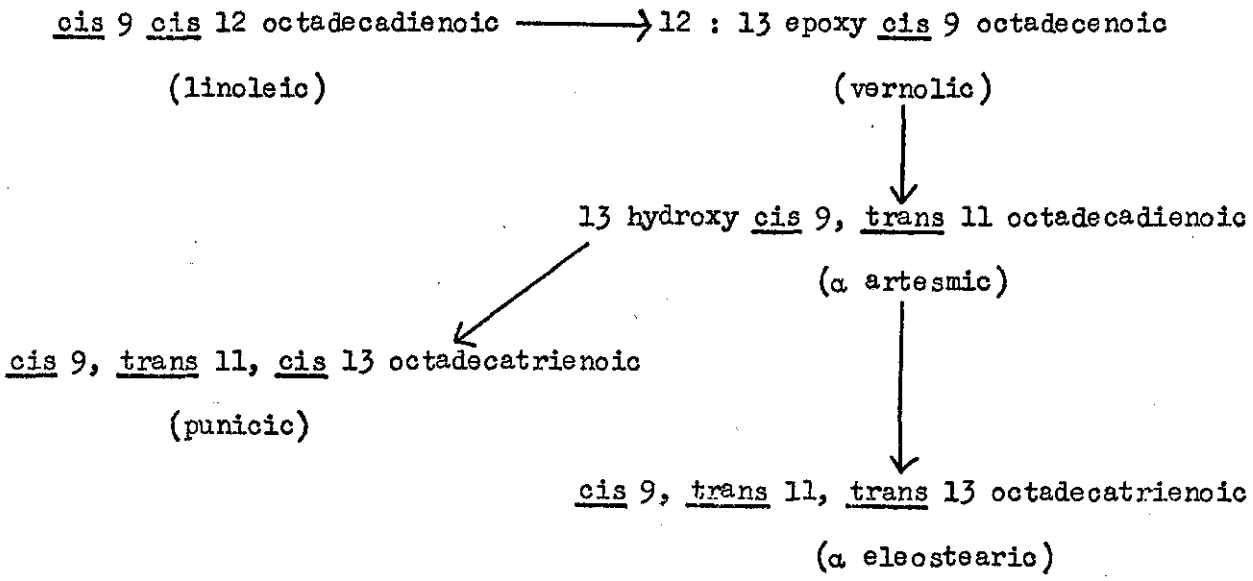


Table V Naturally occurring conjugated ethylenic hydroxy fatty acids

Name	Trivial Name	Source (Seed)	Ref.
9 hydroxy trans 10 trans 12 octadecadienoic	dimorphecolic	Dimorphotheca sinuata	95
13 hydroxy cis 9 trans 11 octadecadienoic	artemesic	Coriaria nepalensis	96
9 hydroxy trans 10 cis 12 octadecadienoic		Calendula officinalis	94

Some fatty acids which occur naturally, contain acetylenic bonds both in conjugated[e.g. 92,97] and methylene interrupted arrangements with ethylenic bonds[e.g. 98,99]. Bu'Lock[100] postulated that acetylenic bonds were formed by

the further desaturation of olefinic acids and Bohlmann and Schulz[101] have demonstrated that linoleic acid is converted to crepenynic acid (octadec-9en-12 ynoic acid) in Chrysanthemum flosculosum and Coreopsis lanceolata.

Haigh Morris and James[102] showed in Crepis ruba that crepenynic acid was not formed from linoleic acid but from oleic acid in a reaction requiring oxygen. It was thus shown that in this plant system Bu'Lock's proposed pathway was not in operation although the usual reservations about linoleate being an irreversibly enzyme bound intermediate must be considered.

Although acetylenic bonds, conjugated bonds and trans double bonds do exist in naturally occurring fatty acids, by far the most common arrangement is of cis double bonds and where more than one bond occurs in a chain, for them to be methylene interrupted.

A general picture of the biosynthetic relationships between these acids is shown below in Fig.II.

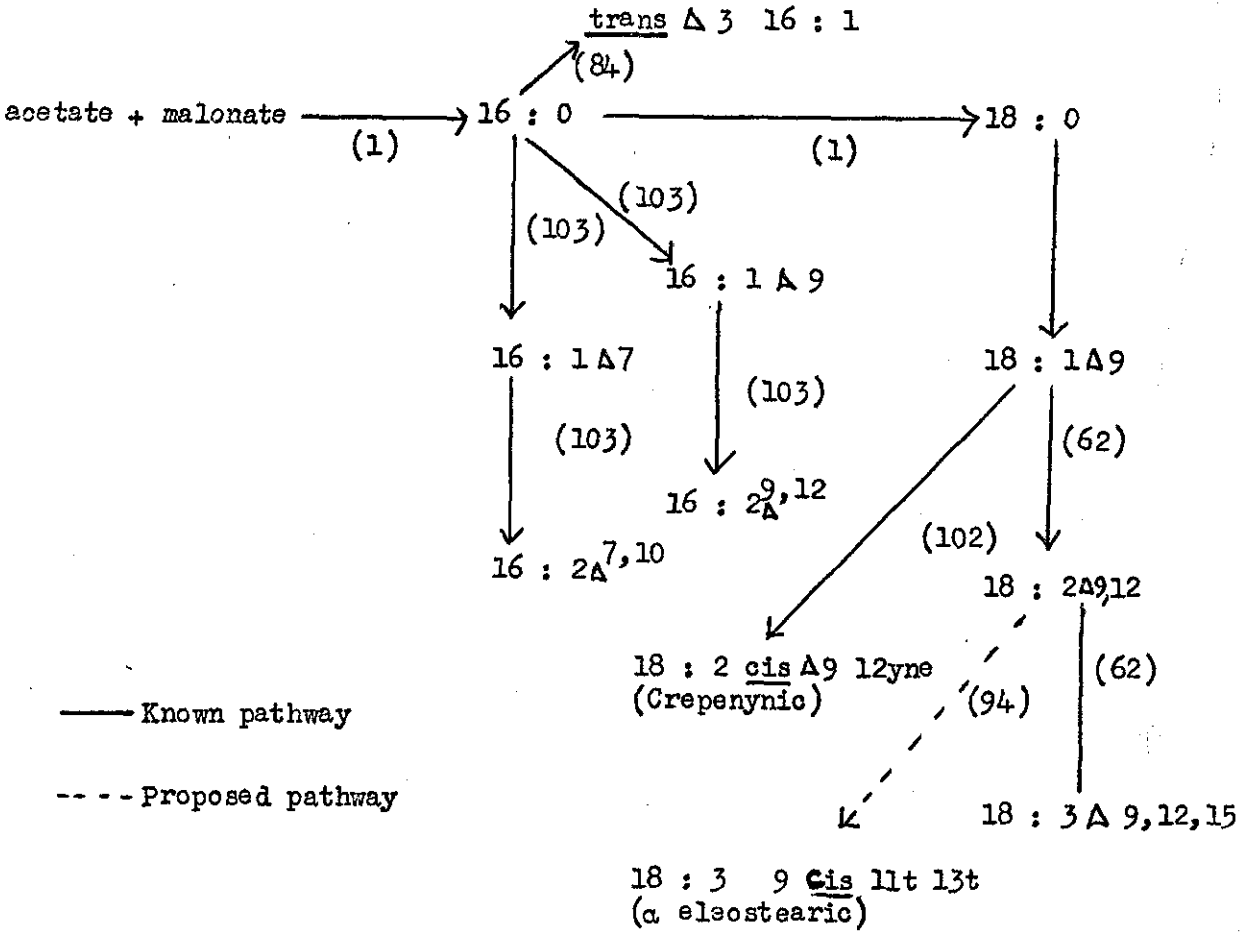


Fig II Defined biosynthetic pathways in plants.

From what has been said before, it is clear that many of the problems surrounding the direct aerobic pathway for the biosynthesis of unsaturated fatty acids have been solved. The enzyme system responsible has been shown to be firmly particle bound to the chloroplasts in plant systems and to the endoplasmic recticulum of animals. The substrates for the enzyme activity have been shown to be the coenzyme A thioester in some systems and the A.C.P. thioester in others. In all systems NADH and oxygen are necessary cofactors.

The mechanism and stereochemistry of the hydrogen removal has been elucidated in some systems and the final products of the reaction i.e. the unsaturated fatty acids have been isolated from many sources and much knowledge has been gained by examining their structure particularly the positions of the double bonds. The close involvement of certain lipid classes with the process of aerobic desaturation has also been observed, both in the chloroplasts of plants and the microsomes of animals. Despite all this there remains many unanswered problems about the process and much work has still to be done and the fact that the enzymes are particle bound and resistent to fractionation makes direct investigations difficult.

One of the questions that remains unanswered is what are the factors which govern the position of the bonds in the chain in aerobic desaturation? For example, why is the first double bond introduced in the position that it is and what governs the position of the subsequent double bonds in the case of polyunsaturated fatty acids?

It was therefore of interest to synthesise unnatural fatty acids and study the position and degree of the subsequent desaturation, if any, and hence attempt to elucidate the factors which influence the positional specificity of the desaturation.

It is along these lines that this thesis is directed.

SECTION 1

Chemical synthesis of Radiolabelled

Fatty acids for use as substrates.

Introduction

Synthesis of fatty acids is necessary for both confirming the structure of naturally occurring fatty acids and for the production of unnatural fatty acids to elucidate their physical, chemical and biological properties.

The literature on the synthesis of these compounds is far too vast for the scope of this thesis and dates back to 1871 when Lieben et al[104a] prepared all the n-saturated acids from acetic to heptanoic by chain extension from methanol, and in the same year Krafft [104b] starting from naturally occurring stearic acid by degradation prepared all the n-saturated acids from C_9 - C_{18}.

The topic has been the subject of many reviews among which the ones by F. D. Gunstone,[105] W. J. Gensler [106] and K. Markley[107] are the better presented and most comprehensive. These cover the synthesis of all the classes of fatty acids i.e. saturated, unsaturated and substituted fatty acids.

Since most of the interest in general, and of this thesis in particular, is the synthesis and biological properties of unsaturated fatty acids it is intended to limit the rest of this introduction to the methods whereby unsaturated fatty acids of the correct chain length, having unsaturation of the required configuration and in the right position can be synthesised. Briefly the methods of synthesis fall into two broad classifications.

The first is the preparation of fatty acids from other fatty acids or closely related compounds. The second is by the build up of the molecule from smaller units - de novo synthesis. Usually where the former method can be used it provides a more convenient synthesis.

1. Synthesis of unsaturated fatty acids from closely related compounds

(i) Reactions involving no change in chain length

These reactions usually involve either elimination from a substituted acid or partial reduction of an acetylenic or polyunsaturated acid. Methods which fall into the former category are the dehydration of hydroxy acids, the dehydrohalogenation of halo acids and the dehalogenation of halo compounds.

In the case of dehydration the position of the hydroxy group affects the product. α-Hydroxy acids have a tendency to undergo several pyrolytic dehydrations to yield aldehydes or cyclic lactides in preference to the αβ-unsaturated acid.[108]

β-Hydroxy acids on the other hand do dehydrate to yield the αβ-unsaturated acid in preference to other products.[109, 110, 111] Where the αβ-unsaturated compound is structurally impossible as in the dehydration of ethyl 3-hydroxy, 2,2 dimethylbutyrate[112] with phosphorus pentoxide the βγ-olefin is produced.

With the hydroxy group in the 3 or 4 position the reactions undergone are much more those of the lactone.[113] As a rule beyond the 4 position the yield of monoenoic acid over lactone formed increases as the hydroxy group becomes more removed from the carboxyl group.

The presence of an isolated double bond does not influence the dehydration and Fokin[114] and other workers[115,116] all found that ricinoleic acid gives a mixture of 9,11 and 9,12 dienes. Baudart[117] also showed that a terminal olefin could be prepared by dehydration.

The main agent used for dehydration is pyrolysis through phosphorus pentoxide; thionyl chloride and phosphorus oxychloride are also used.

Dihydroxy acids dehydrate to give dienoic acids as above, but vicinal dihydroxy acids yield only the conjugated acids e.g. 9, 10-dihydroxy stearic acid yields only 8,10 octadecadienoic acid.

Dehydrohalogenation of a halo compound is another common method of introducing a double bond into a chain. The most common reagents used to effect

the dehydrohalogenation are dry alcoholic solutions of alkali hydroxides[118]. The absence of water in these reactions makes the system less ionic and thus favours elimination over nucleophilic substitution.[119].

These dehydrohalogenations take place without rearrangement of the carbon skeletons[120] and the elimination is trans always.[121] Despite the stereospecificity of the elimination mechanism both cis and trans olefins are produced by this method.

Other non polar dehydrohalogenating agents are pyridine and collidine. Also more recently the heterocyclic bases DBU (1,5-diazabicyclo (5.4.0) undec-5-ene) and DBN (1,5 diazabicyclo (4.3.0) non-5-ene) are being used.[122, 123]

Once again with 2 and 3 halo compounds the products are largely specific yielding the αβ-unsaturated species[124], but when the halogen is mid chain a mixture of products is obtained[125]. With vicinal dihalides, however, acetylenes are formed along with the conjugated dienes. This is especially noticeable with D.B.N. and D.B.U. where the acetylene is the major product.[122]

Dehalogenation can be used also to form fatty acids from vicinal dihalides. The most common agent is nascent hydrogen generated by metal-acid or metal-alcohol[126,127] combinations. Another method is the Finklestein reaction[123], where the di-halo compound is refluxed with dry sodium iodide in acetone. This depends on the fact that the vicinal diiodide is not stable for steric reasons and loses iodine spontaneously to yield the olefin.

The other class of reactions for generating olefinic acids of given bond position involves partial reduction. The initial materials may be either polyenoic fatty acids or acetylenic fatty acids. Reduction of polyenoic acids forms a mixture of products but these can often be separated by thin-layer chromatography.[129] One of the most selective methods of reduction is with hydrazine[130]. Since this is considered later no more will be said here.

The other method of reduction of synthetic importance is the partial reduction of acetylenes. This is especially so since many of the de novo

syntheses involve acetylenic couplings. This reduction may be achieved either catalytically or chemically. Its most important feature is control over the stereochemistry of the olefin formed. Chemical reducing agents that have been used are zinc and hydrochloric acid containing acetic acid and titanous chloride with which Robinson and Robinson [131] reduced stearolic acid to oleic acid, and sodium in liquid ammonia which Howton and Davis [132] used to produce trans-5-octenoic acid from 5-octynoic acid.

By far the most common and useful methods for partial reduction of acetylenes are the catalytic reductions. Half hydrogenation over Raney nickel has been extensively used but the literature varies as to its usefulness. Adkins and Billica [133] and Walborsky et al [134] are among many workers who found it successful. Palladium is the most used reagent in the reduction of acetylenes to cis olefins. The difficulty with this is that there is a tendency for the reaction to continue to give alkanoic acids if the hydrogen is not limited.

In an attempt to stop the reaction at the olefinic stage and improve the stereospecificity Lindlar [135,136] developed a catalyst of palladium in the form of a 5% suspension on calcium carbonate which was poisoned with lead and quinoline. More recently the reproducibility of this catalyst has been improved by Cram and Hallinger [137] who used pure synthetic quinoline to poison palladium in the form of a 10% suspension on barium sulphate. This has the advantage of stopping at the olefin stage and yielding the pure cis isomer.

(ii) Reactions involving degradation by one or two carbon atoms

The first degradation of fatty acids was the pyrolysis of the barium salt. [104b] This is now only of historical interest. The Hofmann degradation [138,139] has also been used.

Alkanoic acids may be degraded by α bromination [140] followed by hydrolysis to the α hydroxy compound. This is then cleaved with potassium permanganate [141] or lead tetraacetate [142]. This method or any method involving

double bond cleavage is not easily applicable to alkenoic acids unless the unsaturated function has been protected before the cleavage.

For unsaturated acids the most generally used method is the Hunsdiecker silver salt degradation.[143] Using this, Nevenzel and Howton[144] have successfully degraded oleic, linoleic, and α and γ linolenic acids.[145]

A more convenient method for degradation is the Cristol and Firth modification[146,147] of the Hunsdiecker reaction. This involves the use of mercuric oxide, removing the need to prepare a metal salt of the acid. Alkenoic acids have been successfully degraded[146] using this method.

(iii) Extension of the chain by one or two carbon atoms

The most usual methods for extending the carbon chain by one carbon atom are the treatment of the alkenyl halide,[147] mesylate,[149] or p-toluene sulphonate,[150] with potassium cyanide in either aqueous alcohol[151] or dimethyl sulphoxide.[152]

Another method for extension of the chain by one carbon atom is to treat the alkenyl magnesium halide or other organometallic compound with carbon dioxide.[153,154] Both these reactions are useful in the preparation of isotopically labelled fatty acids e.g.[155].

Another very useful homologation reaction is the Arndt-Eistert synthesis[156]. This synthesis involves the preparation of the diazoketone which undergoes a Wolff rearrangement with the loss of nitrogen to give the homologous product. This is useful in cases where molecules are complex or sensitive to reducing agents[157] and has been used by Karrer and Koenig[158] to prepare the C_{19} and C_{20} homologues of linoleic acid.

Several methods exist for extending chains by two carbon atoms, among the most useful of which is the treatment of the Grignard reagent with ethylene oxide.[159] Another much used extension reaction is the reaction of malonic ester with alkenyl halides.[160]

Both the Doebner reaction[161] and the Reformatsky reaction[162] have been used to produce unsaturated fatty acids, but these are not so commonly used.

II Build up of the molecules from smaller units

1. Chain extension by larger units

(i) Alkenoic acids can be elongated by five or six carbon atoms at a time by the use of the enamine of cyclopentanone or cyclohexanone on the acid chloride[163] .

There are several other methods of chain extension by four, five or six carbon atoms and these are comprehensively dealt with in tabular form in F. D. Gunstone's most recent book [164] .

(ii) Coupling with acetylenic compounds

The most widely used reaction for the synthesis of alkenoic acids is coupling with acetylenic compounds [165] . Indeed it is the most useful route to some of the polyunsaturated fatty acids especially the methylene interrupted naturally occurring ones. An example of the usefulness of acetylenes as intermediates is the syntheses of the essential fatty acids linoleic and arachidonic by R. T. Holman [166] .

The usefulness of acetylenes stems from their ability to form alkali metal and Grignard derivatives which allows them to be coupled with alkyl halides, carbonyl compounds, carbon dioxide etc. and thus build up long aliphatic chains with multiple bonding at known positions.

Since acetylenes can be semi hydrogenated to give exclusively cis or trans olefins[167, 168], the ability to tailor fatty acids is complete.

Much initial work in this field was done by Ahmad and Strong who first realised the potential of this route when they prepared 6 hendecenoic acid[169] and went on to use it to prepare many other monoenoic fatty acids [170].

(iii) The Wittig Reaction

The Wittig reaction [171] has been a well used route to olefins for many years. The drawback to its use in the fatty acid field has been the fact that in normal non polar solvent systems e.g. benzene, the ylid reacts with the carbonyl compound to form a trans olefin.

More recently however Russian chemists [172,173] have shown that by using more polar solvents (e.g. dimethyl formamide or Lewis bases e.g. the iodide ion), the cis olefin can be produced. This renders the reaction of much more use in the synthesis of fatty acids. Examples of the use of this reaction are the syntheses of cis 8 hexadecenoic acid, cis 7 ocatadecenoic acid and cis 11 eicosenoic. Furthermore this reaction has been used in the synthesis of the very important naturally occurring methylene interrupted acids [174]. The Wittig reaction can also be used to prepare conjugated olefins and the synthesis of α eleostearic acid [175] is an example.

(iv) Kolbé electrolytic synthesis

The mixed Kolbé electrolytic synthesis using a carboxylic acid and the half ester of a dicarboxylic acid yields a mixture of products among which is a monocarboxylic fatty ester (see page 41).

Because the separation of this mixture is easy the method has been used for the synthesis of many and diverse fatty acids despite only moderate yields of about 30%.

The reaction is usually carried out in glass vessels between platinum electrodes [176]. The reactants are dissolved in methanol with enough sodium methoxide added to allow ionisation [177]. An excellent review [178] of this reaction has been published and only its application to the synthesis of unsaturated fatty acids will be considered here.

The presence of a substituent in the α position or unsaturation either αβ or βγ to the acid carbonyl group will inhibit the reaction [179]. Thus maleic [180], fumaric [181] or muconic half esters [182] will not undergo the reaction. trans β Dihydromuconic half ester will not undergo the though protection of the double bond with a dihydroxy function will enable the reaction to proceed [183]. Terminal double bonds do not affect the reaction and both 7-octenoic acid [184] and 10-undecenoic acid [185] have been prepared although there is some evidence [186] that polymerisation occurs at the electrodes

to some extent during electrolysis.

Apart from the above restrictions this method is perfectly satisfactory and has been used in the synthesis of nervonic acid [187] and behenolic acid [188].

In his review of the chemical synthesis of fatty acids [105] Gunstone divides his discussion into the following separate headings: Methods involving no change in chain length; chain extension; methods involving chain degradation; the modification of existing fatty acids; the isolation of fatty acids from natural materials and de novo synthesis. In preparing the precursors necessary for the biochemical studies which form the real point of this thesis, use has been made of all of these methods to some extent. In most cases, because of the need to provide these acids labelled, a chain extension step has been necessary anyway.

The two most usual ways of labelling fatty acids are either to treat the alkyl or alkenyl magnesium halide with $^{14}CO_2$ [144] or to react the mesylate [149] the tosylate [189] or halide [190] with $K^{14}CN$. Both methods have been used successfully with both saturated and unsaturated fatty acids [149,191] For convenience the method used throughout this work was that of labelled potassium cyanide.

Tests with unlabelled potassium cyanide showed that the use of dimethyl sulphoxide (DMSO) at 90°C [152] gave a cleaner reaction yielding fewer by-products and higher yields than more conventional solvents e.g. aqueous alcohol [151].

Conversions have been shown in the literature using potassium cyanide with p-toluene sulphonates [150], alkyl chlorides [152], bromides [148] and iodides [192] All four of these have been used in the course of these syntheses.

With D.M.S.O. as solvent it was unnecessary in the case of alkenyl bromides to use cuprous cyanide in place of the alkali cyanide. This was found by Celmar and Solomons [193] to improve the yield in the case of aqueous alcohol. Similarly the slightly acid medium with copper powder in T.H.F. as used by Bohlman and Viehe [194] was also not used.

Nevertheless the labelling step did give difficulty and yields generally were low. It is not easy to account for the low yields since in all cases the reactions were homogeneous and a considerable excess of alkyl halide was used,

and trial reactions on the same scale but with unlabelled potassium cyanide consistently gave almost quantitative yields.

Two major impurities arose from the labelling step. The first was a very polar material which did not migrate from the origin on T.L.C. when even quite polar developing solvents were used and the second was a slightly less polar (by T.L.C.) compound than the first. These materials gave no mass spot on the plate when the reaction was tried with unlabelled potassium cyanide. Hence it was concluded that they were artefacts of the radio preparation alone. It is not unknown that radio cyanide may react differently from ordinary cyanide.[195]

These materials were isolated and attempts were made to identify them. This identification was made very difficult by the minute mass present $< 1\mu g$. The only hope was to characterise them by T.L.C. against known compounds. The possibility of them being amides arising by some combination with moisture present, isonitriles, or isocyanates was discounted by virtue of a complete resistance to either acidic or basic hydrolysis. Free acid occurring by hydrolysis was discounted by the fact that it did not respond to treatment with diazomethane. It is possible that some formoxy derivative has been formed but in the absence of further material only speculation can be made.

It was a simple matter to isolate the pure ester after methanolysis, so apart from the loss of yield these artefacts were not a serious handicap.

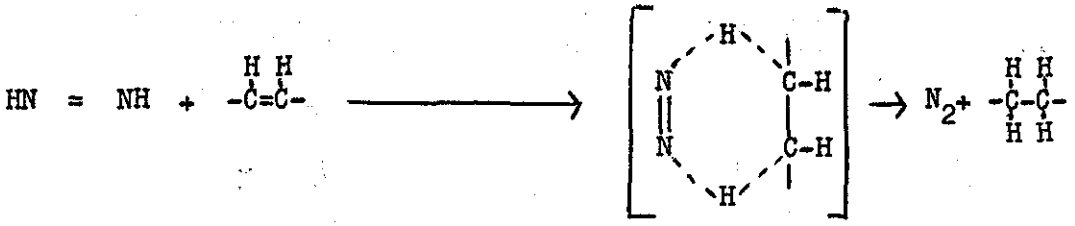
(i) Methods requiring no change in chain length.

Methods used requiring no change in chain length for the sake of this thesis merge into the heading of modification of existing fatty acids. Two such syntheses were carried out. The first was the partial reduction of ($1^{14}C$)-linoleic acid to give ($1^{14}C$) cis 12 octadecenoic acid[130] and the second was the elaidinisation of ($1^{14}C$)- oleic acid to give ($1^{14}C$)- elaidic acid[196].

The reagent used for the partial reduction of methyl ($1^{14}C$)- linoleate was hydrazine hydrate. This reaction was chosen since it does not alter the position or geometrical configuration of the residual bonds.[197,198]

This reduction is effected by the unstable di-imide intermediate I which is formed by autoxidation. The whole reaction proceeds according to the following equations [199]

$$H_2N - NH_2 + \tfrac{1}{2}O_2 \longrightarrow H_2O + \underset{\text{I}}{HN = NH}$$



Variation of reaction time, temperature, solvent and volume of available oxygen allows control of the reaction and hence permits a maximum yield of monoene to be formed.

The solvent used in this case was methanol, faster reaction is brought about with acetoritrile as solvent with pure oxygen bubbling through. Since the prime requirement of this reaction was selectivity for partial reduction advantage was gained by use of the slower solvent system. For the same reason the bubbling of oxygen was substituted by agitation with a wrist action shaker. The temperature used was 60°C. This combination of conditions was tried and found to give the optimum rate of reduction (about two hours).

The reaction was followed by injecting samples on to the R.G.L.C. [200] Here disappearance of the diene peak was observed as monoene and saturated peaks grew. By experience it has been shown that the optimum yield for this reaction is about 40% monoene. This state remains for a while as diene is further converted to monoene and monoene converted to saturated but no greater build up of monoene is achieved.

When this state was attained the reaction was quenched by adding dilute acid to destroy the excess reagent.

The four products from this reaction were separated by the method of Morris, Wharry and Hammond. [201] This consisted of T.L.C. separation on silica

impregnated with 30% silver nitrate developed three times in toluene at - 25°C. The low temperature is used to give greater stability to the bond complex of the unsaturated centre with the silver and hence achieve better separation than at room temperature. Since benzene which is the best solvent at room temperature is solid at this temperature, toluene is used.

This method gave R_f values as shown below:-

Substance	R_f
18 : 0	0.86
18 : 1Δ12	0.53
18 : 1 Δ 9	0.47
18 : 2	0.16

The methyl ($1^{14}C$) *cis* 12 octadecenoate was thus isolated and purified. $1^{14}C$ *cis* 12 octadecenoic acid was prepared from this by hydrolysis in the usual way (see page 45).

The second preparation that requires no elongation and is in fact the modification of an existing compound was the preparation of ($1^{14}C$)- elaidic acid. The method used in this conversion was Griffiths and Hilditch's elaidinisation with the oxides of nitrogen [196].

The nitrogen oxides are generated by the interaction of 6M nitric acid with 2M sodium nitrite. These oxides do not produce a complete isomerisation but set up an equilibrium consisting of about 60% of the *trans* isomer irrespective of which isomer is used as starting material.

This definite ratio of 2 : 1 *trans* to *cis* suggests that the mechanism of the reaction involves the attack of nitro and nitroso radicals on the unsaturated centre followed by the elimination of nitrogen trioxide in such a way as to yield the observed ratio of the isomers. More recent work [202] has suggested that the ratio is 3 : 1 and this is more in line with the yields in this experiment. Despite the incomplete conversion of oleate to elaidate the ease of separation of these isomers by silver ion chromatography [2] makes the

method satisfactory for the purposes of this thesis.

Recently, however, Gunstone et al have published a paper [203] in which they used selenium to effect a similar steromitation which gave a greater percentage of the trans product. Less double bond migration was found if the stereomutation was carried out by irradiation of the ester with ultra violet light in the presence of diphenyl sulphide.

(ii) Methods using chain extension

The preparation of ($2^{14}C$) cis-10-nonadecenoic acid, ($2^{14}C$) pentadecanoic acid or ($2^{14}C$)-nonadecanoic acid are examples of the use of chain extension to prepare a required fatty acid.

Because of the difficulties of labelling compounds with radio active potassium cyanide advantage was taken of using a commercially labelled acid. The chain extension was effected for convenience by the reaction sequence I, see page 43. This was preferred on grounds of convenience to either the Arndt-Eistert homologation or carbonation of the oleyl magnesium halide, the latter being not very suitable for such a small scale.

In the elongation of the commercially labelled alkanoic acids use was made of oleic acid as a carrier. The function of the carrier is to provide a mass of acid large enough to allow the reagents to be used in a visible amount without being in too large an excess and to minimize the losses during extractions and transfers since the mass of the radioactive alkanoic acids was very small indeed. It follows from this that the material chosen to act as a carrier must undergo the same reactions as the radioactive compound but at the end of the reaction scheme must be capable of isolation from the required radioactive product.

In the case of alkanoic acids alkenoic acids are ideally suited to act as carriers since they will undergo all the reactions of the carboxyl group but can be easily separated from the saturated compound by silver ion chromatography.

The following reactions were used not only during these chain extension reactions (Scheme I page 43) but also prior to the labelling with radioactive potassium cyanide (Scheme III page 58).

Although lithium aluminium hydride has been used successfully to reduce carboxylic acids [204] the reduction of esters proceeds more readily [205] and hence the alcohols were prepared from the ester. The ester was prepared by the action of an excess of an ethereal solution of diazomethane on the acid in methanolic solution. The acid was dissolved in methanol in order to ensure methanol was present in the reaction solvent since Gellerman and Schlenk [206] showed that without methanol complete reaction could not be guaranteed. Throughout this thesis wherever an acid is methylated with diazomethane it is done in methanolic solution for this reason.

Although Marcel and Holman [149] use mesylates as the immediate precursors of the homologation step in these syntheses the p-toluene sulphonate has been used. This is because of the difficulty of dealing with Marcel and Holman's procedure on such a small scale and because the extent of conversion of a fatty alcohol to its p-toluene sulphonate can be checked easily by T.L.C. on silica, the tosylate being much less polar than the alcohol in 20% ether in petrol ether for example. On the other hand mesylates behave very similarly to alcohols having similar R_f values. Hence it would be difficult to follow the conversion by T.L.C.

The homologation with potassium cyanide was performed in D.M.S.O. solution [152] for the same reasons as in the case of the labelling with radioactive potassium cyanide, namely that it afforded better yields and fewer reaction products.

Since in all cases following the preparation of the nitrile some purification was necessary the ester was prepared directly from the nitrile. This was done by treating the nitrile with a 25% w/v solution of hydrogen chloride gas in methanol [149].

After purification by either T.L.C. or G.L.C. or both, the acid was obtained by hydrolysis of the ester with 5% potassium hydroxide in methanol. After reaction the solution was acidified with concentrated hydrochloric acid to avoid making the reaction solution heterogenous.

Better yields of the acid were obtained this way. This was important since all carriers had been removed by this stage and very small masses of material were being handled. Following the addition of ether, with the fatty acid still in homogenous solution, the acid and salts were washed out with a minimum of water.

(iii) Preparation of compounds requiring a degradation

Compounds that require a reduction in chain length fall into two categories. The former are those which were kindly donated by F. D. Gunstone and I. A. Ismail which were in fact of the correct chain length but required degradation prior to labelling.

The latter category is represented by (1-^{14}C)-*cis* heptadecenoate which is in itself a degradation product of oleic acid but in order to be labelled had to undergo two degradations.

Despite the papers of Howton, Davis and Nevenzel[144,145] much difficulty was encountered with the Hunsdieker silver salt degradation[207] Although it had been successfully used in trial reactions and heptadecyl bromide had been prepared in 75% yield from stearic acid, with *threo* dibromo octadecanoic acids great difficulty was encountered in the obtaining of a pure dry sample of the silver salt. When some of the salt was prepared none of the desired reaction products could be isolated.

Difficulty in performing the Hunsdiecker reaction with unsaturated compounds has been confirmed by other workers. Corly[208] found difficulty in applying the reaction to unsaturated acids and only obtained reasonable yields when performing the reaction at low temperature. Furokawa et al[209] could only obtain yields of 3% of liberated carbon dioxide in their work with

unsaturated acids.

Attempts were made to substitute the lead salt for the silver salt by refluxing the threo dibromo alkanoic acid with lead tetraacetate in acetic acid. On removal of the acetic acid by vacuum distillation and refluxing with bromine in carbon tetrachloride a yield of 10% of the required tribromo compound was obtained.

The best results however were obtained using the Cristol and Firth modification of the Hunsdiecker technique [146,147]. This reaction is postulated to proceed according to the following equation:

$$2R\,COOH + HgO + 2\,Br_2 \longrightarrow 2R\,Br + Hg\,Br_2 + H_2O + 2CO_2$$

By observation of identical yields of exo and endo products from both the silver salt method and this method [146] a common intermediate RCOOX was postulated (X = halogen). Despite this evidence the mercuric salt when prepared separately did not give good yields [210] and in the case of glutaric acid the reaction did not give the usual product expected from a metal salt viz γ butyrolactane.

The more probable explanation of the reaction mechanism is that the mercuric oxide gives a positive halogen species [211] which reacts with the acid to give the acyl hypohalite.

This reaction has the advantage over the silver salt technique that it does not require the isolation of the metal salt and since water is produced as a by-product, strictly anhydrous conditions are not required.

The reaction had to be done in the dark to minimize polybromination but despite much care some polybromination was observed.

This amounted to about 20% of the total yield and it was isolated as a slightly more polar compound on T.L.C. and identified as tetra and penta bromo alkane by mass spectrometry (see page 62).

The above procedure was slightly modified in the case of the compounds being degraded prior to a labelling step. Because of the small

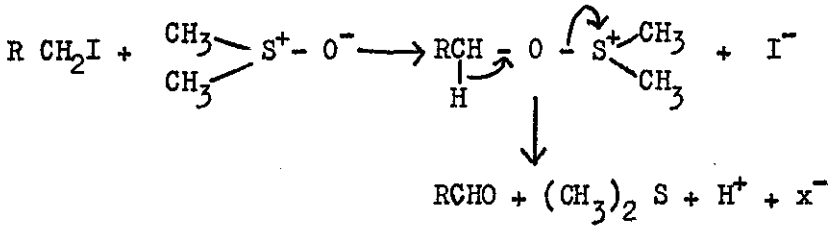
amount of solvent that had to be used stirring was very difficult and some agitation of the slurry was essential for reaction. If a wrist action shaker was used refluxing was difficult but after several test reactions the best yields were obtained using a wrist action shaker and prolonging the reaction time to 72 hours. In the case of these compounds the vicinal bromine atoms were removed using zinc dust in ethanol .

In the preparation of ($1^{14}C$)- cis 8 heptadecenoic acid from oleic acid a double degradation had to be used. Some difficulty was observed in proceeding from the alkyl halide to the carboxylic acid to begin the second degradation.

Following the removal of the vicinal bromine atoms by zinc in ethanol[212] attempts were made to convert the alkenyl bromide to the alcohol with caustic potash. This was not successful since aqueous alkali gave an heterogenous reaction mixture and methanolic alkali favoured elimination.

Two methods that were more successful both required the alkenyl iodide as starting material. This was conveniently prepared from the 1,7,8 tribromoheptadecane by refluxing with anhydrous sodium iodide in dry acetone. The vicinal bromine atoms were removed by a Finklestein reaction [128] whilst a substitution of iodine for bromine was effected at the 1 position.

The cis 8 heptadecenyl iodide thus prepared was first converted to cis 8 heptadecanoic acid by the reaction of Johnson and Pelter[213]. This uses DMSO as an oxidising agent. With sodium bicarbonate present as a proton acceptor the aldehyde is prepared by heating the iodide with DMSO at 150°C under dry nitrogen. The reaction is thought to go via the following mechanism:-



The acid was prepared by oxidation of the aldehyde with chromium trioxide in acetic acid[214].

Although this reaction worked well the preparation was finally achieved by converting the alkenyl iodide to the acetate with potassium acetate in acetic acid .

The acetate thus formed was hydrolysed to give the fatty alcohol which was oxidised to the alcohol with chromium trioxide in acetic acid[214].

The oxidation system was used in order to leave the double bond unaffected.

Because the double bond is in the centre of the chain with very similar groups to either side in all these compounds, the dipole change on stretching the double bond is very small and therefore the infra red peak at 1658cm^{-1} is very weak. N.M.R. was found to be much more useful in determining the structure of compounds during this sequence of reactions (Scheme II page 52). The mid chain double bond gave a characteristic triplet at 4.7τ[216] whilst the threo dibromo compounds gave a doublet at 5.8τ for the protons on the same carbon atoms as the bromine atoms. A terminal bromine atom gave a triplet at 6.7τ whilst the methoxy group of an ester gave a singlet at 6.3τ. The peaks identified from these spectra are listed below for completion.

Peak	Multiplicity	Assignment
variable 0	singlet	acid proton
4.7	triplet	cis - CH=CH -
5.8	doublet	- CHBr-CHBr -
6.3	singlet	ester OCH_3
6.7	triplet	methylene-CH_2Br
7.7	triplet	methylene group α to carbonyl
8.7	broad band	- methylene protons -
9.1	triplet	terminal methyl

Infra red spectrophotometry was used to demonstrate the presence or absence of carbonyl peaks for example before and after the decarboxylation reactions and for identification of the primary hydroxyl group in cis 8

heptadecenol (page 56).

Although the preparation of ($1^{14}C$)-cis 8 heptadecenoic acid is a long one the fact that the starting material (oleic acid) is readily available and that the mass of final product required is small Scheme III is a perfectly satisfactory route for the purpose.

Isolation of fatty acids from natural materials

Many of the fatty acids used both commercially and in the laboratory are not synthesised chemically but are extracted from natural materials, amongst the commonest being fish oils and seed oils.

In the case of labelled acids the particular living system has to be grown with a suitable radioactive precursor. This can be carbon dioxide (usually in the form of sodium bicarbonate in solution), acetate or in the case of the two precursors synthesised here, the saturated fatty acid of the same chain length.

The compounds prepared were ($1^{14}C$) cis-9-heptadecenoic acid and ($1^{14}C$) cis-9-nonadecenoic acid.

From work done which is dealt with in other parts of this thesis it was known that the yeast Torulopsis apicola would convert ($1^{14}C$) heptadecanoic acid into ($1^{14}C$) cis-9-heptadecenoic acid in about 70% yield. Apart from the residual ($1^{14}C$)-heptadecanoic acid there were no other radioactive acids produced by this system. It only required the separation of the product from all the other acids present to obtain a pure sample.

The amount of time consumed and the yield obtained compares very favourably with any of the de novo chemical synthetic routes. The yeast was grown with ($1^{14}C$)-heptadecanoic acid ex. Amersham, in its nutrient medium and incubated at room temperature for six hours. The lipids were then extracted with chloroform-methanol (2 : 1, v/v) and washed with physiological saline to remove the protein material. The lipids were transmethylated to yield the methyl esters of the fatty acids.

The required ($1^{14}C$) cis-9-heptadecenoic acid was isolated by preparative T.L.C. on 10% silver nitrate impregnated silica[129] to remove all but the monoenoic fatty acids. The monoenoic fatty acids were then fractionated into their respective chain lengths by preparative G.L.C. on F.F.A.P. at 230°C. This yielded the methyl ($1^{14}C$) cis-9-heptadecenoate chemically and radiochemically pure. Alkaline hydrolysis in the normal way yielded the required acid.

($1^{14}C$) cis-9-Nonadecenoic acid was also prepared biologically. The yeast used in the previous preparations did not give a good enough yield to be considered. Two systems which would give a moderately good conversion (about 30%) from nonadecanoic acid were the green alga Chlorella vulgaris or a microsomal preparation from hen liver.

Of the two the liver system gave a more specific reaction. The alga being a whole cell system, and a photosynthetic one at that, gave radioactive dienes as well as much breakdown and resynthesis.

The hen liver microsomal system, as the yeast in the previous preparation, gave only ($1^{14}C$) cis-9-nonadecenoic acid as a radioactive product which therefore, apart from the unconverted ($1^{14}C$) nonadecanoic acid, was the only radioactive compound present.

($1^{14}C$) Nonadecanoic acid was synthesised by the action of ^{14}C potassium cyanide on octadecanyl p-toluene sulphonate in DMSO as in Scheme I (page 45). This acid was dispersed in bovine serum albumin for better absorption into the system and incubated for six hours at 37°C with a microsomal preparation from a hen liver suspended in phosphate buffer with the required cofactors added. After the incubation the lipids were extracted, transmethylated and the pure methyl (1-^{14}C) cis-9-nonadecenoate isolated in a manner similar to the methyl (1-^{14}C) cis-9-heptadecenoate before (see page 39).

Bond positions were checked in both cases by oxidative cleavage according to the method of von Rudloff[217]. The radioactive dicarboxylic acids were identified by R.G.L.C.

Synthesis of fatty acids from smaller chain length materials

Only one fatty acid was synthesised from much smaller chain length material. This was ($1^{14}C$) 18-nonadecenoic acid.

Due to the fact that this was required labelled the initial target for synthesis was 17-octadecenoate. The method chosen was a Kolbé electrolytic synthesis.[178] Other possible methods were those used by F. D. Gunstone and I. A. Ishmail[163]. However in this case, bearing in mind that the starting material 10-undecenoic acid was readily available, that methyl hydrogen azelate is easily prepared and that only a small amount (50 mg) was required, the Kolbé route seemed the most convenient.

The Kolbé electrolysis is an anodic oxidation of a mixture of two acids. For the synthesis of the ester of a fatty acid one of the two initial acids must be the half ester of a dicarboxylic acid[218].

From the reaction of a monocarboxylic acid (R.COOH) and a dicarboxylic half ester ($HOOC\text{-}R^1\text{-}COOCH_3$), three products were obtained as shown below:-

$$R.COOH + HOOC.R^1.COOCH_3 \longrightarrow R\text{-}R^1\ COOCH_3 \quad \text{I}$$

$$+$$

$$R - R \quad \text{II}$$

$$+$$

$$CH_3OOC.R^1.R^1.COOCH_3 \quad \text{III}$$

The amount of product III can be minimised by the use of a four molar excess of the monobasic acid R.COOH [219].

The isolation of the pure product I can be easily achieved by silicic acid column chromatography using ether-petroleum ether mixtures.

Thus the Kolbé synthesis is a route to many and diverse fatty acids[187,188].

