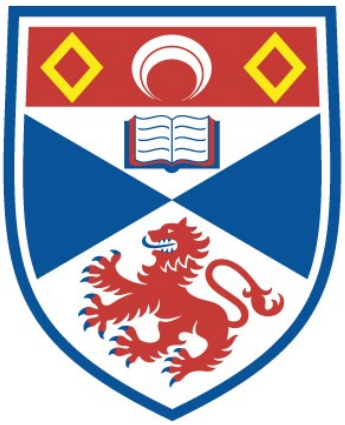
THE SYNTHESIS, ^{13}C NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY, AND ENZYMIC DESATURATION OF SOME UNSATURATED FATTY ACIDS

Michael Roman Pollard

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1977

THE SYNTHESIS, ^{13}C NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY, AND ENZYMIC DESATURATION OF SOME UNSATURATED FATTY ACIDS

A thesis presented by

Michael Roman Pollard

to the

University of St. Andrews

in application for

the degree of Doctor of Philosophy

May 1977

Abstract

A wide range of [1-^{14}C] monoenoic (32) and polyenoic (8) fatty acids and two unlabelled fatty acids containing a 6,7-cyclopropene ring were prepared by existing methods. Several procedures for the conversion of oleic acid to its C_{17} iodide with concomitant decarboxylation were investigated without success.

The CMR spectra of (27) saturated, (65) cis and trans monoenoic, (39) monoynoic, (2) cyclopropene, (20) cis-polyenoic, (14) diynoic and (10) mixed-function diunsaturated acids and methyl esters were recorded and interpreted. An empirical approach, based on the shielding and deshielding influence of functional groups on the rest of the molecule, was employed, and the assignment of ^{13}C resonances by this method agreed with those obtained by other researchers using different methods. The shielding and deshielding effects were generally additive, except when two function groups were close together. CMR spectroscopy can be used to distinguish cis and trans olefins, locate double and triple bond positions and interpret patterns of polyunsaturation.

The specificity of the mammalian desaturases was examined by incubating [1-^{14}C] fatty acids with a rat liver microsomal preparation and analysing the products formed after one hour.

The Δ9 desaturase was believed to hold its substrate by the binding of the CoA moiety at the carboxyl end and by the fit of the alkyl chain into a deep, narrow cleft, where, except at the active centre, it was constrained to take up predominantly trans conformations. This model is confirmed. Trans monoenoic acids were generally Δ9 desaturated to give as high conversions as saturated acids, and the Δ7t and Δ11t monoenoic acids gave the corresponding Δ7t9c and Δ9c11t conjugated dienes. Cis monoenoic acids, which were not expected to fit into the enzyme cleft between C(5) and C(15), were not Δ9 desaturated unless the double bond position was beyond Δ13, and even then they were much poorer substrates than stearic acid.

The Δ6 desaturase shows a much wider substrate specificity than was previously anticipated. The enzyme can accommodate a wide range of substrate chain lengths (at least C_{14} to C_{22}) and the Δ9 *cis* double bond is not obligatory for Δ6 desaturation. The model advanced for the enzyme contains two distinct features. Firstly, a region of steric constraint between C(10) and C(13) on the substrate geometry acceptable to the enzyme is proposed. Secondly, π-bond binding sites on the enzyme surface in the C(9) to C(13) region are envisaged. Dienoic acids with a Δ9c double bond and adjacent olefinic unsaturation (Δ11-Δ13) were much better substrates than monoenoic acids or dienoic acids containing a Δ9c double bond and a second double bond beyond the Δ13 position. Therefore, to be d substrate for Δ6 desaturation the co-operative binding of two nds, separat ed by 0-2 methylene groups, to these π-binding s is essential.

The Δ5 desaturase also shows a wider substrate specificity than was previously anticipated. The principal feature is a high degree of chain length specificity. Within the C_{16}-C_{20} range tested C_{20} was the optimum chain length while minimal Δ5 desaturation occurred when the chain length dropped to C_{16}. A region of steric constraint between C(10) and C(13) on the substrate geometry acceptable to the enzyme is envisaged. However, the range of acids tested was not extensive and an evaluation of the role of π-bonds in substrate-enzyme binding was not possible.

No Δ4 desaturation was observed. This agreed with other studies. Some ideas for the production of a Δ4 *cis* PUFA not involving a direct, microsomal Δ4 desaturation step are advanced.

Δ6 Desaturation was not strongly inhibited by a 6,7-cyclopropene C_{18} fatty acid, though a mild inhibitory effect was discerned. Thus Δ6 desaturation differs from Δ9 desaturation, which is strongly inhibited by low concentrations of sterculoyl-CoA. When Δ9 desaturation was almost totally inhibited by sterculic acid some Δ6 and Δ5 desaturation of saturated fatty acids occurred.

Preliminary investigations into the time course of desaturation were undertaken, using low substrate and high microsomal protein concentrations. An unexpected feature occurred for the Δ6 desaturation of 18:3(9c12c15c) and the Δ5 desaturation of 20:3(8c11c14c). There was a rapid initial removal of substrate into lipids, which stopped abruptly after 1-2 minutes, whereas desaturation continued. An explanation in terms of the acyl-CoA substrate concentration controlling competition between lipid incorporation and desaturation is offered, with low concentrations favouring the latter. BSA had a variable effect on the stimulation of *in vitro* desaturation. Δ5 and Δ6 desaturation of PUFA was enhanced, and this effect was attributed to BSA-substrate binding reducing the concentration of free acyl-CoA substrate, thus favouring desaturation.

To

Seweryn Marek Podolski

and

Betty Margaret Podolska

Declaration

I hereby declare that this thesis is a record of the results of my own experiments, that it is my own composition, and that it has not previously been presented in application for a higher degree.

The research was carried out in the Department of Chemistry, University of St. Andrews, under the supervision of Professor F.D. Gunstone, D.Sc., F.R.I.C., and in Colworth House (Unilever Research Ltd) under the supervision of Dr. L.J. Morris.

University Career

I graduated in 1973 from St. Andrews University with the degree of Bachelor of Science, and was admitted as a research student to the United College, University of St. Andrews in October 1973, with a CASE studentship provided by the Science Research Council. Unilever Research, Colworth House was the co-operating body.

Certificate

I hereby certify that Michael Roman Pollard has completed thirteen terms of research work under my supervision, has fulfilled the conditions of the Resolution of the University Court 1967, No. 1 (St. Andrews) and that he is qualified to submit the accompanying thesis in application for the degree of Doctor of Philosophy.

Research Supervisor

Acknowledgements

I would like to express my sincere gratitude to Professor F.D. Gunstone and to Dr. L.J. Morris for their willingness to listen to my problems, their guidance, counsel, and encouragement throughout my project. I am also deeply indebted to Dr. A.T. James, for his direction, to Mr. Eugene Hammond for helping me to find my feet when I first arrived at Colworth House (and subsequently), and to Dr. Roger Jeffcoat and Dr. Charlie Scrimgeour for much practical advice and discussion at the bench.

I thank all the other lipid research workers and the staff at St. Andrews and Colworth House for their advice, help and good company. I am very grateful to Mrs. Melanja Smith for running so many NMR spectra, and to Mrs. Wilma Pogorzelec for typing this thesis. And I must mention the kindness shown to me by Graham and Gail Collins during my stay in Sharnbrook.

Finally I must thank the Chemistry Department (St. Andrews) and the Society of Chemical Industry for sponsoring my attendance at various meetings, the Science Research Council for my maintenance grant, and Unilever Research for the use of their scientific facilities and for other assistance.

Contents

Abbreviations

Unsaturated fatty acid structure is denoted by A:B ( ), where A is the chain length and B is the number of unsaturated centres in the molecule. The expression in parenthesis shows the nature of the unsaturation and its position relative to the carboxylic acid group C(1). "a" denotes a triple bond, "c" a cis double bond, "t" a trans double bond and "e" a double bond of unspecified configuration. Other functional groups are represented by the appropriate symbol prefixing A.

eg. 18:1(9c) oleic acid

12-OH 18:1(9c) ricinoleic acid

Δ gives the position of unsaturation counting from the carboxyl end, ω the position counting from the methyl end.

Ag^+TLC	Argentation thin layer chromatography
ATP	Adenosine-5'-triphosphate
bp	Boiling point
BSA	Bovine serum albumin
CMR	^{13}C nuclear magnetic resonance
CoA	Coenzyme A
DEGS	Diethylene glycol succinate
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
ECL	Equivalent chain length
EFA	Essential fatty acid
GLC	Gas liquid chromatography
HMPA	Hexamethylphosphoramide
IR	Infra-red
	The symbols used to describe an IR absorption are (s) - strong intensity, (m) - moderate intensity, (w) - weak intensity, (i) - inflexion and (b) - broad.
Mes	Methanesulphonyl (mesyl)
mm	mm mercury pressure (torr)
NADH	Nicotinamide adenine dinucleotide

NADPH	Nicotinamide adenine dinucleotide phosphate
NL	Neutral lipid
NMR	Nuclear magnetic resonance
PE00	A mixture of petroleum (bp 40-60°C) and diethyl ether. The number after the abbreviation indicates the % by volume of ether in petrol.
PL	Phospholipid
PMR	Proton magnetic resonance A typical PMR signal is represented as 2.00, t, 4H. ie. A triplet 2.00 ppm downfield from tetramethylsilane internal standard, with an integral indicating four protons. Coupling constant data is given only where considered necessary (eg. 2.00, t(J=6Hz), 4H). The symbols used to describe the multiplicity of the signal are:- s - singlet d - doublet t - triplet q - quartet qu - quintet sx - sextet m- multiplet b - broad D - distorted
PUFA	Polyunsaturated fatty acid
R_f	Retention index (TLC)
RGLC	Radiochemical gas liquid chromatography
RTLC	Radiochemical thin layer chromatography
THF	Tetrahydrofuran
thp	Tetrahydropyranyl
TLC	Thin layer chromatography
TMS	Trimethylsilyl
Tos	p-Toluenesulphonyl (tosyl)
δ	Chemical shift
$_{\alpha}\Delta^{C}(X)$	Substituent shielding parameter (Section 3:2:1)

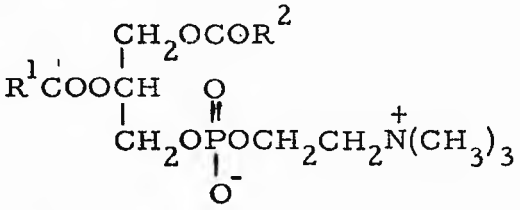
DMAA	N, N-Dimethylaminoacetonitrile
FAD	Flavin-adenine dinucleotide
ACP	Acyl carrier protein

Section 1. General Introduction

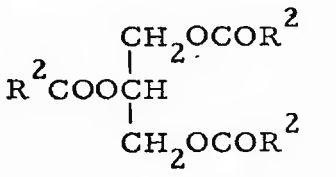
Fatty acids are essential constituents of the living cell. They occur mainly as esters of glycerol which may be neutral or polar. Polar lipids, such as phosphatidylcholine (Figure 1a), have an ionisable functional group which gives the molecule an amphipathic character, and consequently they play an important structural role in the cell. Neutral lipids contain no easily ionisable functional group, and are represented mainly by triacylglycerols (Figure 1b), which serve an important energy storage function. A more extensive classification of lipid molecules, their location, function, and biosynthesis can be found in most biochemistry textbooks [1, 2].

Figure 1: Typical Phospholipid, Neutral Lipid, and Prostaglandin Structures.

(1a) 1-Palmitoyl-2-arachidonoyl-sn-glycero-3-phosphatidylcholine or L 1-palmitoyl-2-arachidonoyl-glycerol-3-phosphorylcholine.



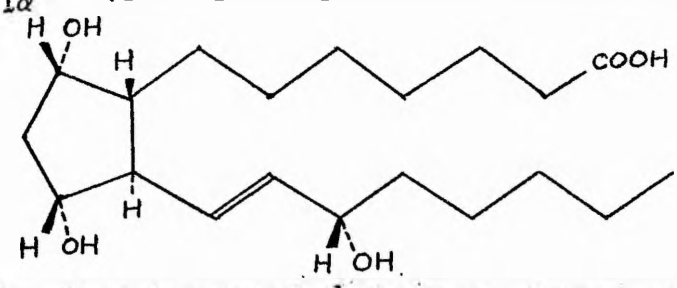
(1b) Tripalmitoyl-sn-glycerol (Triacylglycerol).



$R^1 = CH_3(CH_2)_4(CH{=}^{cis}CHCH_2)_4(CH_2)_2-$

$R^2 = CH_3(CH_2)_{14}-$

(1c) $PGF_{1\alpha}$, a typical prostaglandin, derived from 20:3(8c11c14c)



Fatty acids can be classified as saturated or unsaturated molecules. A list of some of the more common fatty acids, with their trivial names, is given in Table 1. Unsaturated fatty acids found in higher animals usually have an even number of carbon atoms in the chain (C_{16} to C_{22}) and one to six *cis* double bonds in a methylene-interrupted pattern. Unsaturated fatty acids isolated from plants show a greater diversity of structure, including acetylenic, allenic, and *trans* unsaturation, conjugated and polymethylene-interrupted patterns of unsaturation and hydroxy, epoxy, and oxo functional groups [3].

PUFA primarily serve a structural role in biological membranes, where the phospholipid molecules are organised into bilayers or in lipoprotein complexes. However they can also be utilised as an additional energy source since they can be catabolised by the mitochondrial β-oxidation pathway. PUFA are more important in membranes than saturated acids because they give the membrane greater fluidity. The presence of *cis* double bonds destroys the ordered packing of the hydrocarbon chain that occurs with saturated acids.

In higher animals C_{20}PUFA (and also C_{19} and C_{21}PUFA, although these do not occur naturally), with the Δ8c11c14c double bond arrangement as a minimum structural requirement, are converted into prostaglandins by ring closure and oxygenation. Prostaglandins, an example of which is shown in Figure 1c, have marked physiological properties, and these are leading to their pharmaceutical use [191]. Prostaglandin formation correlates very closely with essential fatty acid activity [6], but whether it is the only role of EFA activity is still open to question. EFA is a generic term originally applied to fatty acids which stimulated growth when supplemented to a fat free diet and which prevented or cured dermatitis. The complex nature of EFA activity has been reviewed by Holman [192].

The sources of fatty acids in animals are *de novo* synthesis from acetate and dietary intake, with subsequent metabolism of both *de novo* and ingested acids. *De novo* synthesis is catalysed by the fatty acid synthetase, a cytoplasmic multi-enzyme complex, and produces saturated fatty acids, predominantly palmitic [5]. Higher animals also have a series of enzymes, bound to the endoplasmic reticulum, that can progressively desaturate and elongate dietary and preformed acids. The sequence has

Table 1: Trivial Names and Structures of Some Common, Naturally Occurring Fatty Acids.

Systematic Name	Symbol	Trivial Name
Hexadecanoic	16:0	Palmitic
Hexadec-*cis*-9-enoic	16:1(9c)	Palmitoleic
Octadecanoic	18:0	Stearic
Octadec-*cis*-9-enoic	18:1(9c)	Oleic
Octadeca-*cis*-9,*cis*-12-dienoic	18:2(9c12c)	Linoleic
Octadeca-*cis*-9,*cis*-12,*cis*-15-trienoic	18:3(9c12c15c)	α-Linolenic
Octadeca-*cis*-6,*cis*-9,*cis*-12-trienoic	18:3(6c9c12c)	γ-Linolenic
Octadeca-*cis*-9-en-12-ynoic	18:2(9c12a)	Crepenynic
Eicosa-*cis*-5,*cis*-8,*cis*-11,*cis*-14-tetraenoic	20:4(5c8c11c14c)	Arachidonic

been elucidated from data collected by many *in vivo* and *in vitro* studies and is illustrated by the pathway for $\omega 7$ acids [6, 15].

$$16{:}0 \xrightarrow{\Delta 9 \text{ desaturase}} 16{:}1(9c) \xrightarrow{\Delta 6 \text{ desaturase}} 16{:}2(6c9c)$$

$$\xrightarrow{\text{elongase, +2C}} 18{:}2(8c11c) \xrightarrow{\Delta 5 \text{ desaturase}} 18{:}3(5c8c11c)$$

$$\xrightarrow{\text{elongase, +2C}} 20{:}3(7c10c13c) \xrightarrow{\Delta 4 \text{ desaturase}} 20{:}4(4c7c10c13c).$$

The most important points concerning the biosynthesis of unsaturated acids in animals are:-

(1) Four desaturases are believed to be operative, removing two hydrogen atoms stereospecifically to form *cis* double bonds at the $\Delta 4$, $\Delta 5$, $\Delta 6$ and $\Delta 9$ positions. The desaturases are believed to exhibit carboxyl-end specificity towards the substrate, but whether each

desaturase is a single enzyme is still uncertain.

(2) An elongase(s) of general specificity promotes the addition of two carbon atoms at a time from malonyl-CoA.

(3) Acyl S.CoA is the true substrate in all the reactions. Enzymes are present which can synthesise or hydrolyse acyl-S.CoA and which can incorporate the acyl moiety from the thioester into lipids.

(4) The desaturation reaction is aerobic and irreversible, and has an overall equation:-

$$RCH_2CH_2(CH_2)_nCO.SCoA + NADH + O_2 + H^+ \xrightarrow{\text{microsomes}} RCH{=}CH(CH_2)_nCO.SCoA + NAD^+ + 2H_2O$$

(5) All unsaturated fatty acids found in vertebrates can be explained in terms of these four desaturases and an elongase, plus a retroconversion step which occurs in the mitochondria [6].

It is significant that higher animals do not contain a desaturase which can introduce a double bond beyond the Δ9 or at the Δ7 and Δ8 positions. The main saturated acids present in animals, palmitic and stearic acids, undergo Δ9 desaturation to give palmitoleic and oleic acids respectively. However PUFA produced from these two monoenoic acids (themselves products of the de novo pathway utilising acetate) will not contain the essential C_{20}, Δ8c11c14c structure. Therefore EFA must be obtained from dietary sources. Higher plants contain desaturases which can introduce a double bond at the Δ12 and Δ15 positions in a C_{18} acid, via the pathway:-

$$\underset{}{18{:}0} \longrightarrow \underset{[\omega 9]}{18{:}1(9c)} \longrightarrow \underset{[\omega 6]}{18{:}2(9c12c)} \longrightarrow \underset{[\omega 3]}{18{:}3(9c12c15c)}$$

Dietary linoleic and α-linolenic acids are the EFA in animals. These unsaturated C_{18} acids can be converted to C_{20} acids containing the Δ8c11c14c structure. It is ω6 and ω3 acids that constitute the PUFA found in a healthy animal.

ω 6 Family

18:2(9c12c) ⟶ 18:3(6c9c12c) ⟶ 20:3(8c11c14c)* ⟶

20:4(5c8c11c14c)* ⟶ 22:4(7c10c13c16c) ⟶ 22:5(4c7c10c13c16c)

ω 3 Family

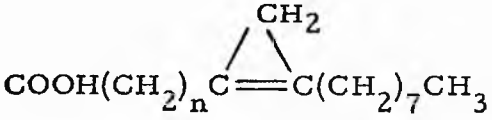
18:3(9c12c15c) ⟶ 18:4(6c9c12c15c) ⟶ 20:4(8c11c14c17c)*

⟶ 20:5(5c8c11c14c17c)* ⟶ 22:5(7c10c13c16c19c) ⟶

22:6(4c7c10c13c16c19c)

* These PUFA can be converted to prostaglandins in animals.

Cyclopropenoid fatty acids occur naturally in the seed oils and fruit fats of the Malvacae, Sterculaceae, Tiliaceae and Bombacaceae plant families. The most common of these unusual acids are malvalic and sterculic, whose structures are given below.



(Malvalic acid, n=6)

(Sterculic acid, n=7)

Although sterculic acid contains 19 carbon atoms it will be referred to in the text as a 9, 10-cyclopropene C_{18} acid. Interest in cyclopropene acids was aroused when animals fed on diets containing them showed a marked reduction in the level of oleate and a corresponding increase in the level of stearate in their tissues. James et al using plant and algal systems [7], Reiser et al using rats [8], and Johnson et al using hens [9] have all shown that biological systems capable of introducing a Δ9cis double bond into a saturated fatty acid were inhibited by sterculic and malvalic acids. The light that this inhibition throws on models for the Δ9 desaturase is discussed in section 2:1. It was of interest to see if a 6,7-cyclopropene fatty acid would inhibit Δ6 desaturation.

The enzymes involved in desaturation and elongation have proved very difficult to isolate in a soluble form or to purify, primarily because

they are membrane-bound. A successful purification of rat liver Δ9 desaturase has only recently been reported, by Strittmatter et al, using detergent solublisation techniques [10]. However much useful information on the nature of the enzyme-substrate interaction of the desaturases has come from substrate specificity studies.

The research described in this thesis involved the synthesis of [1-^{14}C] labelled fatty acids of varying chain length and unsaturation in order to test certain aspects of the substrate requirements and specificity of the Δ4, Δ5, Δ6 and Δ9 desaturases. It was hoped to be able to check, modify, or extend existing models for these enzymes. In particular a series of [1-^{14}C] trans monoenoic acids was prepared, together with several [1-^{14}C] Δ8 cis, Δ9 cis, and Δ9 cis 12 cis acids of varying chain length. Some of these substrates were also used in studies on the specificity of the Δ5 desaturase of the bacterium Bacillus megaterium undertaken by Hammond and Hitchcock at Colworth House [11].

A comprehensive review covering most aspects of desaturase biochemistry in bacterial, plant, and animal systems has been written by M.I. Gurr [12].

To supply materials for biochemical and physical studies and to verify the structures of natural acids by total synthesis a variety of methods for the preparation of labelled and unlabelled unsaturated fatty acids has already been developed [13, 14]. Existing routes were used in this study, with the introduction of the ^{14}C radio label by chain extension with K^{14}CN as the last step.

Allied to the synthetic methods are a range of chromatographic, chemical and spectroscopic techniques which can be used to identify or to check the purity of unsaturated fatty acids [13, 16]. Complete identification is usually accomplished by a combination of techniques. Recently nmr spectrometers which can measure chemical shifts on natural abundance ($\sim$1%) ^{13}C have become commercially available. A limited amount of data on the CMR spectroscopy of fatty acids has been published, so the acids obtained in this work along with others available at St. Andrews were examined and rules for the interpretation of CMR spectra of unsaturated fatty acids derived.

Section 2. In Vitro Studies On The Mammalian Desaturases.

2:1 Introduction

The elucidation of the structure of the major fatty acids present in higher animals (mammals, birds, and fish) laid the basis for and prompted the investigation into the interconversions of unsaturated acids. A survey of ^{14}C and T *in vivo* and *in vitro* tracer work and of feeding studies, done in the 1960s and early '70s, led to the proposition that the interconversions can be explained solely in terms of four desaturases, and two-carbon chain elongation and retroconversion steps [6, 15, 17, 18]. The sequence of events on the pathway to PUFA has already been given in Section 1, but to recapitulate it is Δ9 desaturation followed by Δ6 desaturation, elongation, Δ5 desaturation, elongation and finally Δ4 desaturation. Desaturases exhibiting a Δ7, Δ8 and Δ10 or greater carboxyl-end control or methyl-end control are believed to be absent in animals. This limits the pathways by which PUFA can be produced, and the term "stop point" has been coined by James to describe any branch which (as a result of chain elongation) cannot lead to further desaturation (without retroconversion) [6].
The previous investigations resulted in the following generalisations on desaturase specificity in higher animals:-

(1) A *cis* double bond is always introduced.

(2) The position of the new double bond is specific with respect to the carboxyl end, and can be introduced only in the Δ4, Δ5, Δ6 and Δ9 positions.

(3) The new double bond can be inserted only between the carboxyl group and the first existing double bond in the substrate.

(4) Except for the Δ9 desaturase which recognises saturated fatty acids as substrates the other desaturases recognise only *cis* monoenes or methylene-interrupted polyenes. In particular, the Δ4, Δ5 and Δ6 desaturases extend the all-*cis* methylene-interrupted polyene system.

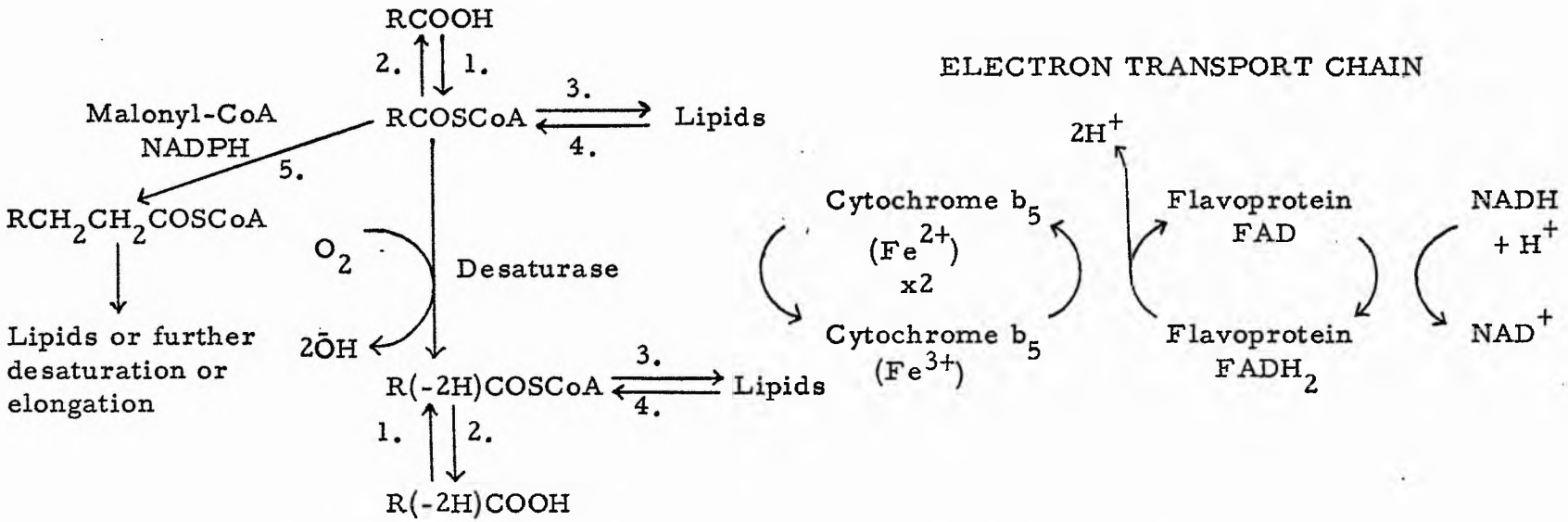
ie. The Δ6 (Δ5,Δ4) desaturase requires Δ9c or Δ9c12c..(Δ8c11c..., Δ7c10c...) substrates to give Δ6c9c or Δ6c9c12c..(Δ5c8c11c..., Δ4c7c10c...) products.