In the case in question, 10-undecenoic acid was used in excess of methyl hydrogen azelate in sufficient methanol to give a 10% w/v solution. 5% of the total acids were neutralised with sodium metal to produce some ions to carry the current. The electrodes used were 2.5 cm square platinum foils, 1 mm apart, between which was 120 volts D.C. In this particular preparation polymeric material (probably due to the terminal olefin) was also generated and periodically

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the electrodes had to be cleaned and the current reversed.

It is important during the Kolbé reaction that the temperature be kept below 50°C.[178] This was achieved by the use of cooling coils around the electrodes. Mixing of the solution is adequately carried out by the escaping carbon dioxide bubbles.

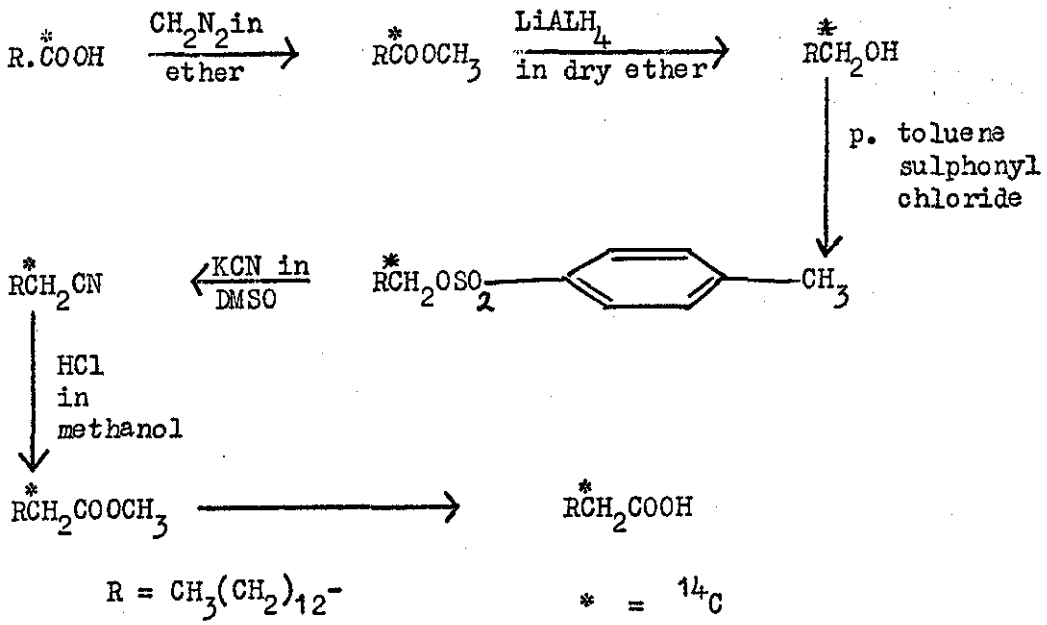
The methyl hydrogen azealate was prepared by the reaction of dimethyl azelate with one equivalent of potassium hydroxide in methanol. This gave a mixture of the diester, the half ester and the diacid. The required half ester was isolated by column chromatography on silica eluted with chloroform-methanol mixtures.

EXPERIMENTAL

I Preparation of substances by elongation of commercially labelled material

1. Synthesis of 2-^{14}C pentadecanoic acid

Reaction Scheme I



R = $CH_3(CH_2)_{12}$- * = ^{14}C

(i) Esterification of the acid with diazomethane [206]

Oleic acid (25 mgm) was mixed with(1-^{14}C)myristic acid (100μc; 15.4 mc/mM). The oleic was acting as "carrier" to provide a mass of acid large enough to allow the reagents to be used in a visible amount without being in too large excess and to minimise losses during extractions and transfers.

The acids were dissolved in methanol (0.2 ml.) and an ethereal solution of diazomethane added until the solution remained yellow. The solution was allowed to stand for 15 minutes and then the excess diazomethane and solvents were removed at the pump to yield the corresponding esters in quantitative yield.

The diazomethane was prepared by placing ether (30 mls) and 40% aqueous potassium hydroxide solution (30 mls) in a 100 ml round bottom flask. N nitroso methyl urea (4 g) was then added to the flask and the ethereal solution of diazomethane which was formed was distilled off into an ice cold receiving flask.

This method for the preparation of diazomethane was used throughout this thesis and wherever diazomethane was used it was prepared as above.

(ii) Reduction of the esters to the alcohols [205]

The methyl esters prepared in (i) above were dissolved in sodium dried ether (5 mls) and refluxed for two hours with lithium aluminium hydride (30 mgm). Water (5 mls) containing dilute hydrochloric acid (5 drops) was added cautiously to destroy the excess lithium aluminium hydride after the refluxing had been completed. The product was extracted into ether (10 mls) which was washed acid free with 5 ml aliquots of water and finally dried by azeotropic distillation with ethanol.

A little of the product was examined by T.L.C. on 0.25 mm silica plates run in 20% ether in petroleum ether. This showed no residual acid at the origin or ester by comparison with suitable standards. The whole product corresponded to the alcohol standard indicating a total conversion to the alcohol.

(iii) Alcohol to p toluene sulphonate [220]

The alcohols were dissolved in pyridine (dried over potassium hydroxide pellets) and to this solution was added p toluene sulphonyl chloride (30 mgm). The mixture was allowed to stand overnight.

The whole was then dissolved in ether (30 mls) and the pyridine washed out with dilute hydrochloric acid. The acid was destroyed and the p toluene sulphonic acid washed out with dilute potassium hydroxide solution. The whole was then washed to neutral pH with water and dried by azeotropic distillation with ethanol.

The product was checked at this stage by T.L.C. of an aliquot on 0.25 mm silica plates. This showed about 90% conversion to the p toluene sulphonate and 10% to the alkyl chloride. No alcohols remained unreacted.

(iv) p toluene sulphonate to nitrile. [189]

The mixture of the p toluene sulphonate and alkyl chloride from (iii) were dissolved in dimethyl sulphoxide (DMSO) (0.5 mls) dried by standing

over calcium hydride and redistilled under reduced pressure (BP 116°-118°C at 5 mm). Potassium cyanide (30 mgm) was dissolved in this solution and heated at 90°C for 3 hours.[150] After cooling, the reaction mixture was dissolved in ether (10 mls) and the DMSO and potassium salts washed out with successive 5 ml aliquots of water. The ether was then removed <u>in vacuo</u> and the nitriles dried by azeotropic distillation with ethanol.

(v) <u>Methanolysis of the nitrile</u>.[149]

The nitriles were converted directly into the methyl esters by the addition of 25% w/v hydrogen chloride in methanol (5 mls). The solution was allowed to stand at room temperature overnight. Ether (20 mls) was added to extract the esters and the acid washed away by successive aliquots of water. The ether was removed at the pump and the esters dried by azeotropic distillation with ethanol.

The radiochemical purity of the esters was tested by RGLC on a PEGA column at 198°C. All the label was shown to be in pentadecanoic acid by comparison with standard material. T.L.C. on silica gel showed quantitative conversion to ester.

(vi) <u>Isolation of the pure ($2^{14}C$)methyl pentadecanoate</u>.

($2^{14}C$)methyl pentadecanoate was separated from the carrier material, now methyl <u>cis</u> 10 nonadecenoate by preparative layer chromatography. This was achieved on 0.25 mm silica plates impregnated with 10% w/w silver nitrate. The solvent system employed was 10% ether in petrol ether. The plate was visualised by spraying with a methanolic solution of dichlorofluoroscein and viewed under U.V. light. The band corresponding to the saturated esters was scraped off and the pure ($2^{14}C$)methyl pentadecenoate eluted from the silica with ether.

(vii) <u>Hydrolysis of the ester to give ($1^{14}C$)pentadecanoic acid</u>.

The ester was dissolved in 5% potassium hydroxide in methanol and the mixture refluxed for 30 mins. Then the solution was cooled and acidified with concentrated hydrochloric acid (0.2 mls). The ($1^{14}C$)pentadecanoic acid

was extracted with 3 x 5 ml aliquots of ether. The ether extracts were bulked and washed acid free with water and dried by azeotropic distillation with ethanol. The acid thus prepared was dissolved in dry benzene at - 8°C. An aliquot of this solution was counted by scintillation in 4% P.P.O. in toluene using a Packard Tri-carb scintillation spectrometer. The overall yield was 30.3μc equivalent to 30.3%.

2. Synthesis of (2^{14}C) nonadecanoic acid from (1^{14}C) stearic acid

This synthesis was achieved by an identical procedure to the one used for (2^{14}C) pentadecanoic acid described above i.e. Reaction Scheme I (Page 43) R = $CH_3(CH_2)_{16}$-. The overall yield was 25.6μc equivalent to 25.6%.

3. Synthesis of (2^{14}C) cis 10 nonadecenoic acid from (1^{14}C) oleic acid.

This synthesis was also achieved by Reaction Scheme I (Page 43) R = $CH_3(CH_2)_7$ CH = $CH(CH_2)_7$-. except that the carrier employed was palmitic acid. The overall yield was 20.1μc equivalent to 20.1%.

II Preparation of Substrates using a biological system to modify commercially available labelled compounds.

(i) The preparation of (1^{14}C) cis 9 heptadecenoate from (1^{14}C) margaric acid.

(1^{14}C) margaric acid (50μc 14.8 mc/mM) was dispersed in water (5 mls) to which had been added 1 drop of 10% sodium bicarbonate solution and 1 drop of Tween 20. The final dispersion was achieved by sonication. This dispersion was added to 20 mls of a culture of Torulopsis apicola (equivalent to 1 gram of dry cells) in the nutrient medium in which it was grown (see Page 117).

This culture was then incubated at room temperature for 6 hours. During this time the flask was shaken to improve aeration. At the end of this period the lipids were extracted by the addition of 2:1 v/v chloroform methanol (100 mls). This was allowed to stand overnight to ensure complete extraction. 0.7% saline (25 mls) was then added to the mixture [260] and the organic layer removed. The aqueous layer was washed once with chloroform (25 mls) and the organic layers bulked. The solvent was removed at the pump and the lipid residue dried by azeotropic distillation with ethanol.

The dry extracted lipids were then transmethylated by refluxing with methanol-benzene-sulphuric acid (20:10:1) v/v/v. (20 mls) for 90 minutes. Ether (100 mls) was added after cooling and the solution washed acid free with water. The ether was then removed at the pump and dried by azeotropic distillation with ethanol to yield the pure methyl esters.

The esters were dissolved in dry ether and spread on to two 20 cm x 20 cm 0.25 mm silica plates impregnated with silver nitrate (10%). These plates were developed in 15% ether in petrol. ether and visualised by spraying with dichlorofluorescein and observed under U.V. light. The monoenoic band was isolated and the esters eluted from the silica with ether. The methyl ($1^{14}C$) cis 9 heptadecenoate was separated from the monoenoic homologues by preparative GLC on an apiezon G column at 198°C.

The ($1^{14}C$) methyl cis 9 heptadecenoate was tested for radiochemical purity by Radio GLC on PEGA at 198°C. It was found to be chemically and radiochemically pure.

½% of the ester thus obtained was oxidised with potassium permanganate potassium periodate according to von Rudloff's method.[217] After decolorisation with sulphur dioxide the half ester was extracted with ether and the ethereal layer washed acid free with water. The ether was removed at the pump and the product dried by azeotropic distillation with ethanol.

The half ester thus produced was dissolved in methanol (0.5 mls)[206] and an ethereal solution of diazomethane added until the solution remained yellow. After 5 mins the solvent and excess diazomethane were removed at the pump and the pure radioactive diester obtained. By comparison with a known standard an RGLC at 198°C (PEGA) the diester was shown to be dimethyl azelate.

The ($1^{14}C$) methyl cis 9 heptadecenoate was dissolved in 5% methanolic potassium hydroxide solution (5 mls) and refluxed for 1 hour. After cooling the solution was made acid with conc hydrochloric acid. The ($1^{14}C$) cis 9

heptadecenoic acid was extracted with ether, washed acid free and dried as before. An aliquot of the acid was counted in 4% P.P.O. toluene solution on a Packard Tri-carb scintillation spectrometer and the yield was found to be 35μc equivalent to a 70% yield.

The acid was dissolved in dry benzene and stored at -8°C.

(ii) Preparation of (1-^{14}C) cis 9 nonadecenoic acid from (1-^{14}C) nonadecanoic acid.

A Warren hen was sacrificed and its liver (39.6g) removed. The liver was homogenised with 0.3M sucrose buffer pH7.4 (120 mls) in a Potter homogeniser. All operations were done in an ice bucket to keep the tissue as cold as possible. The homogenate was centrifuged at 12,000 rpm for 15 mins. The supernatent was taken off and centrifuged at 30,000 rpm for 1 hour in an MSE40 refrigerated centrifuge. The microsomal pellet was separated from the particle free supernatent. The microsomes were stored at -30°C under nitrogen and the particle free supernatent at -8°C.

Microsomes (equivalent to 6g of the original liver) were suspended in particle free supernatent (12 mls) at 4°C by homogenising the system in a Potter hand homogeniser. To this suspension was added the following cofactors; coenzyme A (0.6 mgm), NADH (4 mgm), NADPH (2 mgm), 0.1M ATP (0.8 mls) 0.5M phosphate buffer pH7.4 (2 mls) and a substrate suspension. The substrate suspension was made up as follows : (1-^{14}C) nonadecanoic acid (prepared P 60) (10μc 17.3 mc/mM) suspended on 0.1g/ml Bovine serum albumin (2.5 mls) to which had been added 0.1M magnesium chloride solution (1.0 ml) and 0.5M phosphate buffer pH 7.4 (1 ml).

After the addition of the substrate the incubation medium was gassed with 70% oxygen in air for 1 minute and then incubated for 6 hours at 37°C in a reciprocating water bath. At the end of the incubation period the reaction was quenched by pouring into 2:1 v/v chloroform-methanol (50mls) and the mixture allowed to extract overnight. The precipitated material was filtered off and the lipids extracted and transmethylated as in the previous experiment (page 46).

The mixed methyl esters containing the ($1^{14}C$) methyl cis 9 nonadecenoate were dissolved in ether (5 mls) and spread on to two 20 cm x 20 cm silica plates impregnated with 10% silver nitrate. These were run in 15% ether in petrol ether and the monoene band isolated by visualisation with dichlorofluorescein and viewing under U.V.

The radioactive ($1^{14}C$) methyl cis 9 nonadecenoate was isolated by an identical procedure to that used for ($1^{14}C$) cis 9 heptadecenoate (page 47). Once again von Rudloff oxidation[217] yielded dimethyl azelate as the only radioactive product. Radio GLC on PEGA at 198°C showed the ester to be chemically and radiochemically pure. After hydrolysis with 5% potassium hydroxide in methanol as before (page 45) an aliquot was counted in 4% P.P.O. toluene solution. This showed that there were 3.12µc equivalent to a 31.2% yield.

The acid was dissolved in dry benzene and stored at -8°C.

II Preparation of substrates requiring chemical modification of commercially labelled material.

(i) Preparation of ($1^{14}C$) cis 12 octadecenoic acid by reduction of ($1^{14}C$) linoleic acid[130]

($1^{14}C$) linoleic acid (100µc 5.91 mc/mM) was dissolved in dry methanol (0.2 mls). Diazomethane in dry ether was added until the solution remained yellow. After allowing the solution to stand for 5 mins the excess diazomethane and solvents were removed at the pump to yield ($1^{14}C$) methyl linoleate in quantitative yield. This ester was dissolved in dry methanol (1 ml) and 60% w/w hydrazine hydrate (7.5µl) was added. The course of the reaction was followed by the injection of 1µl aliquots on to a RGLC containing a FFAP column at 230°C. After 2 hours the reaction was stopped by the addition of water (5 mls) and dilute hydrochloric acid (1 ml). The products were extracted with 3 x 5 ml aliquots of ether which were bulked and washed acid free with water. The ether was removed at the pump and the products dried by azeotropic distillation with ethanol.

The products were separated by preparative TLC on silica plates impregnated with 30% silver nitrate and run in toluene at -30°C [201] . The (1-^{14}C) methyl cis 12 octadecenoate was isolated from the plate by elution of the silica band with ether after it had been identified by TLC scanning using the Panax RTLS-1.

The (1-^{14}C) methyl cis 12 octadecenoate was dissolved in 5% potassium hydroxide in methanol (5 mls) and refluxed for 30 mins. After cooling and acidification with conc. hydrochloric acid the product was extracted with 3 x 5 mls of ether. The ether extracts were bulked and washed acid free with water. The ether was removed at the pump and the product dried by azeotropic distillation with ethanol. A portion of the acid was checked for band position by von Rudloff's oxidative cleavage [217] and contained only the 12 octadecenoic acid isomer. An aliquot was also counted by scintillation counting in 4% P.P.O. toluene solution and this indicated that 20µc had been prepared equivalent to a yield of 20%.

A small quantity of the ester prior to hydrolysis was examined by RGL containing a PEGA column at 198°C and was found to be chemically and radiochemically pure.

(ii) Preparation of (1-^{14}C) elaidic acid by isomerisation of oleic acid.

Oleic acid (50µc 40 mC/mM.) was mixed with stearic acid (50 mgm) and dissolved in methanol (200µl). An ethereal solution of diazomethane was added until the solution remained yellow. This solution was allowed to stand for 15 mins and the excess diazomethane and solvents were removed at the pump yielding the corresponding methyl esters in quantitative yield.

The methyl esters were then dissolved in dimethyl cellosolve. To this solution was added 6N nitric acid (75µl) and 2N sodium nitrite (100µl). The solution was then heated at 65°C for 1 hour. After cooling the products were extracted with 3 x 20 mls of petrol ether. The petrol extracts were bulked and washed acid free with 5 x 10 mls of water. The petrol was removed at the pump and the products dried by azeotropic distillation with ethanol.

A sample of the products was run on a 10% silver nitrate impregnated silica plate in 15% ether in petroleum ether. The plate was scanned on the Panax TLC scanner and the peaks corresponding to the two geometrical isomers were observed. On spraying with chlorsulphonic acid and acetic acid and charring, the carrier stearate was also observed having a greater Rf value (0.61) than the trans isomer which in turn had a greater Rf value (0.55) than the cis isomer Rf = 0.48.

Two preparative plates were run exactly as above and the two radioactive bands scraped off separately. The products were eluted from the silica with ether and the elaidate band was run again on a similar plate to ensure all the stearate had been removed. The radiochemical purity of the (1-^{14}C) methyl elaidate was tested on RGLC on FFAP at 230°C. It was found to contain no radiochemical impurities.

The (1-^{14}C) methyl elaidate was dissolved in 5% potassium hydroxide in methanol (5 mls) and the solution refluxed for 30 mins. After cooling the solution was made acid by the addition of concentrated hydrochloric acid and the product extracted with 3 x 5 ml aliquots of ether. The ether extracts were bulked and washed acid free with water. The ether was removed at the pump and dried by azeotroping with ethanol. The pure acid thus prepared was stored in benzene at -8°C.

An aliquot was dissolved in.4% PPO toluene and counted on the Packard Scintillation counter. This showed 12μc (= 24% Radiochemical yield) of (1-^{14}C) methyl elaidate to have been prepared.

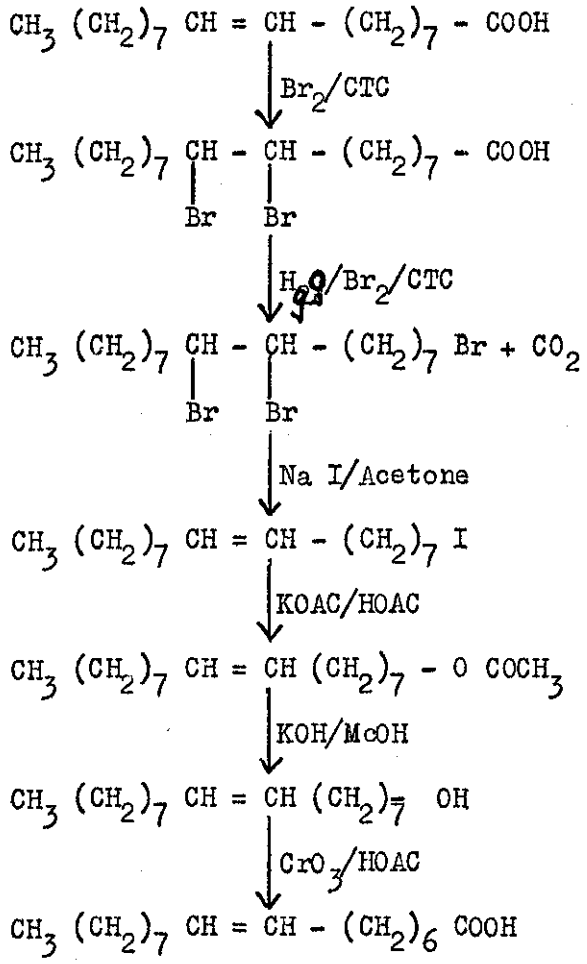
IV Preparation of substrates requiring introduction of the labelled atom

A. Preparation of the precursors to the labelling step.

1. cis 8 heptadecenoic acid

This synthesis was achieved using reaction scheme II.

SCHEME II



(a) Addition of bromine to oleic acid

Oleic acid (20g) was dissolved in carbon tetrachloride, dried over calcium chloride and redistilled BP 76 - 77°C, (200 mls). Bromine (11g) was similarly dissolved in carbon tetrachloride (200 mls). The oleic acid solution was stirred magnetically and cooled in an ice bath. The bromine solution was then added slowly over a period of two hours and the solution stirred overnight. Activated animal charcoal (5g) was then added and the stirring discontinued. After 3 hours the charcoal was filtered off leaving a colourless solution. The carbon tetrachloride was distilled off under vacuum to yield threo 9,10 dibromooctadecanoic acid (32.1g) equivalent to a yield of 99.6%.

The infra red spectrum of the product was determined as a thin film between sodium chloride plates on a Perkin Elmer 625 infra red spectrophotometer. Major peaks were as shown below:-

	Product	Starting Material
Broad OH (acid)	3000 - 3500 cm^{-1}	3000 - 3500 cm^{-1}
Carbonyl	1715 cm^{-1}	1715 cm^{-1}
$-CH_2-$	2950 cm^{-1}	2950 cm^{-1}

The weak peak at 1653 cm^{-1} due to the cis olefin was very difficult to observe but was visible in the case of the oleic acid but not in the product. The N.M.R spectrum was obtained from a Perkin Elmer 60 mcs instrument with TM.S as standard equal to 10%. The spectrum of the starting material was also determined.

Product

Peak (τ)	Multiplicity	Assignment	No. of Protons
- 3.6	Singlet	acid proton	1 (STD)
5.8	Doublet	- CHBr- CHBr-	2
7.5 - 9.1	Multiplet	CH_3- ; CH_2	31

Starting material

Peak (τ)	Multiplicity	Assignment	No. of Protons
- 3.6	Singlet	acid proton	1 (STD)
4.7	Triplet	- CH = CH -	2
7.5 - 9.1	Multiplet	CH_3 ; CH_2	31

(b) Decarboxylation of threo 9,10 dibromooctadecanoic acid

threo 9,10 Dibromooctadecanoic acid (30g) was dissolved in dry, redistilled carbon tetrachloride (100 mls). To this was added red mercuric oxide (15g).

This mixture was slurried by the use of a magnetic stirrer. The whole was then set under reflux in the absence of light and bromine (11.2g) in dry, redistilled, carbon tetrachloride (50 mls) was added dropwise over the period of 1 hour. Finally the refluxing was continued for 4 hours. The excess bromine was removed by the addition of sodium bisulphite and the yellow mercuric bromide and mercuric oxide were filtered off. The organic layer was isolated and washed several times with water and finally dried by azeotropic distillation with ethanol.

A little of this crude product was run on a 0.25 mm layer in 30% ether in petroleum ether with formic acid (1 ml) added. The product was much less polar (Rf 0.9) than the starting acid (Rf 0.15) and appeared in good yield.

The pure product, 1,8,9, tribromoheptadecane was isolated by column chromatography on Davison silica gel and eluted with 10% in petroleum ether. The product was a white, low melting solid (28.3g) equivalent to a yield of 87% based on the acid starting material.

Elemental analysis showed C = 42.12, H = 6.73, Br = 50.30. 1,8,9 tribromoheptadecane requires C =42.72, H = 6.91, Br = 50.37.

Infra red data on a Perkin Elmer 625 showed the absence of the broad bonded-oH at 2500 - 3500 cm^{-1} and the loss of the acid carbonyl peak at 1715 cm^{-1}.

NMR showed the loss of the acid proton at - 3.6 τ.

Peak (τ)	Multiplicity	Assignment	Integration
5.8	Doublet	$-\overset{Br}{CH}-\overset{Br}{CH}-$	2 (STD)
6.8	Triplet	$-CH_2Br$	2
8.0 - 9.2	Multiplet	CH_3, $-CH_2-$	29

(c) Finklestein Reaction of 1,8,9 tribromoheptadecane

1,8,9 tribromoheptadecane (25g) was refluxed in a 14% solution of dried sodium iodide in dry acetone (100 mls) for six hours. The solution was then filtered and added to a solution of sodium thiosulphate in water. The product was isolated by ether extraction and the bulked ether layers washed with water. Finally the solution was dried by standing over anhydrous sodium sulphate.

Infra red spectroscopy showed the presence of a weak band at 1658cm^{-1} due to cis unsaturation. N.M.R. in carbon tetrachloride was also performed

Peak (τ)	Multiplicity	Assignment	Integration
4.7	Triplet	- CH = CH -	2
6.8	Triplet	- CH_2 - Br	2 (STD)
8.0 - 9.2	Multiplet	CH_3; - CH_2 -	27

The product was a viscous, clear liquid (12.6g) equivalent to a yield of 67%.

(d) Preparation of 1, (cis 8) heptadecenyl acetate from the alkyl iodide

1 Iodo cis 8 heptadecene (12.5g) was dissolved in glacial acetic acid (10 mls) and refluxed for $2\frac{1}{2}$ hours with freshly fused, finely ground potassium acetate (5g). The reaction mixture was dissolved in diethyl ether (100 mls) and washed free of acid with water. The ether solution was dried over an hydrous sodium sulphate. The product was obtained pure by column chromatography on Davison silica gel. The eluting solvent being 10% ether in petroleum ether.

I.R. spectroscopy showed an ester peak at 1740 cm^{-1} and a weak peak at 1658 cm^{-1} indicating the retention of the cis double band. This was confirmed by the following N.M.R. data.

Peak (τ)	Multiplicity	Assignment	No. of Protons
4.7	Triplet	- CH = CH -	2
6.8	Triplet	- CH_2 - O	2
7.4 - 9.1	Multiplet	CH_3, CH_3, CH_2	32

The product was a colourless oil (5.0g) equivalent to a yield of 47%.

(e) Hydrolysis of the ester

1, cis 8 heptadecenyl acetate (5.0g) was dissolved in 5% methanolic potassium hydroxide for $1\frac{1}{2}$ hours. At the end of this period the solution was made acid by the addition of concentrated hydrochloric acid. The product was extracted into ether. The ethereal layer was washed acid free with water and finally dried with ethanol. This yielded 3.85g. equivalent to a yield of 91% of a colourless liquid.

The infra red spectrum of a thin film showed the complete absence of a carbonyl peak at 1740 cm^{-1} and the presence of an OH at 3600 cm^{-1}.

(f) Oxidation of the alcohol to the corresponding acid

cis 8 Heptadecenol (3.7g) was dissolved in glacial acetic acid (10 mls) and added to a solution of chromium trioxide (1.5g) in glacial acetic acid (45 mls) and water (5 mls). The solution was stirred magnetically and cooled in ice during the addition. The stirring was continued for 3 hours after the addition had been completed. After this period the solution was poured out on to ice. When the ice had melted the product was extracted with ether. The ether layer was washed acid free with water and dried with ethanol. This yielded a yellow viscous liquid (1.8g) equivalent to 46.1% yield.

A sample of the product (5 mgm) was esterified with diazomethane and the resulting ester run on a PEGA column at 198°C. The product gave a peak corresponding to methyl heptadecenoate by comparison with a standard carbon number plot.

2. Preparation of Methyl 17 octadecenoate by Kolbe coupling

(a) Preparation of methyl hydrogen azelate

Azelaic acid (10g) was dissolved in methanol (50 mls). Concentrated sulphuric acid (2.5 mls) was added dropwise and then the whole solution was refluxed for 90 mins. After this the methanol was reduced in volume to 15 mls and ether (100 mls) was added. This ethereal solution was washed acid free with water and dried by standing over anhydrous sodium sulphate. This yielded (11.25g) of dimethyl azelate (BP 146 - 147°C at 15 mm) equivalent to a yield of 97%.

Dimethyl azelate (10.0g) was dissolved in methanol (100 mls). To this was added a solution of potassium hydroxide (2.6g) in methanol (100 mls). The solution was allowed to stand overnight. The methanol volume was then reduced to 20 mls at the pump. Ether (100 mls) and water (100 mls) were added and the ethereal layer discarded after shaking. The water layer was then made acid and the products extracted into ether, washed acid free and

dried over sodium sulphate. The ether was distilled off to yield a mixture of azelaic acid and the half ester. These were slurried with chloroform and the half ester isolated by column chromatography on silica gel. The eluting solvent was pure chloroform. The acid remained on the column whilst the half ester was eluted in 500 mls of chloroform. On removal of the chloroform at the pump (2.8g) of a clear viscous liquid was obtained. This is equivalent to a yield of 30% based on the diester.

T.L.C. on silica plates eluted with ether, petrol ether formic acid (30 : 70 : 1) and visualised in iodine vapour showed this to be pure and free from any diacid or diester by comparison with standards and having an Rf value between the two.

NMR data showed a ratio of 3 : 1 between the methoxy protons at 6.4 τ and the acid singlet at 0.8 τ.

(b) Kolbé coupling of 10 undecenoic acid and methyl hydrogen azelate

Methyl hydrogen azelate (2.5g), 10 undecenoic acid (10g) and sodium (200 mgm) were dissolved in dry methanol (200 mls). Platinum electrodes 2 cm square were placed 1 mm apart in this solution. Electrolysis at 240v DC was carried out for 32 hours at which time the solution became just alkaline. The current which flowed was 0.4 amps. The solution was kept just below 40°C by the use of cooling coils in the solution. Periodically the current was reversed to help keep the electrode surfaces clean.

After electrolysis the polymer which had been formed was filtered off and the volume of methanol was reduced to 20 mls and ether (100 mls) was added to the solution. This solution was then washed with water to remove the sodium salts. The ether was removed at the pump and the products dissolved in petrol ether (25 mls).

The individual products were separated by silicic acid column chromatography by elution with petrol ether, rising to 5% ether in petrol ether. The yields of products were as follows:-

Polymeric material	2.0g
Hydrocarbon	9.5g
Diester	not detected
Methyl 17 octadecenoate	1.0g equivalent to 25% yield based on half ester.

The above data was obtained by triangulation of GLC traces obtained by injection of the petrol ether solution on to a PEGA column at 198°C.

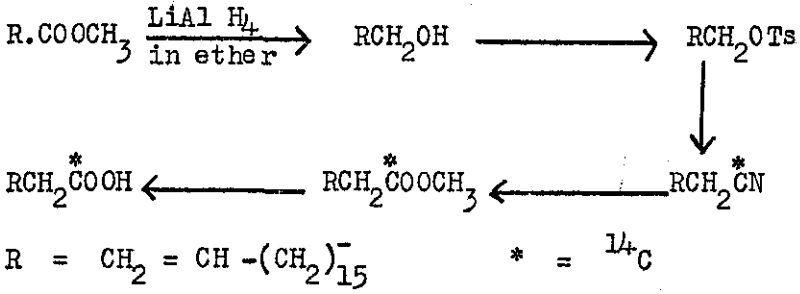
The methyl 17 octadecenoate had a retention volume equal to 18.75 carbon atoms compared with methyl oleates 18.45.

NMR data was as follows:-

Peak (τ)	Multiplicity	Assignment	No. of Protons
6.4	Singlet	$-O-CH_3$	3 (STD)
4.0 - 4.5	Multiplet	$=CH-$	1
5.1	Triplet	$CH_2=$	2
7.6 - 8.8	Multiplet	$-CH_2-$	30

B. Labelling of Precursors requiring elongation of the carbon chain with ^{14}C potassium cyanide

Reaction Scheme III



(i) Reduction of ester to alcohol

Methyl 17 octadecenoate (30 mgm) was dissolved in sodium dried ether (5 mls) and refluxed for 1 hour with lithium aluminium hydride (20 mgm). At the end of this period the excess lithium aluminium hydride was destroyed by the addition of water (5 mls) containing dilute hydrochloric acid (1 ml). The alcohol produced by the reaction was extracted into ether which was washed acid free with 5 ml aliquots of water and finally dried by azeotropic distillation with ethanol. This product was examined by TLC on silica plates run in

20% diethyl ether in petroleum ether. This showed complete conversion to alcohol by comparison with suitable standards.

The yield of cis 17 octadecenol was (26.4 mgm) equivalent to 98.5%.

(ii) Alcohol to p-toluene sulphonate

cis 17 Octadecenol (26 mgm) was dissolved in pyridine (0.5 ml) which had been dried over potassium hydroxide pellets. To this solution was added (26 mgm) of p-tcluene sulphonyl chloride. This mixture was allowed to stand overnight at room temperature. The whole was then dissolved in ether and the pyridine washed out with dilute hydrochloric acid. The solution was then made alkaline by the addition of 5N caustic potash and the excess p-toluene sulphonic acid washed out as the potassium salt with water. Finally the solution was washed to neutral pH with water and dried by azeotropic distillation with ethanol.

The reaction was once again tested by TLC on silica plates developed in 30% ether in petroleum ether. This showed that the reaction had yielded about 80% of the required product and given about 20% of the alkenyl chloride. No starting material remained unchanged.

(iii) Preparation of the alkenyl nitrile - introduction of the label

The reaction products from stage (iii) above were dissolved in D.M.S.O. containing ^{14}C potassium cyanide ex Amersham 100µc (200 µl). The solution was heated at 90°C overnight. After cooling the whole was dissolved in ether (20 mls) and washed five times with water to remove the DMSO and the potassium salts formed as by products. The ether was then removed at the pump and the nitrile and excess starting material dried by azeotropic distillation with ethanol.

(iv) Methanolysis of the nitrile

The products from stage (iii) were dissolved in a solution of 25% hydrogen chloride in methanol (5 mls) and allowed to stand overnight. Ether (20 mls) was then added to extract the products and the ether washed acid free

with successive aliquots of water. The reaction was tested by TLC on silica run in 15% ether in petroleum ether. As well as the usual charring with sulphuric acid the plate was scanned on the Panax radio scanner to identify the position of the label. It was found to correspond to the ester. The active ester was isolated by preparative TLC on a 1 mm silica plate in the same solvent system as above. The active band was identified by scanning and scraped off the plate. The active ester was eluted from the silica with ether.

The radiochemical purity of the ester was tested by RGLC on FFAP at 240°C. Although the major constituent was found to run at 19.75 carbon numbers there were several impurities present also.

The radiochemically pure (1-^{14}C) methyl 18 nonadecenoate was isolated by preparative GLC on FFAP at 230°C.

(v) Hydrolysis of the ester

The pure ester from (iv) was dissolved in 5% potassium hydroxide in methanol (5 mls) and the solution refluxed for 30 mins. After cooling the solution was made acid by the addition of concentrated hydrochloric acid.

The 18 nonadecenoic acid was extracted with 3 x 5 ml aliquots of ether which were bulked and washed acid free with water. The ether was evaporated at the pump and dried by azeotropic distillation with ethanol. The pure acid thus prepared was stored in benzene at -8°C.

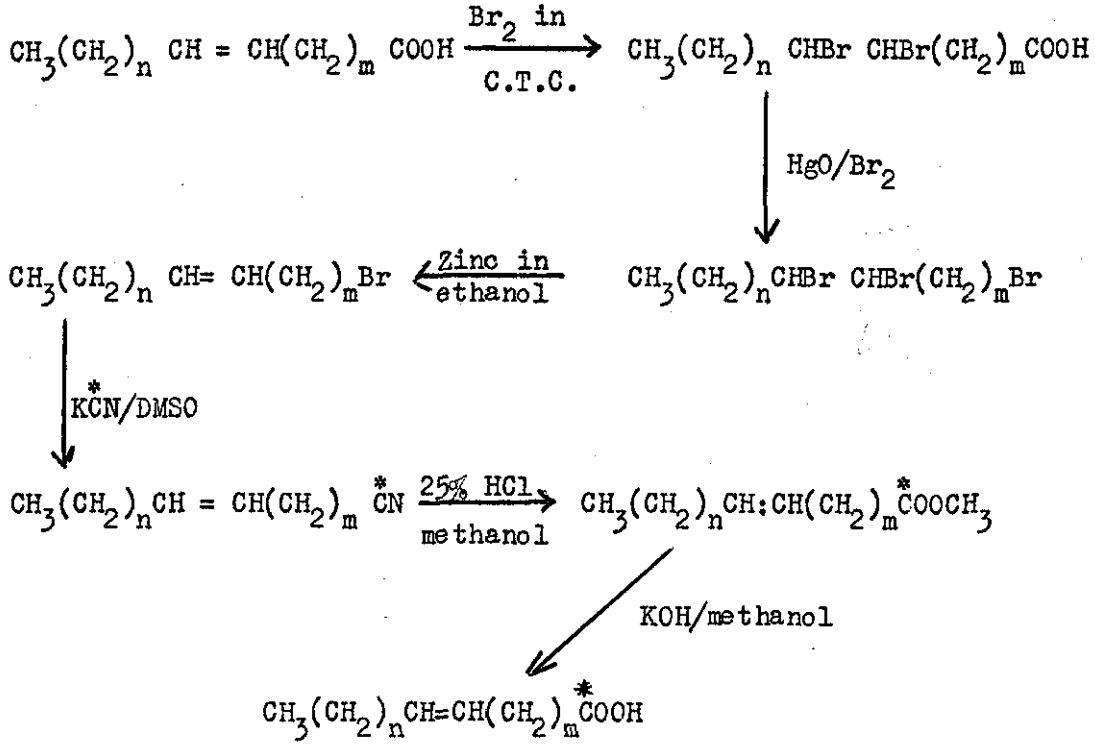
An aliquot of the above benzene solution was dissolved in 4% PPO toluene and counted on a Packard scintillation counter. This showed that 8 μc of the acid had been prepared.

(1-^{14}C) nonadecanoic acid was prepared using reaction scheme III (page 58) R = $CH_3(CH_2)_{16}$-.

Stearic acid was methylated with diazomethane in the usual manner to give the starting material. 10μc of (1-^{14}C) nonadecanoic acid was prepared in this manner and used to prepare (1-^{14}C) cis 9 nonadecenoic acid (see page 48).

C. Labelling of precursors requiring the labelled material to be of the same chain length

Reaction Scheme IV



Compounds prepared using this reaction scheme:-

	m	n	
($1^{14}C$) cis 8 heptadecenoic acid	6	7	- (1)
($1^{14}C$) cis 7 octadecenoic acid	5	9	- (2)
($1^{14}C$) cis 11 octadecenoic acid	9	5	- (3)

(1) was prepared (see page 52) and (2) and (3) were the kind gift of F. D. Gunstone and I. A. Ismail.

(i) Addition of bromine to the cis alkenoic acid

This reaction was carried out exactly as before (page 52).

(ii) Decarboxylation of the threodibromoalkanoic acids

The product from stage (i) above (30 mgm) was dissolved in dry redistilled carbon tetrachloride (0.5 mls). To this was added red mercuric oxide (15 mgm) and bromine (11.2 mgm). This mixture was shaken on a wrist action shaker for 72 hours in the dark at room temperature. The excess

bromine was removed by the addition of sodium metabisulphite and the yellow mercuric bromide filtered off. More carbon tetrachloride (20 mls) was added and the organic layer washed several times with water. The carbon tetrachloride was removed at the pump and the products dried by azeotropic distillation with ethanol.

A small amount of this product was run on TLC on silica plates developed in 2% ether in petrol ether. This showed in each case unreacted acid Rf = o and two reaction products. The major one approximately 60% Rf 0.72 and a minor one approximately 20% Rf = 0.65. Both these products were isolated by preparative TLC in the above solvent system. The bands were visualised by spraying with dichlorofluoroscein in methanol and viewing under U.V. light. The products were eluted from the silica with ether in the usual way.

A mass spectrum of each material was obtained on a MS12 mass spectrometer. This showed the major peak to be the desired tribromo product and the slightly more polar material to be polybrominated, containing four or five bromine atoms.

The major compound had a molecular ion at 476 which is correct for $C_{17}H_{33}Br_3$. Also the splitting pattern of the molecular ion peak showed that there were three bromine atoms in the molecule. This can easily be detected because bromine has two naturally occurring isotopes in similar abundance and therefore a tribrominated compound will split the molecular ion peak into 4 peaks whose heights are in the ratio of 1 : 3 : 3 : 1. Also loss of two bromine atoms could be seen in the spectrum at 397 and 317.

The compound having an Rf value equal to 0.65 had a molecular ions at 557 corresponding to $C_{17}H_{32}Br_4$ and a smaller one at 637 corresponding to $C_{17}H_{31}Br_5$. The splitting of the peaks in the lower region of the spectrum is also in agreement with this.

(iii) Debromination of the tribromoalkane[212]

The desired reaction product from (ii) above i.e. the tribromoalkane Rf 0.72 was dissolved in absolute alcohol (2 mls). This solution was added dropwise to a mixture of finely divided zinc (100 mgm) in refluxing absolute alcohol (2.5 mls) to which had been added 40% w/v hydrogen bromide in water (3 drops). The refluxing was continued for 1 hour. After this time the zinc was filtered off and the filtrate dissolved in ether (20 mls). The ether was washed with 5 x 5 mls of water. The ether was then removed at the pump and the products dried by azeotropic distillation with ethanol.

A sample of the product was run on TLC on silica plates in 2% ether in petroleum ether. This showed that the product had a larger Rf value (0.72) against the starting materials value of (0.63). This was strong evidence in favour of the reaction having proceeded to yield the alkenyl bromide. The overall yield of this reaction was 80%.

(iv) Labelling of the alkenyl bromide

This reaction was performed exactly as the labelling of the tosylates (page 59).

(v) Methanolysis of the nitrile

This reaction was performed exactly as before (page 59).

When the ester had been prepared an aliquot was tested by TLC on a silica plate run in 15% ether in petroleum ether. The plate was scanned on a Panax RTLS-1 thin layer scanner to identify the position of the labelled material then sprayed with dilute sulphuric acid and charred at 250°C to visualise the mass spots. Apart from label in the ester spot there was also label identified at the origin and in a more highly polar material Rf 0.05.