One question that the present work attempted to answer was whether the Δ5 and Δ4 desaturases would recognise Δ8c and Δ7c monoenoic substrates respectively as well as the more conventional PUFA substrates. Evidence, discussed later, had already begun to accumulate that contradicted generalisation (4), and during the course of this work it became apparent that both generalisations (3) and (4) were inadequate.

The Δ9, Δ6 and Δ5 desaturases are known to be microsomal enzymes, but with the recent exception of the Δ9 desaturase [10] these have yet to be purified. Therefore most of the in vitro work cited in the literature has been done with "whole" microsomes (ie. the mitochondrial supernatant or an unwashed microsomal pellet that includes residual supernatant). Figure 2 shows the relevant pathways that operate in the microsomes. The electron transport chain belonging to the Δ9 desaturase is shown, and since the Δ5 and Δ6 desaturases require the same cofactors as the Δ9 desaturase (NADH and molecular oxygen) it may be considered that all three desaturases use the same electron transport process. This remains to be demonstrated. In the microsomes the competing reactions of chain elongation and lipid incorporation may control the desaturation step. Figure 2 is a very generalised scheme: factors such as the organisation of the enzymes on the endoplasmic reticulum or the presence of soluble substrate binding proteins may be significant and may vary from desaturase to desaturase. A discussion of the in vivo controls on the levels of desaturase activity by such factors as diet, health, or hormonal stimulation or depression of the animal or at a molecular level by limiting oxygen tension or cofactor availability is beyond the scope of this thesis [17, 77, 108, 109].

That the immediate substrates for desaturation are the acyl-CoA thioesters and not free fatty acids has been demonstrated by the necessity for ATP, CoASH and Mg^{2+} ions as cofactors when incubating

Figure 2. Fatty Acid Metabolism in the Microsomes



1. Thiokinase (also called acyl-CoA synthetase or fatty acid-CoA ligase), ATP, Mg^{2+}, CoASH
2. Thioesterase (also called acyl-CoA hydrolase or fatty acid-CoA lipase)
3. Acyl-transferase
4. Lipase
5. Elongase

free fatty acids with whole microsomes. These cofactors can be omitted when using the acyl-CoA derivative. The absolute requirement for stearoyl-CoA by the purified Δ9 desaturase system was shown by Strittmatter et al [10, 19]. Using whole microsomes oleoyl-CoA [20] and linoleoyl-CoA [21, 22] were shown to be substrates for the Δ6 desaturase and eicosa-8-cis, 11-cis, 14-cis-trienoyl-CoA to be the substrate for the Δ5 desaturase [23]. At present there is no reason to suspect a further transfer of the acyl moiety to acyl carrier protein, the enzyme or lipid, which would then be the true substrate for either of these desaturases. No suitable in vitro preparation of the Δ4 desaturase has ever been reported so that there is a complete absence of data about its substrate and cofactor requirements.

Marcel and Suzue [24, 25] reported high maximum velocities (150-300 nmole.min^{-1}.mg^{-1} of protein) for the fatty acid-CoA ligase in washed microsomes using a wide range of substrates. Pande [26] concluded that fatty acid-CoA ligase activity is stabilised by high concentrations of phosphate buffer or ATP at 37°C and reported specific activities in the range of 60-130 nmole.min^{-1}.mg^{-1} of protein for 10:0-18:0. Lippel et al observed that the Δ4 to Δ15 trans [27] and the Δ4 to Δ17 cis [28] octadecenoic acids were also rapidly activated using a washed microsomal enzyme preparation, with rates of the order of 100 nmoles.min^{-1}.mg^{-1} of protein. These authors expressed the opinion that there was probably just a single fatty acid-CoA ligase with a wide specificity operative in the microsomes. It is generally accepted that, since the activation reaction is ten to a hundred times faster than the maximum rates for desaturation reactions in the microsomes, the former exhibits no rate control over the latter. Paulsrud et al [29] found that the mean, initial linear rate for stearic acid desaturation by normal rat liver microsomes was 0.4 nmoles.min^{-1}.mg^{-1} of protein, and they compared this with the figure of 0.35-0.4 nmoles.min^{-1}.mg^{-1} of protein quoted by Nakagawa and Uchiyama [30] for stearoyl-CoA as substrate and concluded that

acyl-CoA could be generated *in situ* at a sufficient rate not to be a limiting factor. The acyl-CoA hydrolase(s) has not received much study, but it is active in a crude microsomal system though not a partially-purified one [31]. It is certainly a cytoplasmic enzyme [22] but whether thioesterase activity is also bound to the endoplasmic reticulum is not yet known.

Microsomal chain elongation was investigated by Nugteren [32], who showed the requirement of NADPH and malonyl-CoA as cofactors and demonstrated the following sequence of reactions:-

$$RCOSCoA \xrightarrow{\text{malonyl-CoA (SLOW)}} RCOCH_2COSCoA \xrightarrow{NADPH, H^+}$$

$$RCH(OH)CH_2COSCoA \xrightarrow{-H_2O} RCH^{t}{=}CHCOSCoA \xrightarrow{NADPH, H^+}$$

$$RCH_2CH_2COSCoA$$

For the palmitoyl-CoA elongase the steps were reversible in certain instances, and all the intermediates were reversibly bound to the multienzyme complex. The elongase(s) can cope with a wide range of fatty substrates, and 16:0 and C_{18} PUFA containing a Δ6*cis* double bond are notable for their high rates of elongation [6, 18, 32, 33, 35-37]. The number of elongases that operate in the microsomes is not yet known, but Sprecher proposed at least two, one utilising 16:0 as substrate and the other 18:2(6c9c) and 18:3(6c9c12c), on the grounds of differential stimulation of enzyme activity and competitive inhibition studies [33].

Retroconversion is part of the unsaturated fatty acid interconversion network, and has been demonstrated *in vivo* for a wide range of long chain ($C_{20, 22}$) PUFA [6] and also for 20:1(11c) [15]. It was thought to occur by a reversal of microsomal elongation, coupled with Δ2 or Δ3 hydrogenation where necessary, or by a reversal of the mitochondrial β-oxidation pathway [38], but only a limited amount of *in vitro* work has been done. Ayala *et al* showed that 22:4(7c10c13c16c) was retroconverted to 20:4(5c8c11c14c) in rat testes and liver mitochondria [39]. Stoffel *et al* demonstrated that 18:2, 20:2, 20:3, and 20:4 acids of the ω6 series were not retroconverted, but that 22:3(10c13c16c) and 22:4(7c10c13c16c) were

retroconverted to their corresponding C_{20} acids [40]. This degradation occurred at the inner mitochondrial membrane, not the microsomes, and the authors proposed a partial β -oxidation, with the proviso that there must be mechanisms for the rapid removal of the C_{20} acids produced from the β -oxidation pathway in order to prevent total degradation and for the rapid transport of the product to the microsomes. Kunau and Bartnik demonstrated that a 4-enoyl-CoA reductase, present in rat liver mitochondria, required a Δ4c7c10c13c double bond arrangement as a minimum structural requirement before hydrogenation of the substrate [41]. The retroconversion pathway for 22:5 ω 6 was:-

22:5(4c7c10c13c16c) ⟶ 22:4(7c10c13c16c) ⟶ 20:4(5c8c11c14c)

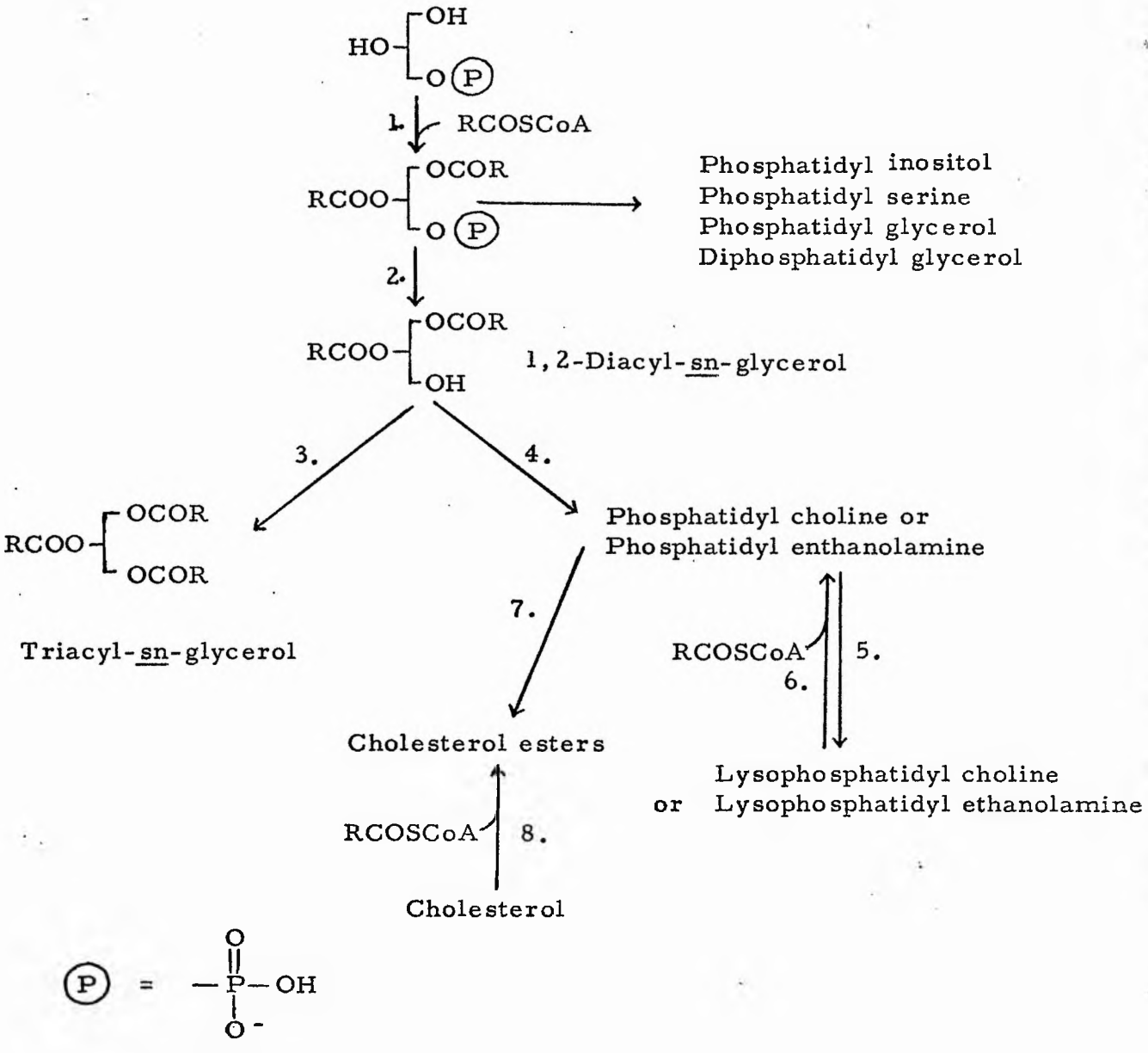
and not chain shortening followed by Δ2 hydrogenation.

The major lipid incorporation pathways present in the microsomes are shown in Figure 3. Acyl-CoA can be removed from the system, and hence from the desaturases by a variety of acyl-transferases exhibiting different specificities. Saturated and trans monoenoic acids are primarily esterified at the 1 or 3 positions in glycerolipids, while PUFA have a strong preference for the 2 position. Oleic acid may be esterified at either primary or secondary positions. The complexity of the acyl-transferase specificities may be gauged from studies on the acyl-CoA: 1-acyl (or 2-acyl)-glycero-3-phosphoryl-choline acyl-transferase in rat liver microsomes where rates between 1 and 30 nmoles.min^{-1} mg^{-1} of protein were recorded for a wide range of fatty substrates [42-44].

2:1:1 The Δ9 Desaturase

Studies, principally in the laboratories of Wakil and Holloway have shown [45-47] that the stearoyl-CoA desaturase is a multicomponent system that requires cytochrome b_5 reductase (a flavoprotein), cytochrome b_5 (a hemoprotein), the desaturase and lipid. Sato and his colleagues were primarily responsible for demonstrating that the process of desaturation involved electron

Figure 3: The Major Lipid Incorporation Pathways in the Microsomes

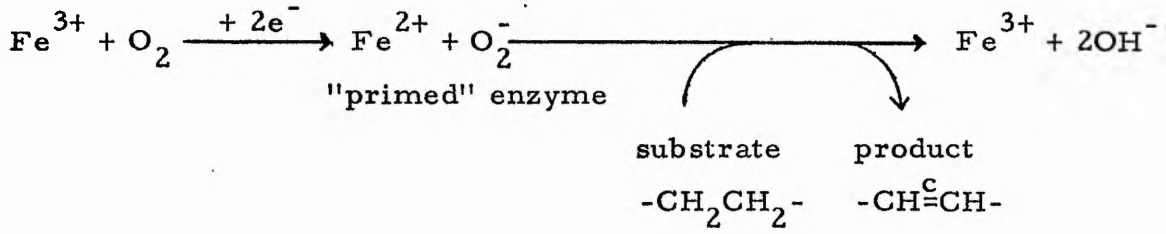


1. Glycerol-3-phosphate acyl-transferase
2. Phosphatidate phosphohydrolase
3. 1,2-Diglyceride acyl-transferase
4. 1,2-Diglyceride:choline (or ethanolamine) transferase
5. Phospholipase
6. 1-Acyl (or 2-Acyl)-glycero-3-phosphorylcholine (or phosphoryl-ethanolamine) acyl-transferase
7. 1,2-Diacylglyceryl phosphorylcholine (or phosphorylethanolamine) acyl-transferase
8. Cholesterol:O-acyl transferase

transport from NADH to cytochrome b_5 via the flavoprotein, and then to the desaturase protein [48,49]. Their earlier work had shown that stearoyl-CoA Δ9 desaturation was inhibited by cyanide ions and they proposed that this inhibition occurred at the desaturase protein, now often termed the cyanide-sensitive factor (CSF) [50]. Cytochrome b_5 and its reductase have been purified for several species of animal, and are well characterised [31,51,52]. Each of these proteins has a hydrophilic and a smaller hydrophobic domain. They are believed to bind to the microsomes by non-polar interactions between their hydrophobic domain and the hydrocarbon region of the lipid bilayer, with their catalytic sites at the surface of the membrane [53]. The binding domain of each can be removed by trypsin digestion, but although the soluble b_5 reductase fragment may still serve in the electron transport chain to give an active Δ9 desaturase the soluble cytochrome b_5 fragment substituted for the intact amphiphilic protein will not [10,49]. The recent purification of the CSF by Strittmatter and his coworkers [10] has enabled them to reconstitute an active Δ9 desaturase system from purified rabbit liver b_5 reductase, calf liver cytochrome b_5, rat liver CSF and either egg lecithin or dimyristoyl lecithin vesicles. Although other workers have reported soluble factors that stimulate Δ9 desaturase activity in whole microsomes [22,54] these are not obligatory [31] and were not used in the reconstituted system. The CSF isolated from rat liver microsomes had a molecular weight of 53,000, contained one mole of non-heme iron, and had 62% non-polar amino-acid residues. The iron atom could only be removed by ferrous chelators on preincubation with NADH and the electron transport chain components, which suggests that the iron changes oxidation state during the process of desaturation, and as it was removed by reagents which do not penetrate the liposomes it must reside at the surface [10,19]. Since the desaturase protein has 62% non-polar amino-acid residues it is deeply embedded in the liposome apolar region, but has a hydrophilic domain containing the iron atom at the surface. The inhibition of Δ9 desaturation by cyanide ions can be explained in terms of

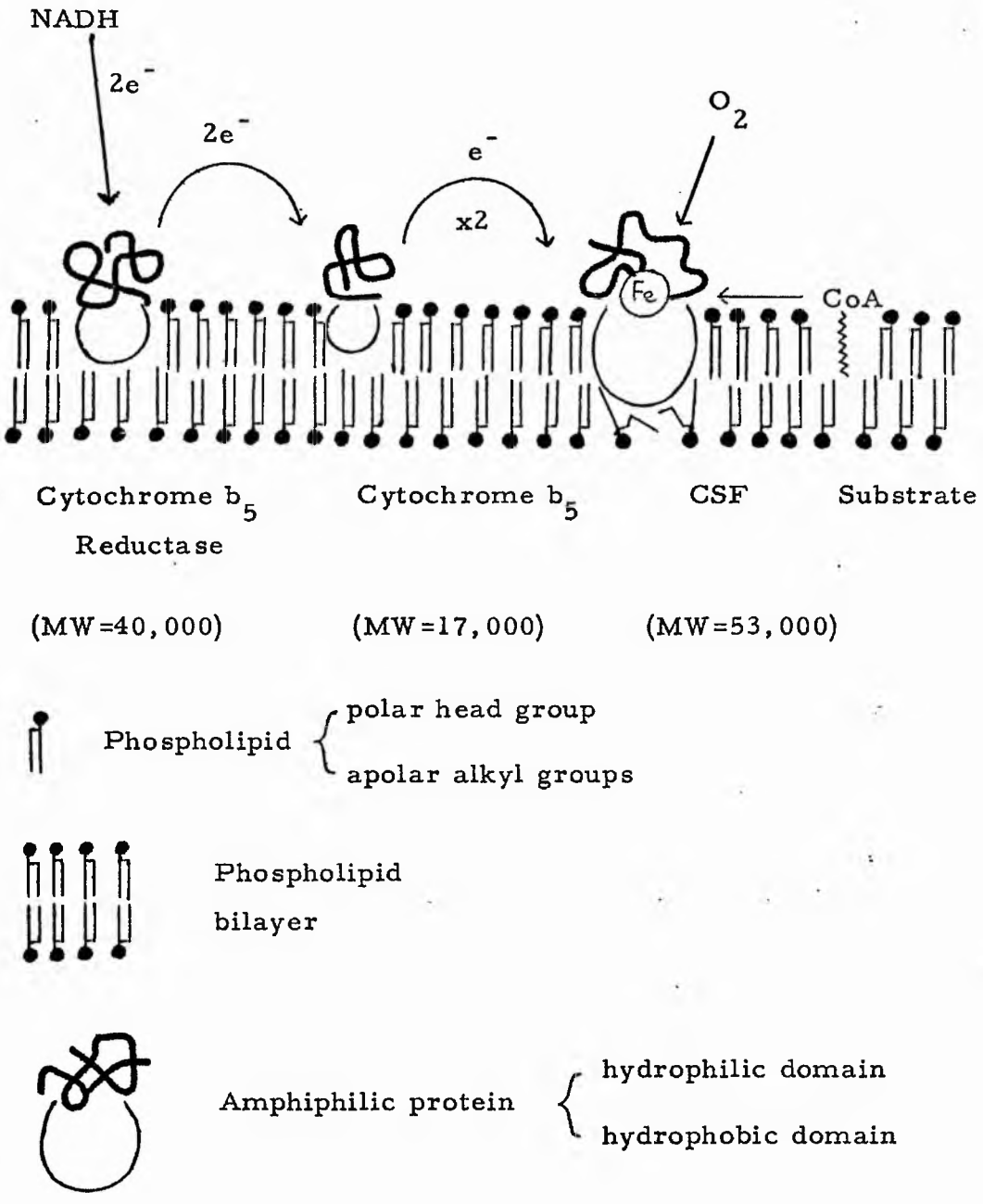
chelation to the iron atom. Strittmatter et al believe that the substrate for the Δ9 desaturase is stearoyl-CoA bound to the liposomes. It has been suggested that amphiphilic acyl-SCoA derivatives may bind to the microsomes [55] and to phospholipid vesicles [56, 57] by insertion of their long, alkyl chains vertically into the hydrocarbon region of the bilayer. Thus we may picture the Δ9 desaturase system as shown in Figure 4.

The mechanism of hydrogen abstraction at the active centre is not known, but molecular oxygen must be utilised at the CSF. To balance the stoichiometry cytochrome b_5 must donate two electrons to the desaturase, but since it is a single electron carrier the process of donation must occur twice. Oxygenated intermediates in the desaturation process have never been isolated, and all the evidence points to direct hydrogen abstraction. Therefore it is proposed that the following reactions give a correct, overall picture of Δ9 desaturation, though the more detailed sequence of events is unknown:-



Using the purified system a primary isotope effect was observed with 9, 9, 10, 10-$^2D_4$-stearoyl-CoA as substrate, so that above the transition temperature of the membrane hydrogen abstraction is rate-limiting [19]. This agrees with earlier results of Morris et al [58] for goat mammary gland and hen liver whole microsomes, when a substantial kinetic isotope effect was found for both 9D and 10D hydrogen atoms. Furthermore, Johnson and Gurr demonstrated that the isotope effect in the removal of the two T atoms at the 9 and 10 positions of stearic acid or stearoyl-CoA in hen liver whole microsomes was twice that for the removal of one T atom at either the 9 or 10 position [59]. These considerations imply that Δ9 desaturation involves a concerted removal of the 9D and 10D

Figure 4. A Schematic Representation of Microsomal Δ9 Desaturation



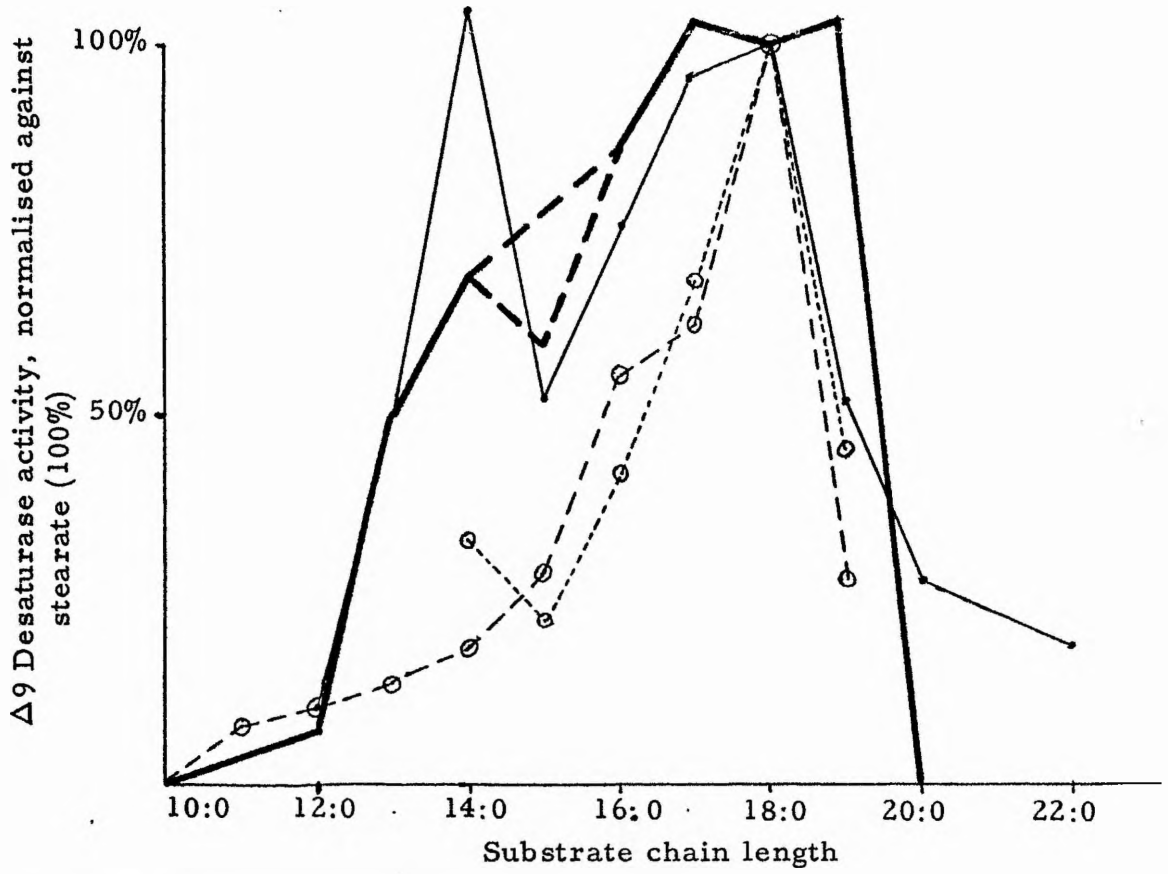
hydrogen atoms. Schroepfer and Bloch found a substantial kinetic isotope effect against only the 9D hydrogen atom, but they were using a bacterial Δ9 desaturase [60].

The nature of the substrate binding site on the CSF has been probed by several specificity and inhibition studies. The concept of an enzyme cleft holding the alkyl chain in a rigidly controlled fashion was put forward by Brett et al [61]. The rigid specificity of the Δ9 desaturase (no Δ8 or Δ10 monoenes are produced) over a wide range of saturated acids suggests carboxyl control via interaction of the CoA moiety of the substrate with the enzyme. Strittmatter et al showed that the Δ9 desaturase had a considerable specificity towards the CoA group, as modified CoA thiolesters were poor substrates and free fatty acids were not desaturated at all [19]. However, a considerable degree of control also needs to be exerted over the alkyl chain in order to explain the positional specificity and the absolute stereochemistry of hydrogen abstraction. Van der Waals interactions between the alkyl chain and an apolar enzyme surface would be insufficient control, so a tight enfoldment of the substrate by the enzyme is called for. The complete series of ^{14}C-labelled (±) methyl-branched octadecanoates were tested as substrates for the Δ9 desaturase in hen liver microsomes [61]. Only the 2, 3, 4, 16, 17 and 18 positional isomers were desaturated, but they were not such good substrates as stearate. A tight spatial constraint from C(5) to C(15) was therefore envisaged, with some latitude at either end. The cleft would have dimensions of about 26 Å in length and 4 Å in width, and a hydrophobic character. The close enfoldment required to lock the substrate into a fixed position implied that a direct insertion of the alkyl chain into a narrow cleft would be unlikely, and a substantial, concerted conformational change in the enzyme structure was proposed as the substrate binds.