(vi) Isolation of the pure ester

A primary separation was made by running a preparative plate in the solvent used in (v) above. The radioactive band corresponding to

methyl ester was identified using the Panax RTLS-1 thin layer scanner. The silica band was scraped off and the radioactive compound eluted from it with ether. The radiochemical purity was checked by RGLC on a FFAP column at 230°C. This showed some impurities but these were removed by preparative GLC under the same conditions as above.

The bond position was also checked by von Rudloff oxidation [217].

(vii) Hydrolysis of the ester

This reaction was carried out as before (page 45). The pure acids were stored in benzene at -8°C. Aliquots were dissolved in 4% PPO in toluene as usual and counted by scintillation counting on a Packard TriCarb scintillation counter. This showed that the following radiochemical yields had been obtained.

Product	Radiochemical Yield
(1-^{14}C) *cis* 9 heptadecenoic acid	4%
(1-^{14}C) *cis* 7 octadecenoic acid	7%
(1-^{14}C) *cis* 11 octadecenoic acid	5%

SECTION II

Studies of the positional specificities of enzymic desaturation of saturated long chain fatty acids

Introduction

Apart from the ubiquitous oleic and palmitoleic acids a wide variety of naturally occurring monoenoic fatty acids have been characterised. These acids have not only a range of different chain lengths but also different positions for the unsaturated centre. Examples to illustrate this point are given with references in the tables below:-

Table I. Naturally occurring monoenoic fatty acids having the unsaturated centre nine carbon atoms from the methyl end of the molecule.

Acid	Trivial name	Source	Ref.
cis 3 dodecenoic	-	Lactobacilli	44
cis 5 tetradecenoic	-	Sperm Whale	222
cis 7 hexadecenoic	-	Euglena Gracilis	223
cis 9 octadecenoic	oleic	In most fats	
cis 11 eicosenoic	cetoleic	Simmodsia Californica	224
cis 13 docosenoic	erucic	Tropæolum seed	225
cis 15 tetracosenoic	nervonic	Brain tissue cerebosides	226

Table II Naturally occurring monoenoic fatty acids having the unsaturated centre in the 9-10 position

Acid	Trivial name	Occurrence	Ref.
cis 9 decenoic	-	Milk fats	227
cis 9 dodecenoic	-	Butter fat	228
cis 9 tetradecenoic	myristoleic	Pycnanthus Kombo - marine fats	229
cis 9 hexadecenoic	palmitoleic	constituents of nearly all natural	
cis 9 octadecenoic	oleic	fats	
cis 9 eicosenoic	gadoleic	marine oils	230

Table III Naturally occurring monoenoic fatty acids which do not fit in the above classes

Acid	Trivial name	Occurrence	Ref.
cis 3 decenoic	-	lactobacilli	44
cis 5 dodecenoic	-	lactobacilli	44
cis 7 tetradecenoic	-	lactobacilli	44
trans 3 hexadecenoic	-	algae and spinach	84,85
cis 5 hexadecenoic	-	B.megaterium	54
cis 10 hexadecenoic	-	M. phlei	43
cis 5 octadecenoic	-	B.megaterium	54
cis 6 octadecenoic	petroselinic	Parsley seed	231
cis 11 octadecenoic	cis vaccenic	brain tissue	232
trans 11 octadecenoic	trans vaccenic	animal and milk fats	19
trans 10 octadecenoic	-	animal and milk fats	19

The series of acids in Table I have their double bonds in the 9-10 position if the methyl group is assigned as the first carbon atom. These acids will be referred to hereafter as W9 acids. The acids in Table II are the carboxyl 9 series of acids since their double bond is 9-10 using the carboxyl carbon as carbon 1. The acids of Table III do not fall into either of these categories.

The concern of this section is the positional specificity of double bond formation by direct aerobic desaturation. Some of the above acids listed in Table III occur in systems where the anaerobic pathway is operative e.g. those isolated from lactobacilli and streptococci, and hence the factors governing the bond position are fully understood[43]. All the others occur under aerobic conditions. This does not mean however that they all occur by a direct aerobic desaturation of the saturated precursor of the same chain length. Many of the acids are formed by a direct aerobic desaturation

of a shorter chain length material followed by a subsequent elongation by units of two carbon atoms in the malonyl CoA pathway [1]. An example of this is the pathway postulated by Nagai and Block for the synthesis of unsaturated acids in photoauxotrophic Euglena [234]. This explains the occurrence of cis 5 tetradecenoic and cis 7 hexadecenoic acids Fig.1, without direct desaturation of myristic and palmitic acids respectively.

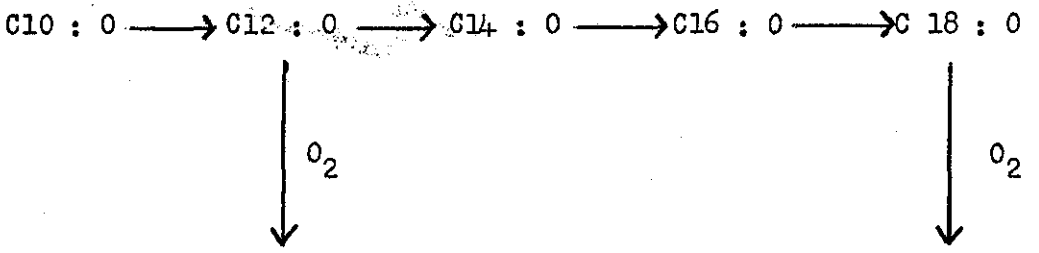


Fig.1 Pathway proposed for the synthesis of unsaturated acids in photoauxotropic Euglena

Jacob and Grimmer [235] demonstrated the presence of a large number of monoenoic acids of varied chain length and double bond position in human depot fat but suggest that many of them arise by either β-oxidation or chain elongation of monoenoic acids of various chain length having their double bond in the usual 9:10 position. Another possibility to account for some of the isomers is that they are assimilated in the diet.

However despite the points made above many acids are formed by direct desaturation of the compound having the same chain length. Bloch [43] showed that whole cells of Mycobacterium phlei would convert palmitic acid directly into cis 10 hexadecenoic acid. The same author showed that Bacillus megaterium [54] converted stearic acid to cis 5 octadecenoic acid and palmitic acid into cis 5 hexadecenoic acid. The conversion of stearic acid to petroselinic acid by parsley has not been finally proved to be a direct desaturation though there is evidence which suggests this [236].

As well as the above examples of double bonds being placed in positions other than 9:10 in the carbon chain there are a great many examples of direct desaturation of fatty acids of several different chain lengths to their

corresponding 9:10 monoenes occurring throughout the whole spectrum of living systems e.g.[47, 49, 56].

In all systems however, where aerobic desaturation occurs, the position of the double bond whether it is in the 9:10 position or not is specific for that system. There is no variation, under identical conditions, in the position of the double bond.

This raises the question of what feature of the substrate molecule determines the position of desaturation.

It is an investigation into that positional specificity with which this section is concerned. Animal, plant and yeast systems have been investigated to determine whether or not direct aerobic desaturation of labelled compounds could be demonstrated. Where this has been established the bond position of the monoene has been determined and attempts have been made to elucidate the factors which cause the bond to be in that position. This has been done by using a homologues series of fatty acids as substrates.

Other factors, for example incorporation into lipid and the rate of desaturation over a given time for all the homologues, were studied. The latter gave an indication of the number of enzymes involved in desaturation and their optimum chain length.

The other work which is described in this section is the effect of chain length of substrate on the inhibition of desaturation by sterculic acid 8-(2-octyl-1-cyclopropenyl) octanoic acid. Sterculic acid is a potent inhibitor of the desaturation of stearate to oleate in both animal[237] and plant systems[63]. The action of the inhibition is believed to be due to the cyclopropene ring since alcohols, methyl esters and hydrocarbons containing this linkage all cause inhibition[63]. The mechanism of inhibition of the stearate to oleate desaturation is thought to be due to the irreversible binding by the cyclopropene ring of an essential thiol group of the total enzyme activation complex. Evidence to support this comes from the fact that sterculic acid when treated with L-cysteine showed evidence of consuming the sulphydryl

groups. Also similar inhibitions to the ones caused by sterculic acid are brought about by iodoacetamide [238] a known sulphydryl inhibitor.

Dietary sterculic acid causes increased amounts of stearic acid and reduced amounts of oleic acid in the lipids of chicken tissue [238-241]. Rieser and Raju [242] showed that an *in vivo* effect of dietary sterculic acid could only be demonstrated if labelled acetate was replaced as precursor by stearic acid. Johnson [237] extended this work to show the inhibition of desaturation of stearic acid to oleic acid in liver preparations. The same author went on to study the effect of chain length from C10 - C20 on the inhibition [244].

Since sterculic acid is a plant product the effect of sterculic acid on desaturations in plants is an interesting problem. It was initially investigated by James Harris and Bezard [63] who also showed that syntheses of unsaturated acids from acetate were unaffected by sterculic acid. They also studied the effect of sterculic acid on decanoic, lauric, myristic, palmitic, stearic and oleic acids and discovered that inhibition increased with chain length and was complete at the stearic acid level. Also they discovered that the effect of the sterculic acid on the enzyme converting oleate to linoleate was not as sensitive to sterculic acid as the stearate desaturase. The whole of this picture was rounded off in this section when all the acids from C14 $\longrightarrow$ C19 were incubated with *Chlorella vulgaris* together with sterculic acid and the total effect of chain length on the sterculate inhibition was studied.

RESULTS AND DISCUSSION

The effect of the structure of the substrate molecule in the desaturation of a saturated fatty acid to produce a monoenoic fatty acid revolves around the chain length of the acid since substituents along the chain form an unnatural complication to the enzyme system. James[49] amongst several workers have used hydroxy and epoxy fatty acids in animal and plant systems and have been unable to demonstrate any desaturation at all. If individual members of a homologous series of fatty acids are incubated with a living system, the position of the resulting bond will tell whether the desaturase system is specific for one chain length, for a given number of carbon atoms from the methyl end, or for a given number of carbon atoms from the carboxyl end of the molecule. The fact that a specificity is involved is assumed from the well recorded observations that a given precursor will, under the same conditions in the same system, always yield the same monoene.

In talking about number of carbon atoms from ends of the substrate molecule, a physical distance along the enzyme surface from a point of reference is implied and this work would hope to show where the point of reference for the substrate molecule on the enzyme surface occurs.

This point of reference for the substrate molecule could be a covalent attachment of the fatty acyl residue by its carboxyl end either directly to the enzyme or indirectly via its ACP or CoA thioesters. Any point of reference involving the distance from the methyl end could only arise by a physical "fit" of the substrate into a cleft in the enzyme surface for example. Clearly it could not involve covalent bonding. If the substrate had to be of a given chain length in order to be desaturated, points of reference for both ends of the molecule would be necessary.

From these studies also data about the actual shape of the enzyme surface in the active region and some evidence of the actual interaction between substrate molecule and enzyme may also be gained.

The close involvement of lipid class in desaturation, as previously described, prompted the investigation in some of the systems of the lipid formation accompanying the desaturation and the amount of freshly desaturated acid found in the classes of polar lipids studied.

The first system studied was the photosynthetic green alga Chlorella vulgaris. The culture was grown in the "rich" medium (Page 137) and then transferred to phosphate buffer in which the incubations were carried out. The reason for this is that in the rich glucose medium the alga exists heterotrophically and under these conditions will not desaturate palmitate or stearate[60,62]. The longest chain acid that it will convert to oleate is myristic acid. When the cells are transferred to phosphate buffer, they undergo a change in fatty acid composition. This is due to a change from the heterotrophic to a photosynthetic mode of existence. This is shown by the fact that more linoleic and linolenic acid is formed. These acids are typical of photosynthetic systems[245-247].

The incorporation of the added saturated precursors is shown in table I. Desaturation, elongation, breakdown and resynthesis are all taking place in this system. Elongation was only noted in the case of even chain length fatty acids and decreased with increased chain length. See fig.I. No direct desaturation was observed in the case of the laurate and the majority of the added precursor was elongated mainly to oleate which showed that activation was not discriminated against by this chain length. It is quite likely that one of the reasons for the lack of desaturation in this case is the fact that elongation or incorporation into lipid is so favoured that direct desaturation has no time to occur rather than being itself prohibited. The increase of direct desaturation of substrate as the extent to which it is elongated decreases, (C12→C18), may in fact be merely a reflection of the relative rates of the two competing reactions. This assumes a dependence of reaction rates on the chain length of the precursor. With this kinetic approach however, it is not easy to explain the complete non-existence of elongation in the case of pentadecanoic acid except that this is not a known intermediate in the malonyl CoA pathway[1] and it may be that as such it cannot be incorporated into it although the yeast Torulopsis apicola did

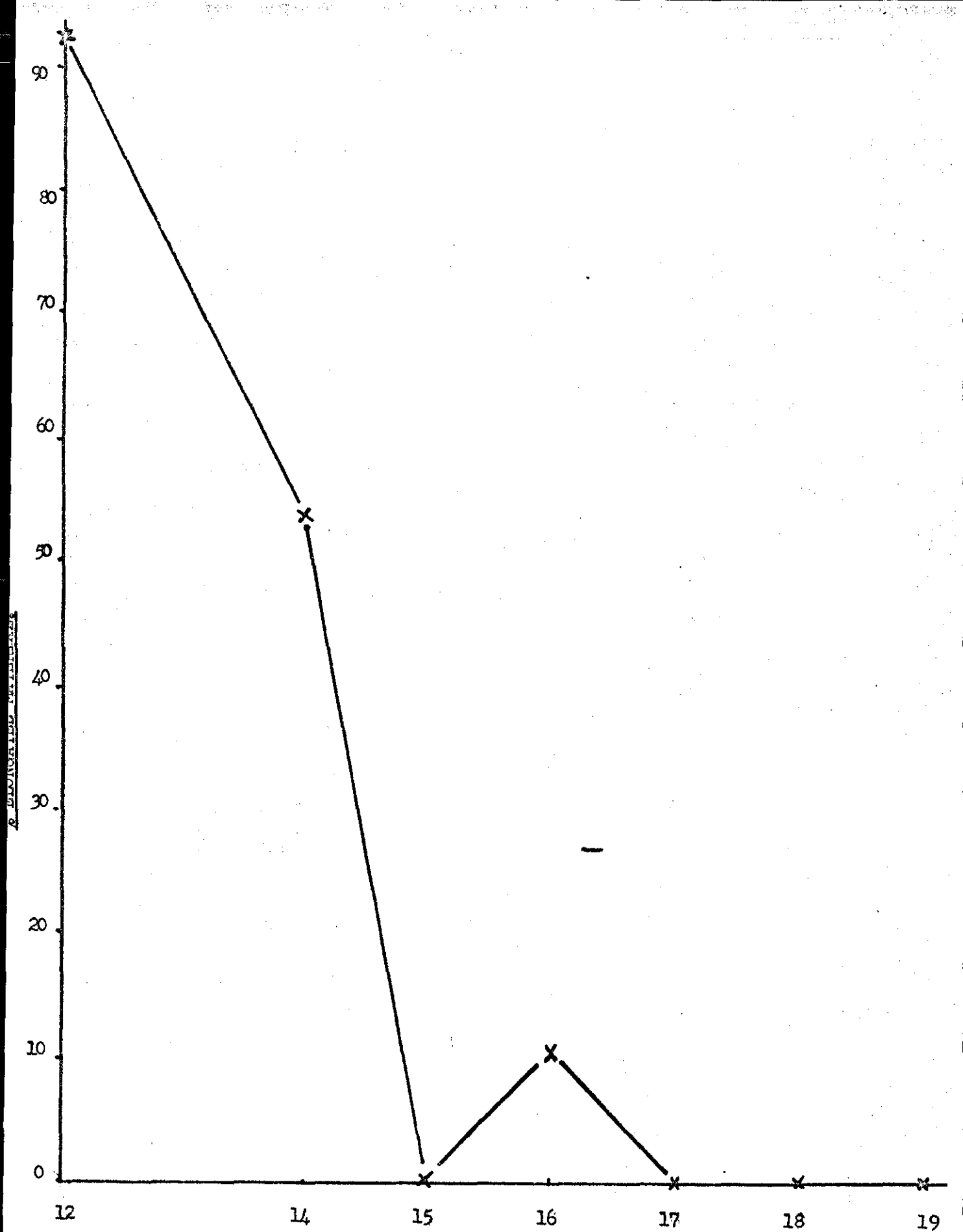


Fig.i. Conversion of saturated fatty acid precursors to longer chain length fatty acids by whole cells of Chlorella vulgaris.

extend pentadecanoic acid to margaric acid (see page 79).

Radioactive Product	Labelled Precursors						
	12:0	14:0	15:0	16:0	17:0	18:0	19:0
12:0	8.5	-	-	-	-	-	-
12:1	-	-	-	-	-	-	-
14:0	Trace	32.5	-	-	-	-	-
14:1	-	15.8	-	-	-	-	-
15:0	-	-	60.5	-	-	-	-
15:1	-	-	33.1	-	-	-	-
15:2	-	-	8.4	-	-	-	-
16:0	8.0	21.1	-	64.2	-	-	6.1
16:1	2.1	5.6	-	17.8	-	-	Trace
16:2	-	2.0	-	6.8	-	-	-
17.0	-	-	-	-	32.3	-	-
17:1	-	-	-	-	48.2	-	-
17:2	-	-	-	-	19.5	-	-
18:0	Trace	Trace	-	Trace	-	9.5	1.0
18:1	25.2	9.0	-	5.0	-	27.9	8.0
18:2	56.2	15.8	-	5.8	-	62.6	2.1
19:0	-	-	-	-	-	-	48.4
19:1	-	-	-	-	-	-	29.1
19:2	-	-	-	-	-	-	6.3

Table I

Labelled fatty acids produced from labelled saturated precursors by whole cells of *Chlorella vulgaris*

However, having said all this every other saturated precursor except the laurate was not only activated by the system but did also yield monoenes of

the same chain length. The fact that these occurred by direct aerobic desaturation alone was indicated by the retention of the radio label in its original position in the desaturated products. All the precursors except laurate and myristate produced dienes of the same chain length as well as monoenes. Since the dienes were produced from monoenes by a further desaturation the figures for saturated to monoene conversion which occur in table II are the sums of the conversion to monoenes plus dienes.

Precursor	Total Desaturation (Monoene + Diene)	
	Δ7 Series	Δ9 Series
(1^{14}C)- dodecanoic	-	-
(1^{14}C)- tetradecanoic	10.6	5.2
(2^{14}C)- pentadecanoic	12.8	28.7
(1^{14}C)- hexadecanoic	8.3	15.5
(1^{14}C)- heptadecanoic	-	67.7
(1^{14}C)- octadecanoic	-	90.6
(2^{14}C)- nonadecanoic	-	35.4

Table II

Total amount of desaturation in individual monoenoic series produced from labelled saturated precursors by whole cells of Chlorella vulgaris.

When the bond positions of the monoenes were determined, all the precursors were found to have given a 9:10 monoene. However myristic, pentadecanoic and palmitic had also produced a 7:8 monoene. The relative amounts of each appear in table II and are plotted in fig.II. Clearly there are at least two enzymes active in this system. The first of these is chain length dependent and introduces the double bond in the 7:8 position. This has an optimum chain length of fifteen carbon atoms but will not accept acids longer than sixteen carbon atoms. The second enzyme system accepts any acid

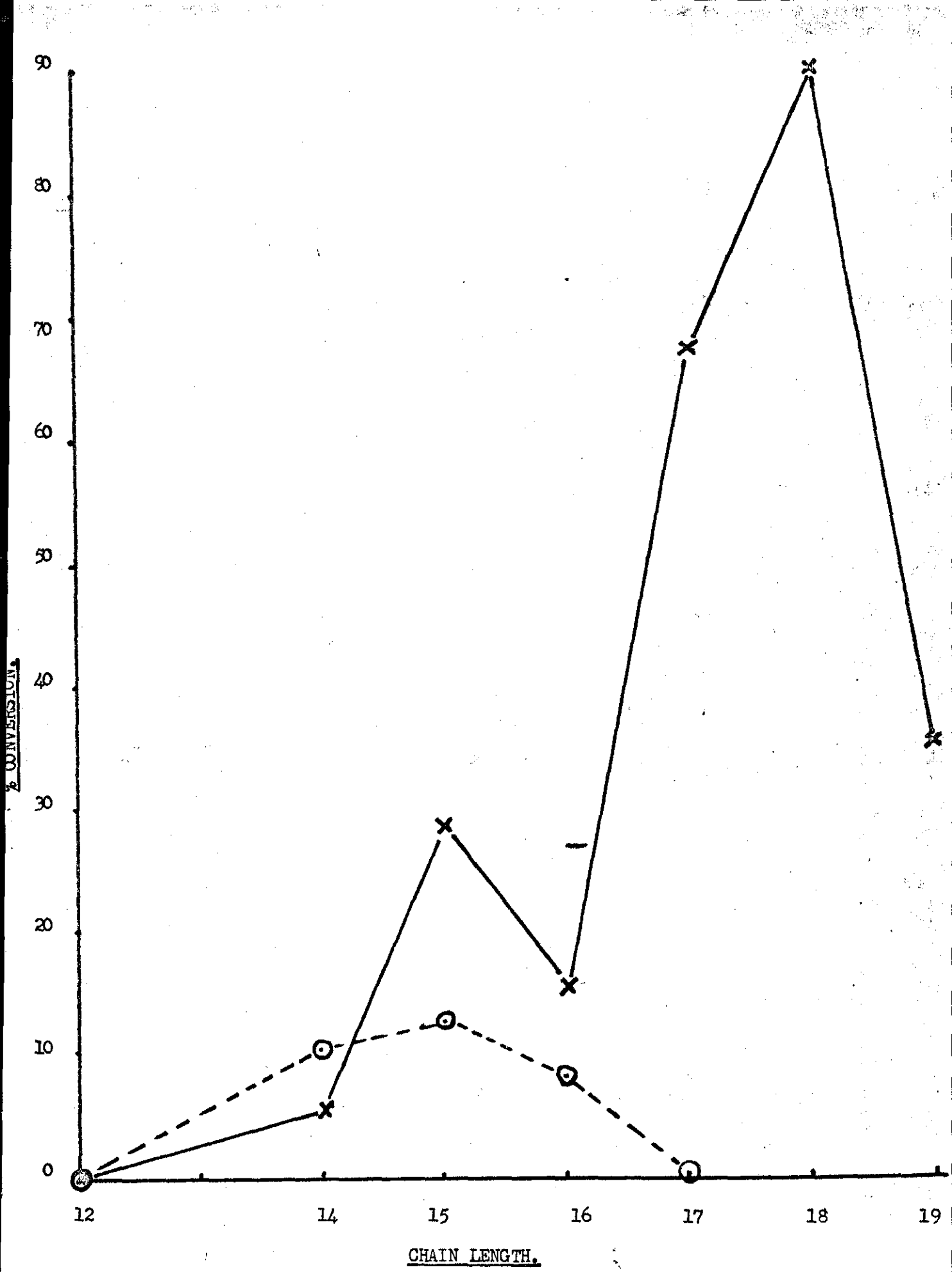


Fig.ii. Conversions of saturated fatty acid precursers to 9:10 monoenoic fatty acids (x—x) and 7:8 monoenoic fatty acids (0 — 0) of the same chain length by whole cells of Chlorella vulgaris.

longer than thirteen carbon atoms and introduces a double bond in the 9:10 position. The fact that the radio label is retained in its original position in all these studies excludes the possibility that any of these products arise from breakdown and resynthesis and verifies that direct desaturation has occurred.

Looking quantitatively at the desaturation caused by this second enzyme (see fig.II) it has two peaks in the curve which indicates that this 9:10 desaturase could, in fact, be two enzymes, one which deals with shorter chain length acids (C14 - C16) which has its optimum at pentadecenoic acid and another which deals with longer chain acids which has an optimum at C18. It is not unknown for more than one enzyme to be used to perform the same reaction from a series of acids depending on their chain length. Wakil[248] showed a similar thing when what was thought originally to be a single enzyme for conversion of the β-hydroxy acid to the αβ monoenoic acid in the malonyl CoA pathway (see page 4), turned out to be three, each responsible for a small range of chain lengths.

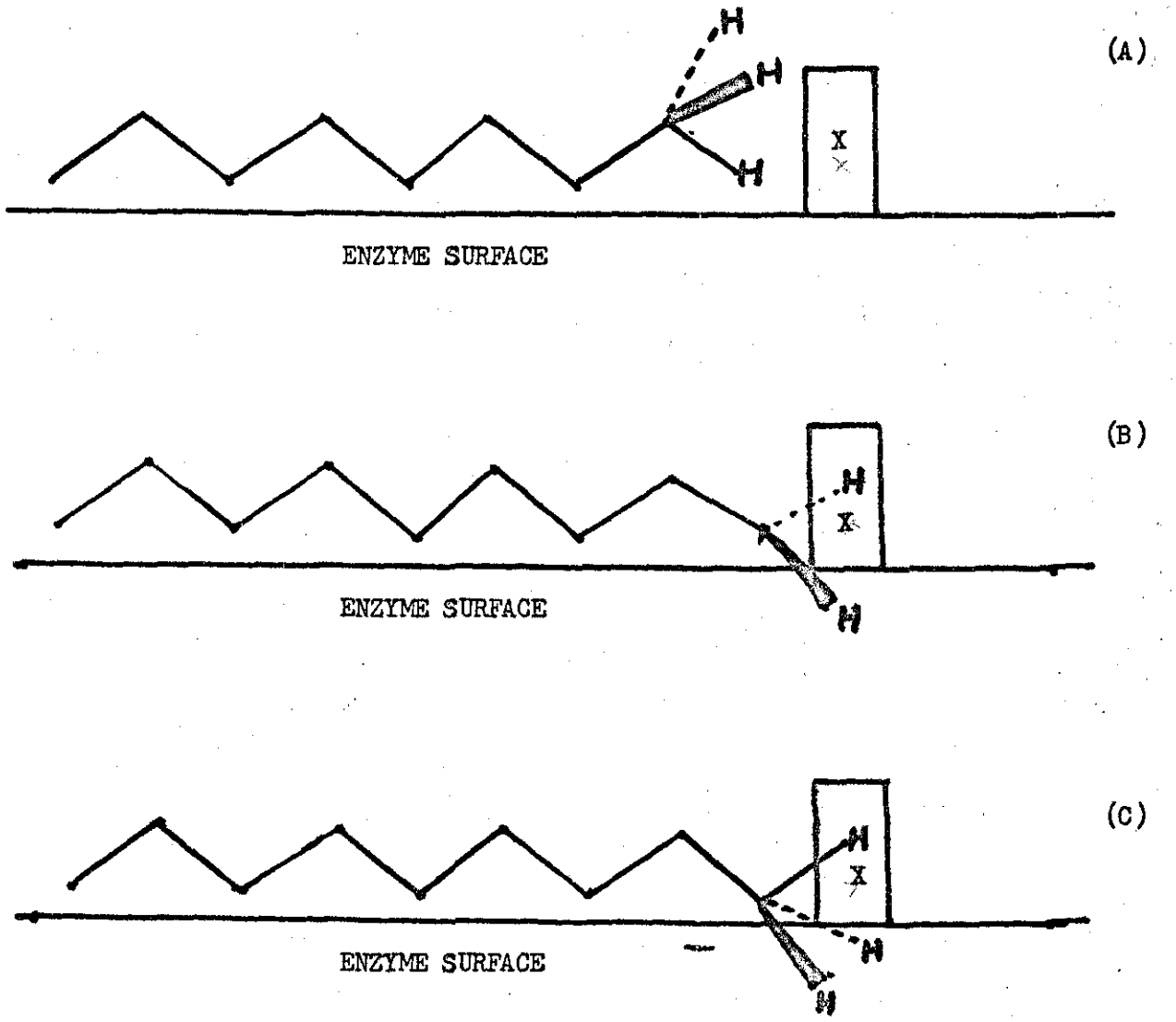
The reason behind the sudden cut-off of the 7:8 desaturase at margaric acid could be due to a side group from the peptide chain of the enzyme projecting out from the enzyme surface at the position the seventeenth carbon atom would occupy, causing a steric block. This would prevent the substrate lying down on the enzyme surface in general and the active site in particular. Alternatively, it could indicate the depth of a cleft in the enzyme tertiary structure if association with the substrate involves enfolding. The implications of these results to the environment of the substrate in the enzyme substrate complex will be dealt with in more detail later.

All the desaturases in Chlorella vulgaris produce their characteristic desaturation at the same position (whether it be 7:8 or 9:10) irrespective of the chain length, providing that this is acceptable at all to it. Thus, providing it does not completely inhibit desaturation, as in the case of the 7:8 desaturase, the number of methylene groups beyond the desaturation centre

does not affect the position of the double bond. In other words it is the distance from the carboxyl group which is specific to the enzyme so far as the position of desaturation is concerned. A physical attachment of the carboxyl end of the substrate molecule to the desaturase enzyme complex is indicated by this, although the precise form of the attachment, whether it is the actual acid that is joined to the enzyme, or either its coenzyme A or ACP thiolester, is not yet known.

The 7:8 desaturase and the shorter chain length requiring 9:10 desaturase are very similar in that they both have the same optimum chain length (pentadecanoic acid) and are unable to deal with lauric acid. This lends strength to the theory that they were originally the same enzyme but that the 7:8 desaturase has arisen by the loss of one amino acid between the active site and the point of attachment of the acyl function.

The loss of one amino acid from the polypeptide chain, as suggested, would reduce the distance by an amount corresponding to two methylene units in the acyl chain. If the difference between the 7:8 desaturase and the 9:10 desaturase is one amino acid residue and since the cut-off point of the 7:8 desaturase is at a substrate chain length C17 then, if the hypothesis is correct, it would mean that the cut-off point of the 9:10 desaturase should be at a substrate chain length of C19. This would place the side group in the enzyme chain, or the limit of the cleft or hole responsible for the cut-off, at a position in the protein molecule corresponding to the C19 position in the chain of an acyl substrate. Although the picture is masked by the presence of the long chain 9:10 desaturase, there is a marked fall off at a substrate chain length of C19. In addition, incubations with ($1^{14}C$) 18- nonadecenoic acid is found to give much more desaturation than nonadecanoic acid, see Table III and the existence of such a side group on the enzyme surface could explain this, see fig.IIIa.



(A) octadecanoic acid

(B) 18-nonadecenoic acid

(C) nonadecanoic acid

X proposed steric block

Fig.iiia. Schematic representation of the substrate molecule on the enzyme surface to illustrate how a steric block in the position indicated could explain the experimental results

Precursor	Total Desaturation monoene + diene	Bond Positions of diene
($1^{14}C$) - heptadecanoic	49.1	9:10, 12:13
($1^{14}C$) - octadecanoic	80.0	9:10, 12:13
($2^{14}C$) - nonadecanoic	24.1	9:10, 12:13
($1^{14}C$)-18 nonadecenoic	72.7	18:19, 9:10, 12:13

Table III

Direct dehydrogenation of labelled precursors by whole cells of Chlorella vulgaris

The presence of a side chain not large enough to completely inhibit the long chain desaturase but enough to reduce its effectiveness must also be present in a similar position to account for the fall off of desaturation at C19 in all the systems studied and also to explain the magnitude of the increase in desaturation of the 18-nonadecenoic acid.

Sterculic acid inhibition has been studied in Chlorella vulgaris by Harris James and Bezard[63]. They incubated acetate and all the even chain fatty acids from C10 C18 with Chlorella in the presence of sterculic acid and studied the distribution of the label amongst the fatty acids.

In this present work the acids from C14 → C19 were similarly studied and the results appear in Table IV. This work confirms the results of Harris et al[63] and shows that the inhibition does steadily increase with increasing chain length. The extent to which a given even chain monoene has been produced by direct desaturation or by β-oxidation of the substrate and resynthesis cannot be stated as distribution of label in these products was not determined but in the production of odd chain monoenes direct desaturation must have taken place. Thus direct desaturation of margaric acid is not completely inhibited by sterculic acid though direct desaturation of stearic and nonadecanoic acids is completely inhibited. Nonadecanoic gives much more label in palmitate, oleate and linoleate formed by β-oxidation and resynthesis

than does margaric acid. This increased tendency to breakdown has been noted in other experiments with Chlorella vulgaris and Torulopsis apicola.

Precursor	14:0		15:0		16:0		17:0		18:0		19:0	
Sterculic	-	+	-	+	-	+	-	+	-	+	-	+
Label												
14:0	32.5	60	-	-	-	Trace	-	-	-	-	-	Trace
14:1	15.8	2.0	-	-	-	-	-	-	-	-	-	-
15:0	-	-	60.5	79.2	-	-	-	-	-	-	-	-
15:1	-	-	33.1	3.6	-	-	-	-	-	-	-	-
15.2	-	-	8.4	-	-	-	-	-	-	-	-	-
16:0	8.0	14.0	-	6.0	64.2	84.0	-	0.5	-	-	6.1	10.8
16:1	2.1	Trace	-	-	17.8	8.3	-	-	-	-	Trace	1.2
16:2	-	-	-	-	6.8	Trace	-	-	-	-	-	-
17:0	-	-	-	-	-	-	32.3	88.0	-	-	-	-
17:1	-	-	-	-	-	-	48.2	7.0	-	-	-	-
17:2	-	-	-	-	-	-	19.5	-	-	-	-	-
18:0	Trace	Trace	-	Trace	Trace	Trace	-	Trace	9.5	100	1.0	-
18:1	25.2	11.3	-	4.8	5.0	3.1	-	1.8	27.9	-	8.0	8.2
18:2	56.2	12.7	-	4.4	5.8	4.6	-	2.8	62.6	-	2.1	10.6
19:0	-	-	-	-	-	-	-	-	-	-	48.4	68.0
19:1	-	-	-	-	-	-	-	-	-	-	29.1	-
19:2	-	-	-	-	-	-	-	-	-	-	6.3	-

Table IV

Radioactive products from the incubation of labelled saturated precursors with whole cells of Chlorella vulgaris with and without added sterculic acid.

The second organism studies was Torulopsis apicola. This is a yeast which grows aerobically and is known to perform aerobic desaturations of long

chain fatty acids. Since this organism is heterotrophic, the radiolabelled precursor had to be introduced to it in the medium in which it had been grown. The results of the experiment are given in Table V. This system is studied more easily so far as monoene synthesis is concerned since it does not form dienes. Being a whole cell culture however it does possess the ability to elongate acids by the malonyl CoA pathway. Decanoic and dodecanoic acids are activated almost completely but form no desaturated product. Here, as in the case of Chlorella, it may well be that the rate at which these acids are elongated or incorporated into lipids prevents any desaturation. In this system also the amount of elongation decreases as the chain length increases but in this case, unlike Chlorella, pentadecanoic acid is elongated.

Radioactive Product	Precursors							
	10:0	12:0	14:0	15:0	16:0	17:0	18:0	19:0
10:0	3.7	-	-	-	-	-	-	-
12:0	-	7.5	-	-	-	-	-	-
14:0	-	-	21.0	-	-	-	-	-
14:1	-	-	4.8	-	-	-	-	-
15:0	-	-	-	37.8	-	-	-	-
15:1	-	-	-	11.5	-	-	-	-
16:0	8.0	8.3	12.0	-	37.2	-	-	1.8
16:1	13.3	18.9	31.5	-	43.2	-	-	1.2
17:0	-	-	-	4.6	-	54.4	-	-
17:1	-	-	-	45.9	-	45.6	-	-
18:0	-	-	-	-	-	-	51.2	-
18:1	74.5	65.3	28.7	-	22.3	-	47.9	7.9
19:0	-	-	-	-	-	-	-	87.0
19:1	-	-	-	-	-	-	-	3.5

Table V

Labelled fatty acids produced from 1 ^{14}C precursors by Torulopsis apicola in its nutrient medium

As mentioned above, nonadecanoic acid provides the only case where β-oxidation has taken place. In this organism, though not in the case of Chlorella, the breakdown of nonadecanoic acid may be only an initial loss of two carbon atoms rather than a complete breakdown to short chain materials. Since the label was at the 2 position of the acid then radiolabelled acetate would be formed in this case and as far as labelled acids are concerned, a normal resynthesis pattern is observed.

If a 3 labelled acid had been used, it is quite possible that no resynthesis would be observed. These statements are based on the work of Tulloch et al[248] who fermented all the even chain saturated acids from C16-C24 and even chain length alkanes from C16-C24 and found that the organism always degraded them to C17 or C18 compounds before converting them to the W-1 hydroxy compound. Jones and Howe[249] confirmed the results in a further series of experiments. In the present work with Torulopsis no hydroxylation was observed at all. The variation with chain length of the amount of elongated products formed is shown in fig.IIIb.

Saturated precursors of fourteen carbon atoms and above, all produced monoenes of the same chain length as the precursor. All these monoenes had the double bond in the 9:10 position. Thus in this system also the position of the double bond is controlled by the distance from the carboxyl end of the molecule. This also indicates that the substrate molecule is joined to the desaturase either directly or via its CoA or ACP thiolester at the carboxyl end. In studying the amount of desaturation there seems evidence that there may only be one desaturase active in this system and that there is a broad optimum for chain length with much better desaturations being obtained with palmitic, margaric and stearic acids than with the shorter chain acids. Nonadecanoic acid is very unfavourable to desaturation in this system. See fig.IV.

Since the amount of lipid incorporation was not determined in these experiments, it could be that the discrimination against nonadecanoic

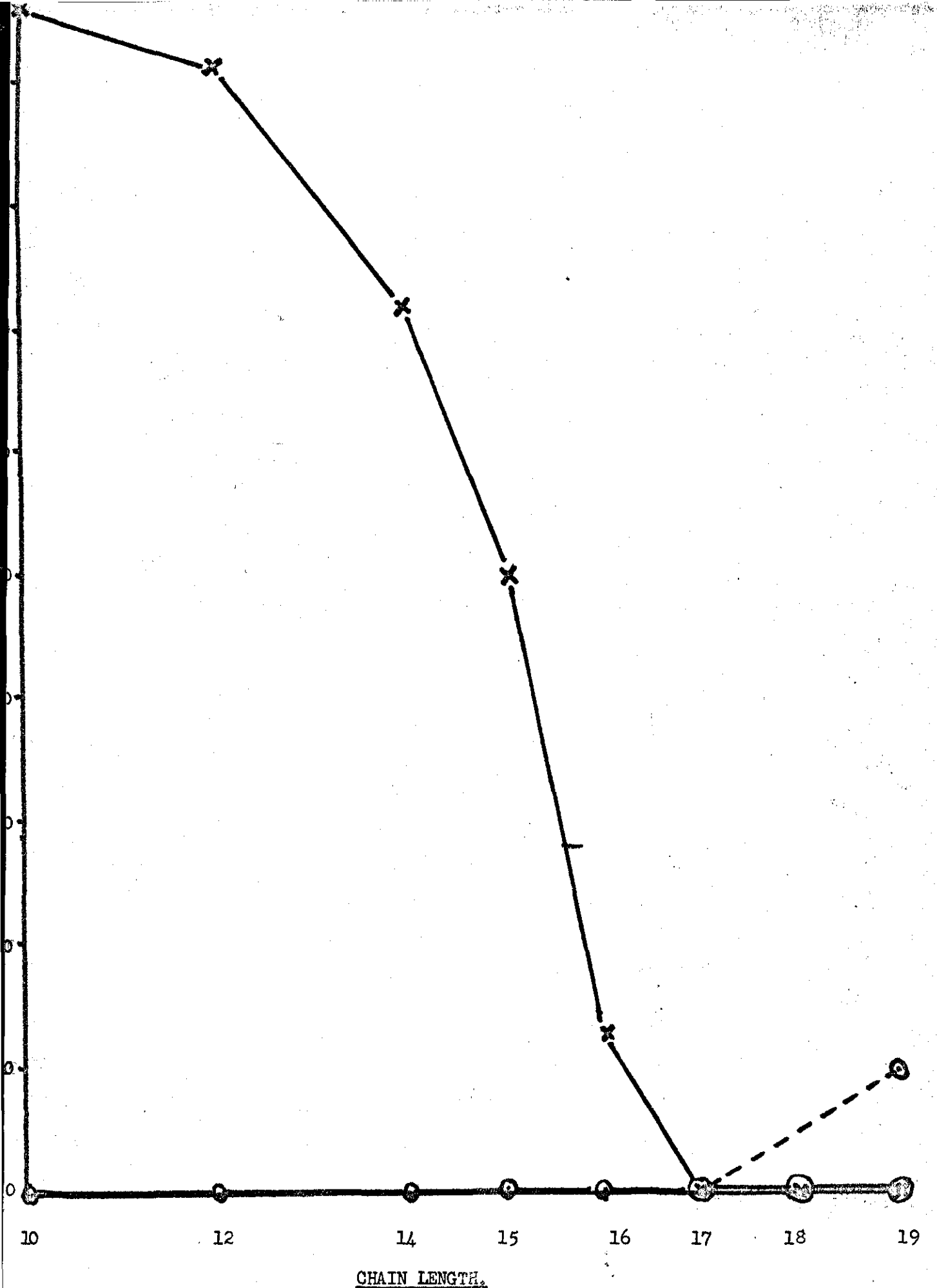


Fig.iiib. Conversions of saturated fatty acid precursors to longer chain length fatty acids (x—x) and to shorter chain length fatty acids (0 - - 0) by whole cells of Torulopsis apicola.