Other specificity data from studies on the Δ9 desaturation are listed below:-

(1) Chain length specificity studies are shown in Figure 5. Rat liver Δ9 desaturase seems capable of handling 11:0 to 19:0 acyl-CoA substrates [19, 29, 31, 62].

Figure 5. Chain Length Specificity Studies on the Δ9 Desaturase



Values of K_m (μM) for acyl-CoA substrates, obtained with the purified Δ9 desaturase (rat) [19] are 8.0 (13:0), 4.7 (14:0), 4.7 (16:0), 5.0(17:0), 4.5 (18:0) and 5.0 (19:0).

——— V_{max}, Purified Δ9 desaturase (rat), acyl-CoA substrates [19]

———•——— % Conversion, Hen liver microsomes, FFA substrates [62]

– – ⊖ – – – V_{max}, Whole microsomes (rat), FFA substrates [29]

···· ⊖ ····· V_{max}, Partially purified Δ9 desaturase (rat), acyl-CoA substrates [31]

(2) 18:1(7c) and 18:1(11c) were not desaturated in either hen liver or goat mammary gland microsomes [61].

(3) 18:2(12c15c) fed to rats maintained on a fat-free diet did not result in 18:3(9c12c15c) or its metabolites appearing in liver lipids [63].

(4) The rat was shown not to possess the ability to Δ9 desaturate 18:1(12c) either *in vitro* [64] or *in vivo* [65]. However, 18:1(12c) was Δ9 desaturated by both hen liver and goat mammary gland microsomes, with a conversion of about 40 to 60% of that of stearate [61].

(5) 19:1(18e) was a better substrate for Δ9 desaturation than 19:0 in hen liver microsomes [61].

The bimodal chain length specificity behaviour of systems which can also desaturate 18:1(12c) has led the Colworth research group to propose the existence of two Δ9 desaturases, with optimum substrate chain lengths of C_{14} (IΔ9 desaturase) and C_{18} (IIΔ9 desaturase) respectively, but with an overlapping chain length specificity [6, 64]. 18:1(12c) is only desaturated by the IΔ9 desaturase, since the Δ12*cis* double bond can lift the tail of the molecule out of the enzyme's cleft such that the substrate mimics a shorter chain substrate. 19:1(18e) is believed to be a better substrate than 19:0 because the IIΔ9 desaturase has an obstruction at the end of the cleft which will hinder the fit of a C(19)methyl group but not a Δ18 terminal double bond. These hypotheses will be discussed in Section 2:2. The conformation of $-CH_2-CH_2-$ bonds assumed by stearoyl-CoA in the IIΔ9 desaturase cleft is believed to be predominantly *trans*. (The term *trans* is used in this thesis in preference to *antiperiplanar*, and *gauche* in preference to *synclinal*.) Therefore fatty acids containing only *trans* unsaturation should be Δ9 desaturated. This hypothesis was tested experimentally during this programme.

Cyclopropene fatty acid inhibition studies have resulted in a considerable confusion of ideas about Δ9 unsaturation. It is well documented that when sterculic or malvalic acids are included in the diet of animals stearic acid accumulates in the lipids at the expense

of oleic acid, and that this is due to a marked inhibition of the stearoyl-CoA Δ9 desaturase by the cyclopropene acids [8, 9]. Studies with hen or rat liver microsomes have shown the following features of this inhibition:-

(1) It was very strong and irreversible. Using either free fatty acids or their CoA derivatives, a ratio of stearate to sterculate of 10:1 to 14:1 gave an inhibition of Δ9 desaturation of about 80-90% [66, 67].

(2) Sterculoyl-CoA, not sterculic acid, was responsible for the inhibition of stearoyl-CoA Δ9 desaturase [67].

(3) With hen liver microsomes the inhibition occurred for all the saturated substrates (12:0-20:0) to a similar extent [62].

(4) Δ6 Desaturation was not inhibited by sterculate [67].

(5) The position of the cyclopropene ring was critical. Fogerty et al showed that C_{17} Δ8, 9-, C_{18} Δ9, 10- and C_{19} Δ10, 11-cyclopropene acids inhibited Δ9 desaturation effectively, whereas the C_{20} Δ11, 12-cyclopropene acid did not [66].

(6) T-sterculate, when incubated with rat liver microsomes at a concentration that caused a major inhibition, bound to the protein but could be totally removed by treatment with mercaptoethanol plus detergent or by solvent extraction. Therefore there was no covalent bonding of sterculate to the Δ9 desaturase [67].

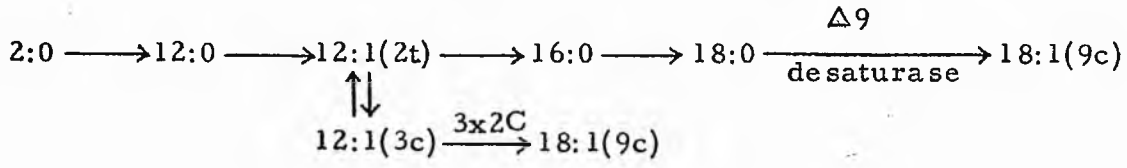
(7) Whole animal experiments showed that the conversion of labelled acetate or laurate to oleate was only mildly inhibited by sterculate, whereas the conversion of stearate to oleate was very strongly inhibited [68, 69].

(8) Brett showed that sterculic acid was preferentially incorporated into the 2-position of phospholipids (a similar specificity to oleate) but that Δ9 desaturase inhibition could not be explained in terms of competition of sterculate or oleate for esterification into lipids [74].

Several theories have been put forward to explain the cyclopropene inhibition of the Δ9 desaturase. One that can immediately be ruled out is the non-specific, detergent type of inhibition proposed by Pande and Mead [70]; this is contrary to observations (1), (4), (5) and (7). Another, frequently quoted idea is that the inhibition stems from the interaction of the cyclopropene ring with an essential sulphydryl group on the desaturase [68, 61]. Cyclopropene fatty acids are known to react with free sulphydryl groups to form thiol

ethers [71-73], and because only acids with a Δ8, 9; Δ9, 10; or Δ10, 11-ring position were effective inhibitors inhibition was often explained by assuming the formation of a covalent C-S bond at an essential sulphydryl group at the active centre. However such a model is invalidated by observation (6).

The most puzzling aspect of cyclopropene fatty acid inhibition is that the conversion of stearate to oleate is inhibited but that the conversion of acetate or laurate to oleate is not. (Experiments to test the non-inhibition of sterculic acid on the acetate to oleate transformation have yet to be done on rat liver in vitro.) Two explanations have been given for this enigma. The first postulated two distinct pathways to oleate, with only one requiring the stearoyl-CoA Δ9 desaturase [68]. However there is little other



evidence to date to support this alternative pathway. It would require a special mechanism to release laurate from the fatty acid synthetase, which normally produces palmitate. Such behaviour has only been reported for anaerobic bacteria [75]. The second explanation, expressed for animal systems, stated that the inhibition was not specific for the stearoyl-CoA Δ9 desaturase, but took place at some transfer stage of exogenous stearate to the enzyme, which could be bypassed by the de novo synthesis pathway [7]. It is unfortunate that Strittmatter and his coworkers have not yet published work on sterculoyl-CoA inhibition using the purified Δ9 desaturase system.

The stearoyl-CoA desaturase can exhibit both substrate and product inhibition. Oshino et al found that oleic acid was not an effective inhibitor whereas oleoyl-CoA was [50]. Using washed microsomes Jeffcoat et al noted a substrate optimum of 10 μM stearoyl-CoA [22], above which substrate inhibition clearly occurred. For the purified, reconstituted Δ9 desaturase the K_m for stearoyl-CoA

binding is similar to the K_i for oleoyl-CoA inhibition [19]. The onset of product inhibition occurred at a lower concentration than for substrate inhibition, with the latter appearing at 15 μM when low amounts of lipid were present. C_{18} acyl-CoA esters with a cis Δ9,10 geometry (eg. cis epoxy or cyclopropyl) were competitive inhibitors of the Δ9 desaturase whereas molecules with a trans or linear Δ9,10 geometry were non-competitive or non inhibitors. Strittmatter et al therefore concluded that a cis Δ9,10 geometry was probably required for the molecule to bind to the active centre, and that the Δ9,10 gauche conformer of stearoyl-CoA was most likely the true substrate for the Δ9 desaturase. Inhibition studies using crude microsomes have given results somewhat at variance to these, but the experimenters did not examine the enzymology in detail and the systems studied were too complex to make an adequate interpretation. Chang et al indicated that only the Δ10 and Δ11 isomers of cis-18:1 acids were effective inhibitors [103], while Brett found that both cis and trans Δ9,10-epimino-stearates were mild inhibitors of the Δ9 desaturase while cis and trans Δ9,10-epoxy- and epithio-stearates were not inhibitors [74].

2:1:2 The Δ6 Desaturase

Substrate specificity studies on the Δ6 desaturase have shown, with one exception, an absolute requirement for the Δ9 cis double bond. C_{16} to C_{18} chain length fatty acids with a Δ9c, Δ9c12c, or Δ9c12c15c pattern of unsaturation were substrates [6]. In a feeding study using EFA deficient rats Sprecher et al failed to observe any conversion of 18:2(9c15c) or 18:2(12c15c) acids to more unsaturated PUFA [63]. Brenner and Peluffo first demonstrated the increase in the conversion rate in the series 18:1(9c), 18:2(9c12c) and 18:3(9c12c15c) for in vitro Δ6 desaturation [76]. Carboxyl-end control, competitive inhibition studies and the similar stimulation or depression of the Δ6 desaturation of each substrate by changes in external factors such as diet, hormonal levels, or temperature, suggested a single Δ6 desaturase [17,77,78]. The results of Schlenk et al also indicated 21:4(9c12c15c18c) to be a good substrate [93]. Two models have been proposed to explain the above

specificity, both assuming that the specificity is a function of the desaturase protein alone.

James has extended the concept of a cleft for the substrate from the Δ9 desaturase to the Δ6 desaturase [6]. No specific interaction of the Δ9cis double bond with the enzyme was envisaged, but the geometry of the Δ9cis double bond lifted the tail of the molecule out of a cleft of about ten carbon atoms in length, thus removing steric hindrance. The series of Δ6 desaturation rates 18:1(9c) < 18:2(9c12c) < 18:3(9c12c15c) was then explained in terms of a more effective removal of the tail of the substrate away from a region of steric hindrance by the additional Δ12cis and Δ15cis double bonds. According to this model short chain saturated or Δ9cis monoenoic acids should be good substrates for the Δ6 desaturase. This was examined during these studies.

Brenner's model also assumed carboxyl-end control, but the alkyl chain was believed to be bound to the enzyme surface by specific interactions rather than to fit in a deep cleft with tight spatial constrictions [77]. Brenner suggested that the tail of the molecule bound to the desaturase by short range London dispersion forces, while the cis double bonds helped to strengthen the binding by either specific π-interactions with polarisable sites on the enzyme surface or by inducing a better fit and therefore increasing the dispersion forces. The Δ9cis double bond interaction was essential, and the desaturase could recognise the all-cis methylene-interrupted polyenoic pattern. The CoA head group was necessary as a long range attractive force to bring the substrate to the enzyme and to anchor the carboxyl-end such that carbon atoms 6 and 7 were brought to the active centre.

Both hypotheses predicted that fatty acids with a Δ9trans double bond would not be Δ6 desaturated, as has been noted experimentally [77, 78, 80] and that 18:2(9c12t) would be Δ6 desaturated, for which there is reasonable evidence [82, 83].

The exception to the Δ6 desaturase specificity pattern outlined above comes from Cook and Spence, who reported that [1-^{14}C]

palmitate and stearate were Δ6 desaturated in homogenates of developing rat brain [81]. This activity was highest in the foetal stage but diminished rapidly after birth and was absent by the twentieth day. At birth 62% of 16:1 produced was the Δ6c isomer and 28% of 18:1 was the Δ6c isomer, with the remainder being the Δ9c isomer in both cases. These results raise some interesting questions. Is the enzyme the same as rat liver microsomal Δ6 desaturase, and if so can rat liver microsomes be induced to Δ6 desaturate saturated substrates? If the Δ6 desaturase proteins for rat liver and developing rat brain are identical where does the control occur that gives rat liver microsomal Δ6 desaturase its characteristic specificity? Complementing the work of Cook and Spence is that of Sand et al on the positional isomers of unsaturated fatty acids present in the rat [84]. 16:1(6c) was found in newborn and suckling rats, and in dam's milk, but its level fell markedly on weaning. Rats fed on a fat-free diet showed an increase in the percentage of the Δ5c8c isomer in the 18:2 fraction. Spence [85], and Kishimoto and Radin [86] reported traces of 16:1(6c) and 18:1(8c) acids in rat lipids and pig brain lipids respectively. Thus, applying the desaturase specificity "rules" to the above data, the pathway

$$16{:}0 \longrightarrow 16{:}1(6c) \longrightarrow 18{:}1(8c) \longrightarrow 18{:}2(5c8c)$$

can be postulated, involving this unusual Δ6 desaturase activity.

Like the Δ9 desaturase the Δ6 desaturase is inhibited by cyanide ions [87], which may be a pointer towards the involvement of iron in the Δ6 desaturase protein. The involvement of molecular oxygen, and the fact that no oxygenated intermediates have ever been isolated, also suggests that a similar mechanism of hydrogen abstraction at the Δ6 desaturase active centre.

Preincubation of whole microsomes with ATP increased Δ6 desaturase activity for both linoleic acid and linoleoyl-CoA substrates, but no satisfactory explanation for this phenomenon was given [17,77].

2:1:3 The Δ5 Desaturase

Substrates of C_{18} to C_{21} chain length, with Δ8c11c, Δ8c11c14c and Δ8c11c14c17c patterns of unsaturation are known to be Δ5 desaturated [6]. However, other acids not obeying the desaturase specificity generalisations can be Δ5 desaturated too. Sprecher showed that the Δ8cis double bond was not obligatory since 20:1(11c), 19:2(11c14c), 20:2(11c14c), 21:2(11c14c) and 20:3(11c14c17c) were all substrates [88, 89]. Although the Δ11cis double bond is common to all the substrates listed so far, it is not obligatory either as elaidic acid, 18:1(9t), was shown to give 18:2(5c9t) *in vivo* [79] and *in vitro* [80] in the rat. No firm data exist on the possible Δ5 desaturation of Δ8cis monoenes, but fragmentary evidence has already been discussed (Section 2:1:2) that points to 18:1(8c) ⟶ 18:2(5c8c) in EFA deficient rats. 16:1, 18:1 and 20:1 Δ8cis monoenoic acids were therefore tested as substrates for the Δ5 desaturase.

A careful examination of the literature reveals a chain length specificity of the Δ5 desaturase, with an optimum at C_{20}. 17:2(8c11c) in EFA deficient rats, fed as a supplement or produced by the retroconversion of dietary 19:2(10c13c), gave no detectable desaturation products [90, 91]. Examination of the fatty acid isomer composition in liver lipids of EFA deficient rats showed 4% 18:2(8c11c) but only traces of 20:2(8c11c), with 5% of ω7 and 22% of ω9 further desaturated products [92], implying that the C_{20} diene is a better substrate for the Δ5 desaturase. This is confirmed by initial rate measurements made by Sprecher [18]. 20:1(11c) was Δ5 desaturated but 18:1(11c) was not [89]. Using a rat liver microsomal preparation exhibiting both desaturase and elongase activity Stoffel and Ach recorded 18:3(8c11c14c) as giving 7% of more desaturated products whereas 20:3(8c11c14c) gave 24%. Ordering substrates according to their relative rates of conversion gives for:-

Δ11c monoenes			18:1	≪ 20:1
Δ8c11c dienes	17:2	≪	18:2	< 20:2
Δ8c11c14c trienes			18:3	< 20:3

The Δ5 desaturase is capable of handling fatty acids of chain length of at least up to C_{23} [93].

A simple relationship between the degree of unsaturation of the substrate and the rate of Δ5 desaturation does not hold. Sprecher reported rates of 0.79 and 0.84 nmoles.min^{-1} mg^{-1} of microsomal protein when incubating 150 nmoles of 20:3(8c11c14c) or 20:2(8c11c) acids respectively with 5 mg of protein for 3 minutes [18]. However, it is not adequate to describe the situation in whole microsomes simply in terms of both substrates having equal rates of Δ5 desaturation. When 100 nmoles of 20:3(8c11c14c) or 20:2(8c11c) were incubated with 5 mg of protein for 10 minutes 13 and 14 nmoles of product were formed respectively, but when 20 nmoles of substrate were used 4.5 and 14 nmoles of product were formed [94]. Detailed kinetic studies on the Δ5 desaturase are lacking.

No suitable model for the Δ5 desaturase has yet been proposed.

A study of the Δ5 desaturation of some (±) methyl-branched eicosa-8-cis, 11-cis, 14-cis-trienoic acids indicated that as the methyl group moved towards the site of desaturation there was a progressive decline in the % conversion, which suggested that methyl branching between positions 2 and 18 would alter the structure of the acid so as to diminish its binding to the desaturase [18, 95]. The results rule out a tightly fitting cleft from C(10) to C(20).

The Δ5 desaturase is cyanide sensitive [96].

2:1:4 The Δ4 Desaturase

A Δ4 desaturase has been postulated from a consideration of the various metabolic transformations of C_{20} to C_{22} PUFA cited in the literature [6, 17, 18]. However, there is increasing evidence that the production of a Δ4cis double bond does not occur via an enzyme(s) resembling the Δ9, Δ6, and Δ5 microsomal desaturases. The problem of Δ4 desaturation will be discussed in Section 2:2:3.

2:1:5 Trans Fatty Acids

The majority of work reported is for elaidic acid or the trans isomers of linoleic acid; However modern diets include industrially hydrogenated fats and oils and also dairy products, which contain minor proportions of a wide range of trans isomers. For example the Δ7t to Δ15t 18:1 isomers could be detected in partially hydrogenated arachis oil [97]. Studies were undertaken on a range of trans fatty acids to test their suitability as substrates for further desaturation.

It is well documented that trans acids are deposited in the tissues, are readily catabolised, and when fed with adequate amounts of EFA have little effect on growth. However, when they are fed as the sole source of dietary fat they give rise to EFA deficiency symptoms. Biologically their behaviour resembles that of saturated fatty acids. 18:1(9t) and 18:2(9t12t) are preferentially incorporated into the primary position in lipids, and the trans alkyl chain can pack as a saturated alkyl chain, making phospholipid membranes less fluid than would the corresponding cis isomers. The subject is reviewed by Kummerow [98].

Only two substantiated instances of the desaturation of trans acids are known. These conversions, 18:1(9t) ⟶ 18:2(5c9t) [79, 80] and 18:2(9c12t) ⟶ 18:3(6c9c12t) [82, 83], have already been discussed, though the latter has not yet been demonstrated in vitro. Knipprath and Mead reported that 18:2(9t12t) gave a 20:4 trans containing acid [99], but this was repudiated by Privett et al [83]. Furthermore, feeding 18:2(9t12t) to EFA deficient rats does not result in any appreciable formation of a 20:4 acid [100]. 18:2(9t12c) [83] and 20:2(11t14t) [101] do not appear to be desaturated in the rat.

Trans acids impair the route to long chain PUFA in vivo. The ω3, ω6 and ω9 series of PUFA have their levels suppressed when animals are fed on diets containing a high proportion of elaidic

or linelaidic acids [79, 82, 100, 102]. This may occur via the inhibition of the Δ6 desaturase, which is believed to be the controlling enzyme on the pathway to PUFA. Brenner and Peluffo demonstrated that elaidic and linelaidic acids had an inhibitory effect on the *in vitro* desaturation of linoleic acid, but the inhibition was very mild [87].

2:1:6 The Role of Soluble Proteins in Desaturation

Rat liver Δ9 desaturase activity in washed microsomes was stimulated by adding back the microsomal supernatant [104], serum albumin or a basic, cytoplasmic protein [22]. The stimulation did not occur when using a partially purified desaturase [31] and soluble factors were not obligatory [10]. Modes of action suggested for the increase in Δ9 desaturase activity in whole microsomes by the addition of soluble, substrate-binding proteins include the protection of the acyl-CoA substrate from the action of the endogenous acyl-CoA hydrolase [31], and the reversible binding of substrate to the soluble protein to give a free substrate concentration approximating to the optimum level [22]. Δ6 desaturation was also stimulated by the addition of BSA or soluble rat liver proteins, and the supernatant had to be added back to washed microsomes to give enzyme activity [22, 54, 104, 105]. Both Δ6 and Δ5 desaturases appear to have an obligatory requirement for a cytoplasmic protein which is excluded from Sephadex G-100 [96]. Recent studies by Baker *et al* [221] provided evidence that the cytoplasmic component required for the desaturation of stearoyl-CoA was catalase. By inference they also suggested that the hitherto unidentified component, partially purified by Brenner's group and required by the Δ6 desaturase might be catalase. The soluble factors did not appear to be affected by dietary modification [104].

The exact function of cytoplasmic proteins has yet to be clearly delineated, but a consideration of their role may be necessary for the future interpretation of specificity work.

2:1:7 The Inhibition of the Desaturases by Fatty Acids

Obviously competitive inhibition between substrates for the same desaturase occurs, but inhibition by other unsaturated acids, including the products of desaturation, is a well recorded feature [77, 106]. At low ratios of substrate plus inhibitor to protein stimulation of desaturation can occur, and this was attributed to a partial blocking of the lipid incorporation pathway by the "inhibitor" [87]. However most studies used high concentrations of fatty acids relative to protein, and from these Brenner proposed a series of feedback, crossed-feedback and product inhibition controls operative on the Δ6 desaturase [77]. Sprecher demonstrated, by feeding studies, that the Δ6 desaturase and acyl-transferases appeared to be the controlling enzymes *in vivo*, and not the Δ5 desaturase and the elongase [18, 106]. However, a control at the Δ6 desaturase operative by a feedback mechanism of PUFA as well as by simple, substrate competition is unlikely. It is doubtful whether the inhibitions noted at high fatty acid levels *in vitro* will apply to an *in vivo* situation where the microsomal enzymes will not be present in limiting amounts. The interpretation of inhibition studies using whole microsomes in order to shed new light on the desaturases will be difficult until more is understood about the microsomes in general (eg. function of soluble proteins, the presence of substrate pools, etc.).

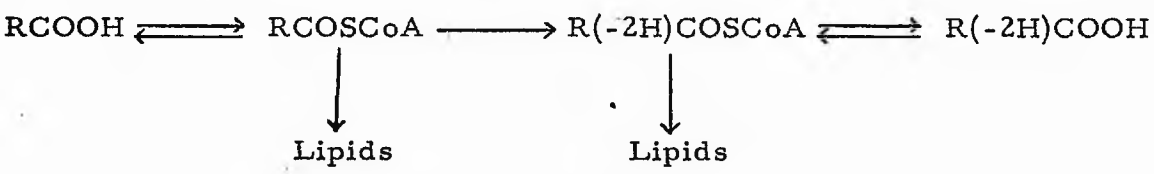
2:2 Results and Discussion

2:2:1 Some General Considerations

The principle aim of this study was to test various unsaturated fatty acids as substrates for the mammalian desaturases and to see if the products formed were explicable in terms of the present models for desaturase specificity or whether the models would require alteration. Measurement was made of the % conversion of substrate to product at the end of a 1 hr incubation: the measurement of initial or maximum rates of desaturation was not attempted. These studies raised questions which could only be answered by a more rigorous kinetic analysis of microsomal desaturation and as a prelude to this the time course of some desaturation reactions was examined.

The understanding of desaturation in a crude microsomal system must be approached in three stages. Firstly certain patterns of specificity will be observed. Secondly these must be related to the desaturase(s) and be distinguished from the influences of the rest of the microsomal system. Finally, the "true" specificity exhibited by the desaturase protein must be explained in terms of enzyme binding constants, molecular fit and specific interactions with the substrates. The isolation and characterisation of the homogeneous enzyme and the other proteins required for desaturase activity will eventually be required to confirm any model constructed.

The reactions to be considered in the microsomal system used here (Section 2:3:1), when represented at their simplest, are:-



The system contained a high ratio of protein to substrate and was selected to give extensive desaturation with negligible chain-elongation. To compare the activity of different substrates for the same desaturase and to interpret the results in terms of desaturase protein-substrate interactions the following assumptions are made:-

(1) Fatty acid activation is not rate limiting.

(2) Substrate and product inhibitions are insignificant.

(3) Once substrate or product is esterified into lipids it is effectively removed from the in vitro system as lipid turnover will be minimal.

(4) Although lipid incorporation via the acyl-transferases competes for substrate it does not affect % desaturation (ie. lipid incorporation specificity is very wide, and has a similar rate for a series of structurally related acids).

(5) % Desaturation is not altered by the binding of FFA or acyl-CoA to protein. This will hold so long as specific transfer proteins or limited metabolic pools do not exist, channeling substrates to specific fates.

In summary, the % desaturation measured after 60 min when incubating FFA with large amounts of microsomal protein plus the appropriate cofactors will be a reflection of the initial rate of desaturation, which in turn will be a reflection of the substrate-enzyme binding constant, providing (1) to (5) hold.

2:2:2 The Choice and Characteristics of the Microsomal Enzyme Preparation

The effect of diet on the mammalian desaturases has been thoroughly investigated [17, 77, 96, 108, 109], as has the effect of age on Δ6 desaturation [77]. Various species and tissues have been screened for Δ9 and Δ6 desaturase activity [77, 96]. An appraisal of these results indicated that rat liver microsomes would yield a sufficiently active preparation for the Δ9, Δ6 and Δ5 desaturases. EFA deficiency stimulates both Δ9 and Δ6 desaturation and a high carbohydrate diet stimulates Δ9 desaturation. Therefore rats fed for several months after weaning on a high sucrose diet containing only a small amount of saturated fat were tried as an enzyme source. The results (Table 1) should be compared with those for weanling rats aged 4-5 weeks (Table 2). Both Δ9 desaturation and Δ6 desaturation of oleate were enhanced in the EFA deficient animals. The Δ6

TABLE 1: Control Desaturations for Liver Microsomes from EFA Deficient Rats

Substrate[1]	Product[1]	% Desaturation	% Lipid Incorporation NL	FFA	PL	a.
18:0	18:1(9c) 18:2(6c9c)	55-66 2-3	20-31	7-8	61-73	4
18:1(9c)	18:2(6c9c)	4-7.5	17-41	3.5-6	53-79	3
18:2(9c12c)	18:3(6c9c12c)	11-16	11-17	2-6	76-87	4
18:3(9c12c15c)	18:4(6c9c12c15c)	32-40	9-20	1-4	75-89	5
20:3(8c11c14c)	20:4(5c8c11c14c)	60.5-70	8-10	2-4	84-89	3

a. No. of determinations

1. Typical ECL values for a 10% DEGS column at 180°C were: 18:1(9c)-18.6, 18:2(6c9c)-19.2, 18:2(9c12c)-19.4, 18:3(6c9c12c)-19.95, 18:3(9c12c15c)-20.4, 18:4(6c9c12c15c)-20.95, 20:3(8c11c14c)-21.85 and 20:4(5c8c11c14c)-22.2

TABLE 2: Control Desaturations for Liver Microsomes from Weanling Rats (A Single Experiment)

Substrate	Product	% Desaturation	Lipid Incorporation (%) NL	FFA	PL
18:0	18:1(9c)	22	21	10	69
18:1(9c)	18:2(6c9c)	1	45	6	49
18:2(9c12c)	18:3(6c9c12c)	15	31	3	66
18:3(9c12c15c)	18:4(6c9c12c15c)	37	41	3	56
20:3(8c11c14c)	20:4(5c8c11c14c)	65.5	18	2	80

desaturation of linoleic and α-linolenic acids and $\Delta 5$ desaturation were effectively identical for the two enzyme sources, and for all the substrates tested the lipid incorporation patterns were broadly similar for each enzyme source. Since the microsomes from EFA deficient rats gave more than adequate levels of $\Delta 9$, $\Delta 6$ and $\Delta 5$ desaturation of the control substrates they were chosen for all further experiments, without recourse to a screening program.

Rat liver microsomes have frequently been used for desaturation studies by other workers.

The results from several experiments showed a good degree of consistency in both % desaturation and lipid incorporation (Table 1).

Unwashed microsomes were recommended as the most active and reliable system for the Δ6 desaturation of linoleic acid [122]. The results in Table 3 confirmed this. The amount of soluble protein present in the enzyme preparation was critical to

TABLE 3: % Δ6 Desaturation and Lipid Incorporation for α-Linolenic Acid in Various Enzyme Preparations

Enzyme Preparation[1]	% Δ6 Desaturation	% Lipid Incorporation NL	FFA	PL	
Mitochondrial supernatant	35	21	22	57	Experiment 1
M + 1/10 S	39	26	9	65	
M + 1/4 S	33	16	14	70	
M + S	30	19	19	62	
Unwashed microsomes	37	41	3	56	Experiment 2
S	9	21	10	69	
M	7	46	13	41	
M + 1/10 S	35	39	3	58	

1. Experiments 1 and 2 used weanling rat liver.
M is the 100,000 g washed microsomal pellet and S the 100,000 g supernatant. The fraction refers to the amount of supernatant added back compared to its original volume.

Δ6 desaturase activity, with the optimum amount being that obtained by adding back 1/10 of the original volume of the 100,000 g supernatant to the total, washed microsomal pellet. This effectively represented

the composition of the unwashed microsomal pellet which was routinely used in this work. The obligatory nature of a soluble factor for Δ6 desaturation was again demonstrated [22, 54, 96, 104, 105]. The 100,000 g supernatant had very little Δ6 desaturase activity and what remained was a consequence of microsomal contamination as further centrifugation at 100,000 g for 2 hr resulted in the sedimentation of a small pellet from the supernatant. A single washing of the crude microsomal pellet did not completely remove the soluble factor as 1/5 of the Δ6 desaturase activity still remained. Full activity was restored by adding 1/10 of the 100,000 g supernatant back to the washed pellet. The soluble factor is highly active in its function, as only small amounts of the 100,000 g supernatant needed to be added back.

1 ml of the enzyme preparation contained the following quantities of endogenous free fatty acids:-

16:0-66, 52 (59) nmole	18:0-42, 38 (40) nmole
16:1-15, 18 (16.5) nmole	18:1-78, 92 (85) nmole
18:2- 8, 5 (6.5) nmole	20:3 ω9-14, 18 (16) nmole

These figures are for two separate determinations using different enzyme preparations. The average value is given in brackets. A total of about 230 nmole of endogenous FFA were present at the start of the incubation. This figure dropped to about 60 nmole after a one hour incubation and compared with 20 nmole of [1-^{14}C] added substrate. Therefore the initial incubation concentrations were 8 μM for [1-^{14}C] acids and 92 μM for endogenous acids. It is not known whether the high concentrations of the latter were caused by lipolysis during the enzyme preparation or if they represented the true concentration, in nmole. mg^{-1} of protein, present in the microsomes <u>in vivo,</u> but it is interesting to note that the profile of these endogenous FFA resembled the depot storage fat rather than the microsomal lipid composition in that much smaller amounts of PUFA were present.

Fatty Acid Profile	Total lipids (per incubation)	Endogenous FFA (per incubation)
16:0	16.6%	18.5%
16:1	7.8%	6.5%
18:0	18.4%	15.5%
18:1	28.0%	38.0%
18:2	2.0%	2.0%
20:3	20.7%	7.5%
20:4	5.7%	trace
22:1	trace	7.0%
Unidentified	0.6%	5.0%

Critical micelle concentrations for acyl-CoA and FFA are usually quoted as being in the 2-5 μM and 100-200 μM regions respectively [25]. However the inhibtion of microsomal desaturation by a non-specific, detergent action was considered unlikely because:-

(1) A protein concentration of 3.5 mg.ml^{-1} would result in the binding of much of the substrate, reducing its free concentration.

(2) The concentration of acyl-CoA, which would produce a more powerful detergent inhibition than FFA, was unlikely to be very high as it would be rapidly removed by the acyl-transferases. Assuming that the behaviour of the endogenous FFA mirrored that of exogenous [1-^{14}C] stearate and oleate the maximum acyl-CoA concentration would not exceed about 30 μM (Section 2:2:9).

(3) The rapid and high desaturations of [1-^{14}C] 18:3(9c12c15c) and [1-^{14}C] 20:3(8c11c14c) indicated that acyl-CoA and FFA non-specific inhibitions were insignificant (Section 2:2:9)

The specific inhibition of Δ9 desaturation by oleoyl-CoA will be discussed in Section 2:2:9. The low levels of endogenous polyunsaturated FFA present in the enzyme preparation were probably helpful, as such acids at higher concentrations might compete for or inhibit Δ5 and Δ6 desaturation.

Cofactor concentrations were not a rate limiting factor. Oxygen tension is discussed in Section 2:3:1. Cofactor concentrations of NADPH (144 μM) and NADH (338 μM) were in a considerable

stoichiometric excess over the total concentration of fatty acids (100 μM) and fairly rapid rates of desaturation were observed for 18:0, 18:3(9c12c14c) and 20:3(8c11c14c). Fatty acid activation was also not rate limiting, as rapid lipid incorporation of [1-^{14}C] substrates occurred. There were ample amounts of acceptor molecules in the microsomes to react with acyl-CoA (Table 4), and the transfer into lipid could occur very rapidly (Section 2:2:9).

TABLE 4: Incubations with High and Low Concentrations of Linoleic Acid

Concentration of [1-^{14}C] linoleic acid		20 nmole (8 μM) Sp. act. =60 μCi.μmole^{-1}	300 nmole (120μM) Sp. act. = 4 μCi.μ mole^{-1}
% Δ6 desaturation		15	14
% Lipid Incorporation	NL	31	41
	FFA	3	4
	PL	66	55

Comparing the incubation conditions with those reported in the literature it is unlikely that saturating conditions with respect to substrate have been employed in this work (ie. the initial rate of desaturation will be dependent on substrate concentration but not proportional to mg of protein). However, this was not tested. The balance between the competing microsomal reactions measured *in vitro* is believed to reflect more accurately the *in vivo* situation when using non substrate-saturating conditions.

The stimulation of Δ9 and Δ6 desaturase specific activity by BSA has been reported [22] and its effect was re-examined (Table 5). Defatted BSA enhanced the Δ6 desaturation of α-linolenic acid. An optimum stimulation occurred at a BSA concentration of 2-4 mg ml^{-1} (30-60 μM). However stimulation of desaturation was not observed with substrates which were not PUFA. Because stimulation was variable and because its mode of action was not fully understood at the time BSA was omitted from the incubations (see also Section 2:2:9).

TABLE 5: Incubations with BSA

Substrate	Product	% Desaturation	[BSA] mg ml^{-1}	Stimulation (+) or Inhibition (-) against a Control without BSA	
18:3(9c12c15c)	18:4(6c9c12c15c)	35.5	-	-	
"	"	44.5	0.68	+25%	
"	"	58	3.29	+63%	
"	"	53.5	7.40	+51%	1.
18:0	18:1(9c)	22	1.32	0%	
20:3(8c11c14c)	20:4(5c8c11c14c)	77	1.32	+17%	
18:0	18:1(9c)	66	2.81	0%	
18:1(9c)	18:2(6c9c)	6	2.81	-10%	
18:1(5t)	18:1(5t9c)	79.5	2.81	- 2%	2.
16:1(8c)	16:2(5c8c)	trace	2.81	-	
18:2(9c12c)	18:3(6c9c12c)	17.	2.81	+40%	
18:3(9c12c15c)	18:4(5c9c12c15c)	56.5	2.81	+47%	

1. Microsomes from weanling rats
2. Microsomes from EFA deficient rats

2:2:3 The Δ4 Desaturase

The liver lipids of fish and mammals normally contain high levels of 22:6(4c7c10c13c16c19c), which is the major acid in the ω 3 PUFA family. The ω6 acid 22:5(4c7c10c13c16c), however, represents only 1-2% of the liver fatty acids of animals receiving a balanced diet though it attains higher values when ω3 acids are absent from the diet [110, 111, 91]. The enigma surrounding Δ4 desaturation is that no direct conversion of 22:4(7c10c13c16c) or 22:5(7c10c13c16c19c) has ever been recorded *in vitro*. Ayala *et al* reported no Δ4 desaturation when incubating T-labelled 22:4(7c10c13c16c) acid

with weanling rat liver or testicular microsomes [39], while Sprecher noted only minute conversions ($\leqslant 1\%$) of 20:3(7c10c13c) and 22:4(7c10c13c16c) in liver microsomes of rats raised on a fat free diet [18].

A rat liver microsomal Δ4 desaturase could not be demonstrated in this work. Liver microsomes from both weanling and EFA deficient rats had good Δ9, Δ6 and Δ5 desaturase activity, but acids which might or should be Δ4 desaturated according to the rationale of the desaturase specificity generalisations (Section 2:1) - [1-^{14}C] 16:1 (7c), 19:3 (7c10c13c) and 22:4 (7c10c1316c)- were not desaturated by these enzyme preparations. In each case the substrate was incorporated into lipids, indicating the activation of the substrate to its acyl-CoA thiolester. The addition of BSA had no effect.

No further work on the Δ4 desaturase was undertaken. Perhaps in future studies to locate the pathway to PUFA containing a Δ4cis double bond it would be better to employ a fish system, along with ω3 substrates, which are preferred to ω6 acids. Owen et al fed [1-^{14}C] 18:3(9c12c15c) to trout previously kept on a fat free diet and recorded that after six days 70% of the radioactivity in liver lipids resided in 22:6(4c7c10c13c16c19c) [112]. Several courses of investigation seem worthwhile.

(1) In order to see if the production of 22:5 ω6 and 22:6 ω3 acids occurs mainly in the liver (the site of most lipogenic activity) tissue slices or homogenates of organs should be screened for Δ4 desaturation.

(2) Kunau and Bartnik have reported a mitochondrial Δ4 hydrogenase operating on C_{22} substrates with Δ4c7c10c13c unsaturation [41]. It is possible that this enzyme might operate in reverse.

(3) Experiments utilising rat liver or testicular microsomes with both an elongating and a desaturating capacity have shown that Δ4c PUFA can be produced in vitro [35, 39].

^{14}C-20:4 ω6 $\xrightarrow[\text{liver microsomes}]{\text{NADPH, malonyl-CoA}}$ 32% 22:4 ω6 and 6% 22:5 ω6 [35]

^{14}C-18:2 ω6 $\xrightarrow[\text{liver microsomes}]{\text{NADH, malonyl-CoA}}$ 10.7% 20:4 ω6, trace 22:4 ω6, 1.3% 24:4 ω6 and 1.3% 22:5 ω6 [39]

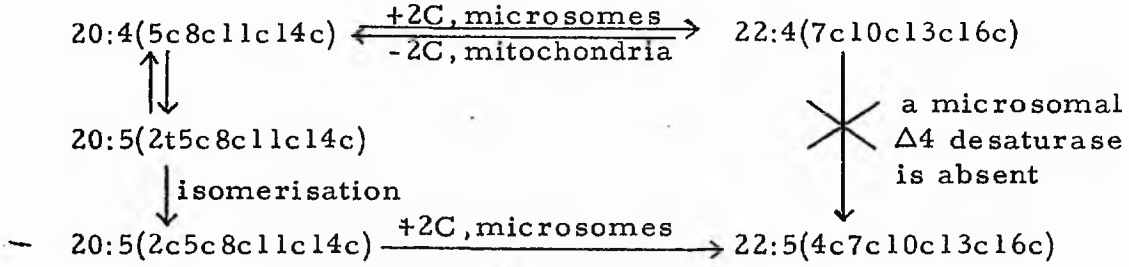
The % of 22:5(4c7c10c13c16c) produced in these experiments was very low. If the pathway to Δ4c PUFA is:

$$20{:}4(5c8c11c14c) \xrightarrow{+2C} 22{:}4(7c10c13c16c) \xrightarrow{-2H} 22{:}5(4c7c10c13c16c)$$

a paradox arises between the results quoted above [35, 39] and the fact that the in vitro Δ4 desaturation of 22:4 ω6 cannot be demonstrated. A possible explanation is that the substrate for Δ4 desaturation is 22:4 ω6 bound to the elongase and not exogenous 22:4 ω6.

An alternative proposal involves the production of a Δ4c double bond by desaturation then elongation of 20:4 ω6.

ie.



The first step requires the Δ2t dehydrogenation of 20:4 ω6 (or 20:5 ω3) and the second step a Δ2t-Δ2c isomerisation, while the third step is the conventional microsomal chain-elongation sequence starting with the addition of malonyl-CoA. The microsomal elongase multienzyme complex is known to contain a reversible Δ2t reductase [32], which could catalyse the first step. The enoyl-CoA hydratase is believed to possess c ⇌ t and Δ2 ⇌ Δ3 isomerising capacities, and could thus catalyse the second step. Overall the production of 22:5 ω6 (or 22:6 ω3) may be viewed as a "malfunction" of the microsomal elongase, with the Δ2c isomer not being released. According to this scheme 20:4 ω6 is the direct precursor for the biosynthesis of a Δ4c double bond; 22:4 ω6 must be chain shortened first. The following points should be considered in relation to this hypothesis:-

(i) The mechanism must be specific for the Δ5c8c11c... pattern of unsaturation otherwise Δ6c9c12c... PUFA would give Δ4c8c11c14c... products on chain elongation.

(ii) Feeding 20:4 ω6 or 22:4 ω6 to rats raised on a fat-free diet

resulted in the latter PUFA producing over twice as high a level of 22:5 ω6 in liver lipids than the former [38]. This is difficult to reconcile with the proposed pathway.

(iii) The injection of [1-^{14}C]20:3(7c10c13c) into EFA deficient rats resulted in 13% of the radioactivity appearing in 20:4(4c7c10c13c) [114]. This conversion would go via unlabelled 18:3(5c8c11c), so it would be necessary to invoke a very limited pool into which ^{14}C-acetate is passed and then ^{14}C-malonate withdrawn.

(iv) When 18:4(3c6c9c12c) was incubated with microsomes plus NADPH and malonyl-CoA cofactors the major product was 20:3(8c11c14c), indicating Δ3c hydrogenation (or isomerisation followed by hydrogenation) as an active process [32].

Mitochondrial contamination was recorded in several experiments (Section 2:3:3) and it was speculated that some of the additional products might have been Δ2c/t or Δ3c/t isomers. However this was not checked at the time as its significance to Δ4 desaturation was not realised. The proposed pathway could equally well involve the Δ2t reductase and the enoyl-CoA hydratase of the mitochondrial β-oxidation pathway.

(4) James and Sprecher have proposed another possible route to Δ4cis PUFA, also utilising an isomerisation step [113].

$$20{:}4(5c8c11c14c) \xrightarrow[\text{microsomes}]{+2C} 22{:}5(2t7c10c13c16c) \xrightarrow{\text{isomerisation}}$$

22:5(4c7c10c13c16c).

The final step in the microsomal chain elongation sequence, the hydrogenation of the Δ2t double bond, is bypassed and replaced by Δ2t $\rightleftharpoons$ Δ4c isomerisation. This hypothetical route can also be viewed as a "malfunction" of the microsomal elongase, and the mechanism for isomerisation must be specific for Δ7c10c13c... unsaturation. Δ2 $\rightleftharpoons$ Δ4 isomerisation has not been observed to date for the enoyl-CoA hydratase. Point (iv) from the previously proposed pathway (3) applies to the James and Sprecher model, as do(ii) and (iii) if the production of a Δ4c double bond can only occur in conjunction with elongation, and not independently via direct Δ2t dehydrogenation then isomerisation.

(5) Another possible route to Δ4c PUFA that may occur *in vivo* and which cannot as yet be ruled out is:-

$$22{:}4(7c10c13c16c) \xrightarrow[\text{microsomes}]{+2C} 24{:}4(9c12c15c18c) \xrightarrow[\text{microsomes}]{\Delta 6 \text{ desaturation}}$$

$$24{:}5(6c9c12c15c18c) \xrightarrow[\text{mitochondria}]{-2C} 22{:}5(4c7c10c13c16c)$$

24:4(9c12c15c18c) and 24:5(6c9c12c15c18c) were produced in small amounts in rat testis [115], while 24:5(9c12c15c18c21c) and 24:6 (6c9c12c15c18c21c) were detected in Baltic herring [116]. Injecting [2-^{14}C]19:4(7c10c13c16c) into rats raised on a fat free diet Schlenk *et al* noted after 12 hr the production of 21:5(6c9c12c15c18c), representing 64% of the total radioactivity in liver lipids. No labelled 19:5(4c7c10c13c16c) or 21:4(9c12c15c18c) were detected [93]. However the same authors noted that when radomly ^{14}C-labelled 21:4(6c9c12c15c) was injected it was retroconverted to 19:4(4c7c10c13c). In a longer term experiment, when cold 22:4(6c9c12c15c) was fed to rats kept on a fat-free diet it was not incorporated into liver lipids but was retroconverted to 20:4(4c7c10c13c) [117].

Clearly much work remains to be done on the biosynthesis of Δ4c PUFA.

2:2:4 The Δ9 Desaturase

Table 6 lists the [1-^{14}C] fatty acids which were Δ9 desaturated. Several of the substrates and/or the products were also Δ5 or Δ6 desaturated. Rf values for Ag^+TLC and ECL values on a 10% DEGS column at 180°C are quoted for substrate and product as points of identification [118, 119]. The conjugated dienes produced, Δ7t9c and Δ9c11t, are particularly distinctive. Conjugated (c, c + t, t) dienes have ECL values considerably greater than those for a methylene- or polymethylene-interrupted diene of the same chain length [118], and elute on Ag^+TLC slightly ahead of oleate [120]. Similar behaviour is reported here for (c, t + t, c) conjugated dienes.

TABLE 6: [1-^{14}C] Fatty Acids which were Δ9 Desaturated

	Substrate ECL; Rf ()	Product ECL; Rf	% Δ9 Desaturation	% Lipid Incorporation NL	FFA	PL
Experiment 1	16:0[a] 16.0;0.57 (1)	16:1(9c)[a] 16.4;0.47	77	21	11	68
	17:0[a] 17.0,0.56 (1)	17:1(9c)[a] 17.4,0.45	65.5	44	3	53
	18:0 18.0,0.57 (1)	18:1(9c)[a] 18.35,0.45	55.5	27	8	65
	16:1(5t) 16.4,0.49 (2)	16:2(5t9c) 17.1,0.25	69.5	27	5	68
	17:1(5t) 17.3,0.49 (2)	17:2(5t9c) 17.75,0.26	73	24	8	68
	18:1(5t) 18.3,0.51 (2)	18:2(5t9c) 18.7,0.27	74.5	23	6	70
	16:1(7t) 16.35,0.48(2)	16:2(7t9c) 17.9,0.40	47	28	20	51
	17:1(12t)[a] 17.35,0.52(2)	17:2(9c12t)[a] 17.85,0.25	18	34	5	61
	18:1(13t)[a] 18.4,0.52 (2)	18:2(9c13t)[a] 18.85,0.25	60	26	7	66
Experiment 2	18:0 18.0; -	18:1(9c)[a] 18.6; -	55	20	7	73
	18:1(5t) 18.5,0.51 (3)	18:2(5t9c) 19.0,0.15	80	28	4	68
	16:1(7t) 16.4,0.48 (3)	16:2(7t9c) 18.2,0.43	23	31	11	58
	18:1(11t)[a] 18.6,0.54 (3)	18:2(9c11t)[a] 20.35,0.46	62	51	3	46
	17:1(12t)[a] 17.7,0.52 (3)	17:2(9c12t)[a] 18.25,0.17	14	31	6	63
	18:1(13t)[a] 18.65,0.52(3)	18:2(9c13t)[a] 19.25,0.17	57.5	37	4	59
	18:1(14t)[a] 18.75,0.51(3)	18:2(9c14t)[a] 19.25,0.21	41.5	38	4	58
	18:1(15t)[a] 18.8,0.51 (3)	18:2(9c15t)[a] 19.35,0.17	48.5	38	4	58
	18:1(13c)[a] 18.75,0.57(4)	18:2(9c13c) 19.3,0.23	1	33	3	64
	18:1(14c)[a] 18.75,0.56(4)	18:2(9c14c) 19.3,0.26	6	30	3	67

TABLE 6 (cont)

	Substrate ECL; Rf ( )	Product ECL, Rf	% Δ9 Desaturation	% Lipid Incorporation NL	FFA	PL
Experiment 2	18:1(15c)[a] 19.1, 0.54 (4)	18:2(9c15c) 19.6, 0.23	4	27	2	72
Experiment 3	17:0	17:1(9c)	40	45	1	54
	17:1(12t)[a]	17:2(9c12t)	5	48	1	51

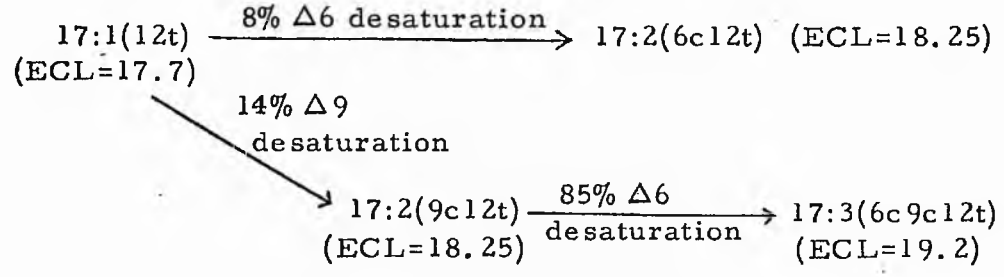
a - this fatty acid was also Δ5 or Δ6 desaturated

(1)-(4) refer to separate Ag^{+} TLC plates. Standard Rf values were:

(1) stearate 0.57, oleate 0.44 (PE20).
(2) stearate 0.56, oleate 0.45, linoleate 0.16 (PE20).
(3) elaidate 0.51, linoleate 0.12 (PE17).
(4) oleate 0.57, linoleate 0.29 (PE27).

As a guide to the polarity of the DEGS columns used linoleate had an ECL value of 19.0 in experiment 1 and 19.4 in experiment 2. Experiment 3 was performed with hen liver microsomes.

For clarity of discussion with respect to the Δ9, Δ6 and Δ5 desaturation information has been tabulated for each desaturase, but this may obscure the fact that several desaturations (occurring in sequence, or competing for the same substrate) can occur in a single incubation. To counter this Tables 6, 7 and 9 are labelled in a consistent fashion such that the overall picture for a single incubation, including the chromatographic properties of the products, can be built up. For example the incubation of [1-^{14}C] 17:1(12t) can be traced in Experiment 2 from two entries in Table 7 and one in Table 6, giving the overall picture:



The sequence of Δ9 desaturation followed by Δ6 desaturation is inferred from the structure of the products.

The results for each incubation are tabulated in this manner in Appendix 1.

	ECL	Rf(Ag^+TLC)	
16:2(7t9c)	16 + 2.2	0.43	Elaidate had an Rf value of 0.51
18:2(9c11t)	18 + 2.35	0.46	
18:2(9c13t)	18 + 1.25	0.17	

The Δ9 desaturation of Δ8 or Δ10 monoenoic acids would have given 8,9- or 9,10-allenes respectively. These have an ECL value on a DEGS column similar to linoleate, and elute approximately with trans monoenes on Ag^+TLC [120]. None were observed.

A plot of %Δ9 desaturation versus double bond position and configuration is shown in Figure 6. A comparison between different positional isomers with different chain lengths (C_{16}, C_{17} or C_{18}) is considered justified, since plotting the Δ9 desaturation for each isomer relative to the %Δ9 desaturation of the corresponding chain length saturated acid gives a similar pattern. The main findings should be grouped as follows:-

(1) No Δ9 desaturation of Δ8 and Δ10 monoenoic acids occurred.