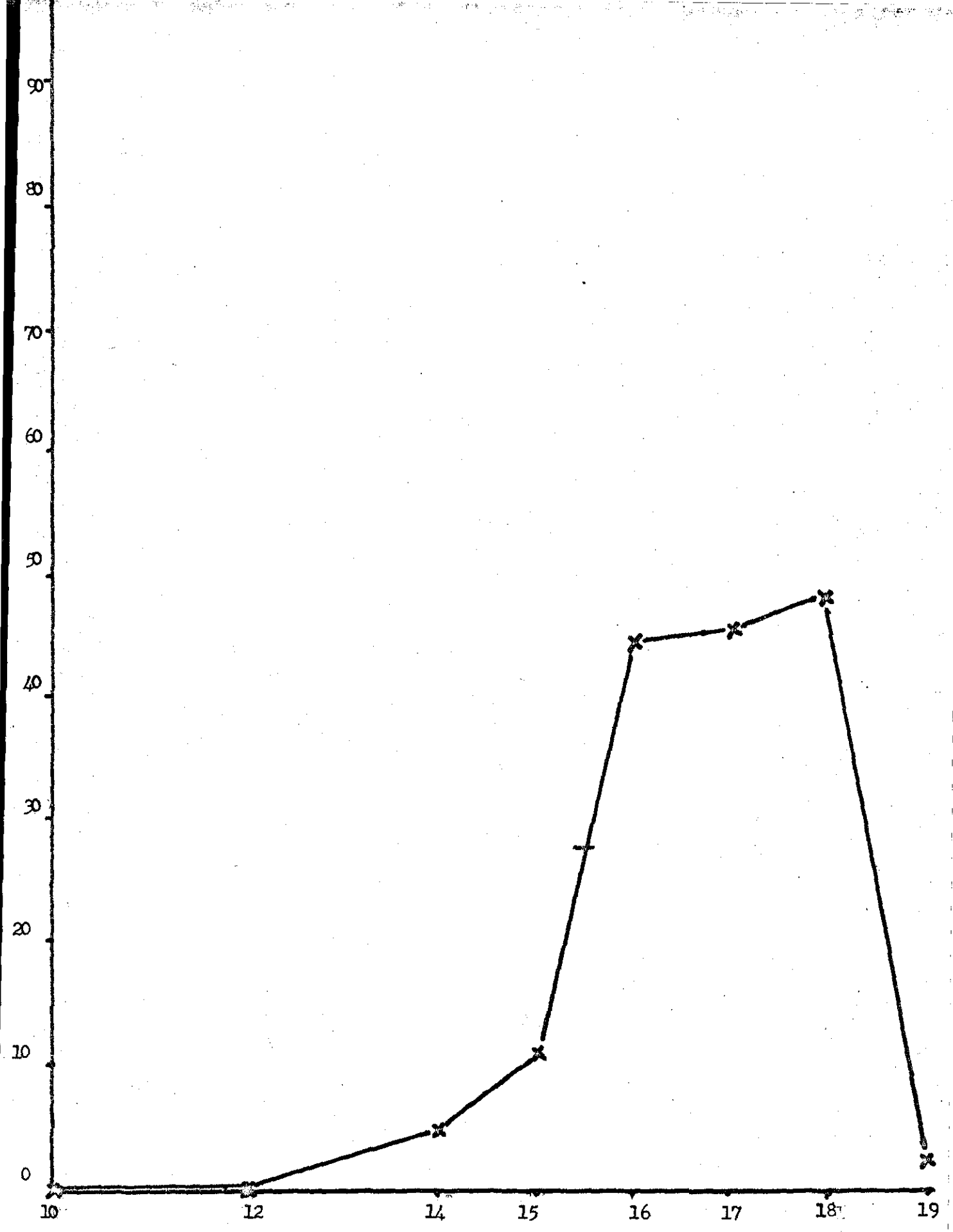


Fig.iv. Conversions of saturated precursors to 9:10 monoenoic fatty acids of the same chain length by whole cells of Torulopsis apicola

acid as a substrate for desaturation is an activation or a permeability problem.

The major product of chain elongation in all the even carbon number acids is oleic acid. The fact that no oleic acid is produced from pentadecanoic acid, however, whilst almost half the precursor is elongated to a seventeen carbon atom monoene, seems good evidence that little breakdown and resynthesis is involved in this system and that the monoenes of the same chain length are formed by direct desaturation.

Since this system is able to elongate substrates by two carbon atoms and form monoenes of more than one chain length from a given saturated substrate e.g. myristic, it is possible that, if the system can elongate monoenoic acids as well as saturated acids, monoenes with the bond in the 11:12 position may be formed. There are three possibilities, either the system elongates saturated precursors only, or the system elongates monoenoic precursors only, or it can do both.

In order to solve this problem the individual monoenes were isolated and the bond position determined. This showed that all monoenes were exclusively 9:10 monoenes and therefore showed that only saturated acids were elongated and that no elongation of the products of desaturation had taken place in this system. This is illustrated in fig.V.

Had the forbidden pathways above been able to operate 11:12 and 13:14 monoenes would have been isolated. On this evidence it is assumed that all the monoenes of longer chain length than the precursor in Table V are 9:10.

The effect of substrate structure on desaturation in animals was studied first of all in the goat mammary gland. Optimum fat metabolism occurs in these tissues when the animal is fully lactating and hence this was the tissue used. Milk production is not only of academic but commercial importance and hence the study of this system was chosen. Although *in vivo* studies with perfused glands is a recognised technique[250], *in vitro* studies of a subcellular fraction has the advantage of more controlled conditions.

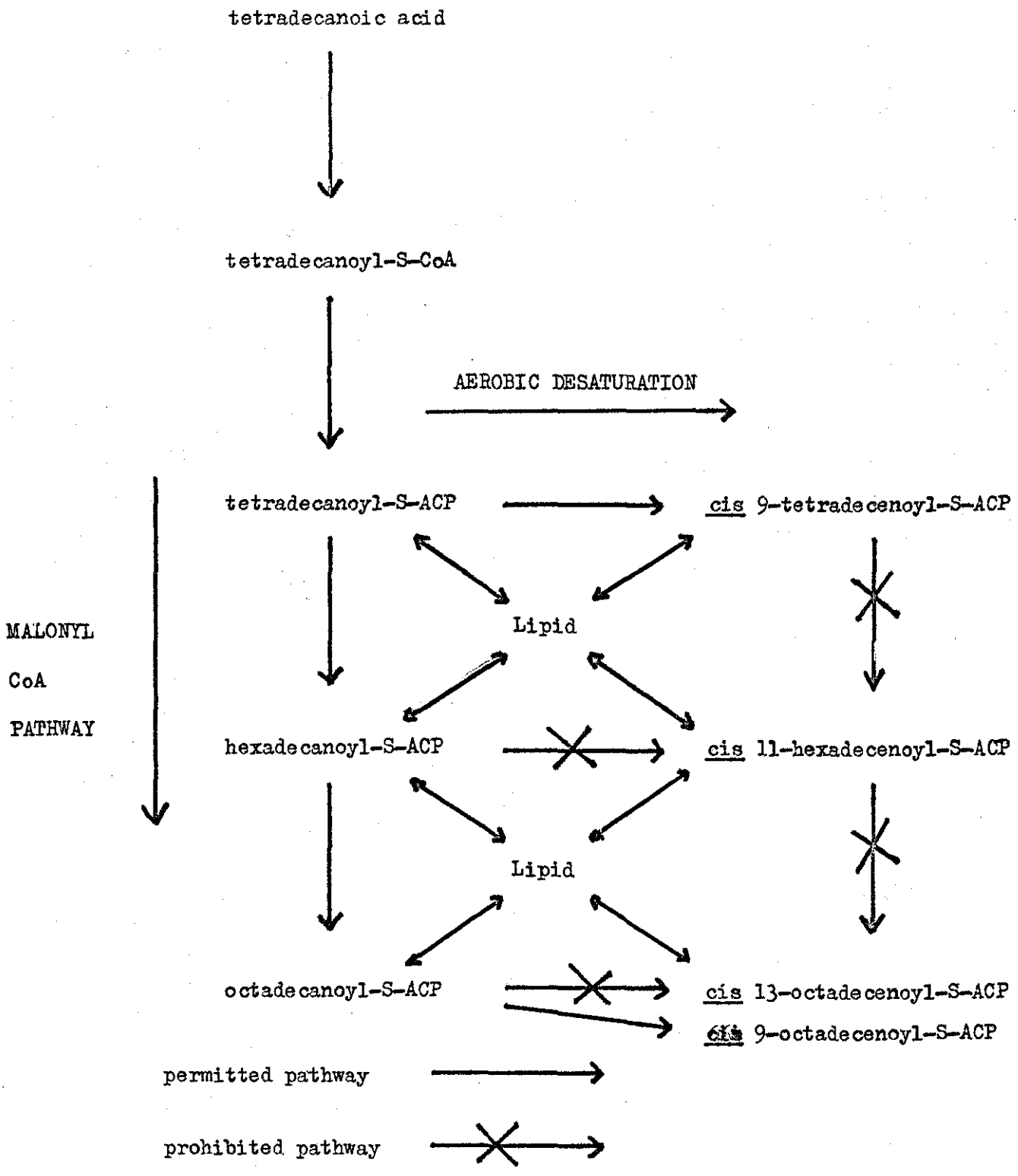


Fig.v. Permitted and prohibited pathways in Torulopsis apicola.

The microsomal fraction was chosen since this is the site of the desaturase[251] and eliminates any chain elongation or β-oxidation which are carried out by mitochondrial enzymes. These latter processes tend to make the study more involved and hence their exclusion here is of some advantage.

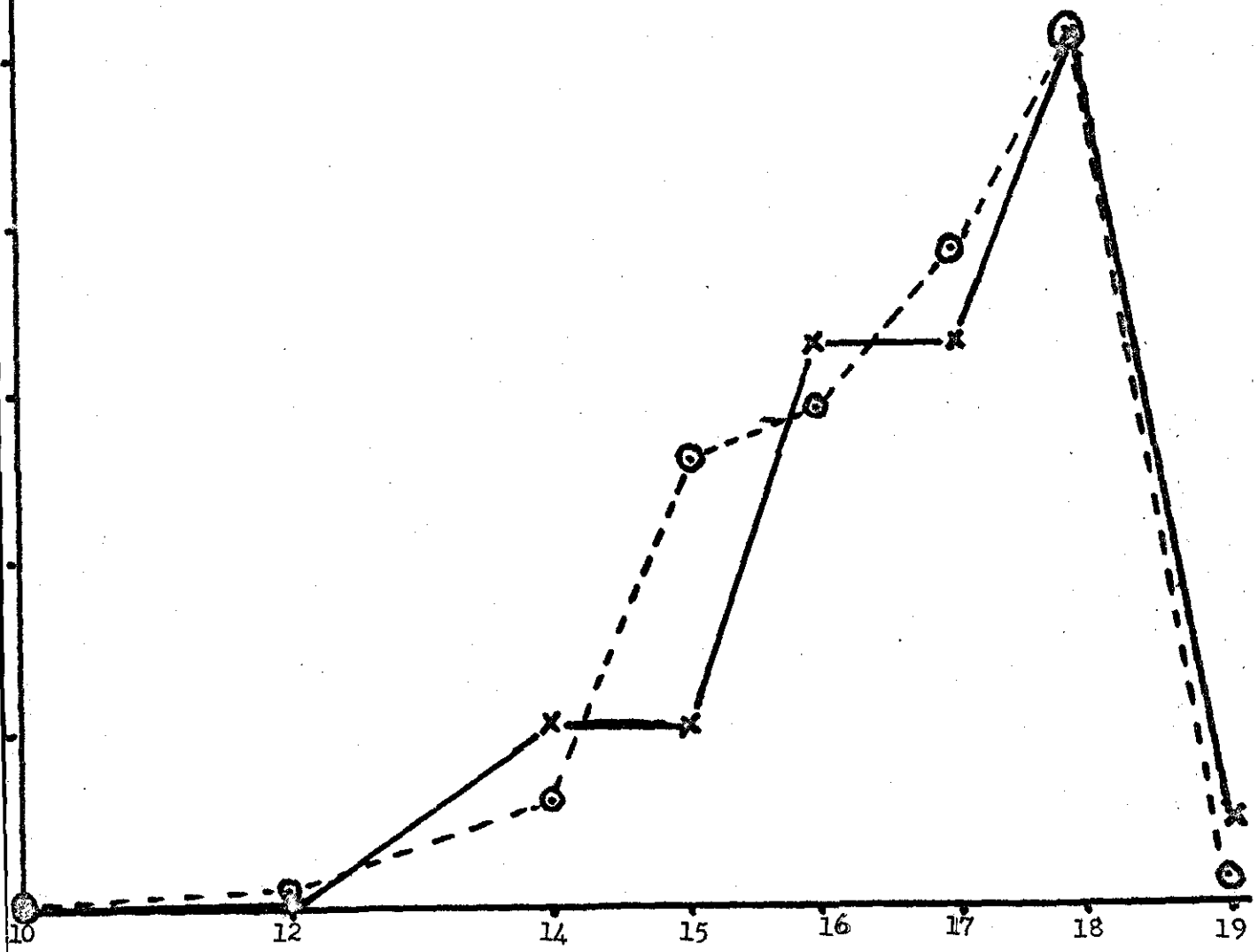
The *in vitro* incubations with labelled C10 - C19 acids were carried out both with and without the presence of particle free supernatent. The amount of desaturation obtained is shown in Table VI

Precursor	Microsomes % Desaturation	Bond Position	Microsomes + P.F.S. % Desaturation
($1^{14}C$) - decanoic	-	-	-
($1^{14}C$) - dodecanoic	-	-	-
($1^{14}C$) - tetradecanoic	5.75	9 - 10	3.5
($2^{14}C$) - pentadecanoic	5.0	9 - 10	3.0
($1^{14}C$) - hexadecanoic	15.5	9 - 10	11.25
($1^{14}C$) - heptadecanoic	20.25	9 - 10	18.75
($1^{14}C$) - octadecanoic	25.5	9 - 10	22.5
($2^{14}C$) - nonadecanoic	2.5	9 - 10	2.5

Table VI

Degree of desaturation of labelled precursors in subcellular fractions of goat mammary gland.

The lipid incorporation of the added substrates was also studied and the results appear in Table VII. Plots of desaturation and polar lipid incorporation appear on the same set of axes below, figs. VI and VII. The bond position of all the monoenes produced was identified and found in all cases to be in the 9 - 10 position. The fact that all the homologues were desaturated in the same position indicates that the number of methylene groups beyond the tenth carbon atom does not affect the position of desaturation. This in turn means that again the specificity of desaturation in this system is the distance from the



CHAIN LENGTH.

Fig.vi. Conversion of saturated fatty acid precursors to 9:10 monoenoic fatty acids of the same chain length(x---x) and incorporation of the same precursors into polar lipids (o - - o) by a microsomal fraction of a fully lactating goat mammary gland.

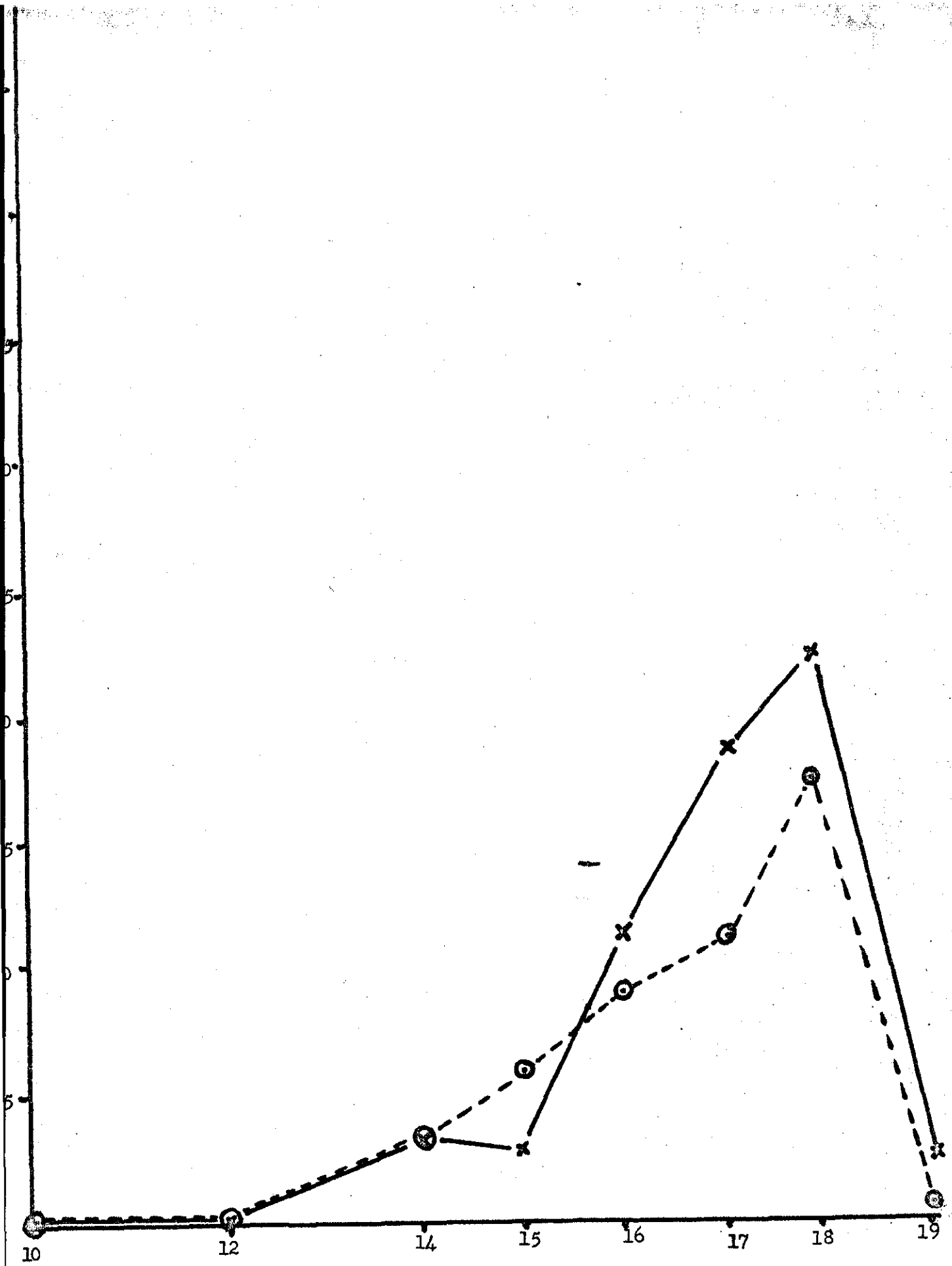


Fig.vii. Conversion of saturated fatty acid precursors to 9:10 monoenoic fatty acids of the same chain length(x——x) and incorporation of the same precursors into polar lipids (o - - - o) by a microsomal fraction of a fully lactating goat mammary gland with added particle free supernatent.

carboxyl end of the molecule. A specific attachment of the carboxyl end of the substrate molecule to the desaturase enzyme complex is indicated by this result though the precise form of the attachment is, of course, still not known.

Precursor	% Count Distribution					
	Microsomes			Microsomes + P.F.S.		
	FFA	TG	PL	FFA	TG	PL
(1^{14}C) 10:0	100	0	0	100	0	0
(1^{14}C) 12:0	95.5	0	0.5	96.5	4.0	0.5
(1^{14}C) 14:0	96.75	0	3.25	83.7	13.2	3.1
(2^{14}C) 15:0	87.0	0	13.0	78.5	15.3	6.2
(1^{14}C) 16:0	85.5	0	14.5	66.0	25.0	9.0
(1^{14}C) 17:0	80.5	0	19.5	77.8	10.8	11.4
(1^{14}C) 18:0	74.5	0	25.5	78.5	4.0	17.5
(2^{14}C) 19:0	99.25	0	0.75	97.5	2.0	0.5

Table VII

Labelled lipids produced by the incubation of saturated precursors with subcellular fractions of goat mammary gland

The microsomal fraction gives no desaturation with decanoic or lauric acids and gives little or no evidence of activation of these substrates. The curve produced, fig.VI, has an optimum desaturation at C18 and the usual steep decline to almost zero desaturation in the case of nonadecanoic acid. The form of the desaturation vs chain length curve appears to be typical of a one enzyme system although the odd chain acids seem to be less favourable than would be expected.

One feature of the microsomal system is the fact that the amount of polar lipid formation is almost identical to the amount of desaturation in most cases. This suggests some close involvement of lipid formation with desaturation,

but, on investigation, it proved that both labelled saturated and monoenoic acyl groups occurred in the polar lipid fraction and also that unsaturated acids occurred in the free form. There was an increased tendency for the unsaturated acids to be bound to lipid as the chain length increased to 18. In the case of myristic acid, most of its monoene was in the free acid form. It appears from this as if the enzymes involved in the formation of polar lipids have some chain length specificity.

The inclusion of the particle free supernatent in the incubations had the effect of decreasing the amount of polar lipid formed but resulted in the formation of an appreciable amount of triglyceride such that overall more lipid formation was achieved. Optimum triglyceride formation occurred at C16. As in the case of the microsomes alone desaturated acids appeared in the free fatty fraction to a similar amount showing that the lipolytic enzyme involved was in the microsomes themselves and not enhanced by the supernatent. All other features of this combined system were the same as for the microsomes alone except the desaturation vs chain length curve did have only one apparently irregular point at C15 and, in the light of the Chlorella and hen liver results, it could be indicative of two enzymes. A repeat of the experiments using only microsomes to check the margaric acid result would have been desirable since it seemed unusually low but further tissue was unavailable,

Since goat tissue was difficult to obtain and as a result of work done by Wakil[252] and Johnson[237] further in vitro studies of subcellular animal systems were performed on the combined microsomal and the particle free supernatent fraction from hen liver. The hens used were either brown Warren hens or white shaver hens. Best results were obtained from hens fed on a balanced diet and the older and more fatty the liver the greater the degree of desaturation obtained. The variation between individual livers as to the absolute amount of desaturation of a given precursor varied greatly but the relative variation from precursor to precursor was very similar in all cases. Thus it was imperative to do all

comparative studies on the same liver. The precise reason for the variation in desaturase activity from liver to liver is not known and only the above observations of the visible state of the liver have been noted.

Since the incubation conditions used in the goat system above differed considerably from those used in the hen liver system by Johnson[237] initial experiments were undertaken to investigate the requirements of the system. The first comparison was of the nucleotide systems used. In the goat system the required reduced nucleotide had been added but in Johnson's system the reduced nucleotide had been generated as required in situ. The method involved the addition of the oxidised form of the nucleotide and sodium lactate. Action of lactate dehydrogenase[253] provided the required reduced nucleotide according to the following reaction :

$$\text{Lactate} + NAD^+ \rightleftharpoons \text{pyruvate} + NADH + H^+$$

The result of this experiment was that there was no advantage in this system over the direct addition of NADH so far as desaturation was concerned (Table VIIIa). Indeed the addition of NADH seemed to result in greater incorporation into lipids.

Precursor	Cofactor System	% Desaturation	% FFA	% TG	% Polar Lipid	% Desaturation in FFA
(1-^{14}C)- stearic	Lactate NAD+	46.6	35.3	7.6	57.0	11.3
(1-^{14}C)- stearic	NADH	46.9	22.0	8.5	69.5	10.7

Table VIIIa

Effect of nucleotide system on desaturation and lipid formation in a hen liver microsomal fraction

The increase in phospholipid produced was a general effect and no particular phospholipid was favoured. The phospholipids labelled in these experiments are listed in Table VIIIb.

Precursor	Cofactor System	Phosphatidyl inositol	Phosphatidyl choline	Phosphatidyl ethanolamine
($1^{14}C$ - stearic	Lactate NAD+	5.8	69.2	25.0
($1^{14}C$) - stearic	NADH	7.4	70.5	22.0

Table VIIIb

Effect of nucleotide system on individual phospholipid composition in a hen liver microsomal fraction

As well as changing the nucleotide requirement, experiments were done to see if the mass of the other cofactors could be reduced. Some cofactors can inhibit at high levels so the mass was reduced. The results are shown in Table IX and they show no advantage in the high level of cofactors, in fact a slight advantage appears to be gained both in lipid incorporation and desaturation with the lower level. The existence of a lipolytic enzyme is indicated with the presence of 6% of desaturated acid appearing in the free fatty acid pool.

Precursor	Cofactor* concn.	% Desaturation	% FFA	% TG	% PL	% Desaturation in FFA
($1^{14}C$)-stearic	High	22.8	66.4	5.0	28.6	5.7
($1^{14}C$)-stearic	Low	25.6	65.8	5.1	29.1	5.9

Table IX

Effect of cofactor concentration on lipid formation and desaturation in a hen liver microsomal preparation

* Cofactor amounts/incubation were as follows:-

	"High"	"Low"
CoA	1 mgm	0.15 mgm
ATP	60.5 mgm	11 mgm
NADH	2 mgm	1 mgm
NADPH	1 mgm	0.5 mgm

With the high levels of free fatty acids (approximately 2/3 of the added label) despite the evidence of lipolytic enzymes, there was some doubt about the ability of this system to activate the added acid. Thus incubations were performed in which the mass of fatty acid substrate added was varied up to forty times the usual amount. The results are shown in Table X. This shows that the mass of added acid does not affect either the desaturation or the lipid incorporation.

Mass of Precursor (µg)	% Desaturation	% FFA	% TG	% Polar Lipid	% Desaturation in FFA
6	25.6	65.8	5.1	29.1	5.9
125	24.8	63.4	4.8	31.8	6.3
250	24.5	64.7	5.0	30.3	6.1

Table X

Effect of mass of stearic acid on the degree of desaturation and lipid formation in a microsomal preparation of hen liver.

Clearly there is no question of the enzyme system being saturated in this case and some selection process must be in operation.

A similar result was obtained by using an increased amount of enzyme (microsomes) and the same mass of acid, Table XI. The same conclusions were drawn i.e. the extra enzyme caused no increase in either desaturation or lipid incorporation, see Table XI.

Quantity of Enzyme Source*	% Desaturation	FFA	TG	Polar Lipid
3 mls	27.0	65.7	5.1	29.2
6 mls	26.1	67.0	5.0	28.0
9 mls	26.7	63.8	5.0	31.2

* See experimental section (page 111).

Table XI

Variation of the amount of desaturation and lipid incorporation with the increase of enzyme source

Having decided upon the optimum conditions for this system, the homologous series of acids from C10 - C19 was incubated under those conditions. In the first experiment the bond position and extent of desaturation and the percentage of activity in polar lipid was measured, Table XIIa. The desaturation data once again show complete carboxyl end control of the positional specificy of the desaturation process with the double bond always in the 9:10 position and is indicative of carboxyl attachment to the enzyme. Quantitatively, the data, fig VIII, show the characteristic pattern of two enzymes, one responsible for the shorter chain acids with an optimum at C14 and a longer chain enzyme with its optimum at C18. In this system for the first time decanoic acid has been directly desaturated. Apart from this, however, the picture is very much the same as the Chlorella 9:10 desaturase.

Precursor	% Desaturation	% Polar Lipid	Ratio $\frac{\%\ \text{Desaturation}}{\%\ \text{Polar Lipid}} \times 100$
($1^{14}C$) - decanoic	2.5	5.1	49
($1^{14}C$) - dodecanoic	8.8	12.7	69
($1^{14}C$) - tetradecanoic	27.0	40.0	67
($2^{14}C$) - pentadecanoic	7.5	19.0	39
($1^{14}C$) - hexadecanoic	18.0	26.4	68
($1^{14}C$) - heptadecanoic	20.8	29.8	70
($1^{14}C$) - octadecanoic	25.0	36.0	69
($2^{14}C$) - nonadecanoic	12.5	22.7	55

Table XIIa

Degree of desaturation produced in labelled saturated precursors by a microsomal fraction of a hen liver

There is a marked cut-off at C19 whereas 18 - nonadecanoic acid is desaturated to the same extent as stearic acid, Table XIIb. Once again this points to a side group projecting from the enzyme chain in such a way as to

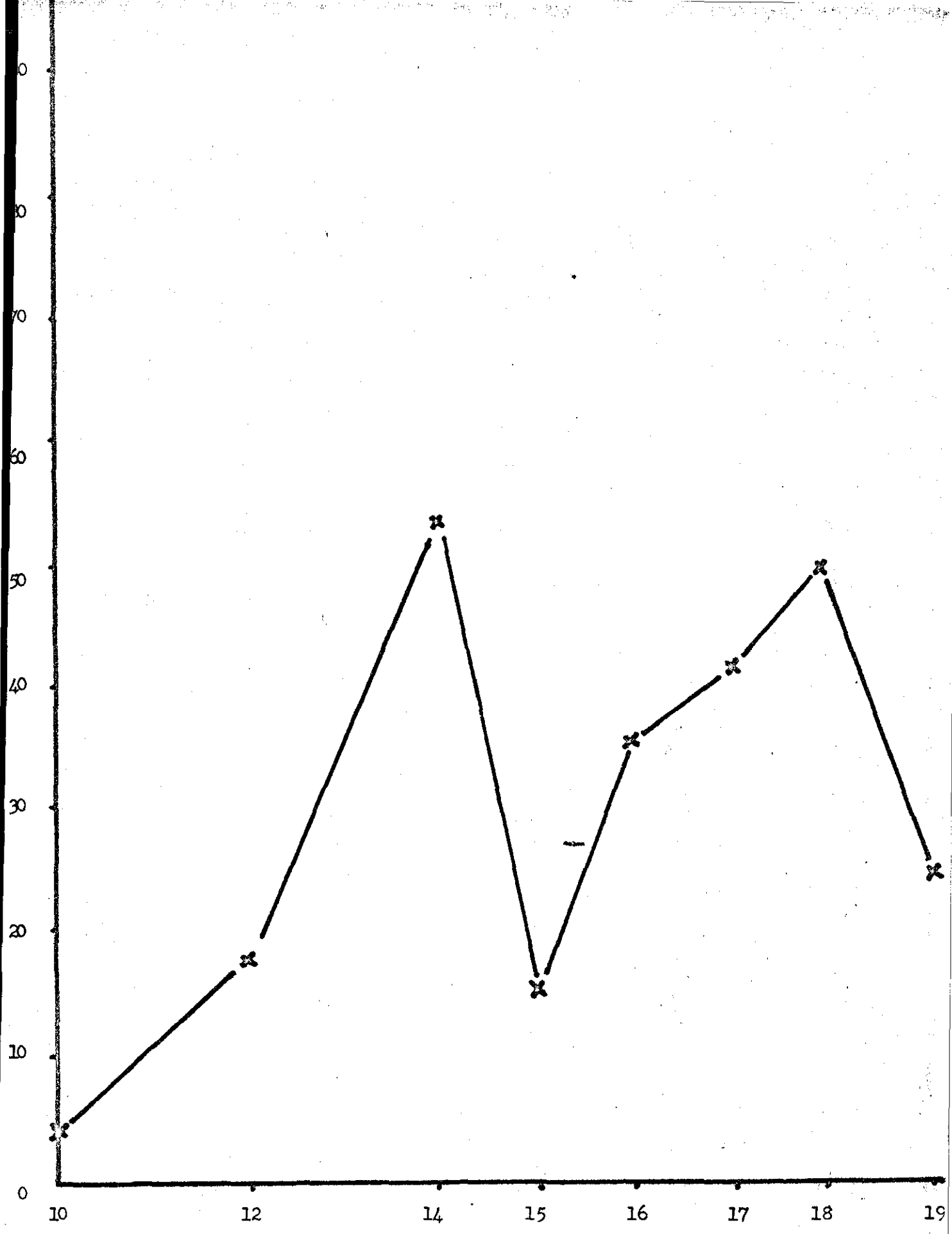


Fig.viii . Conversions of saturated precursors to 9:10 monoenoic fatty acids of the same chain length by a microsomal fraction of hen liver.

interfere with the terminal methyl group of nonadecanoic acid.

Precursor	% Desaturation	Bond Position
($1^{14}C$) - hexadecanoic	45.1	9-10
($1^{14}C$) - heptadecanoic	56.8	9-10
($1^{14}C$) - octadecanoic	57.8	9-10
($2^{14}C$) - nonadecanoic	47.1	9-10
($1^{14}C$) - 18 nonadecenoic	57.0	18-19, 9-10
($1^{14}C$) - cis 7 octadecenoic	-	-
($1^{14}C$) - cis 11 octadecenoic	-	-
($1^{14}C$) - cis 12 octadecenoic	23.6	12-13, 9-10

Table XIIb

Degree of desaturation of labelled precursors by a microsomal fraction of hen liver

cis 12- Octadecenoic acid is also desaturated - this system to give cis, cis linoleic acid although not to the same extent that steareate is desaturated to oleate. Although unlike bonds in the 7:8 and 11:12 position which give no desaturation, the bond at the 12:13 position does not completely prevent desaturation but it does reduce its efficiency. This secondary effect is probably due to the bend in the molecule caused by this double bond reducing the binding efficiency of the substrate to the enzyme surface by reducing the total London dispersion force. Another possible explanation is the simple steric factor that the bend in the molecule produced by this double bond makes interaction between the desaturase active site and the D-9 and D-10 hydrogen atoms of the substrate more difficult.

The production of linoleate by the action of the 9:10 monoene desaturase on cis 12 - octadecenoic acid has been noted in Chlorella vulgaris,

the microsomal fraction from the mammary gland of a fully lactating goat and the microsomal fraction of hen liver. This result in these three systems is of considerable interest for the reasons outlined above. However, the last two are particularly important for they represent known cases of animal systems synthesising in situ an essential fatty acid from which they can ultimately form prostaglandins. Normally, linoleic acid and thence arachidonic acid cannot be synthesised from any other animal product and has to be taken in the diet. This result is not only of great academic interest but it has potential commercial possibilities as an alternative means of supplying essential fatty acids for animals. The only point of reservation is that the effect of large amounts of cis 12-octadecenoic acid in the diet is unknown although the hen liver system incorporates it into lipids to the same extent as oleic acid, Table XIV. If it has no harmful effects, the inclusion of cis 12-octadecenoic acid in the diet of animals or man could substitute to some extent for linoleic acid. Since it is likely that this monoenoic acid could be synthesised industrially more easily than linoleic acid, these results may be of substantial commercial importance.

The fact that cis 7-octadecenoic acid and cis 11-octadecenoic acid are not further desaturated shows the profound effect of a double bond so close to the active site, see Table XIIb. However, these acids are activated and incorporated into polar lipids, Table XIV, as effectively as oleic acid.

In the second experiment with the series of acids C10 - C19, although the overall amount of desaturation was less than in the first series, the results were identical in relative terms, Table XIII and Fig IX. Here it is shown that the total lipid incorporation is always greater than the amount of desaturation but follows the same chain length pattern, Fig.IX. The amount of desaturation product in the free fatty acid fraction and in the polar lipid fraction is shown in Fig.X. Half of the shorter chain length acids tend to remain in the free form after desaturation, whereas the longer chain acids have a much greater tendency to become bound into lipids. This is further evidence

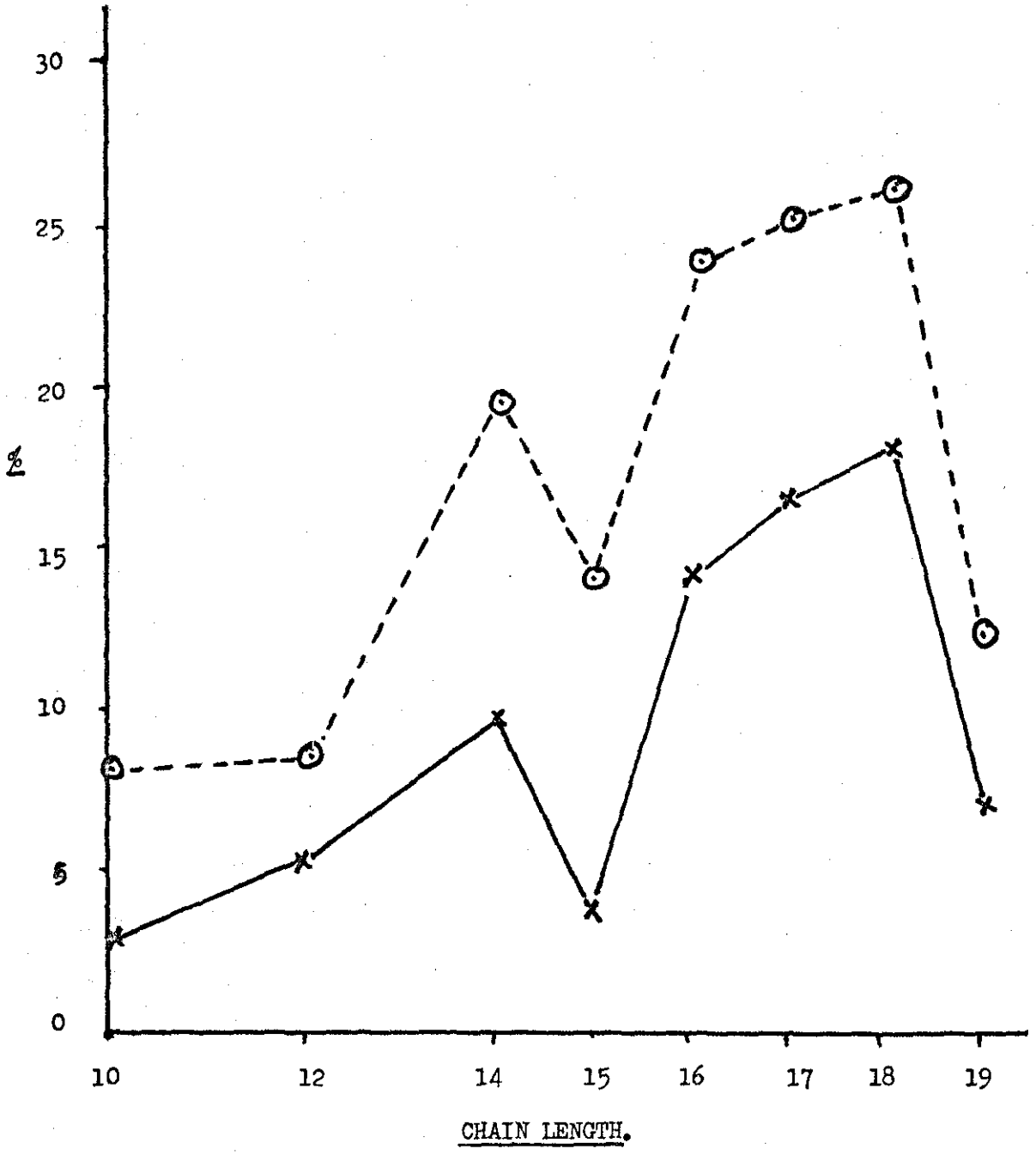
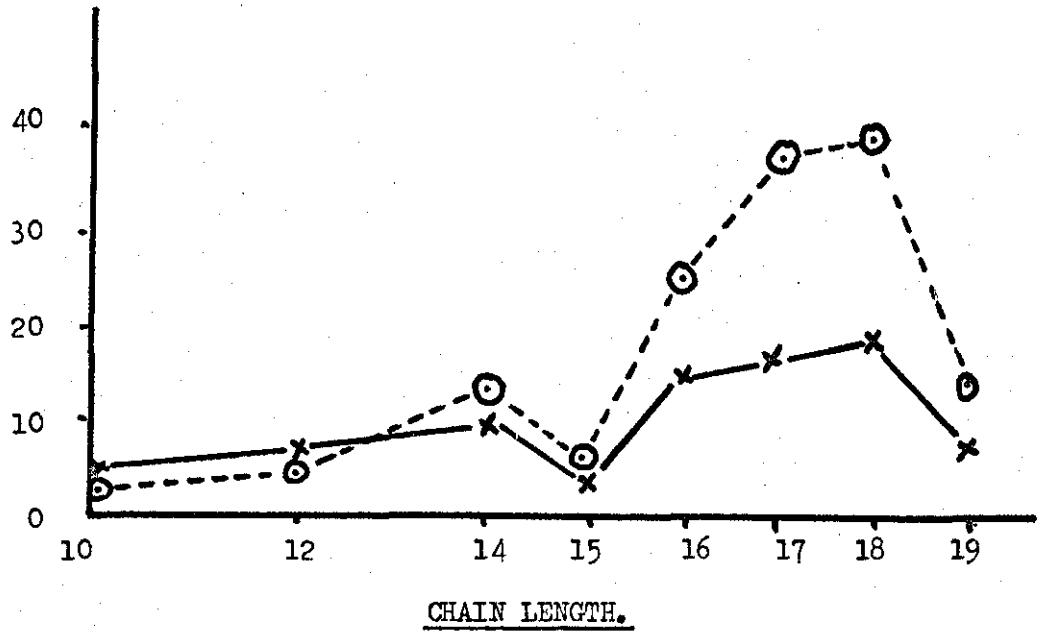


Fig.ix. Conversions of saturated fatty acid precursors to 9:10 monoenoic fatty acids of the same chain length (x—x) and incorporation into polar lipids (O - - O) by a microsomal fraction of a hen liver.



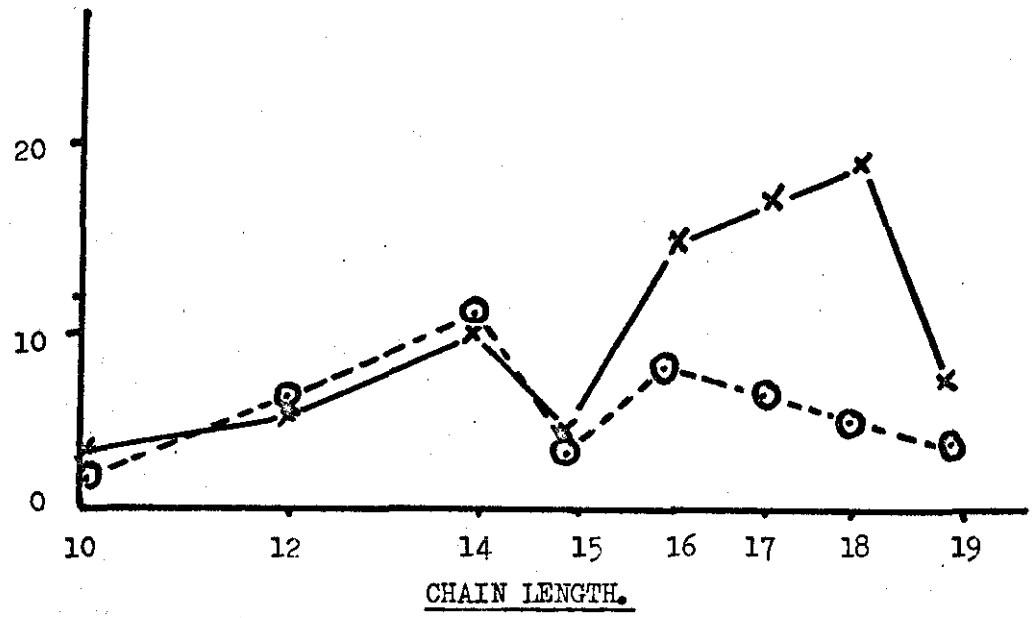


Fig. x. Conversions of saturated fatty acids to 9:10 monoenoic fatty acids of the same chain length(x——x) in both graphs.(0 - - 0) represents the amount of labelled 9:10 monoenoic fatty acid in the polar lipid fraction in the top graph and the amount of labelled monoenoic fatty in the free fatty acid pool in the bottom graph.The enzyme system was the microsomal fraction of a hen liver.

for the existence of two enzymes with the chain length specificities outlined before.

Precursor	Total % Desat.	% Incorporation into Polar Lipid	Ratio $\frac{\%\ \text{Desat.}}{\%\ \text{Polar Lipid}} \times 100$	% as monoene in FFA	% as monoene Polar Lipid
(1-^{14}C)-decanoic	3.1	8.4	36.5	3.6	1.0
(1-^{14}C)-dodecanoic	5.5	8.6	64.0	5.9	2.0
(1-^{14}C)-tetradecanoic	9.9	19.5	50.5	9.1	11.5
(2-^{14}C)-pentadecanoic	3.9	14.0	27.8	3.1	5.6
(1-^{14}C)-hexadecanoic	14.3	23.8	62.0	7.8	24.6
(1-^{14}C)-heptadecanoic	16.7	25.2	66.4	6.1	37.8
(1-^{14}C)-octadecanoic	18.2	27.2	67.0	5.0	39.0
(2-^{14}C)-nonadecanoic	7.2	12.5	60.0	3.9	13.0

Table XIII

Degree of desaturation of labelled saturated precursor in total acid in the individual free fatty acid and polar lipid fractions of a microsomal preparation of hen liver

In the investigation of the fates of exogenous (free) monoenoic fatty acids all the added labelled monoenoic acids were found to be activated and incorporated into polar lipids, Table XIV, whereas when stearoyl CoA was incubated with the system labelled oleic and stearic acid were found in both the polar lipids and the free fatty acid fractions, Table XIV. All the above data involving the fate of acyl groups in this system suggest that the metabolic pathways illustrated in Fig XI are operative.