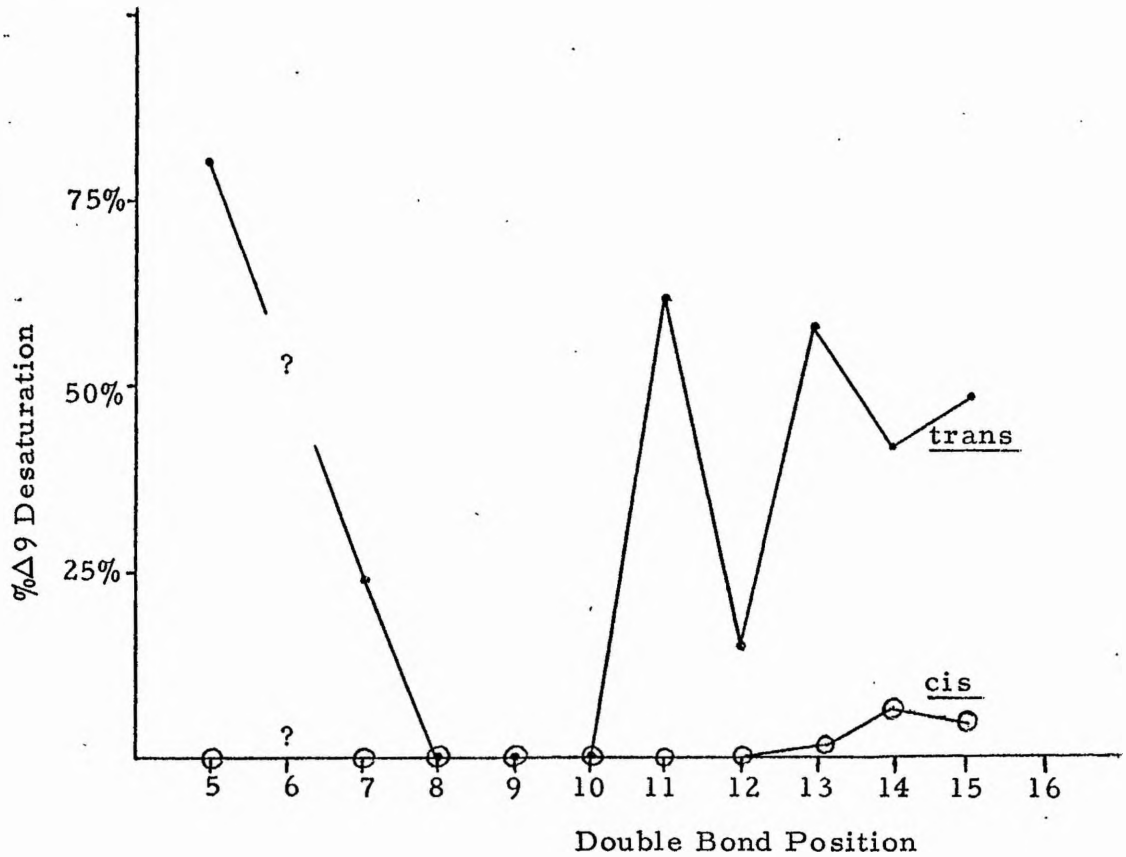
(2) Δ5c, 7c, 11c, 12c, 12a isomers were not Δ9 desaturated, while Δ13c-15c isomers were poor substrates. 18:1(7c) and (11c) were previously demonstrated not to be substrates for hen liver Δ9 desaturase [61], and 18:1(12c) not to be desaturated by rat liver Δ9 desaturase [64].

(3) Δ5t, 7t, 11t, 13t-15t isomers were extensively desaturated. The Δ12t isomer could only be considered as a poor to moderate substrate in comparison.

The detection limit was approximately 1%, so "no desaturation" indicates a conversion of less than 1%. A "poor" substrate represents about a 1-10% conversion to product and a "moderate" substrate 10-25%. These general descriptions will be used throughout this discussion.

In cases where the substrate tested was also Δ5 or Δ6 desaturated the removal of substrate by these pathways was small, and no correction to the %Δ9 desaturation figure is considered necessary.

Figure 6: The Effect of Double Bond Configuration and Position on Δ9 Desaturation of some C_{16}, C_{17} and C_{18} Monoenoic Acids



The results are taken from experiment 2 (Table 6).

18:0 was 55% Δ9 desaturated.

—•—•— trans monoenoic acids

—⊖—⊖— cis monoenoic acids

The chain length of the substrates tested was C_{18}, except for double bonds in Δ7, 8 and 12 positions where it was C_{16}, C_{16} and C_{17} respectively.

? No data for Δ6c or Δ6t monoenoic acids is available.

Such corrections would be minor and would not affect the general arguments.

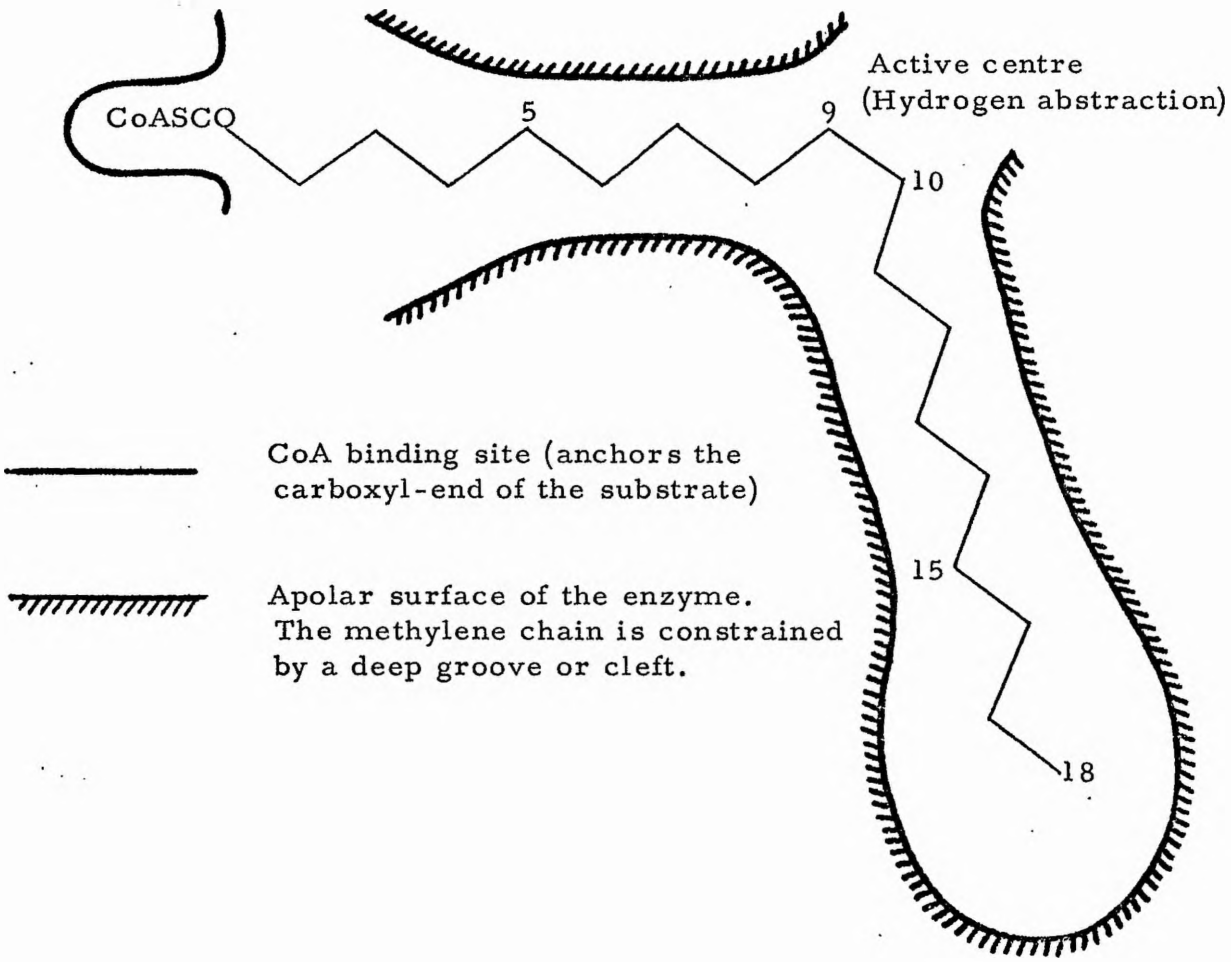
The results fitted the model advanced by Brett et al [61]. The substrate, with an extended alkyl chain, is believed to fit into a deep cleft with a tight spatial constraint between C(5) and C(15). A schematic representation of this is given in Figure 7. The work of Enoch et al pointed to the 9,10-gauche conformer of stearoyl-CoA being the true substrate [10] and this is drawn in Figure 7. The 9D and 10D hydrogen atoms will be in a position to allow their cis elimination [61], producing oleoyl-CoA, which will have a similar geometry to the bound substrate. A trans double bond can mimic the geometry of the extended methylene chain so it may fit into the cleft (Figure 8). However if the correct alignment of the methylene chain is to be conserved cis double and triple bonds cannot fit into the cleft. Because the spatial constraint is partially lifted beyond C(15) 18:1(14c) to 18:1(17e) acids may be Δ9 desaturated. The enlarged cleft beyond C(15) for rat liver microsomal Δ9 desaturase is sufficient to accommodate up to a C_{19} chain length but not greater.

18:1(14c) and 18:1(15c) were poor substrates for the Δ9 desaturase. Their C(16-18) and C(17+18) atoms respectively were the ones which would give a steric perturbation. This indicated there was still considerable spatial restriction in the C(16) to C(18) region of the cleft, and thus confirmed the results of Brett et al [61] with methyl-branched stearates. None of the other cis monoenes tested were Δ9 desaturated.

Substrate	%Δ9 Desaturation relative to stearate(100%)	
15-Me 18:0	⩽ 2%	Hen liver microsomes [61]
16-Me 18:0	15%	
17-Me 18:0	35%	
18:1(13c)	⩽ 2%	Rat liver microsomes [This work]
18:1(14c)	11%	
18:1(15c)	7%	

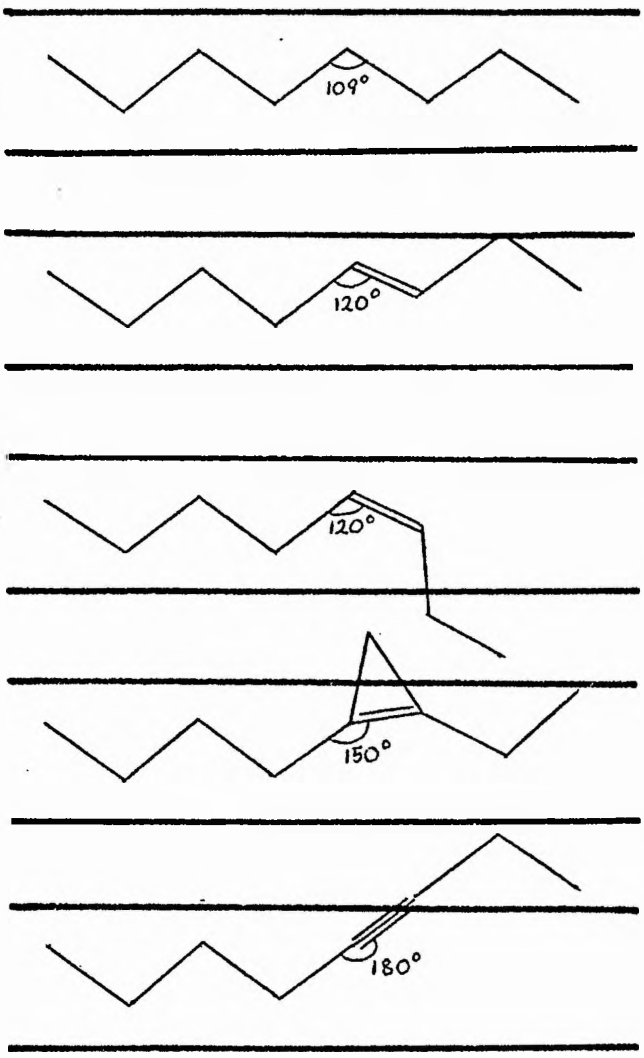
An interesting feature was the production of conjugated dienes, which is believed to be the first recorded instance in animals. Δ9 Desaturation of Δ8t and Δ10t isomers would give allenes. Although

Figure 7: A Schematic Representation of the Stearoyl-CoA:Δ9 Desaturase Enzyme Binding Site



Because of the two dimensional representation the C(9)-C(10) bond appears as the synperiplanar conformer rather than the proposed gauche conformer.

Figure 8: The Fit of Unsaturated Centres into the Substrate Binding-Site of the Δ9 Desaturase



Methylene chain

Trans double bond

Cis double bond

Cyclopropene ring

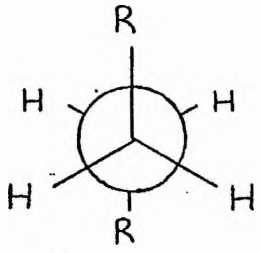
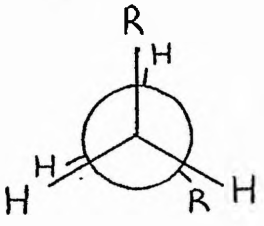
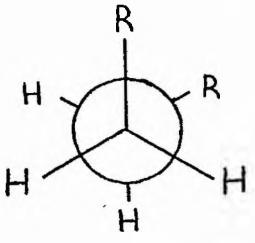
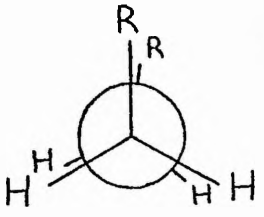
Triple bond

The surface of the enzyme cleft is represented by ——— , with the methylene chain, in all the trans conformation, correctly aligned on the left hand side in order to bring 9D and 10D hydrogen atoms to the active centre.

these potential substrates can fit into the cleft hydrogen abstraction would involve breaking a vinyl C-H bond (D_{dissoc}=452 kJ $mole^{-1}$) in a different orientation to the more usual alkyl C-H bond (423 kJ $mole^{-1}$), and the linear geometry of the allene formed would be incompatible with the required 9,10*cis* geometry. Therefore allene formation was not envisaged. None was observed (p. 44).

Apart from the Δ8t and Δ10t isomers all the other *trans* monoenoic acids tested were Δ9 desaturated. 16:1(7t) was a poorer substrate than 16:0 or 16:1(5t) (Figure 6), but this was not surprising as the *trans* double bond lay between the CoA binding site and the active centre, but adjacent to the latter. As *trans* unsaturation does not have identical geometry to an extended alkyl chain, neither in bond angles or lengths nor in the disposition of hydrogen atoms (Figure 8), perhaps there is insufficient tolerance for the 9D and 10D hydrogen atoms to align exactly at the active centre.

The reduced desaturation of 17:1(12t), relative to 17:0 and to 18:1(11t) and 18:1(13t), also requires explanation, and the following hypothesis is advanced. The conformation of the C(12)-C(13) bond adopted by stearoyl-CoA in the cleft is not *trans*, but *anticlinal* or *gauche*. The following Newman projections for the C(12)-C(13) bond in the n-alkyl chain show the relationship between the conformers and *cis* and *trans* geometry. R represents either the methyl or the carboxyl end of the fatty acid, and the energy of each conformer relative to the *antiperiplanar* conformer is given for the C(2)-C(3) bond of butane as a useful guide [123]. If the C(12)-C(13) bond conformation adopted by stearoyl-CoA is *anticlinal*, but the cleft has a moderate amount of spatial tolerance, a Δ12t but not a Δ12c olefin can be accommodated, though the substrate-enzyme binding constant for the *trans* olefin relative to the saturated acid will be less.

Newman projection		ΔE	
	antiperiplanar or trans	0	Increasing tendency to mimic trans olefin geometry.
	anticlinal	14.7 kJ mole^{-1}	
	synclinal or gauche	3.8 kJ mole^{-1}	
	synperiplanar	20 kJ mole^{-1}	Increasing tendency to mimic cis olefin geometry.

The Δ9 desaturase specificity for 16:0, 17:0 and 18:0 reported here is in agreement with that of Brenner and Peluffo [76], who used almost identical, non substrate-saturating incubation conditions, but is at variance with maximum velocity measurements on whole microsomes [31] or on the purified or partly purified enzyme system [19, 29] (Table 8). This emphasises the difficulty of extrapolating simple, % conversion data from a complex biological system back to the enzyme under investigation. No explanation for the discrepancy is offered, but it is noted that the critical influences on a system operating at non-saturating conditions with respect to substrate will be the binding constants for the desaturase and the competing enzymes, and perhaps pool sizes, and that these criteria may bear

TABLE 8: The Relative Δ9 Desaturation of 16:0, 17:0 and 18:0 in Various Rat Liver Enzyme Preparations

	Assay conditions			Δ9 Desaturase activity relative to stearate (1.0)		
	Substrate	Enzyme Preparation	Measurement	16:0	17:0	18:0
This work	FFA	Whole microsomes	% conversion	1.40	1.18	1.0
Brenner and Peluffo [76]	FFA	Whole microsomes	% conversion	1.46	-	1.0
Paulsrud et al [29]	FFA	Whole microsomes	V_{max}	0.55	0.62	1.0
Jeffcoat et al [31]	Acyl-CoA	Partly purified	V_{max}	0.42	0.67	1.0
Enoch et al [19]	Acyl-CoA	Purified	V_{max}	0.86	1.03	1.0

little resemblance to maximum velocities. In vivo 16:0 is rapidly elongated to 18:0, so oleate and not palmitoleate is the principal monoenoic acid in the tissues. Even the V_{max} specificity for the purified Δ9 desaturase [19] does not tally in all aspects to that for the whole microsomes [29, 31] (Figure 5). The study with the purified enzyme had one important omission; K_m and V_{max} values for 15:0. Two possibilities are shown in Figure 5 for the specificity pattern depending on the position of 15:0 (— — — —). One possibility is a broad range of V_{max} values decreasing slowly in magnitude from 18:0 to 13:0. The other possibility is two distinct maxima. A knowledge of the correct pattern might help to resolve the debate as to whether two Δ9 desaturases exist in certain biological systems [6, 64]. The writer favours the existence of a single Δ9 desaturase per species, with slight difference between species possible, rather than a IΔ9 desaturase for short chain acids and a IIΔ9 desaturase for long chain acids.

The Δ9 desaturase has no need to handle short chain acids in vivo. The fatty acid synthetase produces palmitate, which is rapidly elongated to stearate: these are generally the only two substrates which the Δ9 desaturase will encounter in nature. The purified rat liver Δ9 desaturase can dehydrogenate saturated acids with a chain length down to C_{13} with reasonable efficiency [19] (Figure 5) and this fact also eliminates the necessity for a IΔ9 desaturase with maximum activity at 14:0. According to Gurr et al a species with two maxima (at 14:0 and 18:0) in its chain length specificity should also Δ9 desaturate Δ12c monoenoic acids, as the IΔ9 desaturase will be present. Unfortunately it is not clear whether rat liver Δ9 desaturase, which cannot desaturate Δ12c substrates, exhibits one or two maxima. The data of Paulsrud et al showed a single maximum at 18:0 [29], those of Jeffcoat et al pointed to a small, secondary maximum at 14:0 [31], while those of Strittmatter and his group were inconclusive [19].

An alkyl chain can take up a geometry approximating to that of a cis olefin if the appropriate C-C bond has a gauche conformation. The additional energy required for this conformation, above that of

the trans conformer, is small ($\sim$4 kJ mole^{-1}) and the activation energy for the trans $\longrightarrow$ gauche transition is of the order of 15 kJ mole^{-1}. Calculations based on CMR evidence indicated a high population of gauche conformers in linear alkanes, and hence fatty acids, in organic solvents [121]. Therefore it seems unreasonable to state that a Δ12c monoenoic acid will mimic the behaviour of a short chain acid (13:0 or 14:0) whereas a long-chain saturated acid will not, as the latter can take up a conformation with a Δ12 cis "kink" in free solution or if constrained to do so. This invalidates the argument that 18:1(12c) will fit into the C(14) length cleft of the IΔ9 desaturase by the Δ12c double bond lifting the methyl end of the substrate away from the steric block, whereas the long-chain saturated acids ($\geqslant$15:0) will not fit in it at all. Hen liver microsomal Δ9 desaturase was the best example of the two enzyme hypothesis, as it showed a very distinct bimodal chain length specificity and had a high % desaturation of 18:1(12c) relative to 18:0 [64]. Johnson et al reported a broadly similar behaviour for the inhibition of Δ9 desaturation of 12:0-20:0 in hen liver by sterculic and malvalic acids [62]. At fixed substrate and inhibitor concentrations sterculic acid gave 85-95% inhibition over the range of substrates examined and malvalic acid gave 65-80% inhibition. To explain this uniform inhibition by C_{17} and C_{18} cyclopropene fatty acids in terms of a IΔ9 and a IIΔ9 desaturase it is necessary to postulate that the inhibition occurred at a transfer point prior to desaturation, which was utilised by both enzymes. 20:0 and 22:0 were Δ9 desaturated by hen liver microsomes but rat liver Δ9 desaturase could only accommodate acids up to 19:0, so there must be a difference between the long chain Δ9 desaturases for the two species. If there is one subtle difference, why not two? Rat liver could not Δ9 desaturate 18:1(12c) but hen liver could, and for both species the Δ12t isomer was a substrate for the Δ9 desaturase but a much poorer one than the corresponding saturated acid.

Δ9 desaturation relative to the corresponding saturated acid (1.00) for:-

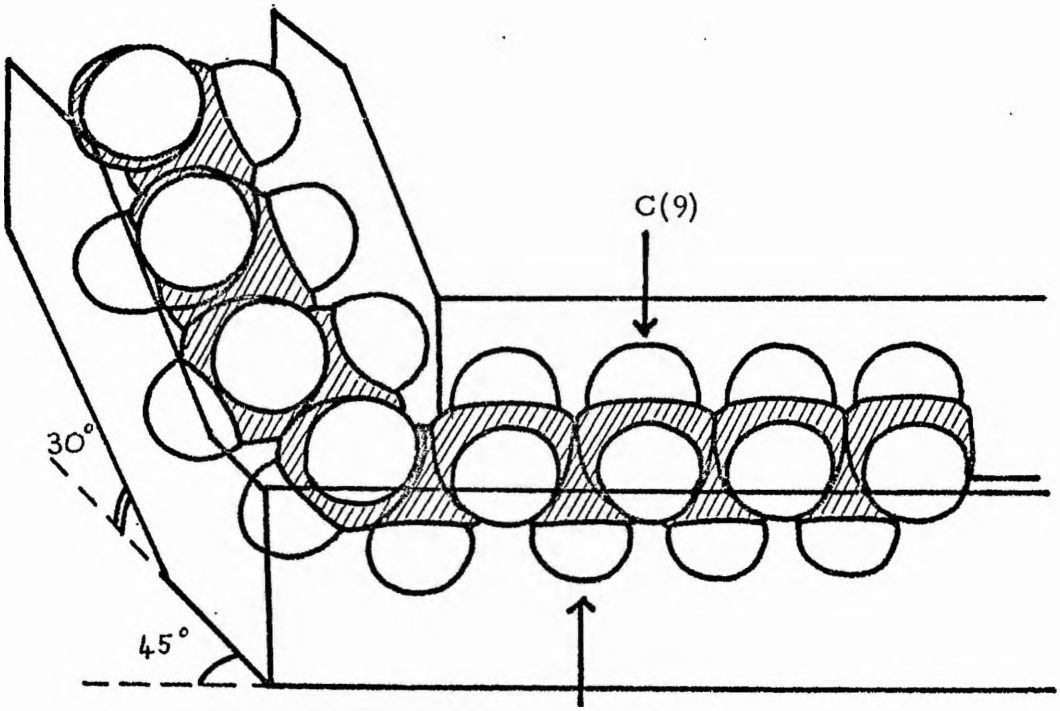
Hen liver		Rat liver		
17:1(12t)	0.125	17:1(12t)	0.28	17:1(12c/t) [This work]
18:1(12c)	0.60	17:1(12c)	<0.02	18:1(12c) [64]
		18:1(12c)	<0.02	

Both hen and rat liver long chain Δ9 desaturases have a constraint at the Δ12 position with respect to trans olefinic substrates, and it is proposed that this is due to cleft geometry at C(11) to C(14) accommodating a 12, 13-gauche or anticlinal conformer.

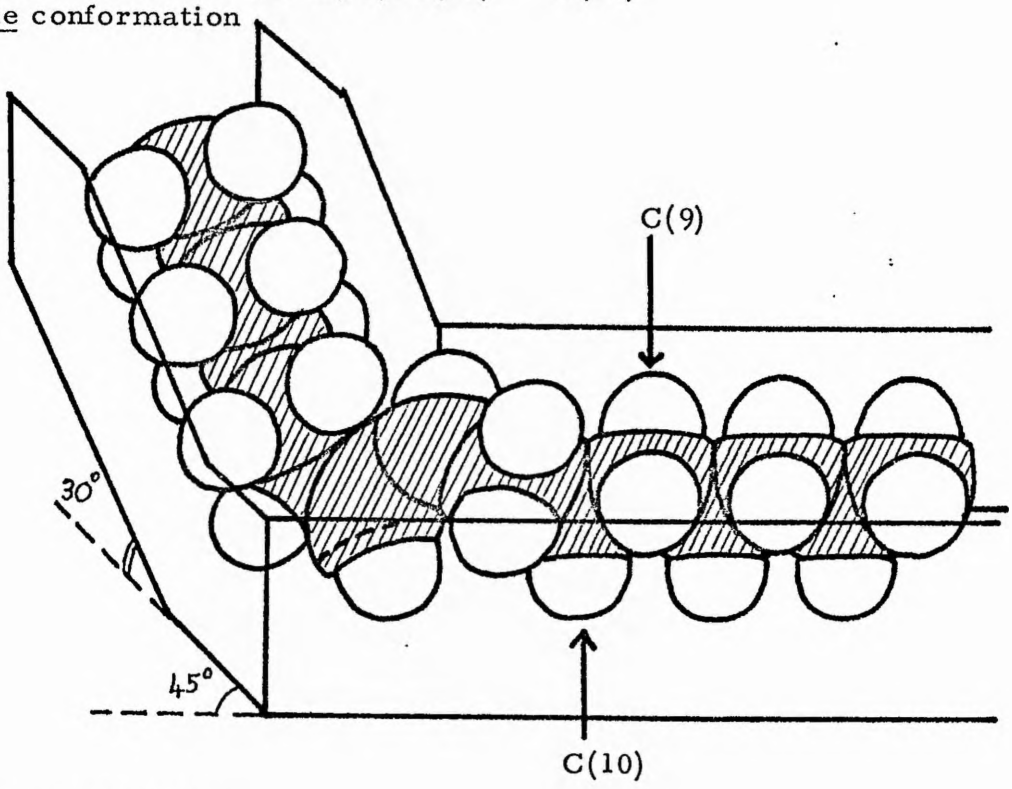
An enzyme cleft that can accommodate a gauche conformer as well as cis and trans olefins is shown in Figure 9. This model explains how a single hen liver Δ9 desaturase can dehydrogenate saturated, Δ12c and Δ12t substrates but not (±) 11 to 14 methyl-branched stearates. The model does not elaborate on the subtle differences in cleft geometry (ie. a non-uniform enzyme surface) and substrate binding that could explain the order of preference of saturate > Δ12c monoene > Δ12t monoene substrates for Δ9 desaturation: it merely shows that all three substrates can take up conformations that fit into a similar space. For rat liver Δ9 desaturase there must be a small difference in cleft geometry to explain the different order of preference - saturate > Δ12t monoene ≫ Δ12c monoene. Perhaps the enzyme differs in a more limited spatial tolerance in the C(11) to C(14) region, or the saturated substrate takes up an anticlinal rather than a gauche conformation.