Assuming that these pathways are correct and that the masses of the activated acyl groups are negligible compared to the masses of the lipids and free fatty acids, then applying simple algebra, it is possible from the three measurements shown in Table XII to calculate the ratio of desaturated acid from

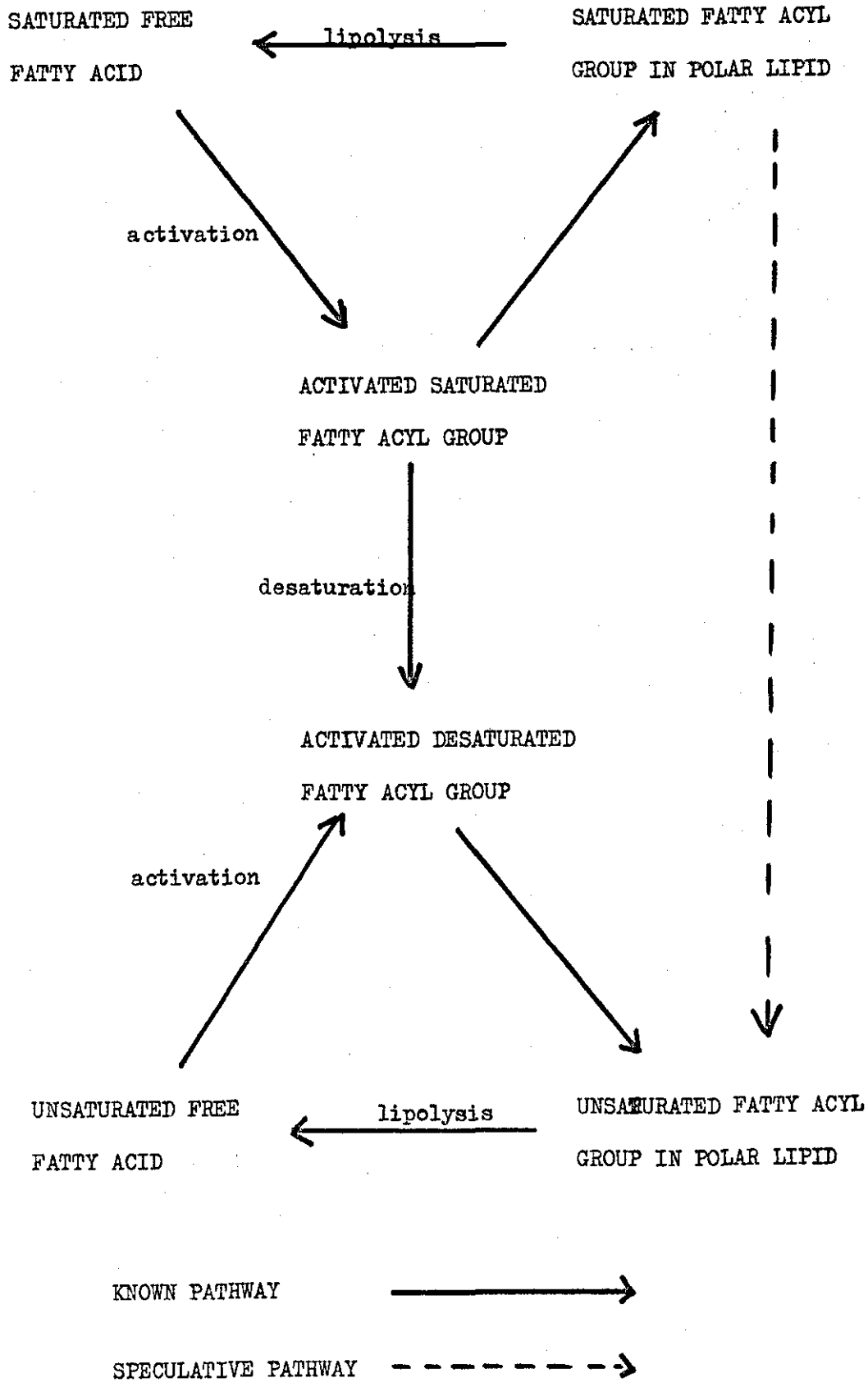


Fig.xi Metabolic pathways in a microsomal fraction of a hen liver.

the added label on lipid to desaturated acid from the added label which occurs free. This ratio for each chain length has been plotted, Fig XII, and shows that the optimum chain length for the desaturated label to occur bound into polar lipid is 18 which confirms quantitatively the facts which appeared qualitatively in Fig X.

Precursor	% Desaturation Total	% Polar Lipid	% monoene in Polar Lipid	% FFA	% monoene in FFA
($1^{14}C$)cis 9-octadecenoic	-	22.5	100.0	71.5	100
($1^{14}C$)cis 7-octadecenoic	-	18.7	100.0	73.7	100
($1^{14}C$)cis 11-octadecenoic	-	19.5	100.0	72.9	100
($1^{14}C$)cis 12-octadecenoic	~~74~~	21.7	~~100.0~~ 12.6	73.0	~~100~~ 3.9
($1^{14}C$) Stearoyl CoA	25.8	32.6	38.1	54.2	11.3

Table XIV

Degree of desaturation of labelled precursors in total and in the individual free fatty acid and polar lipid fractions of a microsomal preparation of hen liver.

The graph of the conversion of saturated precursor to 9:10 monoene of the same chain length with chain length, Fig VIII, shows a depression at C15 which suggests the presence of two enzymes one for C10 - C14 the other for C14 - C19. Certainly the low desaturation at C15 is not an experimental error since it was reproduced some six times and is corroborated by the results of Johnson[244] working with the same system.

It is just possible that this C15 chain length is discriminated against on another ground and one explanation of it might lie in the binding of substrates to the Bovine Serum Albumin. If for some reason pentadecanoic acid is bound more strongly than the other acids this could explain the lower desaturation and lipid incorporation value. The variation of degree of desaturation with chain length would then become typical of a one enxyme system

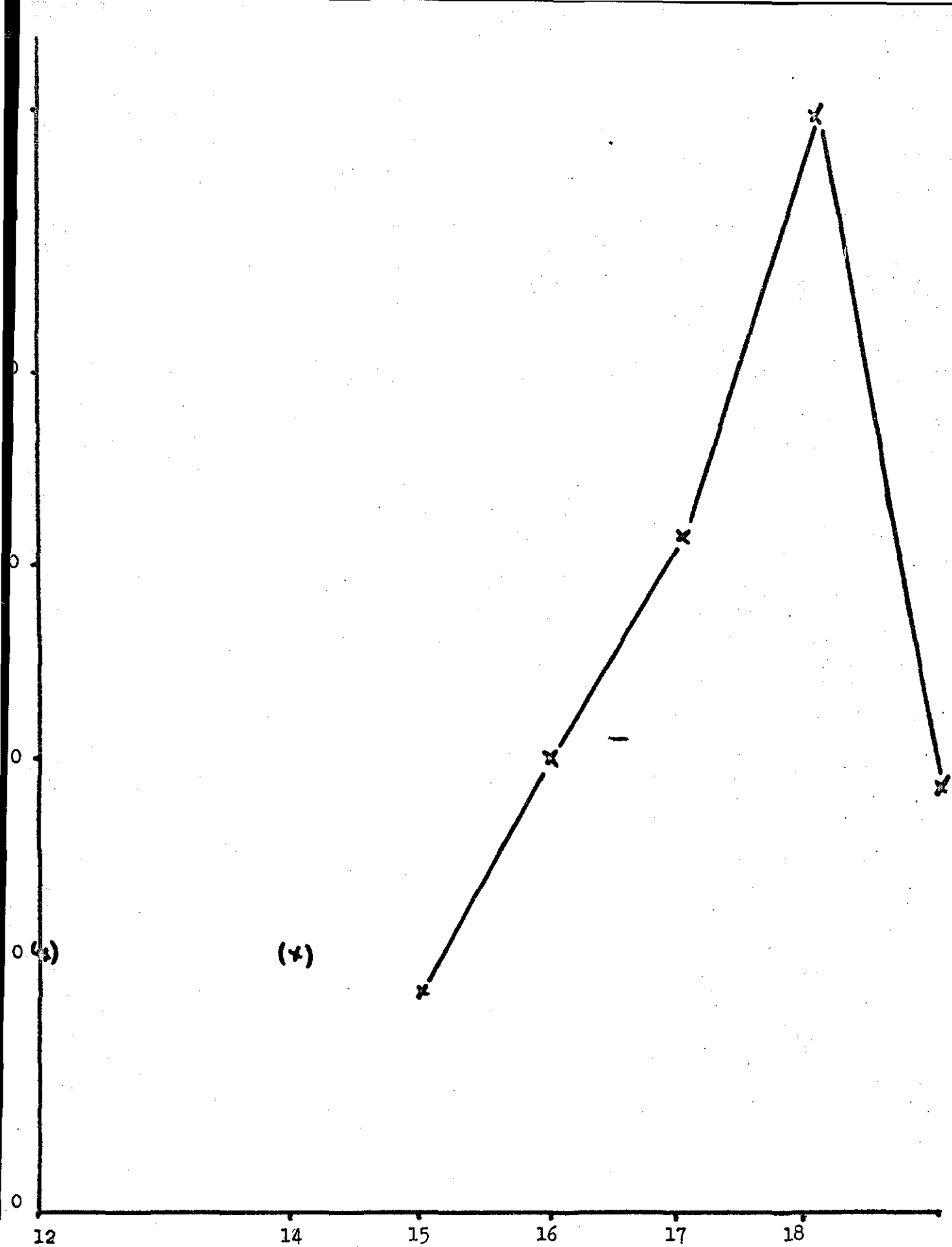


Fig.xii. Ratio of labelled 9:10 monoenoic fatty acids in the polar lipidfraction
o labelled 9:10 monoenoic fatty acid in the free fatty acid pool.

rising steadily to an optimum value at C18.

Goodman[254] and Reynolds et al[255] have investigated the binding of fatty acids to Albumin and have discovered three different classes of site on the albumen molecule where fatty acyl groups can be attached. In the first site the acyl groups are bound most tightly and usually there are two acyl groups per albumin molecule. The secondary sites have a smaller binding force and hold 4 - 6 fatty acyl residues whilst the third class of site which has the smallest binding force tends to have a larger less determinate number of acyl groups. Table XV shows Goodman's values of the association for the even numbered fatty acids for each of the three types of binding site.

Fatty Acid Anion	k_1'	k_2'	k_3'
octanoate *	-	5×10^4	-
decanoate *	-	6×10^4	-
dodecanoate	1.6×10^6	2.4×10^5	6.0×10^2
tetradecanoate	4.0×10^6	1.4×10^6	2×10^2
hexadecanoate	6.0×10^7	3.0×10^6	1×10^3
octadecanoate	8.0×10^7	8.0×10^5	1×10^3

Table XV

The Apparent Association constants for the interaction of human serum albumin with fatty acid anions at pH 7.5

* These results are due to Reynolds et al[255].

Although there is a general increase in binding force with the increase in chain length due to the increased number of short range non specific van der Waals interactions between the non polar portions of the binding ion and the non polar side chains of the albumin molecule, Goodman suggests that there is a great deal of structural specificity for individual chain lengths as well as this general effect. No data for pentadecanoic acid is available and Goodman's

data is for human serum albumin and although there is no evidence to suggest the overall picture is not the same in bovine serum albumin, an experiment was done to investigate the precise effect of the bovine serum albumin in the hen liver system.

The homologous series of fatty acids was again incubated with a microsomal preparation of hen liver and a duplicate series of experiments were done in which the added fatty acid was merely suspended in water to which Tween 20 had been added instead of being suspended on bovine serum albumin in the normal way. The results appear in Table XVI. ~~(and Fig XII)~~ These results show a general reduction in the amount of desaturation by a factor of about 15 - 20% at all chain lengths, although a study of the ratios of the two sets of figures shows that the increase in desaturation with the addition of Bovine serum albumin decreases as the binding force increases i.e. with chain length. Despite this the overall pattern of desaturation with chain length is the same both with and without the albumin. Pentadecanoic acid especially gives its characteristic lower value in both cases showing that any reason for its lower value is not to be found here.

Precursor	% Desaturation With BSA	% Desaturation Without BSA	Ratio $\frac{\text{without}}{\text{with}}$ x 100
($1^{14}C$) - Dodecanoic	51.0	34.0	66.7
($1^{14}C$) - tetradecanoic	80.6	55.0	68.7
($2^{14}C$) - pentadecanoic	59.5	39.0	71.5
($1^{14}C$) - hexadecanoic	73.0	55.5	76.0
($1^{14}C$) - heptadecanoic	79.0	64.0	81.0
($1^{14}C$) - octadecanoic	83.0	66.5	80.0
($2^{14}C$) - nonadecanoic	64.0	50.0	78.0

Table XVI

Effect of bovine serum albumin on the degree of desaturation of saturated precursors in a microsomal fraction of hen liver.

A final study into the rates of desaturation with chain length was undertaken to see whether the overall figures for the degree of desaturation represented the true initial and continuing rates of desaturation or whether the initial rates were similar but that for some reason a chain length specificity for lipid formation, for example, caused a particular chain length to cease being desaturated as its requirement ceased. This is of special interest since the requirement for cis 9 pentadecenoic acid will be less than either myristoleic or palmitoleic and might explain the reason for the low values of the pentadecanoic figure. Though less likely, if this were the case the overall figures quoted throughout this section would have a different meaning and would, in fact, represent the requirement of the system for given monoenoic acids.

Time (mins)	% Desaturation			Ratio $\frac{C14}{C15}$
	(1-^{14}C)-Tetradecanoic	(?-^{14}C)-Pentadecanoic	(1-^{14}C)-hexadecanoic	
5	31.0	22.0	29.3	0.71
10	42.0	30.0	40.7	0.71
20	55.0	37.6	53.5	0.68
30	64.5	42.5	63.0	0.66
60	68.0	51.7	65.0	0.76
120	72.0	52.2	66.2	0.71
240	76.0	53.3	68.2	0.70
480	79.0	56.0	70.0	0.71

Table XVII

Rate of desaturation at various times of saturated precursors by a microsomal fraction of hen liver.

Thus the amount of desaturation produced by myristate, palmitate and pentadecanoate was measured after given periods of time and the individual rates of desaturation at all these times were measured. The figures appear in Table XVII and the myristic and pentadecanoic graphs appear in Fig.XIII.

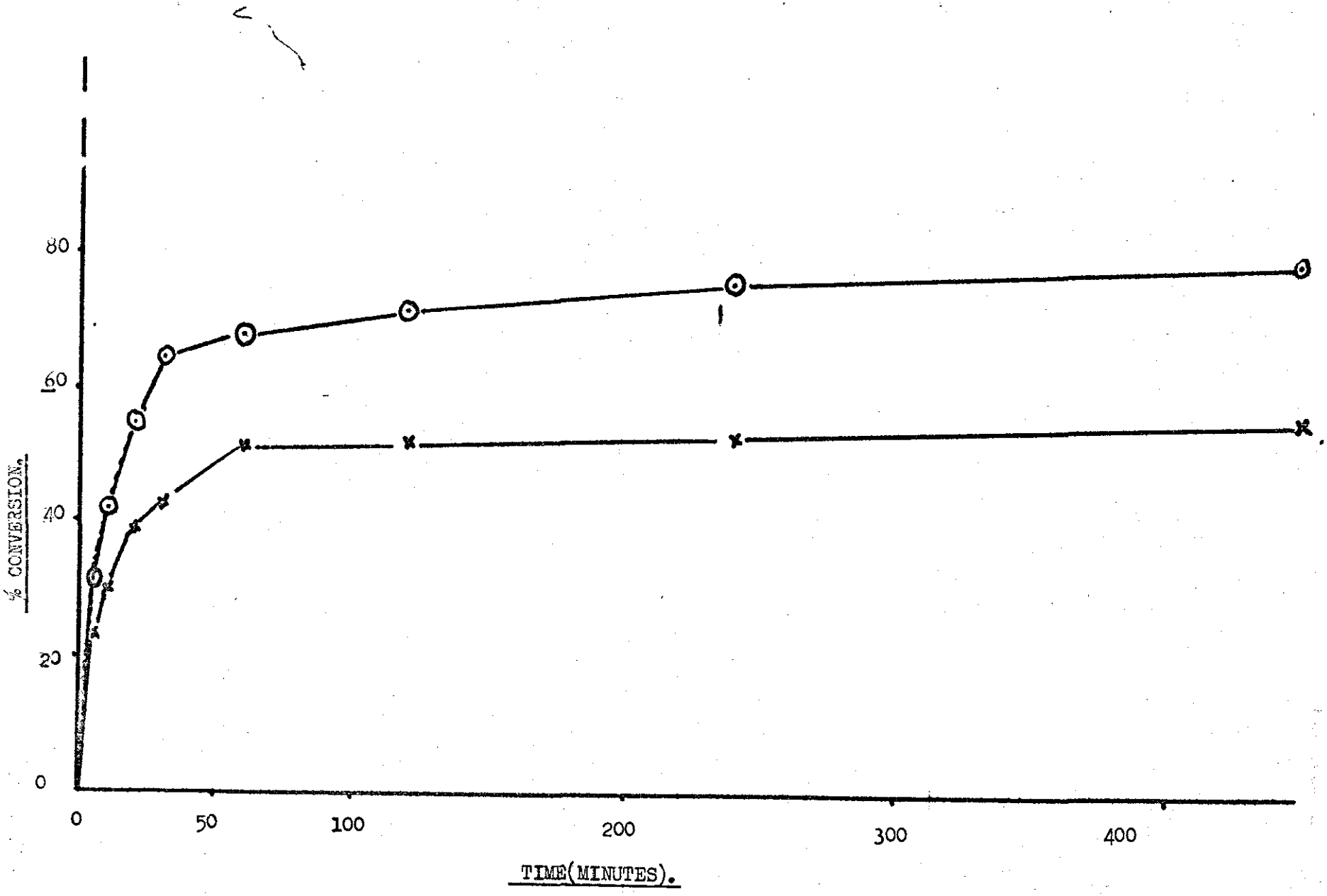


Fig.xiii. Conversion of tetradecanoic acid(0 – – 0) and pentadecanoic acid (x x) to a 9:10 monoenoic fatty acid of the same chain length after various times.

From these results it would appear that the fall off of enzyme activity is exponential with time with the majority of desaturation produced under these conditions in the first 30 mins. The initial rates of desaturation and indeed the rates at all the times during the experiment, are in the same ratio (see the given myristic - pentadecanoic figures, Table XVII) and equal to the ratio of the final samples. This proves that at all times during the life of the enzyme, the specificities indicated by the usual final sample are in action and that the figures quoted throughout this section are a reflection of the rates at any given time during the enzymic activity.

Conclusion

Much knowledge about the effect of bovine serum albumin, rates of desaturation, lipid involvement in desaturation and metabolic pathways in general has been learned from the results in this section and are discussed in the preceeding pages. There is no need to restate them here but it would be useful to bring out the points that have a direct bearing on the specificities, the steric environment and the interaction of the fatty acyl substrate and the enzymes responsible for the introduction of the first double bond in these systems.

Isolation of the pure crystalline enzyme followed by a precise X-ray study will provide the ultimate data about the structure of the desaturase and enzyme-substrate complex. However, since this does not seem possible in the foreseeable future, indirect studies of the desaturase yields knowledge about its structure and relationship with the substrate. This section describes several investigations and would indicate the following situation.

Morris and James[256, 257] have postulated that the active desaturase site may be a strained -S-S- bridge between two peptide chains which, on interaction with the substrate molecule, are converted to two sulphydryl groups. The release of strain between the chains provides the energy necessary to perform the reaction and to expel the substrate from the site. This theory has the advantage that the -S-S- bond distance is of the right order to undergo such a reaction and it would explain the inhibitory action of SH reacting compounds

e.g. iodoacetamide.

The highly specific position of desaturation in a given species clearly involves some specificity of attachment other than just randomly by London dispersion forces. In addition, some feature of the system ensures that it is always the D9 and D10 hydrogen atoms in stearic acid that are situated in such a position as to interact with the hypothetical disulphide bridge. The work described in this section suggests that the positional specificity is brought about either by the direct attachment of the carboxyl group of the substrate to the enzyme either directly or by a specific association of the CoA or ACP activating group with an "attachment" site on the enzyme in such a position as to bring the D9 and D10 carbon atoms of the substrate into action with the desaturase site. This occurs irrespective of the number of methylene groups beyond the tenth carbon atom. However, in general with enzymes capable of accepting the longer chain lengths (17, 18 and 19), there is a secondary chain length effect which suggests that desaturation is more favoured as the chain length increases. This is interpreted as being due to the fact that as the chain length increases, so does the London dispersion forces which hold the substrate to the enzyme surface. Thus myristate is much less tightly held to the desaturase than is stearate and hence the reaction at the active site is less favourable.

This is also illustrated by the fact that cis - 12 octadecenoic acid is desaturated but not as well as stearate. In other words, the primary specificity is satisfied but the secondary effect finds that the bend in the chain at 12:13 position renders the actual binding of the molecule to the substrate less favourable.

In the case of the cis 7 octadecenoic and cis 11 octadecenoic acids, the fact that desaturation is completely inhibited could be due to the steric effect of the actual desaturase centre being too far removed from C9 and C10 by the bend in the molecule being so close. Alternatively, being in conjugation with the actual hydrogen atoms which would be removed, it is more likely that

the chemistry of the desaturation is affected.

The increase in desaturation with chain length reaches a maximum at C18 and falls off, in some cases dramatically, with the addition of one further carbon atom in nonadecanoic acid.

This is probably due to the position of a steric block, e.g. a side group on the enzyme surface interacting with the terminal methyl group. This would tend to lift the substrate molecule off of the enzyme surface and hence weaken the binding forces. This is also indicated by the fact that 18-nonadecenoic acid is desaturated almost as well as stearate and this is discussed more fully on (page 77).

Thus, in the formation of a monoenoic acid from a saturated precursor, the position of the double bond depends on the distance from the carboxyl end of the molecule which is attached to the enzyme and the rate of desaturation depends on the strength of the combined binding forces between the enzyme and the substrate.

EXPERIMENTAL

Materials

In this section all solvents used were redistilled before use and all chemicals used were of the analytical grade.

The specific activity of all radioactive substrates used was approximately 40 mC/mM and where synthetic materials have not been used the precursors were supplied by the Radiochemical Centre, Amersham, Bucks. All radioactive precursors were dispersed in distilled water containing a small amount of sodium carbonate. 1 drop of a 10% aqueous solution of Tween 20 was added and ultrasonication used to aid dispersal. The concentration of each radiosubstrate was thus made up to 10μc 1ml.

Radiochemical Gas liquid chromatography

Throughout this thesis reference is made to radiochemical gas liquid chromatography (RGLC) with little detail given. The radiochemical gas liquid chromatograph is an instrument that separates, quantitatively detects and records both the mass and the radioactivity of compounds labelled with ^{14}C and capable of separation on normal gas-liquid chromatograms. The instrument referred to throughout this thesis is a radiochemical gas liquid chromatograph based on the original design of James and Piper [200, 258] later modified by James and Hitchcock.[259]

The basic principle of the instrument is that the components emerging from a conventional gas liquid column are converted to carbon dioxide and hydrogen. The mass of the products is estimated by the response of a Katharometer detector to the hydrogen in the gas stream and the radioactivity counted as ^{14}C carbon dioxide in a proportional gas flow counter.

The sample is introduced to the column by pipetting a solution into a small loading tube and evaporating off the solvent. This loading tube is then placed at the top of the column where the sample is volatilised at the temperature of the column and is carried on to the column by the stream of carrier gas flowing through the loading tube.

The columns used were normally packed columns of FFAP which is a polar stationary phase obtained from Varian Aerograph AG, or SE30 which is a methyl silicone gum rubber stationary phase having non polar characteristics.

At the end of the column is a furnace tube to combust the eluted materials to carbon dioxide and water. This tube is half filled with copper oxide to oxidise the materials to carbon dioxide and water and the second half of the tube is packed with iron filings to reduce the water to hydrogen.

The Katharometer is sensitive to hydrogen in argon and detects the mass of materials in this way and the message is passed to a 1mV recorder where a trace is obtained.

After passing through the Katharometer the effluent gases have carbon dioxide bled into them to give a concentration of 5% carbon dioxide in argon.

This mixture then flows through a proportional counter tube the signal from which is passed to a ratemeter and hence to a 1 mV recorder.

The output of the ratemeter can be presented as a differential record or electronically integrated.

The sensitivity of this instrument is such that it will detect 1 nc ^{14}C with ease as a differential record and 0.1 nc with ease as an integrated record, counting efficiency being 100%.

The Katharometer response is linear from 1 - 500µg. Once the response to a master compound is known the response to any other compound can be calculated from the molar hydrogen yield relative to that master substance. Some typical traces are shown on the next page.

Scintillation Counting

Unless it is reported to the contrary, all scintillation counting has been done in 0.4% P.P.O. in toluene solution and counting has been on the Packard 4000 series Tri-carb liquid scintillating spectrometer set to count for 10 minutes or 20,000 counts. P.P.O. is 2,5 diphenyl oxazole scintillation

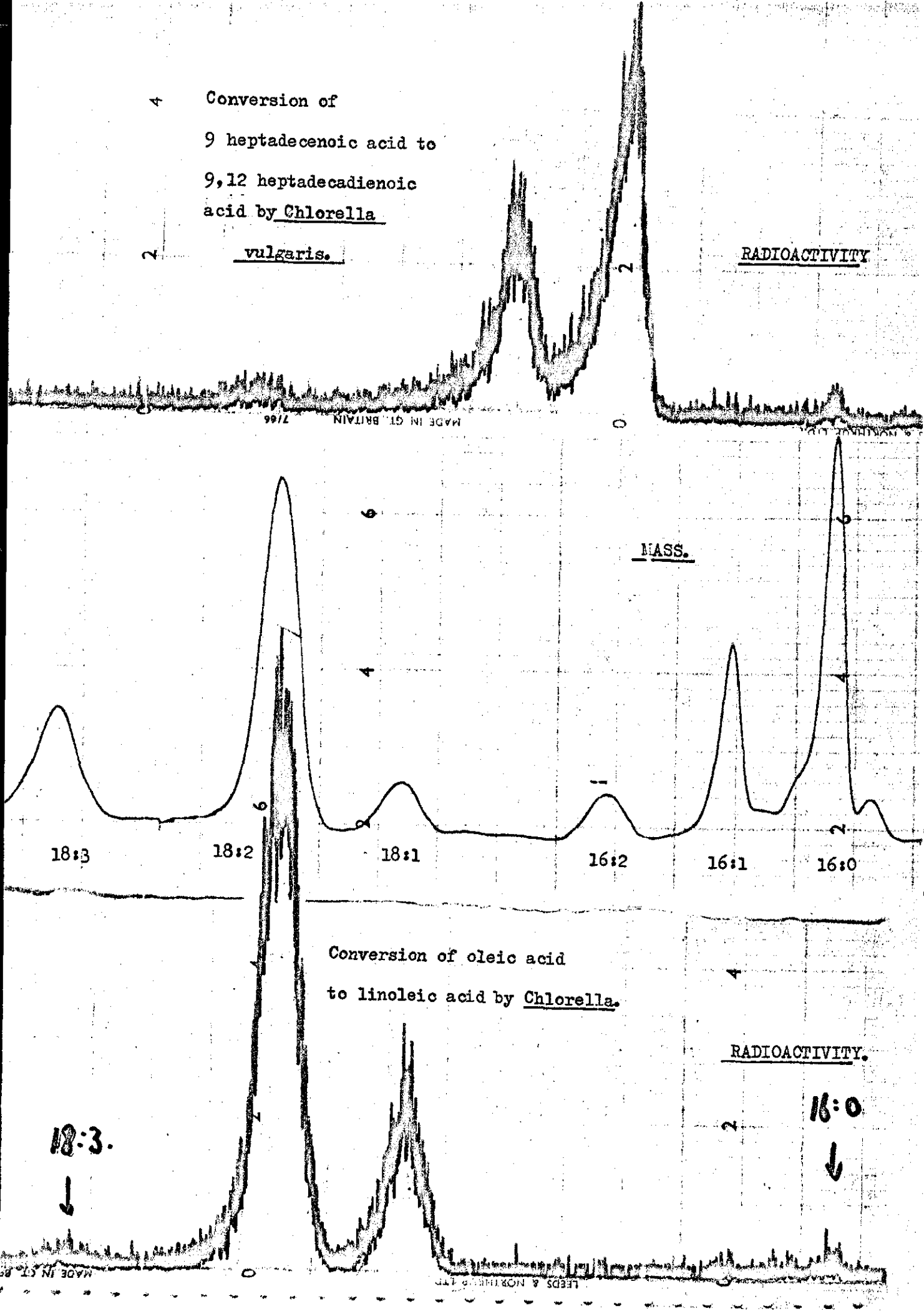


Fig. 1. EXAMPLES OF RADIOCHEMICAL GAS-LIQUID CHROMATOGRAPHY.

fluid supplied by Packard.

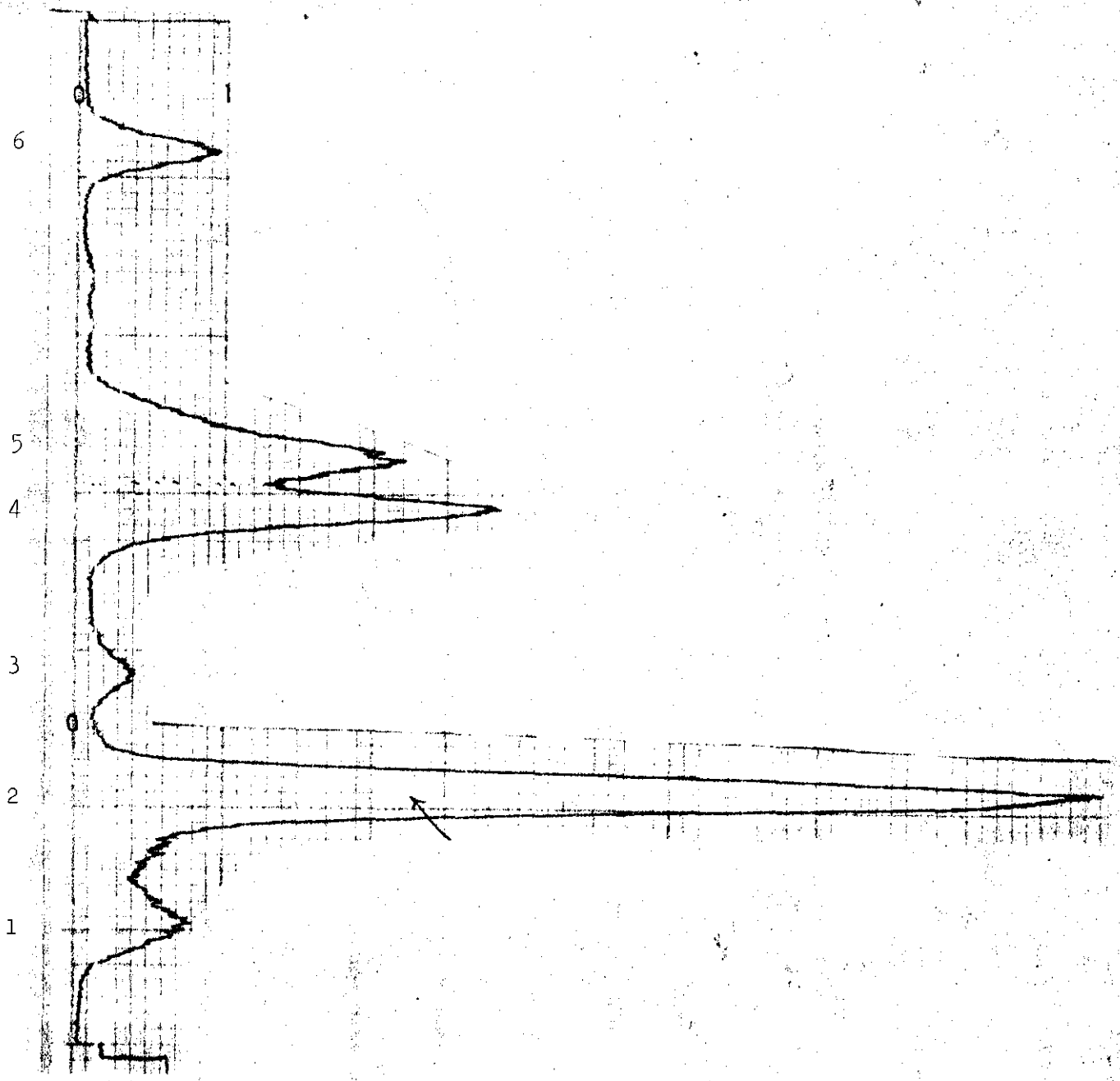
Radiochemical Thin layer Chromatography Scanning

The scanning here was done on either a prototype instrument designed and built by James et al[260] or on a commercially adapted version of it. The latter was the Panax RTLS-1 with a proportional counter tube of dead time 20μsecs, a time constant of 3 seconds and a carrier gas of 2% propane in argon.

The basic principle of both these machines is that a proportional counter tube with an open slit (10 x 1 mm) is placed about 1mm above the surface of the thin layer plate which is traversed slowly under the slit. The pulses are once more fed via a ratemeter to a 1mV recorder, the chart paper of which travels at the same speed as the plate, where they are displayed either as an integrated trace or a differential record. An example of the use of this instrument is shown on the next page.

I Experiment 1 To determine the extent and position of desaturation of various saturated fatty acids by a microsomal preparation from the mammary gland of a fully lactating goat.

Mammary gland tissue (17.0g) was obtained surgically from a fully lactating goat. Care was taken during all the following procedures including the centrifugation to keep the tissue and reagents below 5°C. All solutions were chilled and all operations were carried out in a cold room at about +2°C. The tissue was minced with sharp pointed scissors and washed with sucrose-EDTA tris buffer adjusted to pH 7.6 (50 mls) to remove the milk. The washed tissue was then homogenised with sucrose EDTA tris buffer adjusted to pH 7.6 (80 mls) using a Potter homogeniser. This homogenate was centrifuged for 6 mins in a Serval centrifuge at 8,000 rpm. The supernatent from this spin which removed the cell debris was centrifuged again for 10 mins at 12,000 rpm with a AH 50 head using a MSE super 40 centrifuge. This process removed the mitochandria. The supernatent was



1 Basal material

2 cis, cis linoleate

3 cis, trans linoleate

4 cis 9-octadecenoate

5 cis 12-octadecenoate

6 stearate

Fig. ii Examples of Radiochemical Thin Layer Scanning.

The above scan is of the reaction products from the partial reduction of (1^{14}C)-linoleic acid with hydrazine. The plate is 30% silver nitrate impregnated silica gel and has been developed twice with toluene at -15°C.

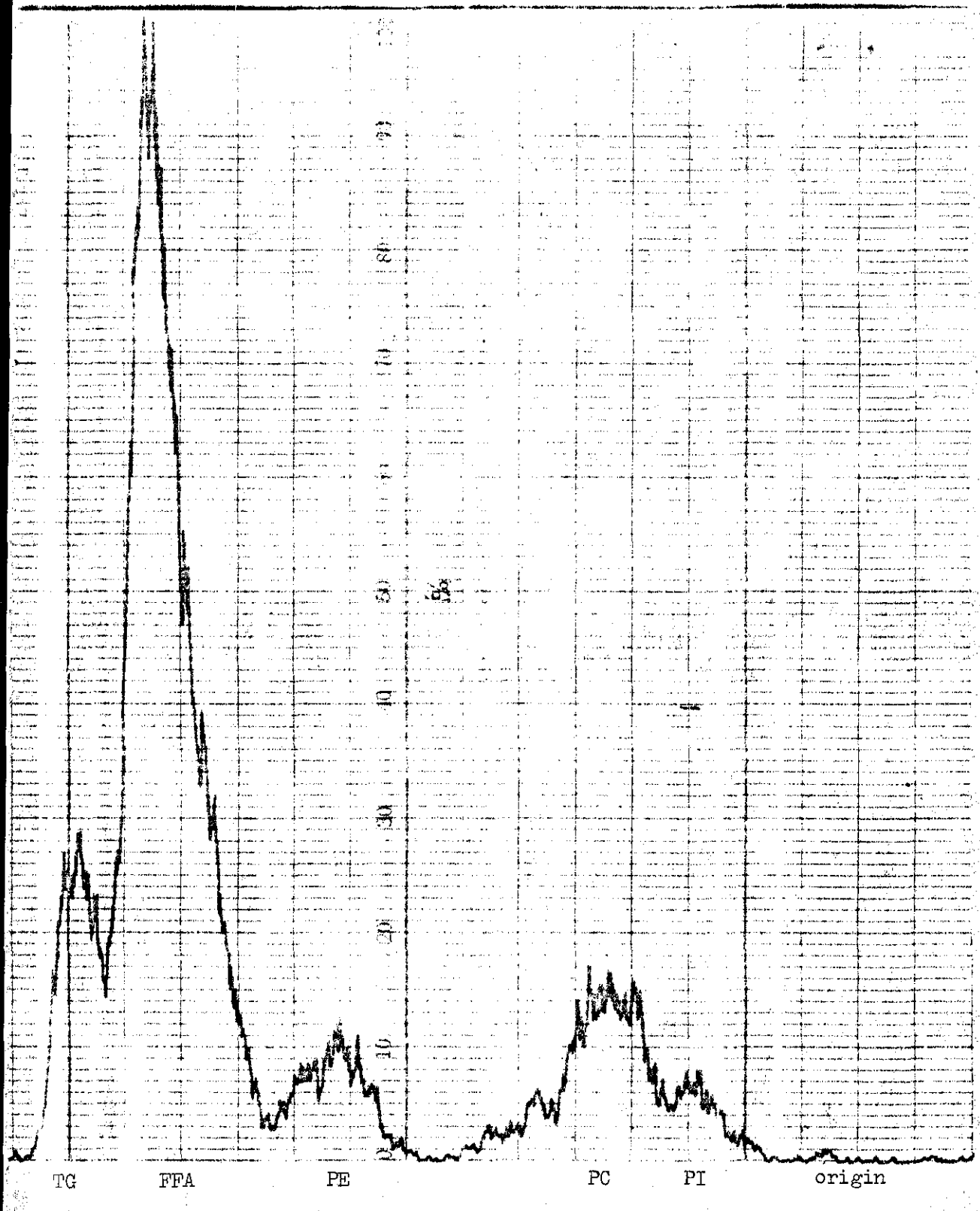
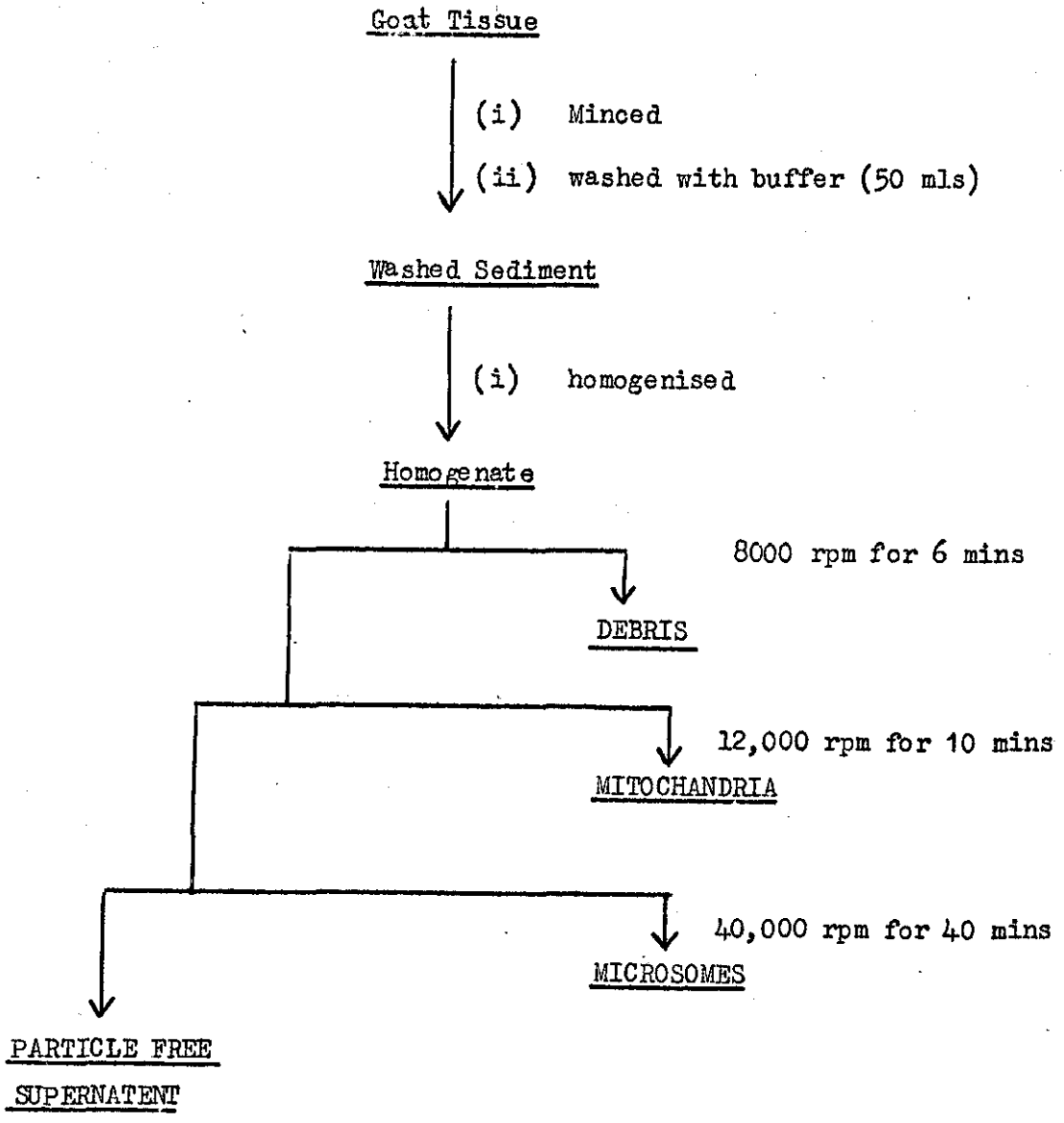


Fig. iii Examples of Radiochemical Thin Layer Scanning

The above scan is of the lipids of hen liver run on a 0.25 mm silica gel plate in 65,25,4 chloroform, methanol, acetic acid.

once more centrifuged this time at 40,000 rpm for 40 mins. This spin produced a microsomal pellet and a particle free supernatent. Both these materials were isolated and kept at low temperature (0°C). The microsomal pellet was rehomogenised in 25 mls buffer using a small Potter hand homogeniser.

The complete isolation procedure is shown diagramatically below



The sucrose tris EDTA buffer used was 0.25M Sucrose, 3M tris and 5M EDTA adjusted to pH 7.6 with sodium hydroxide.

Nine transmethylation tubes were set up each containing the following cofactors dissolved in distilled water.

Cofactor	Concentration	Amount/tube (mls)
phosphate Buffer	0.5M pH 7.4	0.5
ATP	200 mM	0.1
CoA	2 mM	0.1
$MgCl_2$	200 mM	0.1
NADH	10mgm/ml	0.1
NADPH	5 mgm/ml	0.1
WATER	-	3 mls.

To each of these tubes was added a substrate preparation made up of 100 µl of substrate suspension (= 1µc), 0.1g 1 ml bovine serum albumin solution (300 µl) and phosphate buffer pH 7.4 100µl.

The substrates used in this experiment were ($1^{14}C$) - decanoic, ($1^{14}C$) - dodecanoic, ($1^{14}C$) - tetradecanoic, ($2^{14}C$) - pentadecanoic, ($1^{14}C$) - hexadecanoic, ($1^{14}C$) - heptadecanoic, ($1^{14}C$) - octadecanoic, ($2^{14}C$) - nonadecanoic and ($1^{14}C$) - cis 12 octadecenoic.

A second series of nine tubes were set up exactly as above except that the 3 mls of water was replaced by 3 mls of the particle free supernatent.

To each of the eighteen tubes was added 600µl of the microsomal preparation and oxygen gas was bubbled through for 1 minute. The tubes were then sealed and incubated at 37°C for 1 hour in a reciprocating water bath.

Termination of the incubation

At the end of the incubation period the whole of the incubation medium was poured into 2 : 1 w/v chloroform methanol (50 mls) and allowed to stand for 1 hour to extract the lipids and precipitate the protein material. This method of ending incubations was used in all cases and the details will not be referred to again during this section.

Extraction of the lipids

This procedure which is a modification of that used by Folch et al [261] and was used exactly as below for all the incubations performed in this thesis.

The incubation medium quenched by the addition of chloroform methanol as above was filtered to remove any precipitated material. The filtrate was transferred to a separating funnel and 0.75% saline (15 mls) was added and the whole shaken. Two layers separated on standing and the bottom layer was collected in a flask. The upper layer was once more shaken with chloroform (20 mls) and the lower layer added to the flask containing the first extract. The solvent was removed from this flask at the pump to yield the extracted lipids which were dried by azeotropic distillation with ethanol. The lipids thus extracted were dissolved in 2 : 1 v/v chloroform methanol (5 mls) and stored at -30°C.

Transmethylation of the extracted lipids

This method of conversion of lipids to the methyl esters of their component fatty acids is that used by Nichols and James [262] and is standard procedure throughout this thesis.

A portion of the extracted lipids was placed into a 25 ml tube and the solvent removed at the pump. The lipids were redissolved in a mixture of benzene, methanol, sulphuric acid, (10 : 20 : 1) v/v/v (5 mls) and refluxed for 90 mins. After this time the tube was cooled and ether (10 mls) was added. The ether was then washed acid free with 5 x 5 ml aliquots of water. The ether was then removed at the pump and the methyl esters formed in the reaction dried by azeotropic distillation with ethanol. The esters were finally dissolved in ether (0.5 mls).

Identification and measurement of the radioactive products

The radioactive products from incubations were identified by the injection of a sample of the methyl esters on to a radiochemical gas liquid

chromatograph and records of both the mass peaks and the radiochemical peaks were obtained. By comparison of the retention volumes with a standard run of known esters the radiochemical peaks were identified. The relative proportions of the radiochemical peaks were determined by triangulation.

Separation of the monoenoic products from the saturated precursors

A portion of the ester solution was dissolved in ether (5 mls) and streaked on to a 0.25mm silver nitrate impregnated silica plate using a Desaga sample applicator. The plate was developed with a solvent consisting of 15% ether in petrol ether. The bands were visualised by spraying with dichlorofluoroscein solution and illuminating with U.V.light.

The monoene band was isolated and the esters eluted from the silica with ether. The ether was removed at the pump and the monoenes dissolved in von Rudloff's oxidation solutions.

These solutions were made up as follows:-

Solution A — 25.6 mgm K_2CO_3
30 ml
20 mls water

Solution B — 8 mgm $KMnO_4$
427 mgm $NaIO_4$
50 mls water.

The monoenes were first dissolved in solution A (1 ml) and solution B (1 ml) was immediately added. The solution was shaken at room temperature for 2 hours. If during this time the solution lost its pink colouration equal amounts of both oxidant solutions were added to the reaction.

After the 2 hours water (5 mls) was added and the excess reagents were destroyed by bubbling sulphur dioxide gas through the solution until it became colourless.

The products were then extracted with 3 x 5 mls diethyl ether and

the extracts bulked. The bulked extracts were washed acid free with distilled water (5 x 5 mls). The ether was removed at the pump and the products dried by azeotroping with ethanol.

The dried products were dissolved in methanol (200µl) and an ethereal solution of diazomethane was added until the yellow colouration remained. The excess diazomethane and the solvent was removed at the pump to yield a mixture of mono and di carboxylic esters which were dissolved in diethyl ether (0.5 mls).

Since the label was in the 1 on 2 position it was only the dicarboxylic esters that were radioactive. The radioactive dicarboxylic esters were identified by injecting 100µl of the solution on to a RGLC containing an FFAP column at 230°C. The radioactive peaks were identified by comparison with standard dicarboxylic esters run under the same conditions.

Investigation of lipid incorporation

In order to investigate the amount of incorporation into lipids the original lipid solution (1 ml) was concentrated to 100µl and this solution pipetted on to a 0.25mm silica plate drawn into channels 1cm wide. Suitable lipid standards were also placed on the plate which was developed in 15% diethyl ether in petrol ether to which had been added formic acid (1 ml).

By association with the formic acid free fatty acids migrate as a discrete spot; otherwise they smear up from the origin.

After developing the solvent was removed, care being taken to see that all the formic acid had evaporated, and the plate scanned for radioactivity on the Panax RTLS-1 to obtain a differential record or the prototype scanner^{260} for an integrated scan. The plate was then sprayed with dilute sulphuric acid and charred at 250°C to allow the spots to be identified by comparison with the standards.

Investigation of the distribution of labelled precursor and desaturation product in polar lipids and free fatty acids

The above procedure was repeated except that after the plate had been scanned the bands corresponding to the free fatty acid and polar lipid were

scraped off, eluted with ether and 2 : 1 v/v chloroform methanol respectively, and converted to methyl esters with diazomethane or by transmethylation respectively as described before.

The proportion of saturated precursor and monoene produced in each fraction was determined by RGLC as before.

Estimation of the amount of desaturation that has occurred when the conversion is low i.e. < 5%

When the conversion to monoene is small less than 5% errors in triangulation due to peak tailing and triangulation became large. This was overcome by running the ester solution on a 10% silver nitrate impregnated 0.25 mm silica plate channelled into 1 cm channels and developing it in 15% ether in petrol ether. After evaporation of the solvent the plate was scanned on an integrated setting. A second check was done by scraping off the monoene and saturated bands, dissolving the fatty esters in 0.4% P.P.O. toluene and counting the activity on a scintillation counter.

I Experiment with a microsomal fraction of hen liver

1. Determination of the optimum requirements of the system

a) To check the nucleotide requirement

A warren hen was sacrificed and the liver (35 g) removed. From the time of removal until the incubation, care was taken to keep the tissue and reagents at 0-5°C.

The liver was minced with sharp pointed scissors, 0.3 molar sucrose buffer pH 7.4 (90 mls) was added and the mixture was homogenised by means of a mechanically driven Potter homogeniser. The homogenate thus prepared was centrifuged at 12,000 rpm for 15 mins. using an MSE super 40 centrifuge. This process removed the blood cell debris and mitochandria and left a suspension of microsomes in a particle free supernatent with a fatty layer at the top which was removed by straining the suspension through muslin cloth.

Six tubes were set up as follows:-

	Tubes 1 - 3	Tubes 4 - 6
Enzyme	3 mls	3 mls
0.5M phosphate buffer pH 7.4	1 ml	1 ml
CoA	1 mgm	1 mgm
0.1M. ATP solution	1 ml	1 ml
0.5M Lactic acid	1 ml	-
NAD^+	2 mgm	-
NADH	-	2 mgm

All the above solutions were previously adjusted to pH 7.4 with sodium hydroxide solution. To each of the six tubes was added a substrate prepared as follows: ($1^{14}C$) octadecanoic acid suspension (0.2 mls) suspended in 2.5% Bovine serum albumin (4 mls) to which had been added 0.1M magnesium chloride solution (100µl).

After the addition of the substrate the tubes were gassed with oxygen for 1 minute then incubated in a reciprocating water bath at 37°C for 6 hours.

At the end of this period the reaction was stopped and the lipids extracted. A portion of the lipids was converted to the respective methyl esters by transmethylation. A 50µl aliquot of each of the six ester solutions was separately injected on to a radiochemical gas liquid chromatograph with an FFAP column at 230°C. The radioactive esters were identified and measured as before.

The lipid incorporation was investigated by T.L.C. on silica plates developed in ether petrol ether formic acid and scanned as before. An investigation of the incorporation of label into the individual phospholipids was performed by following the above procedure except that the developing solvent was chloroform methanol, acetic acid, water. (85 : 15 : 10 : 3.7) v [263] .

The distribution of labelled precursor and desaturated product in the polar lipid and free fatty acids was determined as before and the results obtained by radiochemical gas liquid chromatography on SE30 at 230°C.

b) To check the mass of reduced nucleotides

Co enzyme A and ATP required

A Warren hen was sacrificed and its liver (39.0g) removed and a microsomal suspension was prepared as described in the previous experiment. Six tubes were set up as follows:-

	Tubes 1 - 3	Tubes 4 - 6
Microsomal Suspension	3 mls	3 mls
0.5M KH_2PO_4 pH 7.4	1 ml	0.5 ml
CoA	1 mgm	0.15 mgm
0.1M ATP	1 ml	0.2 ml
NADH	2 mgm	1 mgm
NADPH	-	$\frac{1}{2}$ mgm

To each tube was added a substrate as follows:-

	Tubes 1 - 3	Tubes 4 - 6
1 ^{14}C octadecanoic	0.2 ml	0.2 ml
Bovine serum albumin	4 ml (2.5%w/v)	0.6 ml (0.1g/ml)
0.1M $Mg\ Cl_2$	0.1 ml	0.2 ml.
0.5M phosphate buffer pH7.4	-	0.2 ml.

After the addition of the substrate the tubes were oxygenated for 1 minute, stoppered and incubated in a reciprocating waterbath at 37°C for 6 hours. After this time the lipids were extracted and a portion of the lipid solution transmethylated to yield the methyl esters. The methyl esters were then identified and measured by RGLC on a SE30 column at 230°C. The lipid incorporation was determined by TLC followed by scanning in the usual way and the individual phospholipids examined as in the previous experiment. The distribution of labelled precursor and desaturated product in the polar lipid and free fatty acid fraction was determined as before by RGLC of the derived methyl esters on SE30 at 230°C.

2. Investigation of the activation reaction.

a) The effect of the mass of substrate on the degree of desaturation

A Warren hen was sacrificed and a microsomal suspension was prepared from its liver as before. Six incubation tubes were set up as follows:-

Microsomal preparation	3 mls
0.5M KH_2PO_4 pH 7.4	0.5 ml
CoA	0.15 mgm
0.1M ATP	0.2 ml
NADH	1 mgm
NADPH	$\frac{1}{2}$ mgm

In addition to the usual ($1^{14}C$) stearic acid suspension which had a specific activity of 0.2 μc/μg non radioactive stearic was mixed with this suspension. A spot of sodium bicarbonate and Tween 20 was added and the whole resonicated to give specific activities of 0.008 μc/μg and 0.004 μc/μg.

1 μc of each of these substrates were suspended on (0.1g/ml) of Bovine serum albumin solution (0.6 ml). 0.1M Magnesium chloride solution (0.2 mls) and 0.5M phosphate buffer pH 7.4 (0.2 mls) were also added. Half of each of these three substrates were added to a separate incubation tube and the whole oxygenated for 1 minute, stoppered and incubated in a reciprocating waterbath at 37°C for 6 hours. After this time the lipids were extracted and a portion of the lipid solution transmethylated to yield the methyl esters. The methyl esters were identified and measured by RGLC on a FFAP column at 230°C. With the 0.2 μc/mg and the 0.004 μc/mgm samples the mass charts from the above RGLC runs were triangulated to estimate the actual mass of oleate produced.

The lipid incorporation and the distribution of labelled precursor and desaturated product in the polar lipid and free fatty acid fractions was determined as in the previous experiments.

b) The Effect of enzyme concentration on desaturation

Using the enzyme source prepared in the previous experiment six tubes were set up as follows:-

	Tubes 1 and 2	Tubes 3 and 4	Tubes 5 and 6
Enzyme Source	3 mls	6 mls	9 mls
0.3M Sucrose Buffer	6 mls	3 mls	-
0.5M $KH_2 PO_4$	0.5 ml	0.5 ml	0.5 ml
CoA	0.15 mgm	0.15 mgm	0.15 mgm
0.M ATP	0.2 ml	0.2 ml	0.2 ml
NADH	1 mgm	1 mgm	1 mgm
NADPH	0.5 mgm	0.5 mgm	0.5 mgm

To each of these tubes was added a substrate consisting of ($1^{14}C$) - octadecanoic acid suspended on (0.1 g/ml) Bovine serum albumin (0.6 ml) to which had been added 0.1M magnesium chloride (0.2 mls) and 0.5M phosphate buffer pH 7.4 (0.2 mls). The contents of each tube were oxygenated for 1 minute and the tubes stoppered and incubated for 6 hours at 37°C in the usual way. The reaction was stopped and the lipids extracted as before. Half the lipid sample was transmethylated and the methyl esters formed were identified and measured by RGLC on an FFAP column at 230°C. The lipid incorporation was investigated by T.L.C. and scanned as in all previous experiments.

3. Investigation of the transfer of exogenous monoenoic fatty acids and the immediate products of desaturation in this system

a) Exogenous monoenoic fatty acids

A Warren hen was sacrificed and a microsomal suspension was prepared from its liver as before. Eight tubes were set up containing enzyme source and cofactors as in experiment 2a (page 107). The substrates used in this experiment were the isomeric monoenes ($1^{14}C$) cis 7 octadecenoic, ($1^{14}C$) - cis 9 octadecenoic, ($1^{14}C$) - cis 11 octadecenoic and ($1^{14}C$) - cis octadecenoic acids. These were suspended on bovine serum albumin as before in experiment 2b (page 109). After the addition of the substrates the incubation media were oxygenated for 1 minute and incubated at 37°C. for 6 hours. The reaction was quenched and the lipids extracted in the usual way and a portion of the lipids transmethylated. The methyl esters were identified by RGLC on an FFAP column

at 230°C. The lipid incorporation was investigated by T.L.C. followed by scanning in the usual way.

The methyl esters were subjected to silver ion chromatography to separate the monoenoic esters from the dienoic esters. The conditions for this separation are identical to those used to separate the monoenoic esters from the saturated esters (page 105).

When the monoenoic esters and the dienoic esters had been isolated they were separately subjected to von Rudloff's oxidative cleavage [217] as before (page 105).

b) The immediate products of desaturation

The enzyme source produced in the above experiment was incubated for 2 hours at 37°C with (1-^{14}C) stearoyl coenzyme A thiolester*(15µg) and NADH (1mgm). The reaction was quenched in the usual way and the lipids extracted. Half the lipid sample was transmethylated and the resulting methyl esters identified and measured by RGLC on an FFAP column at 230°C. The lipid incorporation was investigated by T.L.C. The amount of desaturated product and labelled precursor in the polar lipids and free fatty acid fraction was determined in the usual way.

4. To determine the effect of chain length of substrate on the extent and position of desaturation in a microsomal preparation from the liver of a hen

A Warren hen was sacrificed and the liver (37.5g) removed. From the time of removal until the incubation, care was taken to keep the tissue and reagents as cold as possible.

The liver obtained was minced with sharp pointed scissors and 0.3M sucrose buffer pH 7.4 (100 ml) was added. The above mixture was homogenised by means of a mechanically driven Potter homogeniser. The homogenate thus prepared was centrifuged at 2,000 r.p.m. for 15 minutes using an M.S.E. super 40 refrigerated centrifuge.

This process removed the blood cell debris and microchondria and left

* Kind gift of Dr. M. I. Gurr

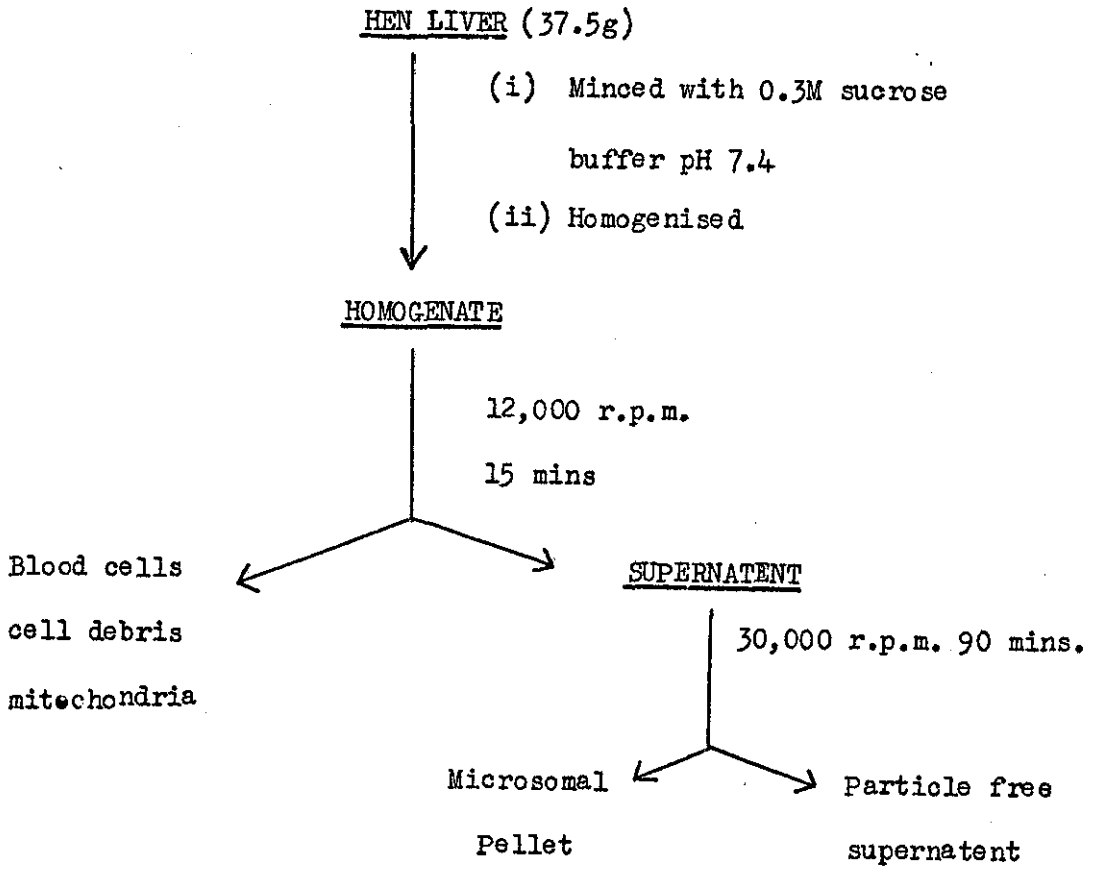
a suspension of microsomes in a particle free supernatent.

The supernatant from this spin was strained through muslin to remove the layer of fat which had collected on the surface during the spin and recentrifuged at 30,000 r.p.m. for 90 minutes.

This spin produced a microsomal pellet and a particle free supernatent. Both these materials were isolated and kept at low temperature, ($0^{\circ}C$).

The microsomal pellet was rehomogenised in 0.3M sucrose buffer pH 7.4 (25 ml) using a small Potter hand homogeniser.

The complete isolation procedure is shown diagramatically below.



Nine transmethylation tubes were set up and to each was added the following co-factors dissolved in distilled water:-

Co-factor	Amount
Microsomal suspension	1 ml
0.5M $KH_2 PO_4$ (pH 7.4)	0.5 mls.
CoA	15 mgm
0.1M ATP	0.2 mls

Co-factor	Amount
NADH	1 mgm
NADPH	$\frac{1}{2}$ mgm
0.3M Sucrose buffer pH 7.4	3 mls

To each of these tubes was added a substrate preparation made up of 100 µl of substrate suspended on bovine serum albumin to which had been added magnesium chloride and phosphate buffer as in experiment 2b. The substrates used in this experiment were ($1^{14}C$) - decanoic, ($1^{14}C$) - dodecanoic, ($1^{14}C$) - tetradecanoic, ($2^{14}C$) - pentadecanoic, ($1^{14}C$) - hexadecanoic, ($1^{14}C$) - heptadecanoic, ($1^{14}C$) - octadecanoic and ($2^{14}C$) - nonadecanoic acids.

Another set of eight tubes were set up exactly as above except that the 3 mls of sucrose buffer was replaced by 3 mls of the particle free supernatent. Also a last tube was set up containing the microsomal suspension (3 mls), NADH (1 mgm) and ($1^{14}C$) stearoyl coenzyme A thioester (15µg)*. All the tubes were aerated for 1 minute with air containing 70% oxygen [264], the tubes stoppered and incubated at 37°C for 6 hours. The reaction was stopped and the lipids extracted in the usual way. Half the lipids were transmethylated in the normal way and the radioactive esters identified and measured after injection of a portion on to a RGLC containing an FFAP column at 230°C. The monoenoic fatty esters were isolated from the rest of the ester solution by silver ion chromatography and the esters subjected to oxidative cleavage by von Rudloff's technique. (see page 105). Comparison of the RGLC trace of the resultant dicarboxylic esters injected on to a FFAP column at 230°C with a standard run of known esters, allowed the monoenoic ester band position to be determined.

The lipid incorporation was determined by T.L.C. as before and the amount of labelled precursor and desaturated product in both the polar lipids and the free fatty acids determined by the RGLC on SE30 at 230°C of the methyl esters prepared from them.

* The kind gift of Dr. M. I. Gurr.

5. To Investigate the effect of Bovine Serum Albumin as substrate carrier, on desaturation

A white shaver hen was sacrificed, its liver removed, and a microsomal suspension prepared as in experiment 2a. Sixteen tubes were set up as follows:-

	Tubes 1 - 8	Tubes 9 - 16
Enzyme source	3 mls	3 mls
0.5M KH_2PO_4	0.7 mls	0.5 mls
CoA	0.15 mgm	0.15 mgm
0.1M ATP	0.2 mls	0.2 mls
NADH	1 mgm	1 mgm
NADPH	½ mgm	½ mgm
0.1M Magnesium chloride	0.2 mls	-
Radioactive Substrate	0.1 ml	-

To tubes 9 - 16 were added substrates prepared as before (see experiment 2b page 109). The radioactive substrates used in this experiment were ($1^{14}C$) - decanoic, ($1^{14}C$) - dodecanoic, ($1^{14}C$) - tetradecanoic, ($2^{14}C$) - pentadecanoic, ($1^{14}C$) hexadecanoic, ($1^{14}C$) - heptadecanoic, ($1^{14}C$) - octadecanoic and ($2^{14}C$) - nonadecanoic acids. All the tubes were aerated for 1 minute with 70% oxygen in air and incubated as usual for 6 hours at 37°C. The reaction was quenched and the lipids extracted as usual. The lipids were transmethylated to yield their corresponding methyl esters and these esters identified and measured by RGLC on an FFAP column at 230°C.

6. To investigate the effect of chain length on the rate of desaturation.

A white shaver hen was sacrificed and a microsomal suspension produced from the liver in the usual way. Three tubes were set up as follows:-

Enzyme Source	6 mls
0.5M KH_2PO_4	1.0 ml
CoA	0.3 mgm
0.1M ATP	0.4 mls
NADH	2 mgm
NADPH	1 mgm

The substrates were set up as follows:-

Radioactive substrate	0.5 mls (= 5 μc)
0.1g/ml Bovine serum albumin	0.4 mls
0.5M phosphate buffer	0.2 mls
0.1M magnesium chloride	0.2 mls

The radioactive substrates used in this experiment were ($1^{14}C$) - tetradecanoic, ($2^{14}C$) - pentadecanoic and ($1^{14}C$) - hexadecanoic acids. The incubation media in the tubes were aerated for 1 minute with 70% oxygen in air and the incubation was begun at 37°C. Samples of 0.5 mls of the incubation medium were removed after 5 mins, 10 mins, 20 mins, 30 mins, 1 hour, 2 hour, 4 hours and 8 hours. As each sample was removed it was quenched by addition to 2 : 1 v/v chloroform-methanol (20 mls) in the usual way. The lipids were extracted and transmethylated to give the corresponding methyl esters which were identified and measured by RGLC on an FFAP column at 230°C.

7. <u>To Investigate the effect of a terminal double band on the amount of desaturation</u>

The microsomal suspension from the liver of a shaver hen was prepared as in the previous experiment. Five tubes were set up containing 3 mls of this suspension and cofactors as in experiment 2a. Radioactive substrates were suspended on bovine serum albumin with magnesium chloride and phosphate buffer added as in experiment 2b. The radioactive substrates used in this experiment were ($1^{14}C$) - hexadecanoic, ($1^{14}C$) - heptadecanoic, ($1^{14}C$) - octadecanoic, ($2^{14}C$) - nonadecanoic and ($1^{14}C$) - 18 nonadecenoic acids. The incubations were carried out exactly as before and after the reactions had been quenched the lipids were extracted and transmethylated as before. An aliquot of the methyl esters was injected on to a RGLC containing an FFAP column at 230°C. The radioactive esters were thus identified and measured. In the case of the esters from the incubation with ($1^{14}C$) - 18 nonadecenoic acid they were oxidatively cleaved by von Rudloff's technique[217] and the position of the double band determined as before.

III Experiments with whole cell cultures of Torulopsis apicola

1. Effects of chain length on degree of desaturation

A culture of Torulopsis apicola was grown from a dried culture in 50 mls of the following medium for 24 hours at room temperature and then the whole transferred to 500 mls of medium in a 2 litre flask equipped with a glass tube to allow the contents to be aerated. This incubation was carried out at room temperature under continuous illumination for 2 - 3 days by which time a healthy growth had been obtained.

The medium used was as follows:-

Glucose	100 gm.
Yeast extract	5 gm.
Urea	1 gm.
$KH_2 PO_4$	1 gm
$Mg SO_4 7H_2O$	3 gm
Metal concentrate	1 ml
Water	to 1 litre

The pH of this solution was adjusted to 5.8.

The metal concentrate was made up as follows:-

$Fe SO_4 7 H_2O$	0.1 gm
$Cu SO_4 5 H_2O$	0.075 gm
$Zn SO_4 7 H_2O$	0.1 gm
$Mn SO_4 4 H_2O$	0.1 gm
$K_2 M_o O_4$	0.01 gm
Water to	100 mls

1 ml of dilute HCl was added to give a clear solution.

After 2 - 3 days when the culture was growing vigorously 5 ml aliquots were removed and placed in eight incubation tubes. To each of these tubes was added a radioactive precursor suspension (0.1 ml). The radioactive substrates used in this experiment were ($1^{14}C$) - decanoic, ($1^{14}C$) - dodecanoic, ($1^{14}C$) - tetradecanoic, ($2^{14}C$) - pentadecanoic, ($1^{14}C$) - hexadecanoic, ($1^{14}C$) - heptadecanoic, ($1^{14}C$) - octadecanoic and ($2^{14}C$) - nonadecanoic acids.

The incubations were allowed to run for 6 hours at 20°C under constant illumination from 4 x 40 watt daylight emission tubes. At the end of this period the reaction was quenched and the lipids extracted in the usual way. The lipids were transmethylated and an aliquot of the resulting methyl ester solution examined by RGLC on an FFAP column at 230°C. From this the radioactive compounds were identified and measured.

The rest of the methyl ester solution was subjected to silver ion chromatography to isolate the monoene fraction. Because there was more than one monoene formed in some of these incubations the required monoene i.e. the one of the same chain length as the labelled precursor was isolated by preparative GLC on an FFAP column at 230°C.

The instrument used for this and all subsequent preparative gas liquid procedures was a Pye 104 flame ionisation instrument equipped with a stream splitter. This allowed 1% to go to the detector whilst the other 99% could be collected in a trap filled with Ballotini beads wetted with 2 : 1 v/v chloroform-methanol. Interchange of these traps allowed different fractions to be collected.

After collection the required compound was eluted from the trap with 2 : 1 v/v chloroform-methanol. When the required monoene had been isolated by this technique it was subjected to von Rudloff's oxidation (see page 105). The dicarboxylic esters obtained after methylation with diazomethane were identified by their relative retention volume on RGLC containing an FFAP column at 230°C.

2. <u>To investigate the band position in monoenes of longer chain length than the precursor saturated fatty acid.</u>

The above experiment was repeated and ($1^{14}C$) tetradecanoic acid suspension (0.5 mls) (≈ 5 μc) was added to the yeast in its nutrient medium (5 mls) and incubated as before. At the end of the incubation the lipids were extracted and transmethylated to yield the methyl esters. The monoenoic esters were isolated by silver ion chromatography and then separated into their

individual chain lengths by preparative GLC as in the previous experiment. The band positionsof all these esters were separately determined by von Rudloff's oxidative cleavage as before.

SECTION III

Studies of the positional specificities of enzymic desaturation and hydroxylation of monoenoic fatty acids.

Introduction

With the exception of bacteria (Pseudomonodales Eubacteriales and Actino mycetales) animals, plants and micro organisms form polyunsaturated fatty acids[264,266]. This section is primarily concerned with the second desaturation to form the dienoic fatty acid.

Dienoic fatty acids of various chain lengths are known in which the two bonds are separated by more than two carbon atoms (Table I below). Most of these acids have a double bond which may be either cis or trans in a position relatively close to the carboxyl group and the other are in a position more usually associated with the methylene interrupted patterns of desaturation.

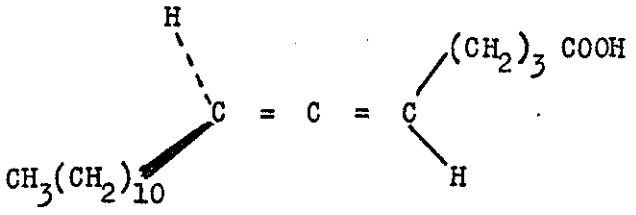
Table I Naturally occurring dienoic fatty acids having their bonds separated by at least two carbon atoms

Acid	Source	Reference
cis 5 cis 9 hexadecadienoic	Dictyostelium discoideum	267
cis 5 cis 9 heptadecadienoic	Dictyostelium discoideum	267
cis 5 cis 9 octadecadienoic	Dictyostelium discoideum	267
cis 5 cis 11 octadecadienoic	Ginkgo biloba	268
trans 5 cis 9 octadecadienoic	Thalictrum vemulosum	269
cis 5 cis 11 eicosadienoic	Ginkgo biloba	268
cis 5 cis 13 docosadienoic	Limnanthis douglasii	270
trans 3 cis 9 octadecadienoic	Aster alpinus	271

Although the majority of fatty acids exhibiting conjugation have more than two unsaturated centres[86] some conjugated dienoic acids are known e.g. trans 10 trans 12 octadecadienoic acid isolated from the seeds of Chilopsis linearis by Hopkins[272].

Perhaps the most unusual dienoic fatty acid isolated from natural sources is laballenic acid I which was isolated from the seed oil of Leonotis nepetaefolia by Bagby and co-workers[273] and since recognised in the seeds of other members of the Labiatae.[274] This acid has the structure as shown below

with an allene grouping between the fifth and seventh carbon atoms.



I Laballenic acid $[\alpha]_D - 47^\circ$

Apart from the examples cited above the vast majority of the dienoic fatty acids which are found to occur naturally have a methylene interrupted sequence of double bonds. The most common dienoic methylene interrupted fatty acid is linoleic acid which occurs in all higher plants and animals and is an essential fatty acid. Besides this acid however several other dienoic fatty acids of various chain length having a methylene interrupted sequence of double bonds are known and examples are shown in table II below.

Table II Naturally occurring methylene interrupted dienoic fatty acids

Acid	Source	Ref.
cis 9 cis 12 octadecadienoic	In many natural fats	
cis 9 trans 12 octadecadienoic	Crepis rubra	275
trans 9 trans 12 octadecadienoic	Chilopsis linearis	276
cis 7 cis 10 hexadecadienoic	Ginkgo biloba	268
cis 11 cis 14 eicosadienoic	Ephedra compylopoda	277

The biosynthesis of all these dienoic acids occurs under aerobic conditions. Several theories have been advanced as to the biosynthetic route to the conjugated fatty acids.[94] The allenic acids are also the subject of speculation but all the other dienoic acids are the result of aerobic desaturations although in several cases either β-oxidation or chain elongation has taken place after the actual desaturation.

Despite this many dienoic fatty acids occur as the direct result of the aerobic desaturation of a monoenoic precursor of the same chain length. Although in different systems the same precursor may be desaturated to give a

different diene product e.g. in Chlorella vulgaris and higher plants oleate is desaturated to linoleate[278] whilst in the slime mould dictyostelium discoideum it is desaturated to cis 5 cis 9 octadecadienoic acid;[279] in a given system under given conditions the same product is always produced. Thus some positional specificity is seen to be acting and it is the object of this section, amongst other things, to investigate the factors which control the position of the second double bond, in particular the features of the substrate molecule.

Two systems, whole cells of Chlorella vulgaris and the embryo and endosperm of the beans of the castor plant Ricinis communis, which are known to produce dienes from monoenes by direct desaturation[280] were studied. Monoenes of various chain length and bond position were incubated with the above systems and where dienes were produced the bond position and amount was determined.

It has been shown by James[281-283], Yamada and Stumpf[284] and Canvin[285] that in the developing castor bean oleic acid is the direct precursor of ricinoleic acid and furthermore that molecular oxygen and N.A.D.H. are obligatory cofactors. Since these conditions are exactly the same as those required in the formation of linoleic acid in the same system it was expected that some synthesis of an hydroxylated species might be noted during the studies of diene formation in this system.

Galliard and Stumpf[286] have shown that a microsomal fraction of the immature beans catalyses the conversion of oleyl-S-CoA to ricinoleate whilst neither linoleic acid nor linoleyl-S-CoA was converted to ricinoleate at all. This work suggests that the mechanism of ricinoleate formation does not involve the hydration of linoleate even though this is synthesised at the same time. However the possibility remains of the hydration of an enzyme bound linoleate species not interchangeable with a pool of exogenous linoleic acid or linoleyl-S-CoA could not be excluded.

Morris[280] however removed any doubt about this enzyme bound theory and proved that the mechanism was one of hydroxyl substitution proceeding with overall retention of configuration at the 12 position. In the same work he

also proved that the stereochemistry of formation of linoleate in this system is the same as in Chlorella vulgaris.

Since the mechanism of the formation of ricinoleic acid does not involve linoleate but is a direct hydroxylation of the parent monoene it follows that any specificity of hydroxylation need not be connected with the specificity of introduction of the second double bond. This means that the factors controlling the position of the introduction of the double bond are not necessarily the same as those controlling the position of hydroxylation. In this section any hydroxylated species formed from the added monoene was isolated and the position of the hydroxyl group determined separately in an attempt to find out which features of the parent monoene determine the position of hydroxylation. This work was carried out as an extension of the work by Galliard and Stumpf who could obtain no hydroxylation in either elaidic or cis vaccenic acids. Other monoenes of various chain lengths and bond positions were incubated with sliced embryos and endosperms of castor bean and any hydroxylation or further desaturation noted.

RESULTS AND DISCUSSION

The work in this section concerns the formation of dienes from monoenes. In the incubation of saturated precursors with whole cells of Chlorella vulgaris not only were monoenes formed but also dienes of the same chain length. These were formed by direct aerobic desaturation as proved by the retention of label in the product. The bond positionsof these dienes were determined by partial reduction with hydrazine[130], isolation of the resultant isomeric monoenes followed by von Rudloff oxidation [217], to give the bond position of the original diene.

In the case of the Chlorella incubations the bond positions and the amounts of the monoenes and dienes formed is given in Table 1. This shows the presence of two enzymes, one which seemed highly specific for converting cis 7 monoenes to 7,10 dienes and another much less chain length specific which converted the more usual 9-10 monoenes into 9,12 dienes. The connection between these two enzymes may be that of the loss of an amino acid between the desaturase site and the point of attachment of the substrate molecule to the enzyme. This was discussed in Section II with reference to the desaturases responsible for the first double bond and the same arguments apply here. (See page 76).

Precursor	Percentage conversion to : 7 monoene	7/10 Diene	9 monoene	9/12 Diene	Ratio Diene to monoene
14:0	10.6	0	5.2	0	0
15:0	12.8	0	20.3	8.4	0.41
16:0	5.3	3	12.5	3	0.24
17:0	0	0	48.2	19.5	0.4
18:0	0	0	27.9	62.6	2.25
19:0	0	0	29.1	6.3	0.22

Table 1

The direct dehydrogenation of saturated acids by Chlorella vulgaris to unsaturated acids of the same chain length.

Ignoring this unusual enzyme and considering only the enzyme that introduces the second double bond in the 12-13 position, it can be seen that this is much less chain length specific being active in all chain lengths from C15 - C19 and having an optimum at C18 as shown by the diene/monoene ratio Table I, Fig.I.

Since variation of chain length does not affect the position of the double bond, it can be said that the position of the double bond is not specific for the distance from the methyl end of the molecule, nor is there any attachment there. These results clearly indicate the attachment of the monoenoic fatty acyl group to the enzyme either directly via the acyl group or the CoA or ACP thiolester as in the case of monoene formation. There are however two factors which may be specific for the position of the second double bond. The first is the distance from the attached carboxyl group as in the case of the monoene formation and the second is that the position of the first double bond fixes the position of the second double bond.

In order to investigate and clarify this issue and to verify that individually added monoenes behaved in the same way as those formed in situ, Chlorella cells were incubated with a series of W9 monoenoic fatty acids of various chain lengths and a similar series of 9-10 monoenoic fatty acids. The results of these experiments are shown in Tables IIa and IIb. As strongly indicated by the first experiment using saturated precursors, the 9-10 monoenes all gave 9-10, 12-13 dienes with an optimum conversion at C18, Table IIb, Fig IIb, which confirms the first experiment, Table I fig I. The more interesting figures are those in Table IIb, Fig.IIb, which show whether the control is solely mediated by the carboxyl end of the molecule as in the case of the monoenes or dependent in anyway on the first double bond. The W9 series had only three members since it was known that cis 7 hexadecenoic acid would yield the 7,10 diene but that this was due to a separate enzyme. The question revolves around whether cis 8 heptadecenoic acid gave the 8,11 diene and the cis 10

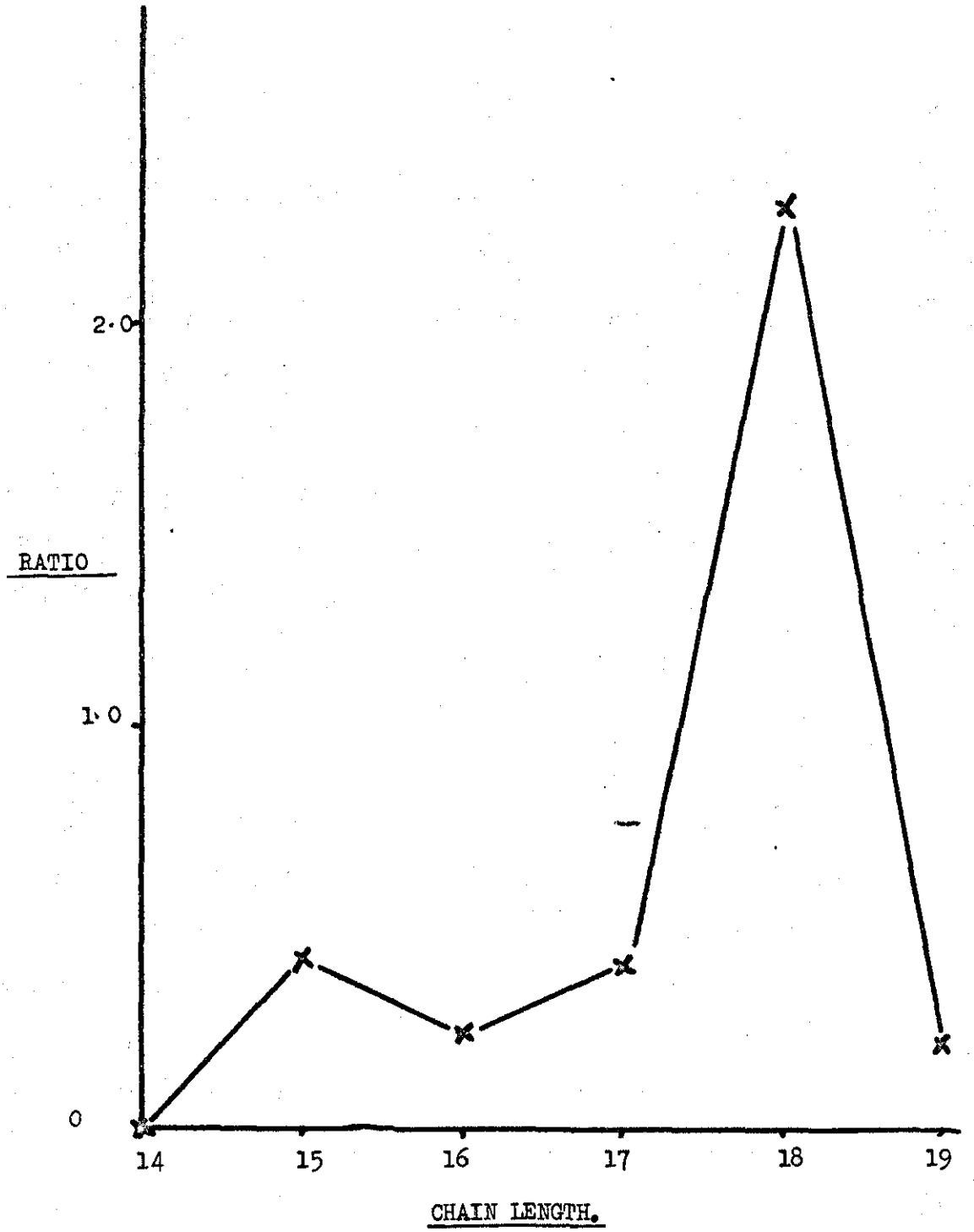


Fig. 1. Ratio of the amount of dienoic fatty acid to the amount of monoenoic fatty acid formed from saturated fatty acid precursors of the same chain length by whole cells of Chlorella vulgaris.