Some of the specificity work accomplished in this programme on rat liver Δ9 desaturase should be repeated on hen liver. 18:1(11c) and 18:1(13c) are not likely to be Δ9 desaturated, as a 12, 13-gauche conformer in the cleft will almost certainly preclude 11, 12- and 13, 14-gauche conformers. The distinctive bimodal pattern of hen liver Δ9 desaturase chain length specificity is not taken as necessarily indicative of two enzymes. The biological system is believed to be too complex and the examination insufficiently rigorous to make any sweeping extrapolation from the specificity pattern back to two distinct enzymes.

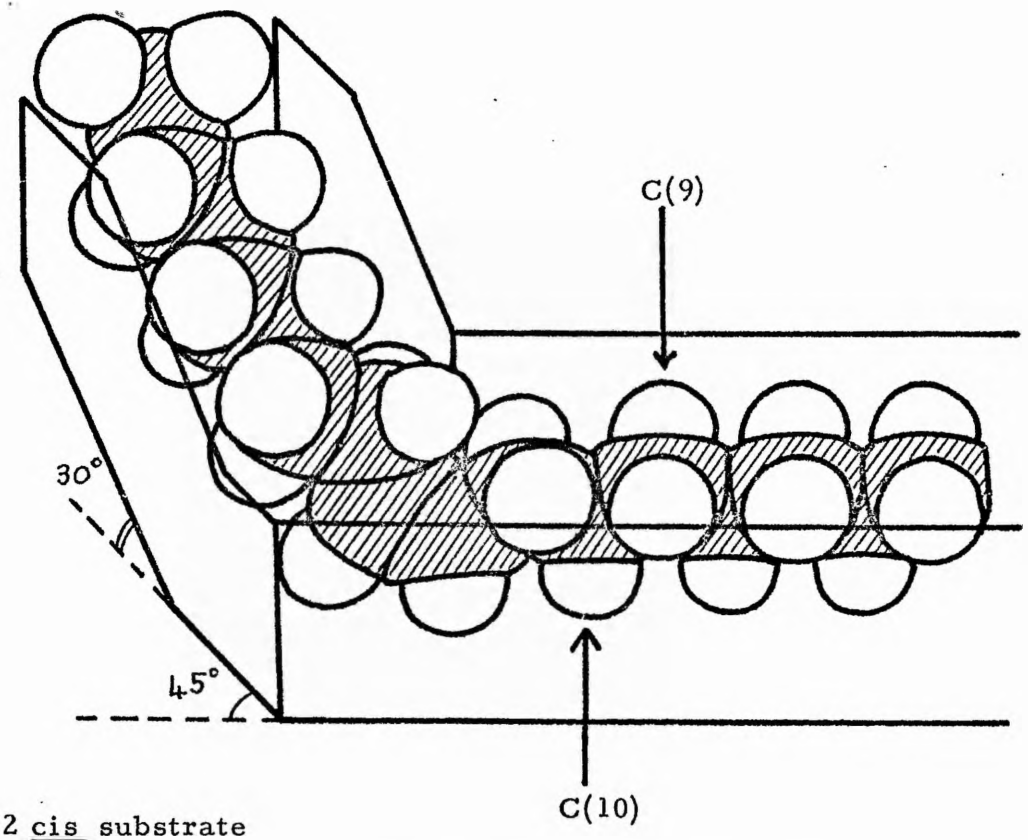
Figure 9: A model of a Δ9 Desaturase Substrate Binding Cleft that Can Accommodate C(12)-C(13) Gauche or Δ12 Cis and Trans Olefinic Substrates



Saturated substrate with the C(12)-C(13) gauche conformation



Δ12 trans substrate



Δ12 cis substrate

The drawings are of a model of a cleft which could accommodate the saturated substrate with a C(12)-C(13) gauche conformation or Δ12 cis and trans olefinic substrates. The cleft was composed of two segments, at angles to each other as shown, and it had a width of 5.2 Å. The methylene chain was aligned so that C(9) and C(10) hydrogen atoms were in exactly the same position at the active centre for each substrate. Although the C(9)-C(10) bond of the substrate is believed to take up a gauche conformation at the active centre, in the model it assumed a trans conformation. This was to simplify the diagram.

Hydrogen atoms

Carbon atoms

2:2:5 The Δ6 Desaturase

Table 7 lists the [1-^{14}C] fatty acids which were Δ6 desaturated, together with the incorporation of their radioactivity into lipids, and the ECL and Ag^+TLC R_f values of substrate and product. To check that a good Δ6 desaturase activity was present in the enzyme preparation the desaturation of linoleic and α-linolenic acids was always examined. In some incubations substrate generated *in situ* by Δ9 desaturation was subsequently Δ6 desaturated, while in others the Δ9 or Δ5 desaturases competed with Δ6 desaturase for the substrate. Where competitive desaturation occurred the figure for % Δ6 desaturation, measured against 100% of substrate available, may need correction if it is to be compared against a Δ6 desaturation where there was no competing desaturation.

The series of Δ9c12c dienoic and Δ6c9c12c trienoic acids produced noteworthy gradations in their chromatographic properties. The incremental ECL values for their unsaturation (Δ_{ECL}=ECL-chain length) increased as the ω double bond approached the methyl end. Δ_{ECL} values are compared with those of Christie for the appropriate ω isomers in a series of c,c-methylene-interrupted octadecadienoates [124] (Table 10). The two sets of data correlate well. The position of unsaturation relative to the carboxyl end is

TABLE 10: Δ_{ECL} Values for Methyl Dienoates and Trienoates

ω Notation	18:2 c,c Diene	Δ_{ECL} [1]	Δ9c12c Diene	Δ_{ECL} [2]	Δ6c9c12c Triene	Δ_{ECL} [2]
ω 2	(13c16c)	+2.25	14:2	+2.5	14:3	+3.1
ω 4	(11c14c)	+1.62	16:2	+1.7	16:3	+2.3
ω 6	(9c12c)	+1.38	18:2	+1.4	18:3	+1.95
ω 8	(7c10c)	+1.23	20:2	+1.1	20:3	+1.7
ω 10	(5c8c)	+1.06	22:2	+1.0	22:3	+1.6

1. Open tubular DEGS column, 180°C [124]
2. 10% DEGS packed column, 180°C [this work]

constant in the series of dienes and trienes examined in this work, so that the ω contribution to the Δ_{ECL} value can be seen on its own.

TABLE 7: [1-^{14}C] Fatty Acids which were Δ6 Desaturated

	Substrate ECL; Rf ( )	Product ECL; Rf	% Δ6 Desaturation	% Lipid Incorporation NL	FFA	PL
Experiment 1	16:0[a] 16.0; 0.61 (5)	16:1(6c) 16.4; 0.48	1	21	11	68
	17:0[a] 17.0; 0.60 (5)	17:1(6c) 17.4; 0.47	1	44	3	53
	15:1(9c) 15.65; 0.49 (5)	15:2(6c9c) 16.2; 0.13	2	37	8	55
	16:1(9c) 16.4; 0.47 (5)	16:2(6c9c) 17.1; 0.14	10.5 (10[b])	35	6	59
	17:1(9c) 17.4; 0.49 (5)	17:2(6c9c) 18.0; 0.13	8.5 (13[b])	25	10	65
	18:1(9c) 18.35; 0.49 (5)	18:2(6c9c) 18.8; 0.14	5 (7[b])	32	6	62
	17:1(12t)[a] 17.35; 0.56 (5)	17:2(6c12t) 17.85; 0.25	15	34	5	61
	16:1(8t) 16.35; 0.57 (5)	16:2(6c8t) 17.9; 0.51	17	13	10	77
	17:2(9c12t)[b] 17.85; 0.25 (2)	17:3(6c9c12t) 18.6; 0.11	~80	34	5	61
	18:2(9c13t)[b] 18.85; 0.25 (2)	18:3(6c9c13t) 19.35; 0.12	35	27	7	66
	18:2(9c12c) 19.0; 0.16 (5)	18:3(6c9c12c) 19.5; 0.0	13.5	12	4	84
	18:3(9c12c15c) 19.9; -	18:4(6c9c12c15c) 20.35; -	37.5	10	2	88
Experiment 2	14:2(9c12c) 16.5; 0.19 (4)	14:3(6c9c12c) 17.1; 0.03	18.5	33	8	59
	Incubated with 2.8 mg ml^{-1} BSA		24	16.5	4	79.5
	16:2(9c12c) 17.7; 0.26 (4)	16:3(6c9c12c) 18.3; 0.05	24	16.5	5	78.5
	Incubated with 2.8 mg ml^{-1} BSA		35	33	5	62
	18:2(9c12c) 19.4; 0.29 (4)	18:3(6c9c12c) 19.95; 0.07	16	17	7	76
	Incubated with 2.8 mg ml^{-1} BSA		13	17	8	75
	20:2(9c12c) 21.1; 0.31 (4)	20:3(6c9c12c) 21.7; 0.09	32.5	39.5	6.5	54
	Incubated with 2.8 mg ml^{-1} BSA		37.5	59.5	5	40.5
	22:2(9c12c) 23.0; 0.34 (4)	22:3(6c9c12c) 23.6; 0.10	75.5	58	8	34
	Incubated with 2.8 mg ml^{-1} BSA		74	76	4	20

TABLE 7 (cont)

Experiment 2

Substrate ECL; Rf ( )	Product ECL; Rf	% Δ6 Desaturation	% Lipid Incorporation NL	FFA	PL
18:3(9c12c15c) 20.4; 0.07 (4)	18:4(6c9c12c15c) 20.95; origin	33	12	2	86
Incubated with 2.8 mg ml^{-1} BSA		46.5	24	4	72
17:1(12a) 19.6; 0.41 (3)	17:2(6c12a) 20.2; 0.09	7	14	7	79
18:2(9c12t) 19.45; 0.28 (3)	18:3 (6c9c12t) 20.0; 0.07	66.5	20	6	74
See text, as the 18:2(9c12t) isomer was co-incubated with 18:2(9t12c)					
18:1(9c) 18.6; 0.57 (4)	18:2(6c9c) 19.2; 0.25	4	17	4	79
18:1(11c)[a] 18.7; 0.57 (4)	18:2(6c11c) 19.1; 0.24	1	37	2	61
17:1(12c) 17.85; 0.57 (4)	17:2(6c12c) 18.4; 0.21	5.5	33	4	64
18:1(13c)[a] 18.75; 0.56 (4)	18:2(6c13c) 19.3; 0.26	1	33	2	65
18:1(14c)[a] 18.75; 0.56 (4)	18:2(6c14c) 19.3; 0.26	3.5	30	3	67
18:1(15c)[a] 19.1; 0.54 (4)	18:2(6c15c) 19.6; 0.23	4.5	26.5	2	71.5
18:1(8t) 18.6; 0.47 (3)	18:2(6c8t) 20.25; 0.40	5.5	20	2	78
18:1(10t)[a] 18.6; 0.49 (3)	18:2(6c10t) 19.15; 0.13	10	42	3	55
18:1(11t)[a] 18.6; 0.54 (3)	18:2(6c11t) 19.0; 0.18	5	51	3	46
17:1(12t)[a] 17.7; 0.52 (3)	17:2(6c12t) 18.25; 0.17	8	31	6	63
18:1(13t)[a] 18.65; 0.52 (3)	18:2(6c13t) 19.25; 0.17	1.5	37	4	59
18:2(9c11t)[b] 20.35; 0.46 (3)	18:3(6c9c11t) 20.9; 0.13	41	51	3	46
17:2(9c12t)[b] 18.25; 0.17 (3)	17:3(6c9c12t) 19.2; 0.07	~ 85	31	6	63
18:2(9c13t)[b] 19.25; 0.17 (3)	18:3(6c9c13t) 19.9; 0.07	27	37	4	59
18:2(9c14t)[b] 19.25; 0.21 (3)	18:3(6c9c14t) 19.6; 0.07	4	38	4	58
18:2(9c15t)[b] 19.35; 0.17 (3)	18:3(6c9c15t) 19.75; 0.07	4	38	4	58

TABLE 7 (cont)

	Substrate ECL; Rf ( )	Product ECL; Rf	% Δ6 Desaturation	% Lipid Incorporation NL	FFA	PL
Experiment 5	17:0[a]	17:1(6c)	8.5	32	3	65
	Coincubated with 20 nmole of sterculic acid					
Experiment 6	18:0[a]	18:1(6c)	1.5	32	1	77
	Coincubated with 20 nmole of sterculic acid					

a - this fatty acid was also Δ5 or Δ9 desaturated

b - this substrate was generated *in situ* by Δ9 desaturation.

(2), (3), (4) and (5) refer to separate Ag^+TLC plates developed once with PE20, PE17, PE27, and PE20 respectively.

See also the footnotes under Table 6.

It is therefore apparent that the principal influence on the Δ_{ECL} value for the isomeric series 18:2(5c8c) to 18:2(13c16c) is the methyl end. Christie has also shown a sinusoidal pattern for the elution profile of the 18:2 c,c isomers on Ag^+TLC [125]. From ω1 to ω8 there was a very slight tendency for the R_f value to diminish. The R_f values (Ag^+TLC) of the 9c, 12c-dienoates and 6c, 9c, 12c-trienoates increased with chain length (and with the ω value) (Table 7).

Generally isomeric dienoic acids produced from a single substrate were not separated by GLC (9ft packed 10% DEGS column) or by Ag^+TLC (5% $AgNO_3$, single development at room temperature) though two examples of separation by Ag^+TLC were observed using dual development with a less polar solvent. The resolved isomers showed different GLC retention times. Since there is little or no information on the separation of c,t-dienoates by Ag^+TLC or GLC the most complete comparative set of information gained in this study is collated as follows:-

Fatty Acid	R_f (5% $AgNO_3$, dual development PE10)	Δ_{ECL} (ECL-chain length) (10% DEGS, 180°C)
18:1(5t)	0.56	+ 0.3
16:1(7t)	0.52	+ 0.4
18:1(8t)	0.56	+ 0.4
18:1(10t)	0.59	+ 0.4
18:1(9c)	0.44	+ 0.45
17:1(12t)	0.59	+ 0.5
18:2(5c10t)[a]	0.19	+ 0.7
18:2(5t9c)	0.14	+ 0.75
18:2(6c10t)[a]	0.15	+ 0.85
17:2(6c12t)[b]	0.16	+ 1.00
17:2(9c12t)[b]	0.21	+ 1.15
18:2(6c8t)	0.50	+ 2.05
16:2(7t9c)	0.40	+ 2.2

a and b mark the resolved pairs of isomers. Other isomer pairs, such as 18:2(6c15c) and 18:2(9c15c) or 18:2(5c15t) and 18:2(9c15t), were not resolved.

The Δ6 desaturase is capable of producing c,t-conjugated dienes. 16:2(6c8t) and 18:2(6c8t) had the highly distinctive chromatographic properties of such systems. Whether the Δ6 desaturase is capable of desaturating a Δ4t acid to give a Δ4t6c conjugated diene was not tested. The Δ6 desaturation of Δ5 or Δ7 monoenoic acids, which would result in allenes, was not detected. No radioactive components with an ECL value on a DEGS column similar to the same chain length methylene-interrupted diene but running on Ag^+ TLC with the trans monoene fraction [120] were observed. The desaturation of the acetylenic acid 17:1(12a) to 17:2(6c12a) was interesting. No other acetylenic acid was examined and it is possible that other ynoic acids could act as substrates.

An equimolar mixture of [1-^{14}C] 18:2(9c12t) and (9t12c), prepared by stereomutation of [1-^{14}C] 18:2(9c12c), could not be separated and was incubated in this form. Since 17:1(12t) was readily desaturated to 17:2(9c12t) and hence to 17:3(6c9c12t) whereas

Δ9t monoenoic acids gave Δ5c9t dienoic acids, the 18:2 mixture was expected to produce the Δ6c9c12t and Δ5c9t12c trienes on the grounds that the double bond nearest the carboxyl end controls the specificity of desaturation. In fact, only the Δ6c9c12t triene was identified as a product. This conclusion was based on the observation that von Rudloff oxidative cleavage of the triene fraction isolated from the incubation yielded only a C_6 ^{14}C-labelled dibasic fragment. The triene fraction contained 33% of the total radioactivity and was represented by a single band on Ag^+TLC. It was therefore assumed to be 18:3(6c9c12t), arising from 18:2(9c12t) by a 66% Δ6 desaturation.

The main findings of this study should be grouped as follows:-

(1) 15:1-18:1 Δ9c acids were poor substrates, and 11:1(9c) and 13:1(9c) were not Δ6 desaturated.

(2) The Δ6 desaturation of 16:0, 17:0 and 18:0 in rat liver was demonstrated conclusively for the first time. However, these saturated acids were all very poor substrates.

(3) The even-numbered C_{14} to C_{22} Δ9c12c dienoic acids were all good substrates for the Δ6 desaturase. 22:2(9c12c) showed a very high % Δ6 desaturation (75%) while linoleic acid was the poorest substrate (15%).

(4) 18:2(9c12t), 18:2(9c11t), 18:2(9c13t), 17:2(9c12t) and 18:3(9c12c15c) were all excellent substrates. 18:2(9c14t) and 18:2(9c15t) gave low % Δ6 desaturations.

(5) A wide range of C_{16-18} monounsaturated acids (Δ8t, 10t, 11t, 12t, 12c, 12a, 9c, 14c and 15c) were Δ6 desaturated, though none were good substrates.

Traces of Δ6 desaturation ($< 2\%$) could be detected for 18:1 Δ11c, 13c and 13t isomers.

(6) No Δ6 desaturation could be shown for Δ10c, Δ8c, and Δ9t isomers.

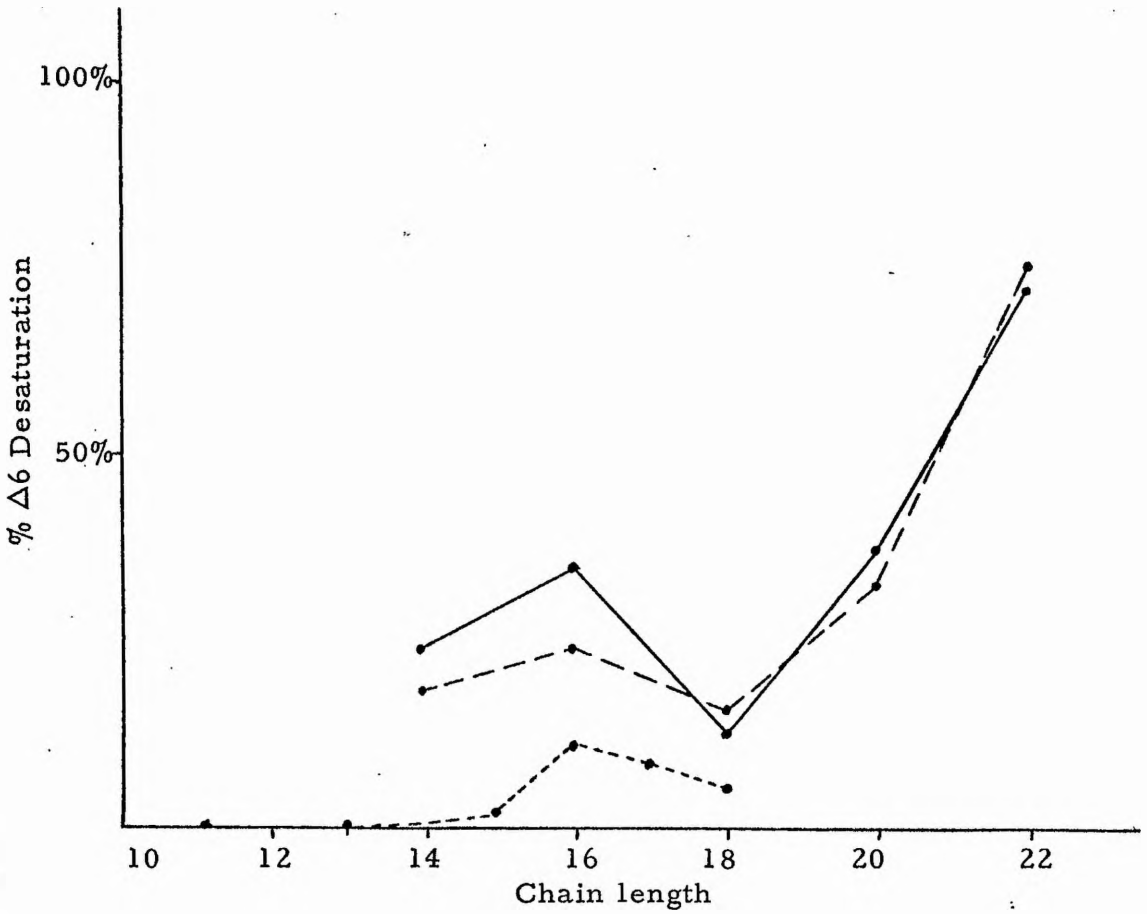
A series of C_{11}-C_{18} Δ9c monoenoic acids was tested for Δ6 desaturation. According to the model advanced by James as chain length decreased Δ6 desaturation should increase [6]. The reverse

was observed, but none of these acids were particularly good substrates (Figure 10). Although 11:1(9c) and 13:1(9c) gave no dienoic acids their extensive metabolism to other, more polar compounds as judged by GLC and TLC occurred. These were not identified. The original James model also predicted that short chain saturated acids (8:0-10:0) would be Δ6 desaturated, but this could not be demonstrated [126].

Under the incubation conditions and dietary regime employed in this work, saturated fatty acids were Δ6 desaturated by rat liver microsomes. The monoene fractions produced from 16:0 and 17:0 were composed mainly of the Δ9c isomer, but traces of the Δ6c isomer could also be detected. More convincing evidence was found when the monoene fractions from the co-incubation of 20 nmole of [1-^{14}C] 17:0 or 18:0 with 20 nmole of sterculic acid were analysed. Δ9 desaturation was markedly inhibited, and von Rudloff oxidative cleavage gave the following isomer composition of the monoene bands:-
17:1 (total 19%) - Δ9c (7.5%), Δ6c (8.5%), Δ5c (3%) (Experiment 5).
18:1 (total 10%) - Δ9c (1.5%), Δ6c (1.5%), Δ5c (7%) (Experiment 6).
Therefore when the Δ9 desaturation reaction is blocked saturated acids can be Δ5 or Δ6 desaturated in vitro in more than just trace amounts. Recent experiments showed that rats fed a diet rich in sterculic acid gave measurable levels of 18:1(6c) in their liver lipids [127]. Since identification was by GLC on a DEGS SCOT column, and the double bond position was not checked by a chemical degradative procedure, perhaps the observed 18:1 peak contained the Δ5c isomer too.

Cook and Spence reported the Δ6 desaturation of 16:0 and 18:0 in homogenates of foetal and immature rat brain. This activity diminished rapidly with ageing[81]. At present there is no reason to suppose that more than one enzyme catalyses the Δ6 desaturation of 16:0, 18:0 and 18:2(9c12c) in rat brain and liver. Brain was observed to have a high linoleoyl-CoA desaturase activity.that fell very rapidly with ageing [128], and it also possessed a much lower stearoyl-CoA Δ9 desaturase activity than the liver [127]. Therefore the unusual behaviour of palmitate and stearate desaturation in developing rat brain, described by Cook and Spence, can be attributed

Figure 10: The Effect of Chain Length on Δ6 Desaturation



Δ9c monoenoic acids

Δ9c12c dienoic acids

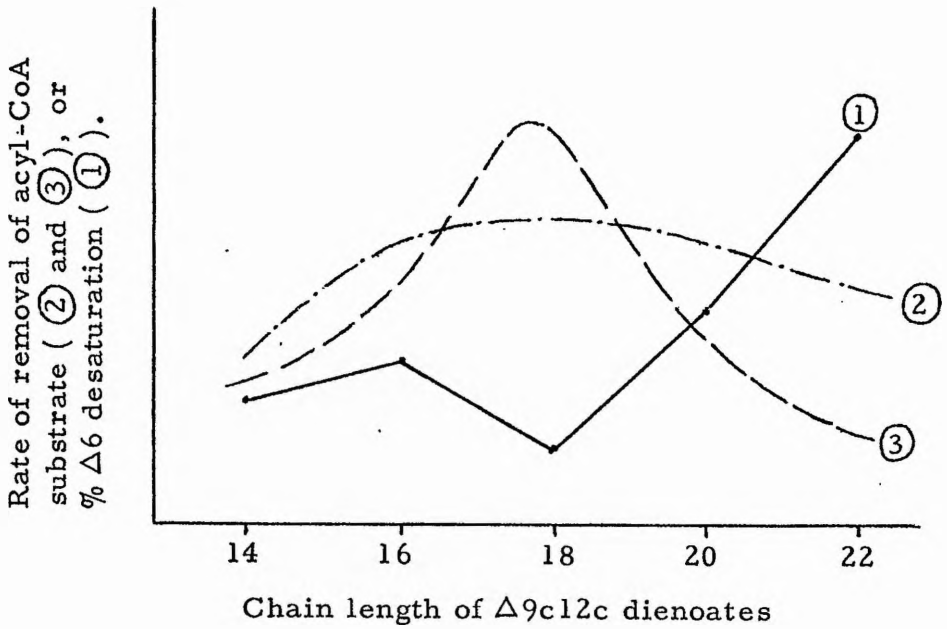
Δ9c12c dienoic acids + BSA

to the balance of Δ9 and Δ6 desaturase activities in this tissue, with the former becoming the dominant enzyme with ageing.