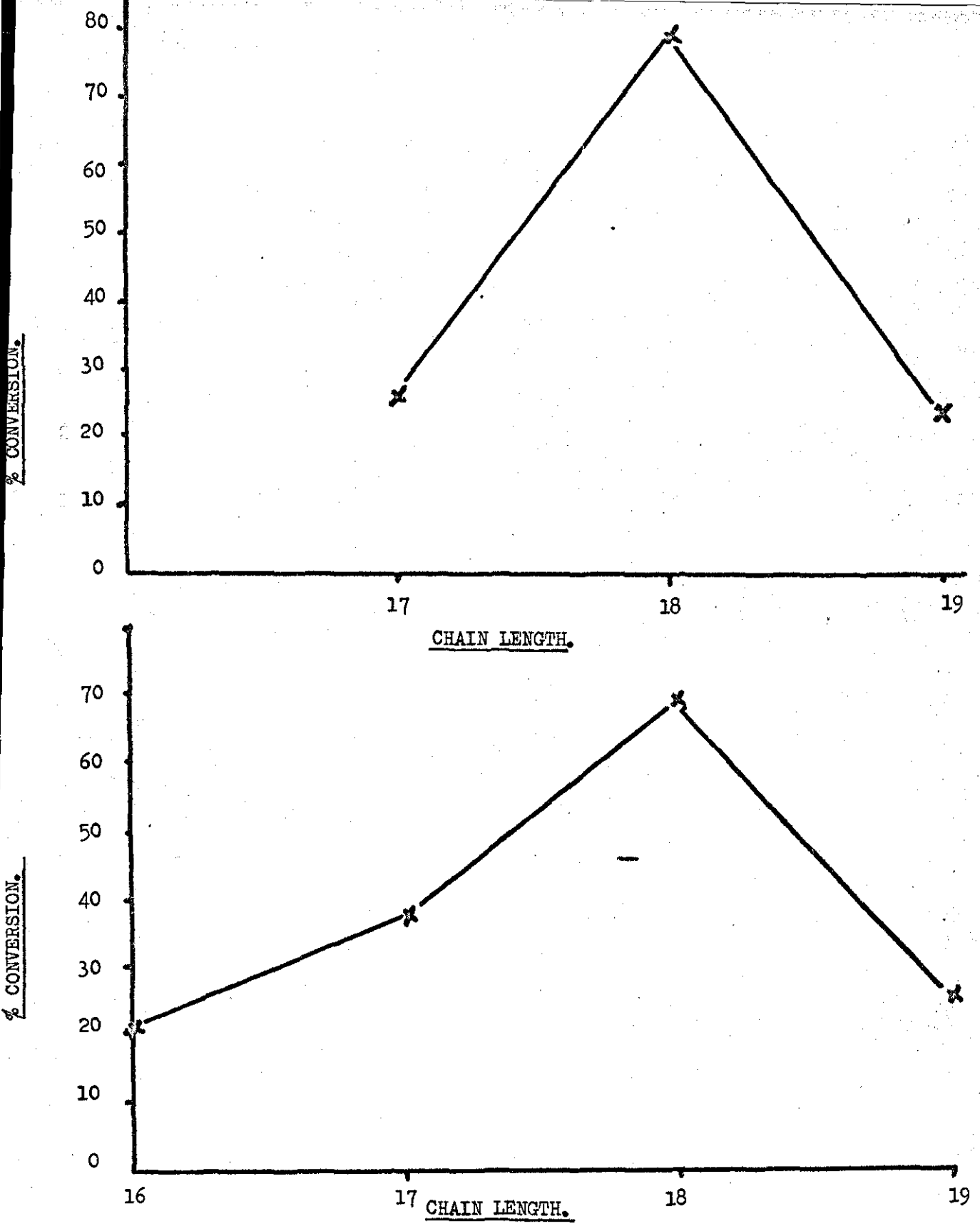


Fig. ii. Conversions of monoenoic fatty acids of the carboxyl 9 series (bottom graph) and the w9 series (top graph) to dienoic fatty acids of the same chain length by whole cells of Chlorella vulgaris.

nonadecenoic acid gave the 10,13 diene showing control by the first double bond or whether cis 8 - heptadecenoic acid gave the 8,12 diene and the cis 10 nonadecenoic gave the conjugated 10,12 diene or nothing which would show carboxyl control. If carboxyl control were the case it would be quite reasonable to expect the cis 10 nonadecenoic acid to give no further desaturation in the light of the experience of the cis 7 octadecenoic and cis 11 octadecenoic acid with the monoene forming enzyme in Section II.

Precursor	% Conversion to Diene	Bond Position
cis 8 heptadecenoic	26.0	8/9, 11/12
cis 9 octadecenoic	79.0	9/10, 12/13
cis 10 nonadecenoic	24.1	10/11, 13/14

Table IIa

The direct dehydrogenation of 1 ^{14}C labelled W.9 monoenoic fatty acids by Chlorella vulgaris

Precursor	% Conversion to Diene	Bond Position
cis 9 hexadecenoic	21.0	9/10, 12/13
cis 9 heptadecenoic	37.4	9/10, 12/13
cis 9 octadecenoic	79.0	9/10, 12/13
cis 9 nonadecenoic	25.5	9/10, 12/13

Table IIb

The direct dehydrogenation of 1 ^{14}C labelled carboxyl monoenoic acids by Chlorella vulgaris

However the result of the experiment was to show that the position of the first double bond is the factor which governs the second double, the second double bond always being methylene interrupted from it. The W9 series of acids gave a series of W6 cis di~~mono~~enes with an optimum conversion at C18, Fig.IIb. These acids have the same terminal structure as linoleic acid and both Schlenk[289] and van Dorp[290] have shown them to have essential properties.

The same experiments were carried out in another system known to convert oleic acid to linoleic acid, namely the sliced embryos and endosperm of the castor bean Ricinis communis[280]. Here the same results were obtained, see Table III, Fig.III.

Precursor	Dienoic Product	Bond Position	Hydroxylated Product	Hydroxyl Position
Carboxyl 9 Series				
cis 9 hexadecenoic	6.7	9/10, 12/13	6.2	12
cis 9 heptadecenoic	6.8	9/10, 12/13	9.0	12
cis 9 octadecenoic	9.2	9/10, 12/13	17.4	12
cis 9 nonadecenoic	4.5	9/10, 12/13	4.8	12
W9 Series				
cis 8 heptadecenoic	2.8	8/9, 11/12	5.6	11
cis 9 octadecenoic	9.2	9/10, 12/13	17.4	12
cis 10 nonadecenoic	2.0	10/11, 13/14	4.3	13

Table III

$1^{14}C$ labelled monoenoic fatty acids incubated with Ricinis communis embryo + endosperms

Since both these systems seemed capable of converting 8, 9 and 10 monoenes to their respective methylene interrupted dienes it was decided to

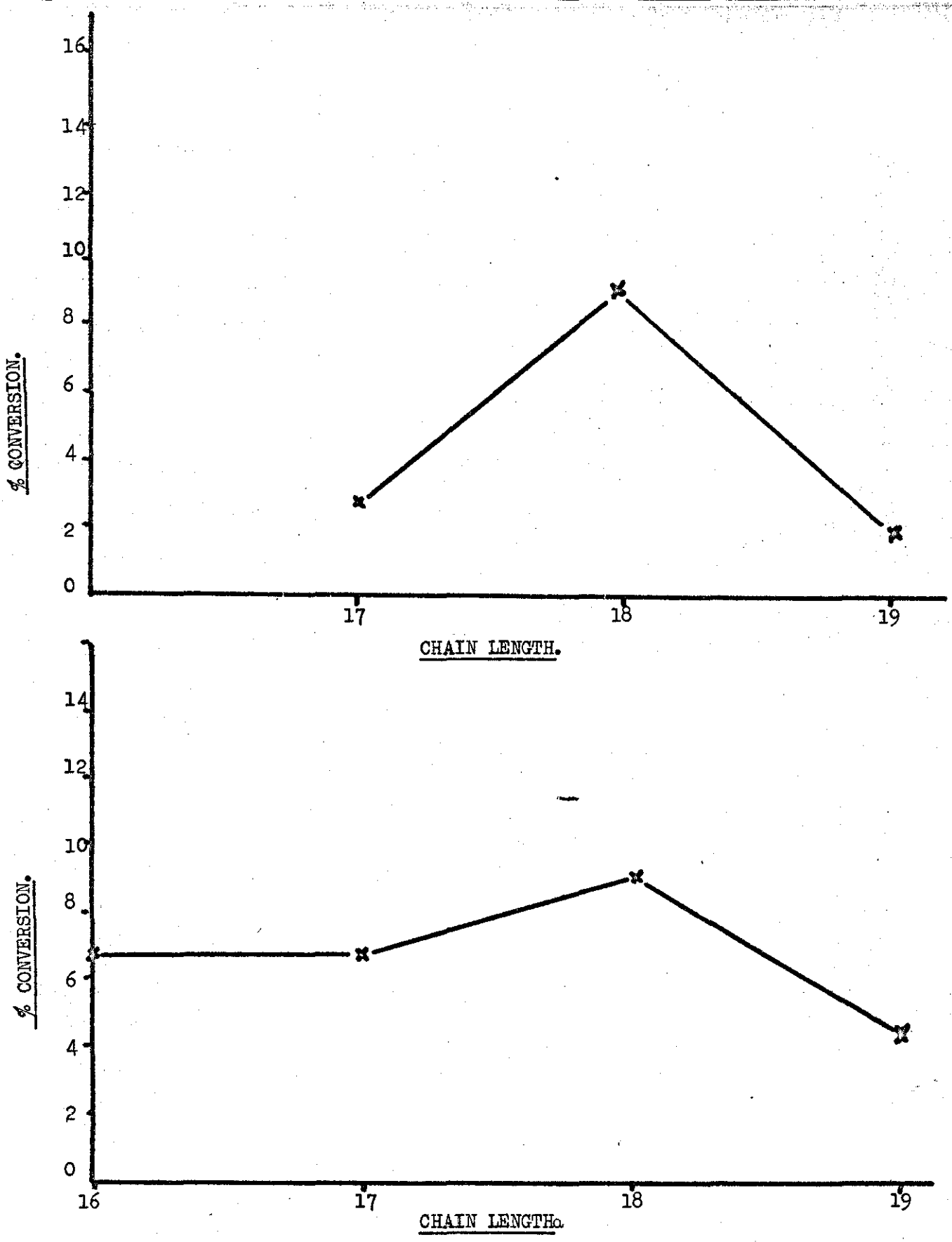


Fig. iii. Conversions of monoenoic fatty acids of the carboxyl 9 series (bottom graph) and the w 9 series (top graph) to dienoic fatty acids of the same chain length by sliced embryos and endosperms of the castor bean (Ricinus communis).

see if they would convert 7 and 11 monoenes. Accordingly, both Chlorella and castor bean systems were incubated with a series of monoenoic acids and stearolic acid, see Table IV. This showed that Chlorella could not desaturate either cis 7 octadecenoic or cis 11 octadecenoic and proved that only double bonds 8, 9 or 10 can be accepted by the system. The optimum bond position was 9-10 (Fig.IV). cis 12 Octadecenoic acid was desaturated but this was in the 9 position not the 15 so the diene produced was not due to the enzyme being studied and was, in fact, due to the 9-10 monoene desaturase in a similar way to that in which linoleate was formed in the hen liver microsomal system (see page 92). The fact that elaidic acid gave no desaturation showed that the bond has to be cis for desaturation to occur. Similarly stearolic acid was not desaturated.

Precursor	% Conversion to Diene	Bond Position
cis 7 octadecenoic	<1%	-
cis 9 octadecenoic	54.0	9/10, 12/13
elaidic	<1%	-
stearolic	<1%	-
cis 11 octadecenoic	<1%	-
cis 12 octadecenoic	48.0	12/13, 9/10

Table IV

The direct dehydrogenation of various labelled precursors by Chlorella vulgaris

As a result of this experiment a precise specificity is difficult to see. For desaturation to occur to form a diene in Chlorella or castor then there must be a cis double bond in the 8, 9 or 10 position. Therefore it would seem once again as if it is a physical distance from the point of attachment of the carboxyl group to the enzyme that really determines the

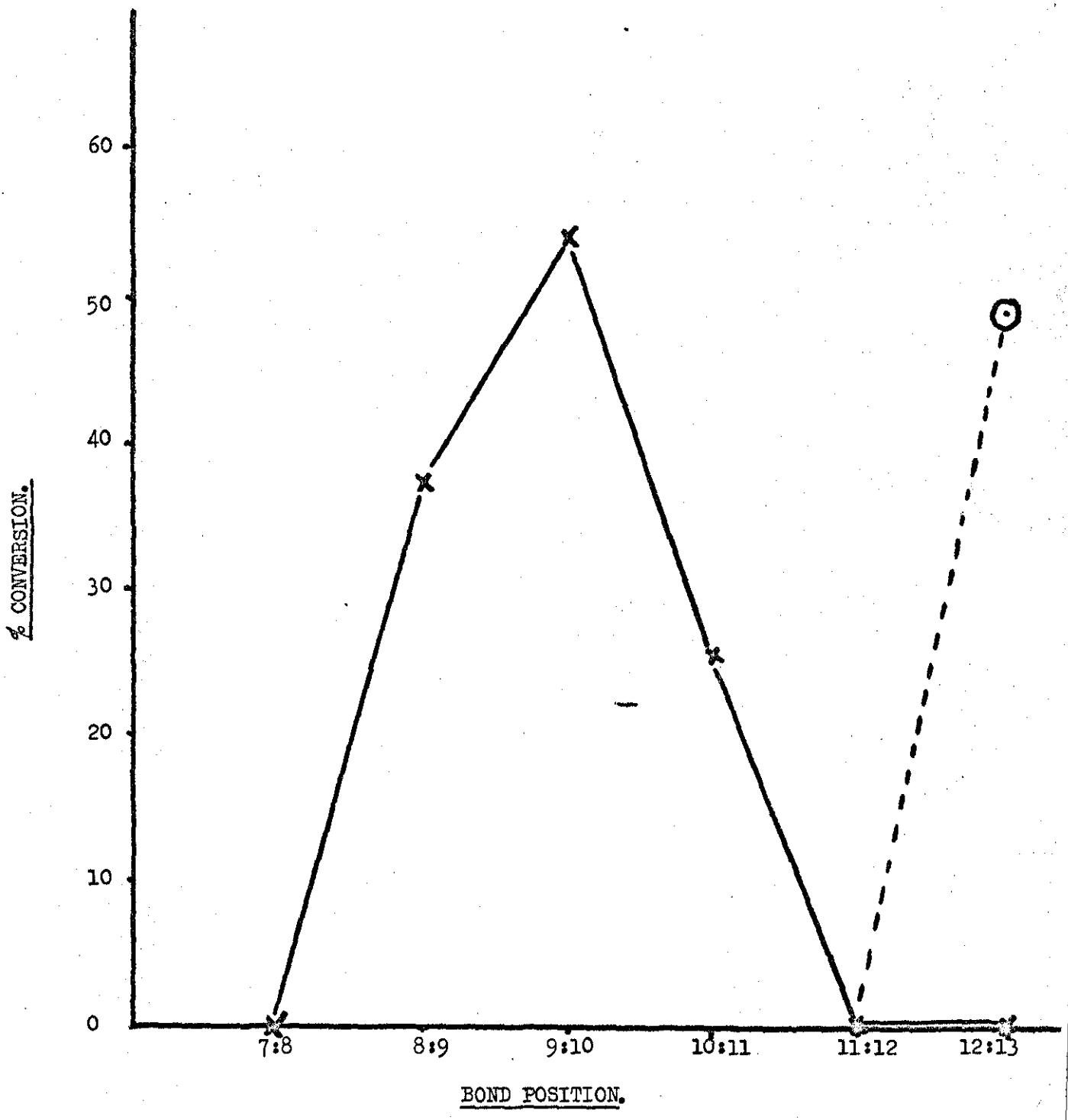


Fig.iv. Conversions of monoenoic fatty acids with double bond position as indicated above to dienoic fatty acids of the same chain length by whole cells of Chlorella vulgaris.

position of desaturation.

Fig.V shows in scale drawing the system as it might be on the enzyme surface. It can be seen that, assuming the aliphatic chain is in the energetically favoured staggered conformation, on rotation of the chain through 180° about its axis, the 11 and 12 carbon atoms of cis 8 heptadecenoic acid and the 13 and 14 carbon atoms of cis 10 nonadecanoic acid fall on either side of the 12 and 13 carbon atoms of oleic acid. Thus, if the active site is directly under the 12 and 13 atoms of oleic acid, the ideal substrate, there is the possibility of some overlap with the 11 and 12 or 13 and 14 carbon atoms of a cis Δ 8 or cis Δ10 system respectively.

Although the 10 and 11 hydrogen atoms of cis Δ 7 octadecenoic acid fall alongside those of the cis Δ 8 variety they are sufficiently far away from the actual desaturase site for there to be no desaturation. The same arrangement holds for cis 11 octadecenoic acid or cis 12 octadecenoic acid.

Having said this it is clear why neither elaidic or stearolic acids gave any desaturation since they have no carbon atoms anywhere near the desaturase site.

Thus the real factor that governs the position of the second double bond is that the hydrogen of the substrate chain should fall within the sphere of influence of the desaturase site. The reason why this system appears less specific than the first double bond system is that, having a bend in the molecule, it is possible to get two different structures having carbon atoms close enough together for either to be in the sphere of influence of the active site. In the monoene case since the saturated precursor is linear, there can only be one structure capable of placing hydrogen atoms within the sphere of influence of the active site.

The magnitude of the sphere of influence of the desaturase site is in keeping with the suggestion of a disulphide bridge. To conclude,

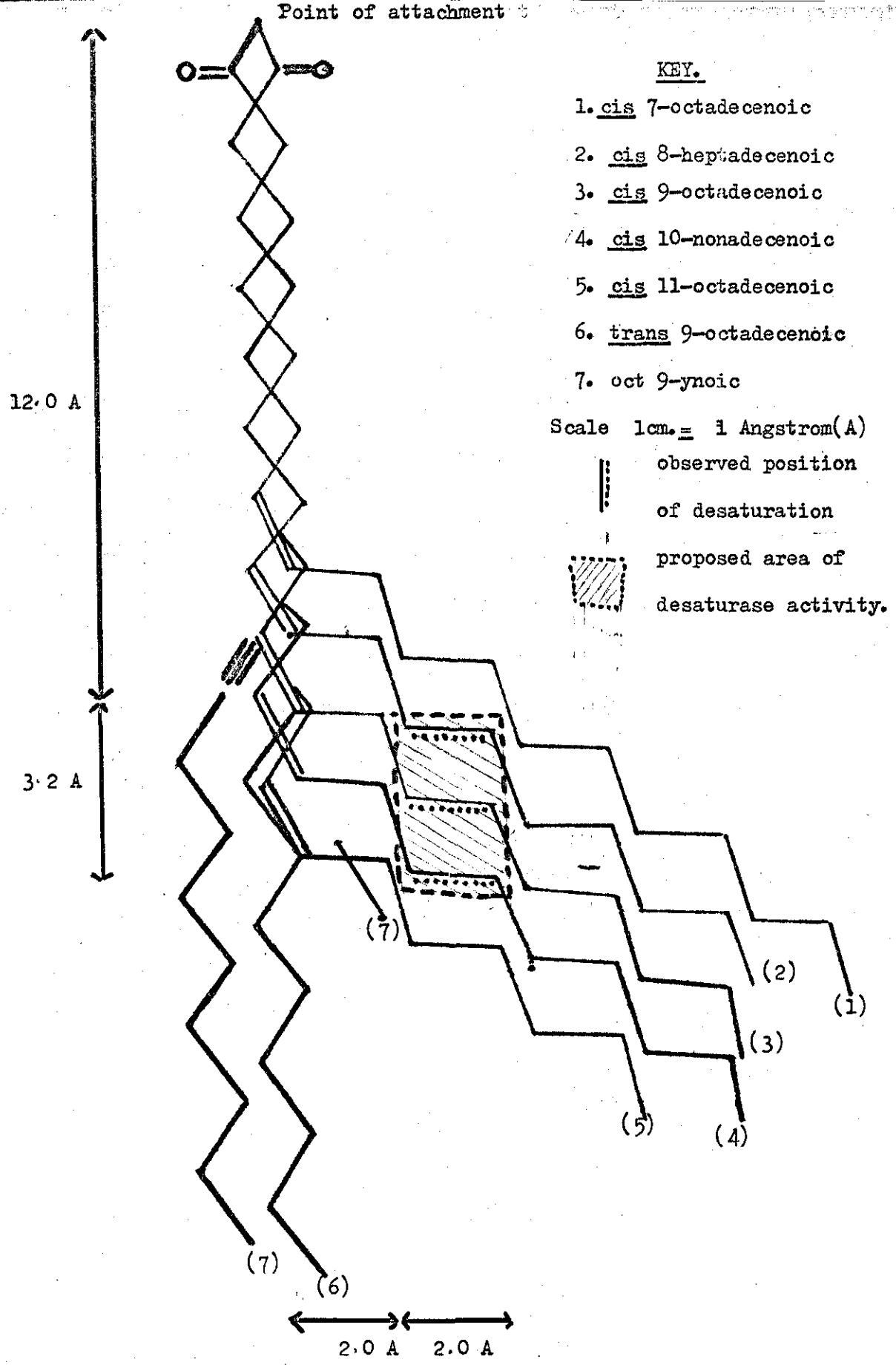


Fig. v. Schematic representation of monoenoic fatty acids illustrating the position of the active site of the desaturase responsible for introducing the second double bond.

the precise position of the second double bond is that it is formed between two adjacent carbon atoms which fall in the rectangle of enzyme surface shown, Fig.V, when the carboxyl group is attached to the binding site of the enzyme. So in a less obvious way, this is also a case of carboxyl control. Quantitatively speaking, it is easy to see why C18 is the optimum chain length for the W9 series, Tables IIa and III, as this gives the best cover of the active site, whilst the optimum of C18 in the carboxyl-9 series can only be explained by saying that up to 18 better binding is obtained by having a longer chain and hence a greater London dispersion force up to 18 but an adverse interaction with the enzyme at the 19th carbon atom causes a less favourable arrangement in the case of C19.

The results obtained in the experiments with the carboxyl 9 and W9 series of monoenes with castor and Chlorella vulgaris, Tables III and IV and Figs III and IV respectively could also be explained in another way. Desaturation was only observed in either the W9 or carboxyl 9 series and never with any other bond position. Therefore it is possible that there are two enzymes responsible for the introduction of the second double bond, one that is carboxyl end controlled and the other that is controlled by the distance from the methyl end of the molecule. Qualitatively this would explain the results as satisfactorily as the previous theory expounded above but quantitatively the results lend an extra strength to this theory.

The previous theory explains quite satisfactorily the qualitative data and also the reason for the 18 carbon atom substrate being the optimum point in the W9 series. The previous theory has a less obvious explanation of the reason why the C18 monoene should be so favoured in the carboxyl 9 series. This second theory would explain this in this way. In castor bean, for example, if the carboxyl 9 enzyme was capable of converting 6.5% of the label to dienoic fatty acids and the W9 enzyme capable of converting 2.5% of the label to dienoic fatty acid irrespective of chain length, then the reason oleic acid is favoured in both series is because it is the only

acid capable of being a substrate for both enzymes and hence is desaturated to 9%. This agrees well with the observed individual components in the seventeen and nineteen carbon atom cases. Similar results were obtained in the Chlorella vulgaris incubations and a comparison of the data appears in Table V below.

Enzyme Length System	% Desat. by W.9 controlled enzyme	% Desat. by carboxyl controlled enzyme	Total	observed Desat. for oleic
Ricinus communis	2.4	6.6	9.0	9.2
Chlorella vulgaris	22.0	31.5	53.5	79.0

Table V

Comparison of the individual conversions of the W9 and carboxyl 9 desaturases with the overall conversion in the case of oleic acid

The postulation of two enzymes responsible for the introduction of the second double bond in the conversion of oleate to linoleate raises the question of whether the substrate is the same for both. If it is not then it could explain the close involvement of lipid in these desaturations (of Chlorella) with the actual lipid as a possible substrate for one of the enzymes[81,83], most likely the W9 desaturase for steric reasons. The carboxyl controlled desaturase could then have the same substrate, the CoA or ACP thiolester, as has been more generally observed to be the case in desaturation studies[47,61].

No lipid involvement has yet been demonstrated in the castor bean but it has not been looked for to date.

Incubations of these systems with cis-11-eicosenoic, cis 8-octadecenoic and cis 10 octadecenoic acids would help to differentiate between these two theories of the specificities of desaturation.

In experiments with castor beans, the actual beans were picked 35 - 40 days after flowering to ensure maximum conversion to linoleate[283]. At this time the same workers found that ricinoleate was also being actively synthesised. Since the conditions of ricinoleate synthesis are the same as those for aerobic desaturation[286] viz molecular oxygen and reduced pyridine nucleotides formation of an hydroxylated species was noted in the desaturation studies. This was of great interest since Galliard and Stumpf[286] working with a microsomal preparation of castor bean had been unable to demonstrate hydroxylation of any substrate other than oleate.

A series of 9:10 monoenes of varying chain length were incubated with sliced embryos under conditions similar to those used by Morris et al[280] in their stereochemical studies of ricinoleate formation in Ricinis communis. All the substrates tested i.e. those possessing carbon chain lengths between 16 and 19 carbon atoms produced an hydroxylated species Table III. The optimum chain length for this was at 18, see Fig.VI. The series of W9 acids also yielded an hydroxylated species, Table III, and, once again, the optimum was at C18, see Fig.VI.

It was unknown, at this stage, at what position the hydroxylation had occurred and there have been observations by Galliard and Stumpf that a β hydroxy species is formed in experiments with castor[286] in the absence of NADPH. However von Rudloff oxidation[217] of the acids, gave the regular non hydroxylated decarboxylic diesters and this eliminated the possibility of the hydroxylation occurring between the double bond and the carboxyl end of the molecule.

These results did not tell where the exact position of hydroxylation occurred and what effect the substrate chain length and position of the double bond had on the enzyme which produced the hydroxylation. The position of the hydroxy group was determined by first reducing the hydroxy alkenoic acid to an hydroxy alkanoic acid since cleavage at the double bond would yield a regular dicarboxylic acid and yield no information about the

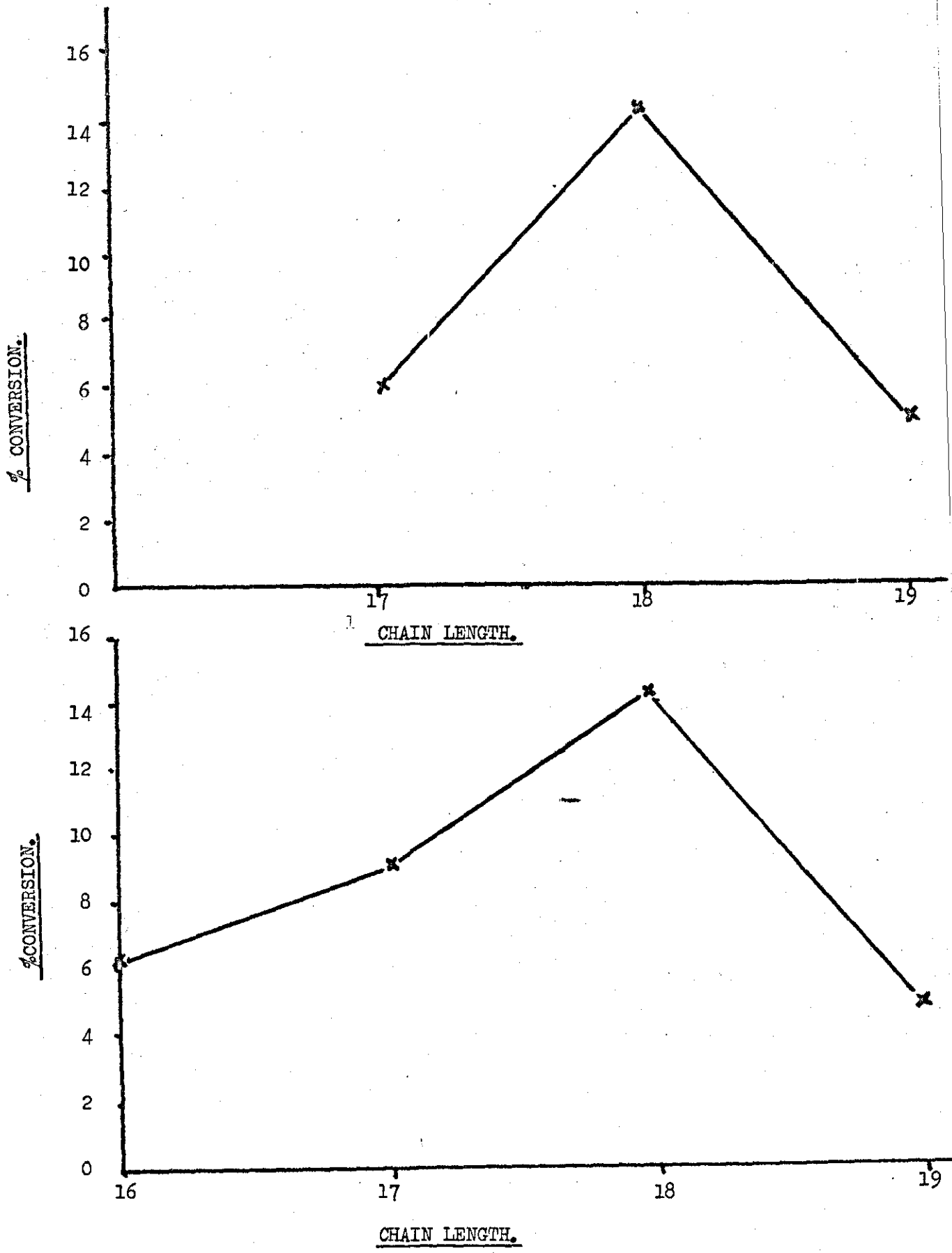


Fig. vi. Conversions of monoenoic fatty acids of the carboxyl 9 series (bottom graph) and the w 9 series (top graph) to hydroxylated monoenoic fatty acids of the same chain length by sliced embryos and endosperms of the castor bean (Ricinus communis).

position of the hydroxyl group. The hydroxy alkanoic acid was then cleaved by strong oxidative conditions[287] which first of all oxidises the hydroxy acid to the corresponding keto acid and then causes cleavage on either side of the carbonyl group. This yields two dicarboxylic acids as products differing by one carbon atom (see page 144). Thus a 12 hydroxy acid yields dodecanedioic and undecanedioc acids in this reaction. By studying the dicarboxylic acids formed the position of hydroxylation can be determined.

The hydroxy alkanoic acids formed by reduction from the products of the incubations were all oxidised in this way and the individual dicarboxylic diesters formed from the products. The individual diesters were then separated and the activity counted by scintillation counting. The radioactive dicarboxylic esters gave the position of the hydroxy group in the molecule.

These results are given below in Table VI in detail and are also included in Table III. However, despite the occurrence of hydroxylated species in these above incubations, no desaturation or hydroxylation could be demonstrated in either cis 7 octadecenoic acid or in cis 11 octadecenoic acid. This latter result confirms the findings of Galliard and Stumpf[286].

Thus it appears that the specificity for hydroxylation in castor beans is similar to that for the formation of a diene. That is that the enzyme shows a primary specificy for the hydroxylation to occur β to the double bond and then only in the direction of the methyl end of the molecule. The work of Galliard and Stumpf shows that this has to be a cis double bond and the work of this thesis shows that that double bond must be in the 8, 9 or 10 position in the chain and that double bonds in the 7 and 11 positions do not yield hydroxy species.

Table VII summarises the total work done on this system.

Precursor	Counts Dicarboxylic Esters				OH position
	C_{10}	C_{11}	C_{12}	C_{13}	
cis 9 hexadecenoic	871	20,136	20,605	423	12
cis 9 heptadecenoic	908	30,110	27,578	519	12
cis 9 octadecenoic	417	63,883	60,191	334	12
cis 9 nonadecenoic	631	23,192	21,544	449	12
cis 8 octadecenoic	47,415	30,279	1,971	853	10
cis 10 octadecenoic	716	1,313	23,071	22,015	13

Table VI

Total counts in the collected dicarboxylic esters from the cleavage of the hydroxy acids formed in the incubation of labelled precursors with sliced embryos of Ricinis communis

Precursor	Hydroxylation	Reference
cis 9 hexadecenoic	Yes	a
cis 9 heptadecenoic	Yes	a
cis 9 octadecenoic	Yes	a b c
cis 9 nonadecenoic	Yes	a
cis 8 heptadecenoic	Yes	a
cis 10 nonadecenoic	Yes	a
cis 7 octadecenoic	No	a
cis 11 octadecenoic	No	a b
trans 9 octadecenoic	No	b
octadecanoic	No	b
cis 9, cis 12 octadecadienoic	No	b c

Table VII

Possible precursors for hydroxylation in Ricinis communis

a) This thesis b) Galliard and Stumpf[286] c) A.T.James, H.C.Hadaway and J.P.Webb[283]

Thus, to conclude, this work suggests that the fatty acyl residue is connected to the enzyme at the carboxyl end though the precise form of attachment is not known and that the real specificity of hydroxylation is that the carbon atom must be β to a cis double bond in such a position in the chain that it falls within the area shown in Fig. VII which is the area of influence of the active site. The optimum bond position is 9:10 since this gives the optimum orientation of the chain on the active site. There is an optimum chain length of 18 carbon atoms among the 9:10 acids. The secondary chain length effect is best explained by once again postulating that the greater the number of van den Waals interactions the better the binding to the active site although there must be a further unfavourable interaction in the case of the 19 carbon atom chain.

The possibility of two enzymes being responsible for the hydroxylation, one carboxyl controlled and one methyl end controlled as postulated in the case of the desaturases responsible for the introduction of the second double bond (see Page 131) is not ruled out. Qualitatively and semi quantitatively, it would fit the data although the precise agreement of the figures does not quite bear it out completely. Here oleic acid is more favoured than the sum of the two seventeen isomers for example. Also, no lipid involvement has been studied in the case of ricinoleate formation and so the position is a little less certain here although this work provides a useful basis for further work on this system.

Once again however, these two theories could be tested and differentiated by using cis 8 octadecenoic and cis 10 octadecenoic acids.

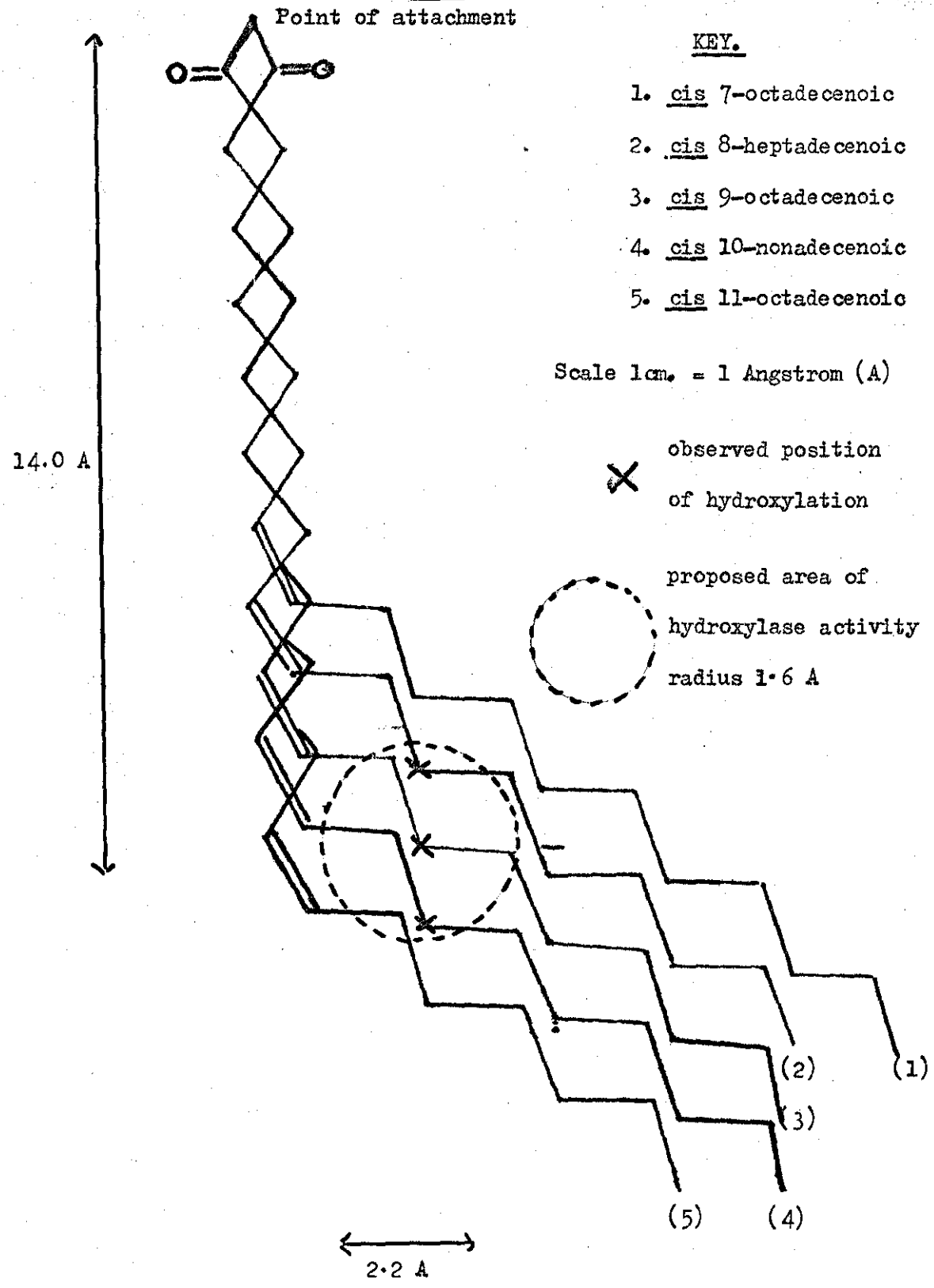


Fig. vii. Schematic representation of monoenoic fatty acids illustrating the position of the active site of the hydroxylase in the embryos and endosperms of the castor bean (Ricinus communis).

EXPERIMENTAL

I Experiments with whole cell cultures of Chlorella Vulgaris

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

Chlorella vulgaris was grown by the following procedure.

One loop of cells was inoculated from an agar slope into 5 mls of "rich" medium (see below) and incubated for 24 hours at 30°C under continuous illumination 18 inches from 4 x 40 watt fluorescent tubes (daylight emission). The 5 ml culture was then poured into 250 ml of "rich" medium in a Roux bottle and incubated for 2 - 3 days in the light incubator at 30°C.

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

Final pH 6.5

Poor Medium	Protoose peptone	1 g.
	KNO_3	2 g.
	K_2HPO_4	200 mgm
	$MgSO_4\ 7H_2O$	200 mgm
	Agar	10 g.
	Water to	1 litre

Final pH 6.5

1a) To investigate the effect of chain length of substrate on the extent and position of desaturation in a whole cell culture of Chlorella vulgaris

The culture of Chlorella vulgaris was grown in the "rich" medium as before and harvested according to the method of Harris et al.[62] This involves spinning down the cells at 1,000 r.p.m. for 10 mins. The cells were then resuspended in the same volume of 0.2M phosphate buffer pH 7.4 and the centrifuging repeated to obtain the cells free of the rich medium. The cells were finally resuspended in 0.2M phosphate buffer pH 7.4 at a concentration of 1 gm of cells to 10 mls of buffer. This suspension of cells was then preincubated for 1 hour at 27°C at a distance of 1 ft from three 250 watt photo flood lamps with a 6" deep water filter to remove the heat.

3 ml aliquots of this suspension were placed in eight tubes. To each was added a radioactive precusor suspension (0.2 mls) and the whole incubated for 6 hours at 27°C in an illuminated incubator. The radioactive substrates used in this experiment were ($1^{14}C$) - decanoic, ($1^{14}C$)- dodecanoic, ($1^{14}C$) - titradecanoic, ($2^{14}C$) - pentadecanoic, ($1^{14}C$) - hexadecanoic, ($1^{14}C$) - heptadecanoic, ($1^{14}C$) - octadecanoic, ($2^{14}C$) - nonadecanoic acids. At the end of the incubation period the reaction was quenched and the lipids extracted with 2 : 1 v/v chloroform methanol as in section II. The lipids were transmethylated and the radioactive esters identified and measured by RGLC on an FFAP column at 230°C. The monoenes and dienes were isolated from the esters by silver ion chromatography and then the ester having the same chain length as the labelled precursor isolated by preparative G.L.C. on FFAP at 230°C as before. The band position of the monoenes was determined by von Rudloff's[217] oxidative cleavage technique and the band positions of the dienes was determined as below.

The determination of the diene band positions

The dienoic ester isolated above was dissolved in methanol (0.5 mls). Where there was little endogenous cold acid present e.g. in the case of methyl heptadecadienoate, methyl linoleate (100µg) was added as carrier. 60% hydrazine hydrate (50µl) and hexanoic acid (2 mgm) were added to the solution

which was shaken in an unstoppered tube at 50°C. The course of the reaction was followed by G.L.C. on a Pye 104 instrument containing an FFAP column at 230°C. The disappearance of the diene peak is accompanied by the appearance of a split monoene peak due to the two isomeric monoenes and a saturated ester peak. These peaks correspond in chain length to the carrier where it was added.

The reduction was quenched when an optimum yield of monoene had been achieved by the addition of dilute hydrochloric acid (1 ml). The reaction mixture was dissolved in ether (50 mls) and washed acid free with successive aliquots of water. The ether was finally removed at the pump and the products dried by azeotropic distillation with ethanol. The esters were dissolved in ether (5 mls) and the monoenoic esters isolated in the usual way by silver ion chromatography. The band positions in these esters were determined by von Rudloff's oxidative cleavage technique followed by RGLC on an FFAP column at 230°C.

Since much breakdown resynthesis had occurred in this system it was thought necessary to determine the position of the radiolabel in the final dienoic product. This was done as follows.

Determination of the position of the radiolabel.[288]

The saturated esters from the partial reduction above were isolated by silver ion chromatography and dissolved in acetone (0.5 mls). Potassium permanganate (50 mgm) was added and dissolved. The solution was placed in a glass tube cooled in dry ice and sealed. The sealed tube was heated at 60°C for 4 hours. The tube was then cooled and opened with a glass file. The contents were added to ether (20 mls) and washed with sodium bisulphite solution, followed by dilute sulphuric acid and finally washed acid free with water. The ether was then evaporated at the pump and the products dried by azeotropic distillation with ethanol. After methylation with diazomethane an aliquot of the products was injected on to a RGLC containing an FFAP column at 230°C.

A single radioactive peak of the appropriate chain length was indicative of retention of label.

b) To investigate the effect of the chain length of substrate on the inhibition produced by sterculic acid.

Chlorella vulgaris cells were grown in the rich medium, harvested, washed and resuspended in 0.2M phosphate buffer as in the previous experiment. After preincubation as before 3 ml aliquots were added to each of six tubes. Radioactive precursor (0.1 ml) was added. To each of an identical set of six tubes was added sterculic acid suspension prepared as below equivalent to (3 mgm) giving an overall sterculic acid concentration of 3×10^{-3}M and radioactive precursor suspension (0.1 ml). The radioactive precursors used in this experiment were ($1^{14}C$) - tetradecanoic, ($2^{14}C$) - pentadecanoic, ($1^{14}C$) - hexadecanoic, ($1^{14}C$) - heptadecanoic, ($1^{14}C$) - octadecanoic and ($2^{14}C$) - nonadecanoic acids.

The sterculic acid suspension was prepared by dissolving a urea clathrate compound of methyl sterculate (50 mgm)* in IN potassium hydroxide in methanol (5 mls) and refluxing for 30 mins. When cool the solution was carefully acidified to pH5 with IN sulphuric acid and the product extracted three times with ether (10 mls) and the combined ether phases washed acid free with water. The ether was removed at the pump and water (0.6 mls) was added to the acid which was emulsified by the addition of 1 drop of 10% w/v sodium carbonate and 1 drop of a 1% aqueous solution of Tween 20 followed by ultrasonication.

The tubes set up as above were incubated for 6 hours at 27°C under constant illumination as before. At the end of this period the reaction was quenched and the lipids extracted and transmethylated in the usual way. The methyl esters thus formed were identified and measured by RGLC on an FFAP column at 230°C.

* The kind gift of Dr. A. R. Johnson of the C.S.I.R.O. Division of food preservation, Ryde, N.S.W. Australia.

2a) Investigation of the position and degree of desaturation produced in a series of monoenoic fatty acids whose double band is in the 9 - 10 position by whole cells of Chlorella Vulgaris

Whole cells of Chlorella vulgaris were grown, harvested and resuspended as in the previous experiments. Four tubes were set up each containing 3 mls of the 0.2M phosphate buffer suspension of cells and a suspension of radioactive precursor (0.1 ml) was added. The radioactive precursors used in this experiment were (^{14}C) - cis 9 hexadecenoic, ($1^{14}C$) - cis 9 heptadecenoic, ($1^{14}C$) - cis 9 octadecenoic and ($1^{14}C$) - cis 9 nonadecenoic acids. The incubations were carried out at 27°C for 6 hours in an illuminated incubator. At the end of this period the reaction was quenched, the lipids extracted and transmethylated in the usual way. The methyl esters were identified and measured by RGLC on FFAP at 230°C. The dienes formed were isolated by silver ion chromatography and the diene of the same chain length as the precursor isolated by preparative G.L.C. on FFAP at 230°C. The band position of the dienes formed were determined by von Rudloff's oxidative cleavage [217] following partial reduction with hydrazine as before.

b) Investigation of the position and degree of desaturation produced in a series of W9 monoenoic fatty acids by whole cells of Chlorella vulgaris

The suspension of cells used for this experiment was the same as prepared for experiment 2a above. That experiment was repeated exactly with the following three radioactive precursors ($1^{14}C$) - cis 8 heptadecenoic, ($1^{14}C$) - cis 9 octadecenoic and ($1^{14}C$) cis 10 nonadecenoic acid.

3a) Investigation of the effect of stearolic acid and positional and geometrical isomers of oleic acid on the extract and position of desaturation in whole cells of Chlorella vulgaris.

Whole cells of Chlorella vulgaris were grown, harvested and resuspended as in the previous experiments. Aliquots (3 mls) of this suspension were placed in tubes and incubated with a radioactive substrate suspension (0.1 ml)

for 6 hours at 27°C in an illuminated incubator.

The radioactive substrates used in this experiment were ($1^{14}C$) - cis 7 octadecenoic, ($1^{14}C$) cis 9 octadecenoic, ($1^{14}C$) cis 11 octadecenoic, ($1^{14}C$) cis 12 octadecenoic, ($1^{14}C$) - elaidic and ($1^{14}C$) - stearolic acid*. When the incubations had been quenched and the lipids extracted half were transmethylated in the usual way, the methyl esters were identified and measured by RGLC on an FFAP column at 230°C. A 10% silver nitrate impregnated silica plate was channelled into 1 cm channels and aliquots of the extracted methyl esters were run in 15% ether in petrol ether and the plates scanned as a second check to establish whether any desaturation had occurred. In the two cases where it had the dienes were isolated by silver ion chromatography and their band positions determined by von Rudloff's oxidative cleavage[217] following partial reduction with hydrazine as before. The lipid incorporation was determined by silver ion chromatography in the usual manner except that the developing solvent was chloroform-methanol acetic acid (65 : 25 : 4) v.

4. Investigation of the effect of a terminal double band in the substrate to the degree of desaturation in whole cells of Chlorella vulgaris

Whole cells of Chlorella vulgaris were grown, harvested and resuspended in the usual way and 3 ml aliquots of the suspension were incubated with radioactive substrate suspension (0.1 mls) for 6 hours at 27°C in an illuminated incubator. At the end of the incubation the lipids were extracted and transmethylated in the usual way and the methyl esters identified and measured by RGLC on FFAP at 230°C.

A double check was done to identify the products. The monoenes, dienes, trienes and tetraenes were isolated by chromatography on a 10% silver nitrate impregnated silica plate which was run in 30% ether in petrol ether. A portion of each of these fractions was separately run on RGLC at 230°C and their retention volume and carbon number determined. These were compared with the unseparated chart and its peaks more positively identified. The fractions

* The kind gift of Mr. M. Crouchman

isolated above were each subjected to band cleavage by von Rudloff's method[217] to determine the position of the double band nearest the carboxyl end of the molecule.

II Experiments with the embryos and endosperms of the castor bean Ricinis communis).

The castor oil plants (Ricinis communis) were grown in the greenhouses of the Basic Horticulture Section Unilever Research Laboratory, Colworth House, Bedford. 35 days after flowering when the seed coat was a deep maroon colour test incubations with ($1^{14}C$) oleic acid were done to check the stage which the seeds had reached in their maturity. These incubations were carried out exactly as the experimental incubations later. These test incubations were repeated daily until the seed was synthesising ricinoleic acid vigorously (about 20% of the label being incorporated into ricinoleic acid). When this stage had been reached beans of a similar age from the same stem of the same plant were picked and the experimental incubations performed as follows.

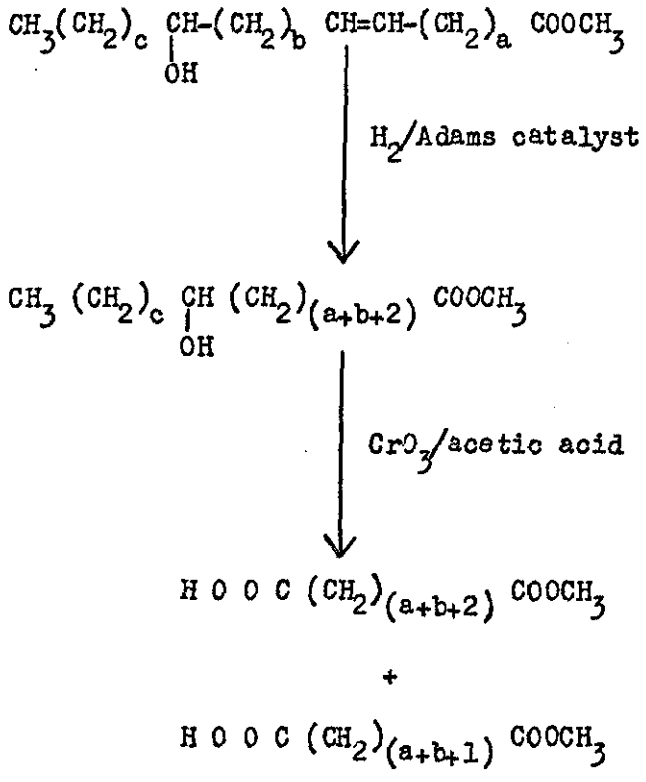
The bean pod was opened with a sharp razor blade and the three beans removed, the three individual beans were then sliced down the middle. The three embryos and one of the endosperms were sliced with the razor blade and placed in a tube to which had previously been added 0.2M phosphate buffer pH 7.4 (2 mls) and radioactive substrate suspension (0.3 mls) dispersed in it by sonication. Eight tubes were set up in this fashion and incubated for 24 hours in a reciprocating waterbath at 30°C under subdued light. The radioactive precursors used in this experiment were ($1^{14}C$) - cis 9 hexadecenoic, ($1^{14}C$) - cis 9 heptadecenoic, ($1^{14}C$) - cis 9 octadecenoic, ($1^{14}C$) - cis 9 nonadecenoic, ($1^{14}C$) cis 8 heptadecenoic, ($1^{14}C$) - cis 7 octadecenoic, ($1^{14}C$) - cis 11 octadecenoic and ($1^{14}C$) - cis 10 nonadecenoic acids. At the end of this period the reaction was quenched and the lipids extracted with 2 : 1 v/v chloroform-methanol as before and the lipids transmethylated. An aliquot of the methyl esters thus formed was run on 10% silver nitrate impregnated silica plate divided into 1 cm channels and developed 8 cm in 50% ether in petrol ether.

The solvent was then evaporated and the plate developed twice in 20% ether in petrol ether. The channels were then scanned on the Panax RTLS-1 and the monoenoic, dienoic and hydroxy bands scraped off. The esters were dissolved in 0.4% PPO in toluene and counted on the Packard Tri-Carb series 4000 scintillation spectrometer.

Preparative T.L.C. plates were run under the same conditions as above and the monoenes, dienes and hydroxy species isolated. The bond positions of the monoenes and dienes were determined as before in the experiments with Chlorella vulgaris. An aliquot of the hydroxy compound was subjected to RGLC on SE.30 at 230°C to check the chain length of the compound and a further aliquot was subjected to von Rudloff's oxidative cleavage[217] to identify the position of any double bond present and also to prove that the hydroxy group had been introduced beyond the double bond. Finally the exact position of the hydroxy group was determined as follows.

Determination of the position of the hydroxyl group

The position of the hydroxyl group was determined by the following set of reactions:-



$$\downarrow CH_2N_2 \text{ in ether}$$

$$CH_3OOC - (CH_2)_{(a+b+2)} COOCH_3 + CH_3OOC(CH_2)_{(a+b+1)} COOCH_3$$

The remaining amount of the hydroxy esters were dissolved in absolute alcohol (10 mls) and Adams platinum oxide catalyst (25 mgm) added. The flasks containing the above mixture were connected to a system which allowed an atmosphere of hydrogen gas at atmospheric pressure to be placed over the solvent surface. The flasks were agitated at room temperature for 3 hours. After this time the flask was removed and the catalyst filtered off. The ethanol was then removed at the pump and the ester redissolved in glacial acetic acid (3 mls) in which had been dissolved chromium trioxide (90 mgm). This solution was heated at 40°C for 24 hours. At the end of this time water (15 mls) was added and the products extracted with 3 x 10 mls of ether. The ether extracts were bulked and washed acid free with water. The ether was removed at the pump and the products dried by azeotropic distillation with ethanol. The products were then methylated with diazomethane. Dimethyl undecanedioate (20 mgm) and dimethyl tridecanedioate (20 mgm) were added to the products and the whole subjected to preparative G.L.C. The dicarboxylic esters having carbon chains 10, 11, 12 and 13 atoms long respectively were collected and dissolved in 0.4% PPO in toluene and counted on the Packard Tri-Carb scintillation spectrometer. By identification of the radioactive esters the position of the hydroxyl group was determined.

BIBLIOGRAPHY

1. F. Lynen, Federation Proc. 20, 941 (1961).
2. M. G. Horning, D. B. Martin, A. Karmen and P. R. Vagelos, J. Biol. Chem., 236, 669 (1961).
3. G. O. Burr and M. M. Burr, J. Biol. Chem., 82, 345 (1929).
4. H. Thomasson, Intern. Rev. Vitamin Research, 25, 62 (1953).
5. D. Chapman, "Introduction to Lipids", McGraw Hill, London 1969, page 40.
6. S. Bergstrom, H. Danielsson and B. Samuelsson, Biochem. Biophys. Acta, 90, 207 (1964).
7. S. J. Wakil, Ann. Rev. Biochem., 31, 369 (1962).
8. S. J. Wakil, J. Amer. Chem. Soc., 80, 6465 (1958).
9. P. R. Vagelos, Ann. Rev. Biochem., 33, 139 (1964).
10. R. O. Brady and S. Gurin, J. Biol. Chem., 187, 589 (1950).
11. R. O. Brady and S. Gurin, J. Biol. Chem., 199, 421 (1952).
12. van Baalen and S. Gurin, J. Biol. Chem., 205, 303 (1953).
13. R. Y. Hsu, G. Wasson and J. W. Porter, J. Biol. Chem., 240, 3736 (1965).
14. P. Goldman, A. W. Alberts and P. R. Vagelos, J. Biol. Chem., 238, 1255 (1963).
15. P. W. Maserus, A. W. Alberts and P. R. Vagelos, Proc. Nat. Acad. Sci., U.S.A., 51, 1231 (1964).
16. D. B. Martin and P. R. Vagelos, Biochem. Biophys. Res. Comm., 5, 16 (1961).
17. P. Goldman and P. R. Vagelos, Biochem. Biophys. Res. Comm., 5, 414 (1962).
18. P. Goldman, A. W. Alberts and P. R. Vagelos, J. Biol. Chem., 238, 3579 (1963).
19. A. W. Alberts and P. R. Vagelos, Federation Proc., 20, 273 (1961).
20. A. W. Alberts, P. Goldman and P. R. Vagelos, J. Biol. Chem., 238, 447 (1963).

21. P. W. Majerus and P. R. Vagelos, Federation Proc. 23, 166 (1964).

22. P. Goldman, A. W. Alberts and P. R. Vagelos, Biochem Biophys. Res. Comm., 5, 280 (1961).

23. P. W. Majerus, A. W. Alberts and P. R. Vagelos, Proc. Nat. Acad. Sci., U.S.A., 53, 410 (1965).

24. M. Kusunose, E. Kusunose, Y. Kowa and Y. Yamamura, J. Biol. Chem. (Japan), 46, 525 (1959).

25. W. J. Lennarz, R. J. Light and K. Bloch, Proc. Nat. Acad. Sci. U.S.A., 48, 840 (1962).

26. H. Den and H. P. Klein, Biochem. Biophys. Acta., 49, 429 (1961).

27. E. L. Pugh, F. Sauer, S. J. Wakil, Federation Proc. 23, 166 (1964).

28. S. J. Wakil, E. L. Pugh and F. Sauer, Proc. Nat. Acad. Sci., U.S.A., 52, 106 (1964).

29. F. Sauer, E. L. Pugh, S. J. Wakil, R. Delaney and R. L. Hill, Proc. Nat. Acad. Sci. U.S.A., 52, 1360 (1964).

30. E. L. Pugh and S. J. Wakil, J. Biol. Chem., 240, 4727 (1965).

31. P. Overath and P. K. Stumpf, J. Biol. Chem., 239, 4103 (1964).

32. E. J. Barron, C. Squires and P. K. Stumpf, J. Biol. Chem., 236, 2610 (1961).

33. J. L. Brooks and P. K. Stumpf, Biochem. Biophys. Acta., 98, 213, (1965).

34. P. K. Stumpf and A. T. James, Biochem. Biophys. Acta., 70, 20 (1963).

35. G. M. Cheniae, Biochem. Biophys. Acta., 70, 504 (1963).

36. P. Goldman, A. W. Alberts and P. R. Vagelos, Biochem. Biophys. Res. Comm., 5, 280 (1961).

36a. P. R. Vagelos, P. R. Majerus, A. W. Alberts, A. R. Larrabee and G. P. Ailhaud, Federation Proc., 25, 1485 (1966).

36b. R. D. Simoni, R. S. Criddle and P. K. Stumpf, J. Biol. Chem., 242, 573 (1967).

37. P. Goldman, J. Biol. Chem., 239, 3663 (1964).

38. W. J. Lennarz, R. J. Light, K. Bloch, Biochemistry, 48, 840 (1962).

39. A. W. Alberts, Federation Proc. 23, 166 (1964).

40. K. Bloch, P. E. Baronowsky, P. E. Goldfine, W. J. Lennarz, R. Light, A. T. Norris and G. Scheuerbrandt, Federation Proc. 20, 921 (1961).

41. H. Goldfine and K. Bloch, J. Biol. Chem., 236, 2596 (1961).

42. G. Schouerbrandt, H. Goldfine, P. E. Baronowsky and K. Bloch, J. Biol. Chem., 236, PC71 (1961).

43. G. Scheuerbrandt and K. Bloch, J. Biol. Chem., 237, 2064 (1962).

44. W. M. O'Leary, Biochemistry, 4, 1621 (1965).

45. P. E. Baronowsky, W. J. Lennarz and K. Bloch, Federation Proc., 21, 288, (1962).

46. A. T. Norris, S. Matsumura and K. Bloch, J. Biol. Chem., 239, 3653 (1964).

47. D. K. Bloomfield and K. Bloch, J. Biol. Chem., 235, 337 (1960).

48. K. Bernhard, J. von Bulow Koster and H. Wagner, Helv. Chim. Acta., 42, 152 (1959).

49. J. B. Marsh and A. T. James, Biochem. Biophysica. Acta., 60, 320 (1962).

50. Y. Imai, J. Biochem. (Tokyo), 49, 642 (1961).

51. A. J. Fulco and K. Bloch, J. Biol. Chem., 239, 993 (1964).

52. R. J. Light, W. J. Lennarz and K. Bloch, J. Biol. Chem., 237, 1793, (1962).

53. J. Erwin and K. Bloch, Science, 143, 1006 (1964).

54. A. J. Fulco, R. Levy and K. Bloch, J. Biol. Chem., 239, 998 (1964).

55. F. Davidoff and E. D. Korn, J. Biol. Chem., 239, 2496 (1964).

56. A. T. James, R. V. Harris and P. Harris, Biochem. J., 95, 6P (1965).

57. A. T. James, Biochem. Biophys. Acta., 70, 9 (1963).

58. J. C. Hawke and P. K. Stumpf, J. Biol. Chem., 240, 4746 (1965).

59. A. J. Fulco, Biochem. Biophysica Acta., 106, 211 (1965).

60. R. V. Harris, B. J. B. Wood and A. T. James, Biochem. J., 94, 22P (1965).

61. J. Nagai and K. Bloch, J. Biol. Chem., 241, 1925 (1966).

62. R. V. Harris, P. Harris and A. T. James, Biochem~~ica~~ Biophys~~ica~~ Acta., 106, 465 (1965).

63. A. T. James, P. Harris and J. Bezard, Eur.J.Biochem., 3, 318 (1968).

64. A. T. James, J. P. W. Webb and T. D. Kellock, Biochem.J., 78, 333 (1961).

65. F. Lynen, Collogues Internationaux du Centre National de la Reserche Scientifique, 99, 71 (1961).

66. M. Keeney, I. Katz and D. P. Schwartz, Biochem. Biophysica Acta, 62, 615 (1962).

67. K. Bloch in The Control of Lipid Metabolism 1 - 1b (J. K. Grant Ed. Academic Press London and New York, 1963).

68 F. Meyer and K. Bloch, J. Biol. Chem., 238, 2654 (1963).

69. G. J. Shroepfer and K. Bloch, J. Biol. Chem., 240, 54 (1965).

70. L. J. Morris, R. V. Harris, W. Kelly and A. T. James, Biochem.J., 109, 673 (1968).

71. L. J. Morris and R. Bickerstaffe in press.

72. D. Brett, Ph.D. thesis, Loughborough University of Technology 1969.

73. L. J. Morris and C. Hitchcock in press.

74. J. F. Mead, Federation Proc., 20, 952 (1961).

75. E. D. Korn, C. I. Greenblatt and A. M. Lees, J. Lipid Res., 6, 43 (1965).

76. J. Erwin and K. Bloch, Biochem. Z., 338, 496, (1963).

77. J. L. G. Ellerman and H. Schlenk, Experientia, 20, 426 (1964).

78. J. P. Riley, J.Chem. Soc. 2728 (1949).

79. R. Kleiman, F. R. Earle, I. A. Wolff and Q. Jones,J. Am. Oil Chem. Soc., 41, 459 (1964).

80. H. Wagner and H. Konig, Biochem Z., 339, 213 (1963).

81. B. W. Nichols, A. T. James and J. Breuer, Biochem. J., 104, 486 (1967).

82. W. E. M. Lands and P. Hart, J. Amer. Oil. Chem. Soc., 43, 290 (1966).

83. M. I. Gurr, M. P. Robinson and A. T. James, European Journal of Biochem. 9, 70 (1969).

84. B. W. Nichols, P. Harris and A. T. James, Biochem. Biophy.Res.Comm., 21, 473 (1965).

85. F. Haverkate, J. de Gier, and L. L. M. van Deenan, Experientia, 20, 511 (1964).

86. F. D. Gunstone. An Introduction to the Chemistry and Biochemistry of Fatty Acids and their Glycerides, 2nd Edition, Chapman and Hall Ltd., London, 1967, P.23.

87. M. G. Chisholm and C. Y. Hopkins, J. Org.Chem., 27, 3137 (1962).

88. M. G. Chisholm and C. Y. Hopkins, Canad. J. Chem., 38, 2500 (1960).

89. L. Crombie and A. G. Jacklin, J. Chem. Soc., 1632 (1957).

90. C. Y. Hopkins and M. J. Chisolm, J. Chem. Soc., 1632 (1962).

91. F. D. Gunstone and A. J. Sealy, J. Chem. Soc., 5772 (1963).

92. F. D. Gunstone, D. Kilcast, R. G. Powell and G. M. Taylor, Chem.Comm. 295 (1967).

93. R. C. Badami and L. J. Morris, J. Amer. Oil Chem.Soc., 42, 1119 (1965).

94. F. D. Gunstone, Chem.and Ind., 1551 (1966).

95. C. R. Smith, M. O. Bagby, T. K. Miwa, R. L. Lohmar and I. A. Wolff, J.Org. Chem., 25, 1770 (1960).

96. W. H. Tallent, J. Harris, I. A. Wolff and R. E. Lundin, Tetrahedron letters, 4329 (1966).

97. L. J. Morris, J. Chem.Soc., 5779 (1963).

98. K. L. Mikolajczak, C. R. Smith, M. O. Bagby, I. A. Wolff, J.Org. Chem., 29, 318 (1964).

99. J. D. Bu'Lock and G. N. Smith, J. Chem. Soc., 332 (1967).

100. J. D. Bu'Lock in Comparative Phytochemistry, ed. T. Swain, Academic Press N. York, 1966, P.79.

101. F. Bohlmann and H. Schulz, Tetrahedron letters, 15, 1801 (1968).

102. W. G. Haigh, L. J. Morris and A. T. James, Biochem. Biophys. Acta., 137, 391 (1967).

103. D. Howling, L. J. Morris and A. T. James, Biochem. Biophys. Acta., 152, 224,(1968).

104a. A.Lieben and A. Rossi, Ann. 159, 58, 70 (1871).
A. Lieben and G. Janecek, ibid 187, 126 (1877).

104b. F. Krafft, Ber., 12, 1664 (1879); 15, 1687 (1882).

105. F. D. Gunstone, Quart. Revs., 7, 175 (1953).

106. W. J. Gensler, Chem. Revs. 57, 191 (1957).

107. K. S. Markley, "Fatty Acids" Part III, Interscience London, Sydney and New York, 1964, page 1769.

108. H. R. LeSueur, J. Chem. Soc., 85, 827 (1904).
87, 1888 (1905).

109. W. Moldenhauer, Ann., 131, 323 (1864).

110. R. Fittig, J. G. Spenzer, Ann., 283, 66 (1894)

111. L. Braun, Monatsh, 17, 207 (1896).

112. J. Colonge and P. Dumont, Bull Soc. Chim. France, 38 (1947).

113. H. Johansson and H. Sebelius, Ber., 51, 480 (1918).

114. S. Fokin, J. Russ Phys. Chem. Soc., 46, 224 (1914).

115. A. Grun and W. Czerny, Ber., 59, 54 (1926).

116. P. H. Bergemann, J. G. Keppler, H. A. Boekenoogen, Rec. Trav. Chim., 69, 439 (1950).

117. P. Baudart, Bull Soc. Chim. France, 13, 87 (1946).

118. R. S. Sweet and F. L. Estes, J. Org. Chem., 21, 1427 (1956).

119. E. D. Hughes and C. K. Ingold, Trans. Faraday Soc., 37, 657 (1941).

120. E. R. Alexander, Principles of Ionic Organic Reactions, Wiley, New York, 1950 page 108.

121. C. C. Price and J. V. Karabinos, J. Am. Chem. Soc., 62, 1159 (1940).

122. K. Eiter and H. Oediger, Ann. 682, 62 (1965).

123. F. D. Gunstone and G. M. Hornsby, in press.

124. H. R. LeSueur, J. Chem. Soc., 85, 1708 (1904).

125. S. Fokin, J. Russ Phys. Chem. Soc., 44, 653 (1912).

126. B. H. Nicolet, J. Am. Chem. Soc., 43, 2122 (1921).

127. E. Erdmann and F. Bedford, Ber., 42, 1324 (1909).

128. H. Finklestein, Ber. 43, 1528 (1910).

129. L. J. Morris, J. Lipid Res., 7, 717 (1966).

130. C. R. Scholfield, E. P. Jones, J. Nowakowska, E. Selke and H. J. Dutton, J. Amer. Oil Chem. Soc., 38, 208 (1961).

131. G. M. Robinson and R. Robinson, J. Chem. Soc., 127, 175 (1925).

132. D. R. Howton and R. H. Davis, J. Org. Chem. 16, 1405 (1951).

133. H. Adkins and H. R. Billica, J. Am. Chem. Soc., 70, 695 (1948).

134. M. Walborsky, R. H. Davis and D. R. Howton, J. Amer. Chem. Soc., 73, 2590 (1951).

135. H. Lindlar, Helv. Chim. Acta., 35, 446 (1952).

136. L. Crombie, J. Chem. Soc., 3510, (1955).

137. D.J.Cram and N.L.Hallinger, J.Amer.Chem.Soc., 78, 2518 (1956).

138. A. W. Hofmann, Ber., 17, 1406 (1884).

139. E. S. Wallis and J. F. Lane, Org. Reactions, 3, 267 (1946).

140. N. D. Zelinsky, Ber., 20, 2026 (1887).

141. A. T. James and J. Webb, Biochem. J., 66, 515 (1957).

142. D.H.S.Horn, F.W.Hougen, E. von Rudloff, D.A.Sutton, J.Chem.Soc.177 (1954)

143. A. Borodine, Ann., 119, 121 (1861).

144. D.R.Howton, R. H. Davis and J. C. Nevenzel, J. Amer. Chem. Soc., 76, 4970 (1954).

145. J. C. Nevenzel and D. R. Howton, J. Org. Chem., 23, 933 (1958).

146. S. J. Cristol and W. C. Firth, J. Org. Chem., 26, 280 (1961).

147. S. J. Cristol, L. K. Gaston and T. Tiedman, J.Org. Chem., 29/30, 1279, (1964).

148. K.S.Markley, "Fatty Acids" Interscience New York, 2nd Edition,Part 1, 1960, page 123.

149. Y. L. Marcel and R. T. Holman, Chem. Phys. Lipids, 2, 173, (1968).

150. J. M. Rountree and J. C. Smith, Chem. and Ind., 190 (1954).

151. G. Eglington and M. C. Whiting, J.C.S., 3052 (1963).

152. R. A. Smiley and C. Arnold, J. Org. Chem., 25, 257 (1960).

153. D. R. Howton, R. H. Davis and J. C. Nevenzel, J. Amer. Chem. Soc., 74, 1109 (1952).

154. S. Bergstrom, K. Paabo and M. Rottenberg, Act. Chem. Scand., 6, 1127 (1952).

155. H. T. Harwood and A. W. Ralston, J. Org. Chem., 12, 740 (1947).

156. F. Arndt, B. Eistert and S. Amende, Ber., 61, 1949 (1928).

157. A. C. Wilds and A. L. Meader Jnr., J. Org. Chem., 13, 703 (1948).

158. P. Karrer and H. Koenig, Helv. Chem. Act. 26, 619 (1943).

159. J. Casson and W. R. Winnans, J. Org. Chem., 15, 139 (1950).

160. K. K. Carroll, Canad. J. Chem., 35, 757 (1957).

161. J. R. Johnson, Org. Reactions, 1, 234 (1942).

162. E. F. Jenny and C. A. Grob., Helv. Chim. Acta., 36, 1936 (1953).

163. F. D. Gunstone and I. A. Ismail, Chem. Phys. Lipids, 1, 209 (1967).

164. F. D. Gunstone, An Introduction to the Chemistry and Biochemistry of Fatty Acids and their Glycerides, Chapman and Hull, London, p.59.

165. W. F. Huber, J. Amer. Chem. Soc., 73, 2730, (1951).

166. R. T. Holman, Progress in the chem. of fats and lipids, II (1966).

167. B. B. Elsner and P. F. M. Paul, J. Chem. Soc., 3156 (1953).

- Prog. Chem. Fats and Lipids (1966) 91

168. F. D. Gunstone and W. Russell, J. Chem. Soc., 3782 (1955).

169. K. Ahmad and F. M. Strong, J. Amer. Chem. Soc., 70, 1699 (1948).

170. K. Ahmad, F. M. Bumpus and F. M. Strong, J. Amer. Chem. Soc., 70, 3391 (1948).

171. von G. Wittig, Experientia, 12, 41 (1956).

172. L. D. Bergelson and M. M. Shemyakin, Tetrahedron, 19, 149 (1963).

173. S. Trippett, Quarterly Reviews, 17, 406 (1963).

174. L. D. Bergelson, V. A. Vaver, M. M. Shemyakin, Izvest. Akad. Nauk S.S.S.R., Otdel. Khim. Nauuk, 1894 (1962).

175. L. D. Bergelson, V. D. Solodovnik, M. M. Shemyakin, Izvest. Akad. Nauk S.S.S.R., Otdel, Khim. Nauk., 1315 (1962).

176. S. Glasstone and A. Hickling, J. Chem. Soc., 820 (1936).

177. D. G. Bounds, R. P. Linstead and B. C. L. Wheedon, J.Chim.Soc., 2393 (1953).

178. B. C. L. Wheedon, Quarterly Reviews, 6, 380 (1952).

179. S. Swann, Trans. Electrochem. Soc., 56, 457 (1929).

180. W. J. Petersen, Z.Electrochem., 18, 711 (1912).

181. F. Fitcher and A. Petrovitch, Helv. Chem. Acta., 24, 549 (1941).

182. P. Karrer and M. Stoll, Helv. Chem. Acta., 14, 1189 (1931).

183. D. G. Bounds, R. P. Linstead and B. C. L. Wheedon, J.Chem.Soc.4219, (1954)

184. L. Ruzicka, M. Stoll and H. Schinz, Helv. Chem. Acta., 11, 670 (1928).

185. R. Stoll, W. Scherrer, H. Schinz and C. F. Seidel, Helv. Chem. Acta., 15, 1459 (1932).

186. D. Howling, Unpublished observation.

187. B. G. Bounds, R. P. Linstead and B. C. L. Wheedon, J. Chem. Soc., 448 (1954).

188. B. W. Baker, R. P. Linstead and B. C. L. Wheedon, J. Chem. Soc., 2218 (1955).

189. D. Howling, Unpublished observations.

190. W. Stoffel and E. Bierwirth, Angew Chem., 74, 904 (1962) Internat. Ed. 2, 94 (1963).

191. W. Stoffel, Ann., 673, 26 (1964).

192. W.J.Hickinbottom, "Reactions of organic compounds" Longmans London 1957

193. W. D. Celmar and I. A. Solomons, J. Amer. Chem. Soc., 75, 3430 (1953).

194. F. Bohlmann and H. G. Viehe, Ber., 87, 712 (1954).

195. J. Catch, "Carbon 14 compounds", Butterworths, London, 1961, p.27.

196. H. N. Griffiths and T. P. Hilditch, J. Chem. Soc., 2315 (1932).

197. O. S. Privett and E. C. Nickell, Lipids, 1, 98 (1966).

198. C. R. Scholfield, R. O. Butterfield and H. J. Dutton, Anal. Chem., 38, 1694 (1966).

199. E. D. Bitner and H. J. Dutton, J. Amer. Oil Chem. Soc., 45, 603 (1968).

200. A. T. James and E. A. Piper, Anal. Chem., 35, 515 (1963).

201. L. J. Morris, D. M. Wharry and E. W. Hammond, J. Chromatog., 31, 69 (1967).

202. C. Litchfield, J. E. Lord, A. F. Isbell and R. Rieser, J. Amer. Oil Chem. Soc., 40, 53 (1963).

203. F. D. Gunstone and I. A. Ismail, Chem. Phy. Lipids, 1, 264 (1967).

204. R. F. Nystrom and W. G. Brown, J. Amer. Chem. Soc., 69, 2548 (1947).

205. W. G. Brown, "Organic Reactions", Wiley New York, 1951, Vol.VI, p.478.

206. H. Schlenk and J. L. Gellerman, Anal. Chem., 32, 1413 (1960).

207. C.V.Wilson, Organic Reactions, 9, 332 (1957).

208. J. C. Conly, J. Amer. Chem. Soc., 75, 1148 (1953).

209. S. Furukawa, K. Narvchi, S. Matsui and M.Yuski, J. Bull Soc. Chem., (Japan), 40, 594 (1967).

210. H. Hunsdiecker and C. Hunsdieker, Ber. 75, 1148 (1953).

211. W. Brenshede and H. J. Shumacher, Z. Anorg. Chem., 226, 370 (1936).

212. H. Silberman and S. Silberman-Martyncewa, J. Org. Chem., 13, 709 (1948).

213. A. P. Johnson and A. Pelter, J. Chem. Soc., 520 (1964).

214. C. L. Leese and R. A. Raphael, J. Chem. Soc., 2725 (1950).

215. W. J. Hickinbottom, "Reactions of organic compounds", Longmans Green and Co., London, 1957, page 549.

216. F. D. Gunstone and I. A. Ismail, Chem. Phys. Lipid, 1, 337 (1967).

217. E. von Rudloff, Canad. J. Chem., 34, 1413 (1956).

218. W. S. Greaves, R. P. Linstead, B. R. Shepherd, S. L. S. Thomas and B. C. L. Wheedon, J. Chem. Soc., 3326 (1950).

219. W. J. Gensler, Chem. Rev. 57, 216 (1957).

220. J. Cram, J. Amer. Chem. Soc., 71, 3863 (1949).

221. T.W.Abbott and D.Althousen, Organic Syntheses, 2nd Edition, Coll.Vol.II page 270.

222. T. P. Hilditch and J. A. Lovern, J. Soc. Chem. Ind., 47, 105T (1928).

223. D. Hulanicka, J. Erwin, and K. Bloch, J. Biol. Chem., 239, 2778, (1964).

224. T. G. Green, T. P. Hilditch and W. J. Stainsby, J. Chem. Soc., 1750, (1936).

225. T. P. Hilditch and M. L. Meara, J. Chem. Soc., 1608 (1938).

226. E. Klenk, Z.Physiol. Chem., 166, 287 (1927).

227. A. Grun and T. Wirth, Ber., 55, 2206 (1922).

228. T. P. Hilditch and H. E. Longenecker, Biochem. J., 122, 497 (1938).

229. D. Atherton and M. L. Meara, J. Soc. Chem. Ind., 58, 353 (1939).

230. H. Bull, Ber., 39, 3570 (1906).

231. E. Vongerichten and A. Kohler, Ber., 42, 1638 (1909).

232. I. D. Morton and A. R. Todd, Biochem. J., 47, 327 (1950).

233. S. S. Gupta, T. P. Hilditch, S. Paul and R. K. Shrivastava, J. Chem. Soc., 3484 (1950).

234. J. Nagai and K. Bloch, J. Biol. Chem., 240, PC3702 (1965).

235. J. Jacob and G. Grimmer, J. Lipid Research, 9, 730 (1968).

236. P. Harris and A. T. James, Unpublished results.

237. A. R. Johnson, J. A. Pearson, F. S. Shenstone, A. C. Fogerty, Nature, 214, 1244 (1967).

238. P. K. Raju, Dissertation abstracts, 237, 2064 (1962).

239. R. J. Evans, S. C. Bandemer, and J. A. Davidson, Poultry Science, 39, 1199 (1960).

240. R. J. Evans, J. A. Davidson and S. L. Bandemer, J. Nutn., 73, 282 (1961).

241. R. J. Evans, S. L. Bandemer, M. Anderson and J. A. Davidson, J. Nutn., 76, 314 (1962).

242. R. J. Evans, J. A. Davidson, J. N. LaRue and S. L. Bandemer, Poultry Science, 42, 875 (1963).

243. R. Rieser and P. K. Raju, Biochem. Biophys. Res. Comm., 17, 8 (1964).

244. A. R. Johnson, A. C. Fogerty, J. A. Pearson, F. S. Shenstone and A. M. Bersten, Lipids, 4, 265 (1969).

245. H. Debuch, Z. Naturforsch, 16b, 246 (1961).

246. J. Erwin and K. Bloch, Biochem Z., 338, 496 (1963).

247. J. Erwin and K. Bloch, Science, 143, 1006 (1964).

248. A. P. Tulloch, J. F. T. Spencer and P. A. J. Gorin, Canad. J. Chem., 40, 1326 (1962).

249. D. F. Jones and R. Howe, J. Chem. Soc., 2801 (1968).

250. H.E.C.Cargill-Thompson, A.N.Drury, D.C.Hardwick, J.L.Linzell and E.M.Tucker, J.Physiol., 143, 74P (1958)

251. J. B. Marsh and A. T. James, Biochemica Biophysica Acta., 60, 320 (1962).

252. S. J. Wakil, Private Communication.

253. G. W. Schwert and A. D. Winder, "Lactate dehydrogenase", Chapter 6, The Enzymes, Vol.7, Academic Press New York, 1963 page 127.

254. Dewitt S. Goodman, J. Amer.Chem. Soc., 80, 3892 (1958).

255. J. Reynolds, S. Hobert and J. Steinhardt, Biochemistry, 7, 1357 (1968).

256. A. T. James, Chemistry in Britain, 4, 484 (1968).

257. L. J. Morris, Plenary Lecture XIIth ICBL Loughborough 1968.

258. A. T. James and E. A. Piper, J. Chromatog., 5, 265 (1961).

259. A. T. James and C. Hitchcock, Kerntechnik., 7 5 (1965).

260. J. R. Ravenhill and A. T. James, J. Chromatog., 26, 89 (1967).

261. J. Folch, M. Lees and G. H. Sloane-Stanley, J. Biol. Chem., 226, 497 (1957).

262. B. W. Nichols and A. T. James, Fette Seifen Anstrichmittel, 66, 1003 (1964).

263. B. W. Nichols and A. E. Walsby, Nature, 221, 673 (1969).

264. P. Harris and A. T. James, Biochem. Biophys. Acta., 187, 13 (1969).

265. J. F. Mead, Ann. Rev. Biochem., 32, 241 (1963).

266. E. D. Korn, J. Lipid Res., 5, 352 (1964).

267. F. Davidoff and E. D. Korn, J. Biol. Chem., 238, 3199 (1963).

268. H. Schlenk and J. L. Gellerman, J. Amer. Oil Chem. Soc., 42, 504 (1965).

269. M. O. Bagby, L. R. Smith, K. L. Mikolajczak and I. A. Wolff, Biochemistry 1, 632 (1962).

270. R. W. Miller, M. E. Daxenbichler and F. R. Earle, J. Amer. Oil Chem. Soc., 41, 167 (1964).

271. L. J. Morris, M. O. Marshall and E. W. Hammond, Lipids, 3, 91 (1968).

272. C. Y. Hopkins and M. J. Chisolm, Chem. and Ind. (2064) (1962).

273. M. O. Bagby, C. R. Smith, I. A. Wolff, J. Org. Chem., 30, 4227 (1965).

274. J. M. Hagemann, F. R. Earle, I. A. Wolff and A. S. Barclay, Lipids, 2, 371 (1967).

275. L. J. Morris and M. O. Marshall, Chem. and Ind., 1493 (1966).

276. M. J. Chisolm and C. Y. Hopkins, Can. J. Chem., 41, 1888 (1963).

277. R. Kleiman, G. F. Spencer, F. R. Earle and I. A. Wolff, Chem. and Ind., 1326 (1967).

278. R. V. Harris and A. T. James, Biochem. Biophys. Acta., 106, 456 (1965).

279. F. Davidoff and E. D. Korn, J. Biol. Chem., 238, 3210 (1963).

280. L. J. Morris, Biochem. Biophys. Res. Comm., 29, 311 (1967).

281. A. T. James, Bull. Soc. Chim. Biol. Paris, 44, 951 (1962).

282. A. T. James, Symp. Biochem. Soc., 24, 17 (1963).

283. A. T. James, H. C. Hadaway and J. P. W. Webb, Biochem. J., 95, 448 (1965).

284. M. Yamada and P. K. Stumpf, Biochem. Biophys. Res. Comm., 14, 165 (1964).

285. D. T. Canvin, Canad. J. Bot., 43, 49 (1965).

286. T. Galliard and P. K. Stumpf, J. Biol. Chem., 241, 5806 (1965).

287. G. D. Meakins and R. Swindells, J. Chem. Soc., 1044 (1959).

288. K. E. Murray, Aust. J. Chem., 12, 657 (1959).

289. R.Adams and V.Voorhees, Organic Syntheses, 2nd Edition Coll.Vol.I page 60.

290. H. Schlenk and D.M.Sand, Biochem. Biophysica.Acta, 144, 305 (1967).

291. R. K. Beerthuis, D. H. Nugteren, H. J. J. Pabon and D. A. van Dorp, Rec. Trav. Chim. Pays Baas, 87, 461 (1968).