The Δ6 desaturation of 16:0, 17:0 and 18:0 proves that cis unsaturation is not obligatory in order for the enzyme to recognise its substrate. As π-binding sites on the enzyme surface are believed to enhance the Δ6 desaturation of a fatty acid with π-bonds between C(9) and C(14), and such a "locking device" is absent in the saturated acids which were Δ6 desaturated, a steric constraint is envisaged to hold C(6) and C(7) opposite the active centre. Good evidence for a tight fit of the enzyme over the C(2) to C(7) region would be a hydrogen abstraction which exhibits absolute stereo-specificity. Unfortunately no data on the stereochemistry of Δ6 desaturation have been reported yet.

The Δ6 desaturation of C_{14}-C_{22} Δ9c12c dienoic acids produced a surprising specificity pattern (Figure 10). A bimodal profile was obtained, with linoleic acid, the natural substrate, showing the lowest % Δ6 desaturation and 22:2 (9c12c) showing a remarkable 75% conversion. The addition of BSA did not affect the specificity pattern to any extent. The bimodal profile may indicate two enzymes, with chain length optima of C_{16} and C_{22} respectively. However, the writer prefers to interpret the data in terms of a single Δ6 desaturase. C_{18} acids would presumably be substrates for both the C_{16} and C_{22} optimum chain length Δ6 desaturases if two enzymes did exist, but Brenner has produced evidence for a single enzyme catalysing the Δ6 desaturation of oleic, linoleic and α-linolenic acids [17, 77, 78]. Competitive inhibition studies are needed to confirm or refute the two enzyme hypothesis.

The biological system examined was complex and the index of specificity was a simple % conversion measured at the end of a 1 hr incubation period. A straightforward explanation of the bimodal specificity is possible if acyl-transferase specificity is also considered. There are two, competing pathways for acyl-CoA removal, so that the experimental chain length specificity measured is the sum of both Δ6 desaturase and acyl-transferase specificity (see schematic diagram on the next page). Certainly the lipid incorporation pattern for total radioactivity varied dramatically over



① Experimental specificity (% Δ6 desaturation after 1 hr)

② Initial rate of Δ6 desaturation of acyl-CoA substrate

③ Initial rate of lipid incorporation of acyl-CoA substrate

② and ③ are drawn to be simple, single maxima specificities, which when added together to give ① result in the bimodal experimental specificity.

the chain length range. Increasing chain length resulted in a much higher proportion of radioactivity in neutral lipids (Table 7). This is not believed to be attributable to the higher % Δ6 desaturations for 20:2(9c12c) and 22:2(9c12c), as incubations with 18:2(9c12c), 18:3(9c12c15c) and 18:3(9c12c15c) plus BSA gave increasing Δ6 desaturation (12%, 38% and 57% respectively) with little change in the % of total radioactivity in neutral lipids (12.5%, 18.5% and 10% respectively). Measurements of the initial rates of removal of acyl-CoA substrate by the Δ6 desaturase and the acyl-transferases are needed to resolve the situation.

The Δ6 desaturase can handle a wide range of chain lengths. On first sight the experimental specificity pattern might indicate a hydrophobic region on the enzyme surface where London dispersion

forces with the C(20) to C(22) region of the substrate enhance enzyme-substrate binding and hence Δ6 desaturation. However, the argument cited in the previous paragraph implies that this is not the case if the composite specificity diagram is drawn as shown, revealing a broader "true" Δ6 desaturase specificity. The Δ6 desaturase recognises primarily Δ9c12c unsaturation, not chain length.

Brenner proposed a model for the Δ6 desaturase where sites on the enzyme surface may recognise Δ9c, Δ12c and Δ15c unsaturation respectively, either via π-bond interactions or by a specific fit increasing the London dispersion forces between the alkyl tail of the substrate and a hydrophobic enzyme surface [77]. This model requires modification in the light of results presented in Table 7. Even when taking into account the competition between the Δ9 and Δ6 desaturases for some of the monoenoic acid substrates it is apparent that C_{16}-C_{18} monoenoic acids are all poor substrates, if they are Δ6 desaturated at all. An approximate order of C_{18} substrate preference exhibited by the Δ6 desaturase is:-

Δ9c12t $\rangle$ Δ9c11t~Δ9c13t~Δ9c12c15c $\rangle$ Δ9c12c $\rangle$ Δ9c14t~Δ9c15t ~ monoenoic acids.

18:2(9c13c), 18:2(9c14c) and 18:2(9c15c) acids were generated in situ in insufficient amounts to make possible an analysis of their Δ6 desaturation. However, in vivo experiments failed to demonstrate any noticeable conversion of 18:2(9c15c) or 18:2(12c15c) to more unsaturated acids [63], so that they can be assumed to be very poor substrates, if at all, for the Δ6 desaturase.

The conclusions reached on the substrate-enzyme interaction are:-

(1) There is a degree of steric constraint on the configuration of the substrate between the active centre and C(12). Δ8t, 9c and 10t monoenoic acids were all Δ6 desaturated, whereas the corresponding Δ8c, 9t and 10c isomers were not. Therefore a saturated fatty acid being Δ6 desaturated will be required to take up a 8,9-trans, 9,10-gauche, 10,11-trans conformation. Certainly beyond C(13), and perhaps beyond C(12), this steric constraint is lifted, as the enzyme can accommodate Δ12c/t/a isomers.

(2) π -Binding sites between C(9) and C(14) are proposed. These sites must be moderately flexible in the C(12)-C(14) region in order to accommodate Δ12c/t/a unsaturation. Since monounsaturated acids were poor substrates whereas the dienoic acids were good substrates if the double bonds were separated by 0-2 methylene groups a large degree of co-operation must exist between the π -binding sites. It is possible that other dienoic acids with unsaturation in the C(9) to C(14) region may be good substrates for Δ6 desaturation, and since the Δ9c double bond is not obligatory for Δ6 desaturation isomers such as Δ8t11t, Δ8t11c, Δ8t12c, Δ10t12c etc. should be tested as well as Δ9c11c, Δ9t13c and Δ9c14c. Δ8c11c, Δ9t12c and Δ10c13c dienoic acids are not Δ6 desaturated. This fits in well with the proposal of a steric constraint over C(8) to C(12) controlling the specificity of Δ6 desaturation. If the double bonds of the substrate are separated by more than two methylene groups it appears that the co-operation effect is lost, as 18:2(9c14t), 18:2(9c15t) and 18:2(9c15c) are poor substrates. Because co-operation occurs when double bonds are separated by 0-2 methylene groups it is difficult to know whether to regard the π -interactions with the enzyme as operating at a single large site or two closely-spaced sites. Another π -binding site at C(15) and/or C(16) can be postulated from the observation that α-linolenic acid was a better substrate than linoleic acid. However, such a site would not have co-operation with either the Δ9c or Δ12c π -binding sites unless all three sites are utilised together.

A more detailed consideration of the substrate-enzyme interaction is not appropriate. Firstly, it is not known how well the Δ6 desaturase can handle Δ11c and Δ13c double bonds, in relation to their trans isomers in a dienoic substrate containing a Δ9c double bond. 18:1(11t) was a better substrate than 18:1(11c), with 5% and ~1% Δ6 desaturations respectively, and allowing for the fact that in the former case much of the substrate was removed by the competing Δ9 desaturase and that 18:2(9c11t) produced competed with 18:1(11t) for the Δ6 desaturase, then the contrast became more marked. Therefore a partial steric constraint at C(12)-C(13) seems plausible, favouring Δ11 trans geometry. However the Δ6 desaturation of 18:2(9c11c) needs to be examined in order to test this. Secondly, since the Δ6 desaturase

can handle a range of substrate geometries and appears to have some flexibility with respect to the π-binding sites, the construction of a model with the correct balance between geometrical restrictions, π-binding energies and London dispersion forces with the methylene chain, is impossible. To do this requires making the very dubious assumption that the % Δ6 desaturation is an exact measure of K_m. Although some general comparisons have been made between substrates such highly specific comparisons between individual substrates have not. Anyway the number of variables that must be fitted together to build such a model is too large.

Summarising, the Δ6 desaturase can accommodate a wide range of chain lengths, and also a much wider range of unsaturation than was previously believed. The Δ9c double bond is not an obligatory feature for a fatty acid to undergo Δ6 desaturation, and the desaturation of saturated acids is also noted. A tight enfoldment of the enzyme between the carboxyl-end of the substrate and about C(11) exists, and there must be strong binding forces between the double bonds of the substrate and the enzyme, particularly in the C(9) to C(14) region, with a co-operative effect between adjacent double bonds critical for this strong binding.

The mode of action of the obligatory, soluble protein required for Δ6 desaturation is not yet fully understood. Recent studies at Colworth House showed that the obligatory requirement for the 100,000 g supernatant by the Δ6 desaturase could be completely supplied by purified bovine catalase at concentrations which were equivalent to those in the supernatant [96]. This confirmed the speculation by Baker et al [221]. It is not clear yet whether the function of catalase is an enzymic one, to remove peroxide (presumably generated by side reactions in whole microsomes rather than by Δ6 desaturation), or if it acts as a soluble protein. In the latter case if it is required to transport or transfer the acyl-CoA substrate to the Δ6 desaturase it may have its own specifity and Δ6 desaturase specificity experiments will need reinterpretation.

2:2:6 The Δ5 Desaturase

Table 9 lists the [1-^{14}C] fatty acids which were Δ5 desaturated, together with lipid incorporation data and the ECL and Ag^+TLC R_f values of both substrate and product. To check that a good Δ5 desaturase activity was present in the enzyme preparation the desaturation of 20:3(8c11c14c) was always examined. With several substrates Δ9 or Δ6 desaturation competed with Δ5 desaturation.

RGLC analysis, on a 10% DEGS packed column, of the methyl esters from the incubations of [1-^{14}C] 17:1(9t), 18:1(9t) and 18:2(9t12t) acids, where low % Δ5 desaturations were recorded, did not resolve substrate and product. Instead a trailing shoulder was observed, c,t-Dimethylene-interrupted dienes have a lower ECL value than the corresponding c,c-methylene-interrupted dienes, so that a small ECL difference between substrate and product coupled with the low % conversions served to mask the Δ5c9t dienoate and Δ5c9t12t trienoate products on the RGLC trace. The following ECL values are from experiment 2 (Tables 6, 7 and 9):

18:1(9t)	- 18.6	18:2(5c9t)	- 19.0	Δ_{ECL} = 0.4
18:1(5t)	- 18.5	18:2(5t9c)	- 19.0	0.5
18:1(8c)	- 18.55	18:2(5c8c)	- 19.1	0.55
18:1(9c)	- 18.6	18:2(6c9c)	- 19.2	0.6
18:2(9t12t)	- 19.35	18:3(5c9t12t)	- 19.75	0.4
18:2(9c12c)	- 19.4	18:3(6c9c12c)	- 19.95	0.55

These considerations probably explain why Brenner did not report the Δ5 desaturation of [1-^{14}C] elaidic and linelaidic acids, but just noted that they were not Δ6 desaturated [77]. His only form of analysis was RGLC on packed DEGS or PEGA columns, and not Ag^+TLC.

The results in Table 9 confirmed the Δ5 desaturation of 18:1(9t) [80] and 20:2(11c14c) [88, 89] reported by other workers. These were conversions that did not follow the generalisations on desaturase specificity (Section 2:1). However this study showed that Δ8c monoenoic acids are Δ5 desaturated, as predicted by these generalisations.

TABLE 9: [1-^{14}C].Fatty Acids which were Δ5 Desaturated

	Substrate ECL; R_f ( )	Product ECL; R_f	% Δ5 Desaturation	% Lipid Incorporation NL	FFA	PL
Experiment 1	16:1(8c) 16.4; 0.43 (2)	16:2(5c8c) 16.95; 0.14	1	38	4	58
	17:1(9t) 17.35; 0.57 (5)	17:2(5c9t) 17.75; 0.24	3	30	4	66
	18:1(9t) 18.35; 0.57 (5)	18:2(5c9t) 18.7; 0.24	10	21	4	75
	18:2(9t12t) 18.95; 0.46 (2)	18:3(5c9t12t) 19.35; 0.14	9	22	5	73
	20:2(11c14c) 20.9; -	20:3(5c11c14c) 21.3; -	63	18	10	72
	20:3(8c11c14c) 21.4; -	20:4(5c8c11c14c) 21.8; -	65.5	9	3	88
Experiment 2	17:1(9t) 17.65; 0.51 (3)	17:2(5c9t) 18.05; 0.15	3	6	10	84
	18:1(9t) 18.6; 0.51 (3)	18:2(5c9t) 19.0; 0.15	7.5	11	2	87
	20:1(9t) 20.4; 0.51 (3)	20:2(5c9t) 20.8; 0.17	55	45	2	53
	16:1(8c) 16.8; 0.55 (4)	16:2(5c8c) 17.4; 0.25	1	22	6	72
	18:1(8c) 18.55; 0.57 (4)	18:2(5c8c) 19.1; 0.26	7	27	7	65
	20:1(8c) 20.4; 0.58 (4)	20:2(5c8c) 20.9; 0.28	18.5	37	7	56
	20:3(8c11c14c) 21.75; -	20:4(5c8c11c14c) 22.2; -	60.5	9	2	89
	18:2(9t12t) 19.35; 0.40 (3)	18:3(5c9t12t) 19.75; 0.08	5	22	2	76
	Incubated with 2.8 mg ml^{-1} BSA		6	25	5	70
	18:1(11c)[a] 18.7; 0.57 (4)	18:2(5c11c) 19.1; 0.24	1	37	2	61
	18:1(10t)[a] 18.6; 0.49 (3)	18:2(5c10t) 19.0; 0.18	5.5	42	3	55
	18:1(14t)[a] 18.75; 0.51 (3)	18:2(5c14t) 19.25; 0.21	10	38	4	58
	18:1(15t)[a] 18.8; 0.51 (3)	18:2(5c15t) 19.35; 0.17	10	38	4	58

TABLE 9 (cont)

	Substrate ECL; R_f ( )	Product ECL; R_f	% Δ5 Desaturation	% Lipid Incorporation NL	FFA	PL
Experiment 5	17:0[a] Coincubated with 20 nmole of sterculic acid	17:1(5c)	3	32	3	65
6	18:0[a] Coincubated with 20 nmole of sterculic acid	18:1(5c)	7	22	1	77

See the footnotes under Tables 6 and 7

a - this fatty acid was also Δ9 or Δ6 desaturated

The principal feature demonstrated by this work was the high degree of chain length specificity exhibited by the Δ5 desaturase. Fragmentary evidence for such a specificity from a survey of data available on the Δ5 desaturation of Δ11c monoenoic, Δ8c11c dienoic and Δ8c11c14c trienoic acids has already been presented in Section 2:1:3. Firm evidence is now available to substantiate this trend from the series of Δ8c and Δ9t monoenoic acids tested.

Substrate	% Δ5 Desaturation
13:1(9t)	<1
17:1(9t)	3
18:1(9t)	7.5
20:1(9t)	55
16:1(8c)	1
18:1(8c)	7
20:1(8c)	18.5

Although the range of chain lengths examined was not extensive it was clear that those of C_{20} (or greater) were optimum, with a rapid drop in enzyme activity with decreasing chain length until it was almost absent by C_{16}. 21:2(11c14c) [89], and 22:4(8c11c14c17c) and 23:5(8c11c14c17c20c) [93] were known to be fairly good substrates for the Δ5 desaturase, so that a sharp decline in enzyme activity with increasing chain length beyond C_{20} could be ruled out.

Saturated fatty acids were Δ5 desaturated if the competing Δ9 desaturation was inhibited by sterculic acid (Table 9). It would be

interesting to check the desaturation of 20:0 in rat liver microsomes, This has not been reported as yet. 20:0 is not a substrate for rat liver Δ9 desaturase [19], and since it has the optimum chain length for Δ5 desaturation a reasonable % conversion to 20:1(5c) is possible.

The Δ8c, Δ11c and Δ14c double bonds are not obligatory for Δ5 desaturation so a model similar to that proposed by Brenner for the Δ6 desaturase [77] where highly specific cis π-bond interactions or geometrical fit at the enzyme surface result in the binding of the substrate can be ruled out. Also a model based on the co-operative binding of adjacent double bonds to flexible π-binding sites on the enzyme seems unlikely on present evidence. 20:1(9t) is almost as good a substrate as 20:3(8c11c14c) or 20:2(11c14c). 18:2(9t12t) is as poor a substrate as 18:1(9t), while 18:2(9t12c) [This work, 83] and 20:2(11t14t) [101] do not appear to be Δ5 desaturated. At the moment the full specificity of the Δ5 desaturase cannot be gauged. Future work should utilise C_{20}, not C_{16-18}, fatty acids for a more detailed picture to be constructed. For example, the 16:1(7t) and 16:1(8t) acids used in this work were hardly likely to be Δ5 desaturated on account of their short chain length, so 20:1(7t) and 20:1(8t) will have to be tested in order to see whether the enzyme can handle Δ7t and Δ8t unsaturation.

From this and other work it seems as if the Δ5 desaturase can accommodate Δ8c, 9t, 10t, 11c, 12t, 14c, 14t, 15t and 17c unsaturation but not Δ8t, 9c, 10c, 11t and 12c unsaturation. This list is somewhat tentative, and also incomplete. Since for Δ8-12 positions either a cis or a trans double bond, but not both, can be accommodated by the Δ5 desaturase, some steric restriction is required between C(10) and C(14). Do and Sprecher examined the Δ5 desaturation of seven positional isomers of (±) methyl-branched 20:3(8c11c14c) acids, and compared them to the Δ5 desaturation of 20:3(8c11c14c) [95]. The (±) 5-methyl-branched isomer was hardly desaturated, while the 2, 10 and 13 positional isomers were only Δ5 desaturated to 20%, 15% and 35% respectively of the level of the control. Therefore a tight but not absolute steric constraint between C(2) and C(13) seems plausible. The fact that saturated fatty acids are Δ5 desaturated also argues for tight enfoldment of the enzyme around the substrate, between the carboxyl-binding site and the active centre, as such acids

do not have π-bonds to act as a "locking device" on the enzyme surface. At present there is insufficient evidence to confirm or deny the existence of such π-binding sites on the $\Delta 5$ desaturase. However a region of hydrophobic binding extending from C(16) to C(20) seems essential for the binding of substrate to the enzyme.

A thorough comparison of the $\Delta 5$ desaturation of 20:3(8c11c14c) and 20:1(9t) acids should be made in order to check whether the assertion that "20:1(9t) is almost as good a substrate as 20:3(8c11c14c)" is valid in terms of initial rates of desaturation and competitive inhibition. Radioactivity in the incubation of 20:3(8c11c14c) with rat liver microsomes entered PL almost exclusively (89%), whereas for 20:1(9t) it was evenly distributed between NL (45%) and PL (53%). This behaviour of 20:1(9t) is reminiscent of that of the Δ9c12c dienes, where increasing chain length accompanied increasing % $\Delta 6$ desaturation and increasing incorporation of radioactivity into NL. The possibility of acyl-transferase control on desaturase specificity, where desaturation is measured as a simple % conversion, has already been mentioned for the $\Delta 6$ desaturase.

2:2:7 Trans Fatty Acids

Unusual isomers of unsaturated fatty acids are present in partially hydrogenated fats and oils and in dairy produce, mutton and beef which constitute part of the modern diet. The presence of such isomers in the tissues will depend on their absorption through the gut, ease of transport via the bloodstream, lipid incorporation and β-oxidation relative to the natural fatty acids. However, once in the microsomes, where they will be exposed to a variety of lipogenic enzymes, their further metabolism may occur. Desaturation-elongation sequences can be written for these unusual isomers.

eg. 18:1(13t) $\longrightarrow$ 18:2(9c13t) $\longrightarrow$ 18:3(6c9c13t) $\longrightarrow$

20:3(8c11c15t) $\longrightarrow$ 20:4(5c8c11c15t)

eg. 18:1(7t) $\longrightarrow$ 20:1(9t) $\longrightarrow$ 20:2(5c9t)

$\downarrow$

18:2(7t9c) $\longrightarrow$ 20:2(9t11c) $\longrightarrow$ 20:3(5c9t11c)

In a diet containing adequate amounts of EFA competition between the $\omega 3$ and $\omega 6$ families of PUFA and unusual unsaturated fatty acid isomers will occur, and it is very unlikely that high levels of unnatural long-chain PUFA will build up. However they may occur in trace amounts if sufficient quantities of partially hydrogenated fats or dairy products are ingested. Their physiological consequences are not likely to be marked if adequate amounts of EFA are consumed since no adverse changes of health have ever been reported owing to the consumption of processed fatty foods.

The very high % $\Delta 6$ desaturation of $\Delta 9c12t$ dienoic acids explained the results of Privett et al [83] and Anderson et al [82] who noted that when rats, raised on a fat-free diet or on one containing only a partial dietary requirement of linoleic acid were fed 18:2(9c12t), it was not noticeably incorporated into lipids but was rapidly converted to a 20:4 acid containing trans unsaturation.

ie.

$$18{:}2(9c12t) \xrightarrow{\text{fast}} 18{:}3(6c9c12t) \xrightarrow{\text{fast}} 20{:}3(8c11c14t)$$

$$\xrightarrow{\text{fast}} 20{:}4(5c8c11c14t)$$

2:2:8 Cyclopropene Fatty Acid Inhibition of the Desaturases

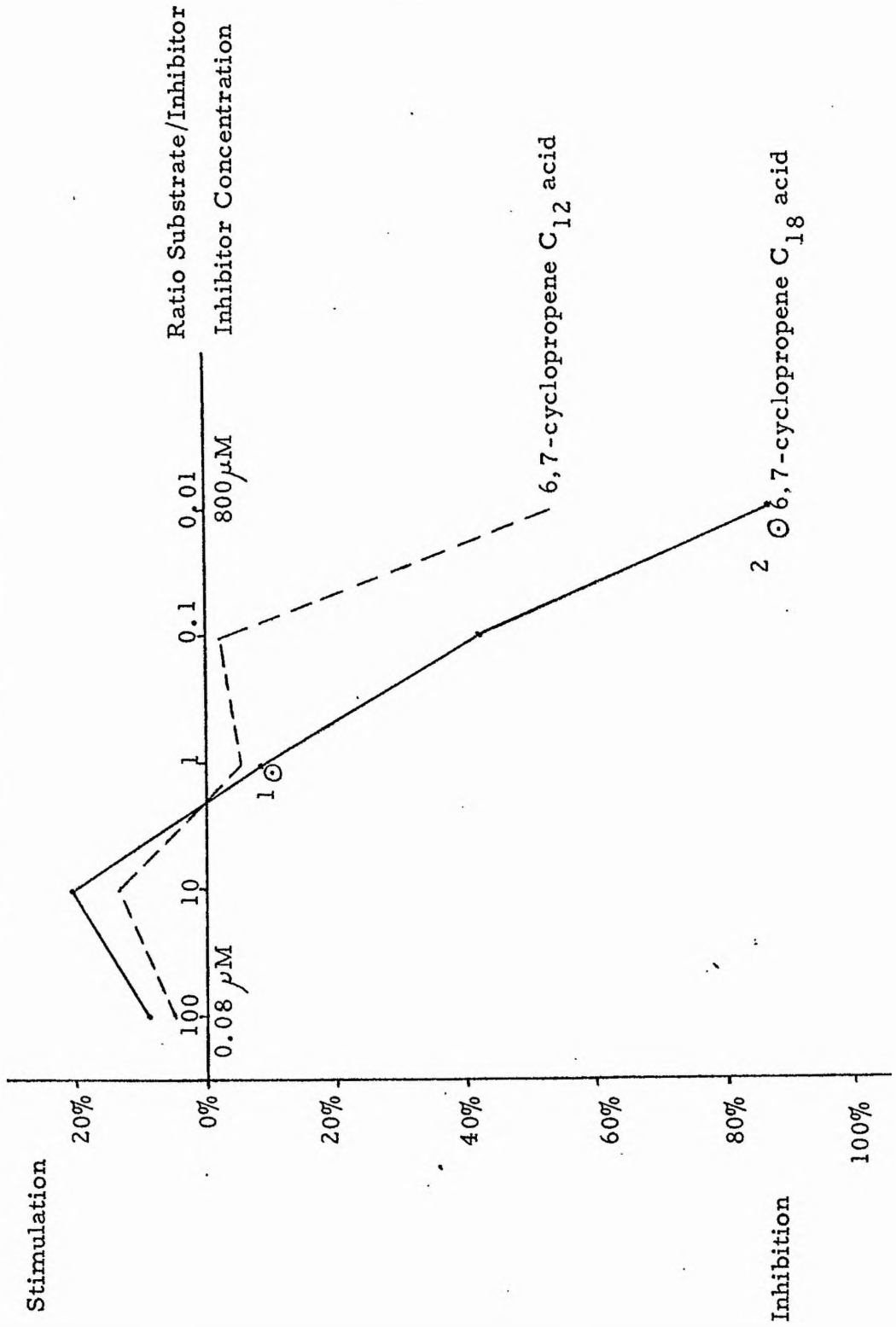
The reasons for investigating the effect of 6,7-cyclopropene fatty acids on $\Delta 6$ desaturation were twofold. When this study commenced it was believed that sterculate inhibition of $\Delta 9$ desaturation probably occurred via a covalent binding of the cyclopropene ring to essential sulphydryl groups at the active centre of the CSF [61, 68], so that if a similar inhibition of the $\Delta 6$ desaturase by a 6,7-cyclopropene acid occurred then it would be possible to label the enzyme by incubating it with a ^{14}C-labelled 6,7-cyclopropene acid. The inactive $\Delta 6$ desaturase could then be isolated. However later work at Colworth House demonstrated that sterculoyl-CoA inhibition of $\Delta 9$ desaturation did not occur via a covalent linkage to the enzyme [67]. The second reason was to make a comparison of the cyclopropene fatty acid inhibition of $\Delta 9$ and $\Delta 6$ desaturation.

The effect of various concentrations of 6,7-cyclopropene C_{12} and C_{18} acids on the % Δ6 desaturation of 20 nmole of α-linolenic acid was examined first in order to establish at what concentration the onset of inhibition was reached (Table 11 and Figure 11). The C_{18} cyclopropene acid was the better inhibitor and consequently used in the following experiments. A strong inhibition of Δ6 desaturation was not observed, since at high α-linolenic acid:6,7-cyclopropene C_{18} acid ratios (100:1-10:1) a stimulation of desaturation occurred and a 50% inhibition was only reached when the ratio diminished to 1:10. Δ9 Desaturation was dramatically inhibited at a ratio of 1:1 [1-^{14}C] saturated acid:sterculic acid. However, as approximately 100 nmole of endogenous saturated acids were present per incubation the true substrate:inhibitor ratio was nearer 6:1. Δ9 Desaturation was not very sensitive to 6,7-cyclopropene acid inhibition, as at a 1:70 [1-^{14}C] substrate:6,7-cyclopropene C_{18} acid ratio only a 36% inhibition occurred and at a 1:1 ratio Δ9 desaturation was stimulated.

The incubation of α-linolenic acid with high concentrations of 6,7-cyclopropene C_{18} acid or 18:1(6c) demonstrated that inhibition by the former was clearly attributable to the cyclopropene ring and was not merely a general free fatty acid or acyl-CoA inhibition. 18:1(6c) was chosen as a suitable comparison because of its 18-carbon chain length and unsaturation at the Δ6 position, and because it was readily available (whereas cis and trans 6,7-cyclopropyl C_{18} acids were not). The incubation of 1.4 μmole of 18:1(6c) with 20 nmole of [1-^{14}C] α-linolenic acid had little effect on the distribution of radioactivity between lipid classes. Therefore the blocking of lipid incorporation by high concentrations of 6,7-cyclopropene or sterculic acids, indicated by the increase of radioactivity in the FFA fraction, was also attributable to the cyclopropene ring. The stimulation of Δ6 and Δ9 desaturation noted at low concentrations of 6,7-cyclopropene acids may be explained by a partial blocking of the competing acyl-transferases by a general cyclopropene inhibition.

At a substrate:6,7-cyclopropene C_{18} acid ratio of 1:70 the inhibition of % Δ6 desaturation of 16:1(9c), 18:2(9c12c) and

Figure 11: Inhibition of Δ6 Desaturation of α-Linolenic Acid by 6,7-Cyclopropene C_{18} and C_{12} Fatty Acids and by Sterculic Acid



⊙ 1 and 2 are the points for sterculic acid inhibition, measured in experiments 6 and 5 respectively.

TABLE 11: Cyclopropene Fatty Acid Inhibition Studies

	Substrate	Inhibitor[1]	[Substrate]/[Inhibitor][2]	Desaturation	% Inhibition[3]	% Lipid Incorporation NL	FFA	PL
Experiment 4	18:3(9c12c15c)	-	-	35% Δ6	—	18	1	81
	"	6,7-CP,C_{12}	100/1	37% Δ6	- 6			
	"	"	10/1	40% Δ6	- 14.5			
	"	"	1/1	33.5% Δ6	5			
	"	"	1/10	34% Δ6	2			
	"	"	1/100	16.5% Δ6	53	25	12	63
	"	6,7-CP,C_{18}	100/1	38.5% Δ6	- 10	19	4	77
	"	"	10/1	42.5% Δ6	- 21.5			
	"	"	1/1	32.5% Δ6	8	13	6	81
	"	"	1/10	19.0% Δ6	41.5			
	"	"	1/100	4.5% Δ6	87	22	59	19
Experiment 5	18:3(9c12c15c)	-	-	39.5% Δ6	—	16	4	80
	"	6,7-CP,C_{18}	1/70	4% Δ6	89	34	23	43
	18:2(9c12c)	-	-	14.5% Δ6	—	18	5	77
	"	6,7-CP,C_{18}	1/70	⩽2% Δ6	⩾85	32	21	47
	16:1(9c)	-	-	6.5% Δ6	—	28	10	62
	"	6,7-CP,C_{18}	1/70	⩽1% Δ6	⩾85	14	56	30
	20:3(8c11c14c)	-	-	69% Δ5	—	8	3	89
	"	6,7-CP,C_{18}	1/70	32% Δ5	53.5	5	55	40
	17:0	-	-	80% Δ9	—	40	8	52
	"	9,10-CP,C_{18}	1/1	7.5% Δ9[4]	90.5	32	3	65
	"	6,7-CP,C_{18}	1/70	53% Δ9	36	24	21	55
	18:3(9c12c15c)	9,10-CP,$^{18}C_{18}$	1/70	5% Δ6	87	16	65	19

TABLE 11 (cont)

Experiment 6

Substrate	Inhibitor[1]	[Substrate]/[Inhibitor][2]	Desaturation	% Inhibition[3]	% Lipid Incorporation NL	FFA	PL
18:3(9c12c15c)	-	-	32% Δ6	—	14	2	84
"	9,10-CP,C_{18}	1/1	29% Δ6	9	2.5	2.5	81.5
"	18:1(6c)	1/70	24.5% Δ6	23	24	2	74
20:3(8c11c14c)	-	-	60.5% Δ5	—	10	2	88
"	6,7-CP,C_{18}	1/1	56% Δ5	7.5	14	3	83
"	9,10-CP,C_{18}	1/1	62% Δ5	-2.5	15	3	82
18:2(9c12c)	-	-	15% Δ6	—	16	4	80
"	6,7-CP,C_{18}	1/1	11.5% Δ6	23	17	7	76
16:1(9c)	-	-	11% Δ6	—	42	6	52
"	6,7-CP,C_{18}	1/1	5% Δ6	54.5	37	2	61
18:0	-	-	55% Δ9	—	46	2	52
"	9,10-CP,C_{18}	1/1	1.5% Δ9[4]	97	21	1	78
"	6,7-CP,C_{18}	1/1	70.5% Δ9	-28	35	2	63

1. 6,7-CP,C_{12}:Δ6,7-cyclopropene C_{12} fatty acid etc.
2. [1-^{14}C] fatty acid concentration was constant at 20 nmole (8 μM), so only the inhibitor concentration varied, between 0.2 and 2,000 nmole (0.08-800 μM).
3. $$\% \text{ Inhibition} = \frac{\% \text{ Desaturation} - \% \text{Desaturation in the presence of inhibitor}}{\% \text{ Desaturation}} \times 100$$
   A negative inhibition value is therefore a stimulation of desaturation.
4. These saturated acids were also Δ5 and Δ6 desaturated (Tables 7 and 9, Appendix 1)

18:3(9c12c15c) was high (approximately 85-95%), and the inhibition of the % Δ5 desaturation of 20:3(8c11c14c) was 53% (Table 11). However, at the ratio of 1:1 a selective inhibition occurred. Inhibition was greatest for the poorest substrate.

Substrate	16:1(9c)	18:2(9c12c)	18:3(9c12c15c)	20:3(8c11c14c)
Desaturation (no inhibitor)	11% Δ6	15% Δ6	35% Δ6	60.5% Δ5
Inhibition at a 1:1 ratio	54.5%	23%	8%	7.5%

These results are taken as good evidence for the reversible binding of the inhibitor, and also demonstrate the diminution of K_m across the series 16:1(9c) > 18:2(9c12c) > 18:3(9c12c15c).

It is not known if the mild inhibitory effect on Δ6 desaturation is a result of a general cyclopropene acid inhibition or more specifically a 6,7-cyclopropene acid inhibition. Very limited evidence suggests the former. The two points for sterculate inhibition of α-linolenic acid desaturation lay on the curve for 6,7-cyclopropene C_{18} fatty acid inhibition (Figure 11). In order to resolve this problem of interpretation an experiment was undertaken to compare the effect of sterculic and 6,7-cyclopropene C_{18} acid inhibitors over a range of concentrations on the Δ6 desaturation of 16:1(9c), 18:2(9c12c) and 18:3(9c12c15c). However stored microsomes were used for this experiment and the results obtained were erratic. There was insufficient time to repeat the experiment using fresh microsomes.

One criticism of the work was that the 6,7-cyclopropene C_{18} Δ9c12c dienoic acid should have been employed as the inhibitor. However, to synthesise such a compound was a daunting proposition, so the simpler 6,7-cyclopropene C_{18} acid was prepared and tested first. Saturated acids were Δ6 desaturated, and it was believed that if sterculic acid inhibition of the Δ9 desaturase was the result of a strong interaction between the cyclopropene ring and the active centre of the enzyme and that the inhibition of Δ6 desaturation by a 6,7-cyclopropene acid occurred by an identical mechanism, then the strong interaction between the 6,7-cyclopropene ring and the active centre would offset the loss of binding forces due to the absence of

Δ9c and Δ12c double bonds. The only situation where this argument seems likely to break down is if the obligatory soluble protein required for Δ6 desaturation is shown to act by transferring the substrate directly to the desaturase (ie. free linoleoyl-CoA is not a substrate), and this protein contains the site of the π-bond specificity.

The results of Fogerty et al, where only 8,9-, 9,10- or 10,11-cyclopropene acids were effective inhibitors of Δ9 desaturation, led to the belief that, owing to the co-incidence of position, inhibition took place by an interaction between the cyclopropene ring and the active centre of the enzyme [66]. However, from a consideration of the geometry of the cyclopropene ring (Figures 8 and 26), the fact that the inhibition can occur over the 8,9- to 10,11-positions, and the very restrictive spatial nature of the cleft (Section 2:2:4) it is apparent that these cyclopropene acids cannot fit into the "closed" enzyme cleft. Therefore, if sterculoyl-CoA inhibition occurs at the CSF, with the CoA head group binding to the usual CoA receptor site, then the cyclopropene ring must interact with a site remote from the active centre or at the active centre prior to enfoldment. The Δ6 desaturase has certain features in common with the Δ9 desaturase which suggest a similar mechanism of hydrogen abstraction. These common features are the NADH cofactor requirement (and hence probably the same electron transport chain), the molecular oxygen requirement, the fact that oxygen-containing acyl-CoA intermediates have never been isolated, and a reversible ^{-}CN inhibition. If the lack of inhibition of Δ6 desaturation by the 6,7-cyclopropene C_{18} acid is due to the absence of a strong cyclopropene ring-active centre interaction and if a similar hydrogen abstraction mechanism exists in both the Δ6 and Δ9 desaturases then this is indicative of a sterculoyl-CoA inhibition of Δ9 desaturation at a site remote from the active centre.

As already noted in the introduction (Section 2:1:1) sterculate inhibited the Δ9 desaturation of exogenous stearate but not the production of oleate from either acetate or laurate [68,69]. Two explanations were advanced; either there were two pathways to

oleate or sterculoyl-CoA inhibition acted at a transfer point of the substrate prior to desaturation. From either acetate or laurate the immediate source of 18:0 for the Δ9 desaturase in the rat is stearoyl-CoA produced by the microsomal elongation of palmitate. Evidence points to stearate produced by the elongase being preferentially channeled to the Δ9 desaturase, since *in vivo* labelled acetate gave labelled oleate first, with labelled stearate only accumulating later [129, 130]. Therefore the elongase may transfer stearoyl-CoA directly to the Δ9 desaturase as well as releasing it. The Δ9 desaturase is known to accept liposomal bound stearoyl-CoA as substrate [19]. Thus, if two entirely separate pathways to oleate are not to be postulated, sterculoyl-CoA inhibition must occur at the CSF, as there are no absolute transfer points for it to attack. The microsomal elongase is not believed to be sterculate sensitive, though this has not been checked *in vitro*, because when sterculate is fed to animals the level of oleate in the tissues is depressed but the level of stearate elevated. Therefore it seems reasonable to ask if stearoyl-CoA produced by the microsomal elongase can bypass sterculoyl-CoA inhibition of the CSF by a direct transfer of the substrate.

ie. Elongase. 18:0-SX $\rightleftharpoons$ Elongase + 18:0-SCoA

18:0-SCoA + Desaturase. Sterculoyl-SX $\not\longrightarrow$ Desaturase. 18:0-SX + Sterculoyl-SCoA

Elongase. 18:0-SX + Desaturase. Sterculoyl-SX $\longrightarrow$ Desaturase. 18:0-SX + Sterculoyl-SCoA + Elongase

Experiments on the production of 18:0 and 18:1(9c) when incubating [1-^{14}C] 16:0 with rat liver microsomes in the presence and absence of sterculate are suggested.

X may be CoA, ACP or a covalent linkage to the enzyme *via* a thiol group.

2:2:9 Time Course Studies

Some studies on the time course of desaturation of the natural substrates 18:0, 18:1(9c), 18:3(9c12c15c) and 20:3(8c11c14c) were undertaken. There was insufficient time to proceed to such studies on some of the novel substrates tested. To recapitulate, the reactions of interest occurring in the microsomes were:

$$\text{RCOOH} \rightleftharpoons \underset{\downarrow}{\text{RCOSCoA}} \xrightarrow[\text{Desaturation}]{\text{NADH, O}_2} \underset{\downarrow}{\text{R(-2H)COSCoA}} \rightleftharpoons \text{R(-2H)COOH}$$

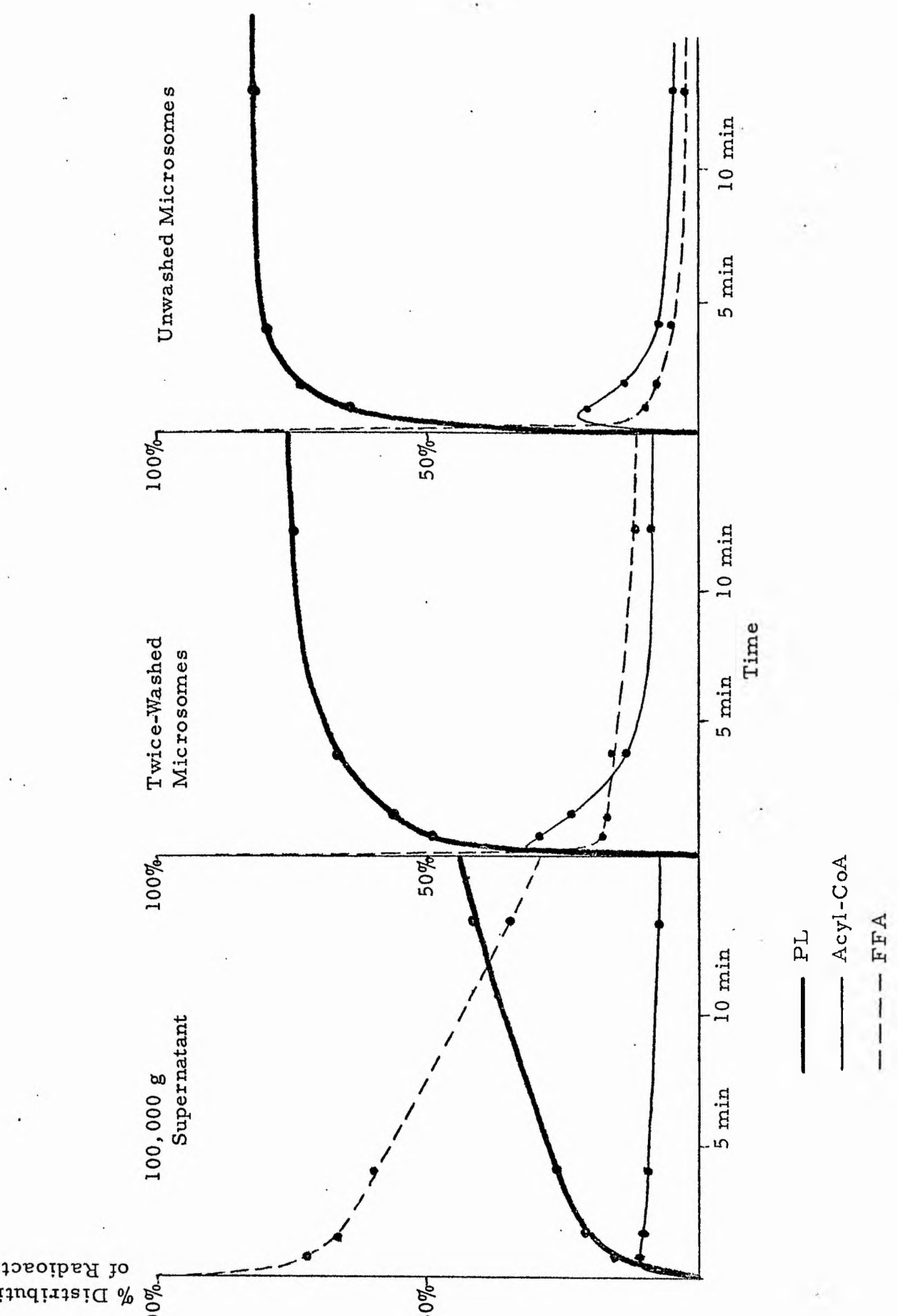
Lipids Lipids

[1-^{14}C]RCOOH was added to the incubation medium and these reactions followed over 1 hr. Section 2:2:1 has already covered some of the general considerations required for the interpretation of the results.

The first experiment was a study of the activation and lipid incorporation of [1-^{14}C] α-linolenic acid in whole microsomes, twice-washed microsomes or the 100,000 g supernatant (Figure 12). ATP, Mg^{2+} and CoASH were present, but the cofactors for desaturation NADH and NADPH were absent. In whole microsomes the activation of FFA to acyl-CoA was very rapid. After 1 min only 10% of [1-^{14}C] 18:3(9c12c15c) remained as FFA, with most of the radioactivity being present in the PL fraction. The initial rate of activation of α-linolenic acid was at least 3.6 nmole $min^{-1} mg^{-1}$ of microsomal protein. Twice-washed microsomes also contained a highly active fatty acid-CoA ligase. The 100,000 g supernatant, re-centrifuged for a further 2 hr to remove microsomal contamination, had only limited fatty acid-CoA ligase activity. Whether this was due to a low ligase activity or to a high ligase activity countered by a high lipase activity is not known. Lipid incorporation of [1-^{14}C] α-linolenate in washed or unwashed microsomes was also fast.

The results of the time-course studies of the desaturation of [1-^{14}C] 18:0, 18:1(9c), 18:3(9c12c15c) and 20:3(8c11c14c) highlighted the different way in which the microsomes handle stearate and oleate as opposed to PUFA (Figures 13-17). Palmitate, stearate, palmitoleate and oleate are the principal energy storage fatty acids transported to and deposited in the adipose tissue. A function of the Δ9 desaturase

Figure 12: Activation and Lipid Incorporation of [1-^{14}C] α-Linolenic Acid with Time



is to keep the depot fats fluid. by producing 16:1(9c) and 18:1(9c) from 16:0 and 18:0. The Δ5 and Δ6 desaturases convert linoleic and α-linolenic acids to C_{20} PUFA, for incorporation into PL (the major constituents of biological membranes) or for prostaglandin biosynthesis. They do not handle saturated or Δ9c monoenoic acids as substrates under normal circumstances.

Incubating 8 μM [1-^{14}C] fatty acid substrate with 3.4 mg of protein ml^{-1} the following measurements were taken at various time intervals over a 1 hr incubation period:-

(1) % Desaturation, measured by RGLC

(2) % Distribution of total radioactivity among the lipid classes (NL, FFA, PL, acyl-CoA), measured by TLC

(3) The ratio of substrate to product in each lipid class, as measured by Ag^{+}TLC.

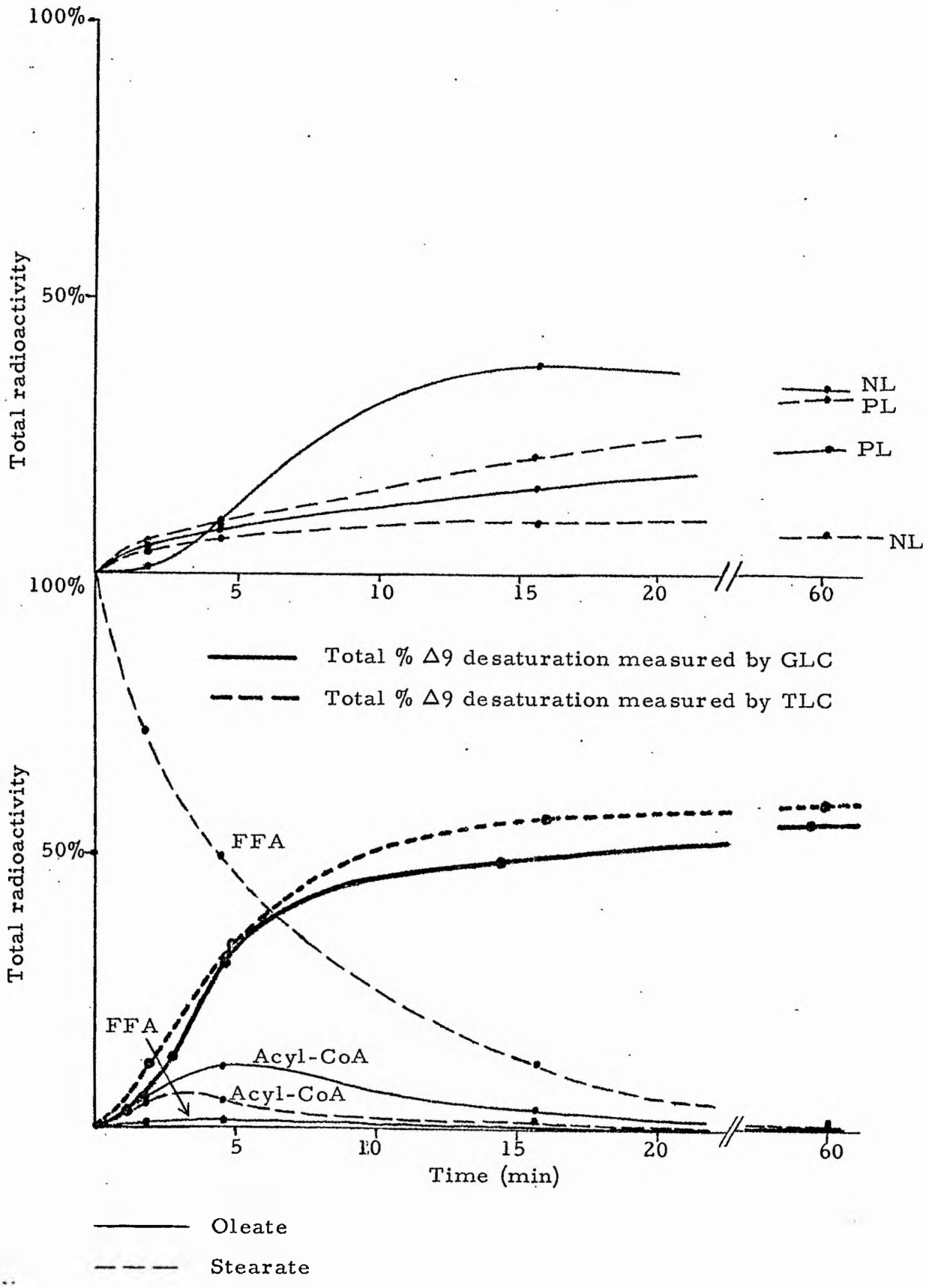
A figure for % desaturation can also be computed using the % distribution of radioactivity between lipid classes and the ratio of substrate to product in each class. This computed value is also shown in Figures 13-17 and compares favourably with that measured by RGLC, indicating good internal consistency for the data comprising the time course.

The interpretation of the time-course studies was complicated by a lack of knowledge of the fate of the endogenous saturated (50 μM) and monoenoic (50 μM) acids. It was not known whether the kinetics of desaturation and lipid incorporation of these endogenous acids could be assumed to be the same as for [1-^{14}C] stearic or oleic acids.

The desaturation of [1-^{14}C] 18:2(9c12c) was also examined, but with stored microsomes and atypical behaviour was observed. Unfortunately there was insufficient time to repeat the time course experiment with fresh microsomes.

Figure 13 shows the time-course experiment for the desaturation of [1-^{14}C] stearate to [1-^{14}C]oleate. The acyl-CoA synthetase was not very active as the stearic acid concentration fell only slowly.

Figure 13: Time Course for the Desaturation of [1-^{14}C] Stearate to Oleate



This was surprising in the light of the results for other [1-^{14}C] substrates, where at least 75% of substrate FFA was removed by the end of the first minute. The acyl-CoA hydrolase was not very active either, as little oleoyl-CoA was converted to oleic acid. The rate of Δ9 desaturation reached a maximum of 0.35 nmole product min^{-1} mg^{-1} of microsomal protein in the fourth minute. However, each incubation contained approximately 40 μM endogenous stearate plus palmitate and if these acids are behaving in a similar fashion to the 8 μM exogenous [1-^{14}C] stearic acid the true maximum rate of Δ9 desaturation is nearer 2 nmole product min^{-1} mg^{-1} of microsomal protein. Maximum initial rates of Δ9 desaturation by liver microsomes from rats raised on a fat free diet, for saturating amounts of [^{14}C]stearic acid plus the appropriate cofactors, are reported as 0.8-1.7 nmole min^{-1} mg^{-1} by Paulsrud et al [29] and 3.3 nmole min^{-1} mg^{-1} by Sprecher [18]. Therefore it seems as if the Δ9 desaturase may be working at or near to its full capacity for a limited period during this incubation. The maximum rate of Δ9 desaturation, using [1-^{14}C]stearoyl-CoA as substrate, was reported as 10 nmole min^{-1} mg^{-1} by Jeffcoat et al [22], but these authors used microsomes from rats where Δ9 desaturation was stimulated by feeding the animals a fat-free, high carbohydrate diet followed by a period of starving then refeeding.

The incorporation of [1-^{14}C] stearoyl-CoA into NL and PL occurred concurrently with Δ9 desaturation and [1-^{14}] oleoyl-CoA was also incorporated into NL and PL. The higher concentration of oleoyl-CoA over stearoyl-CoA probably resulted from the fact that the latter had Δ9 desaturation as an additional pathway for removal, and resulted in the higher rate of incorporation of oleate into lipids over the 3-10 minute period.

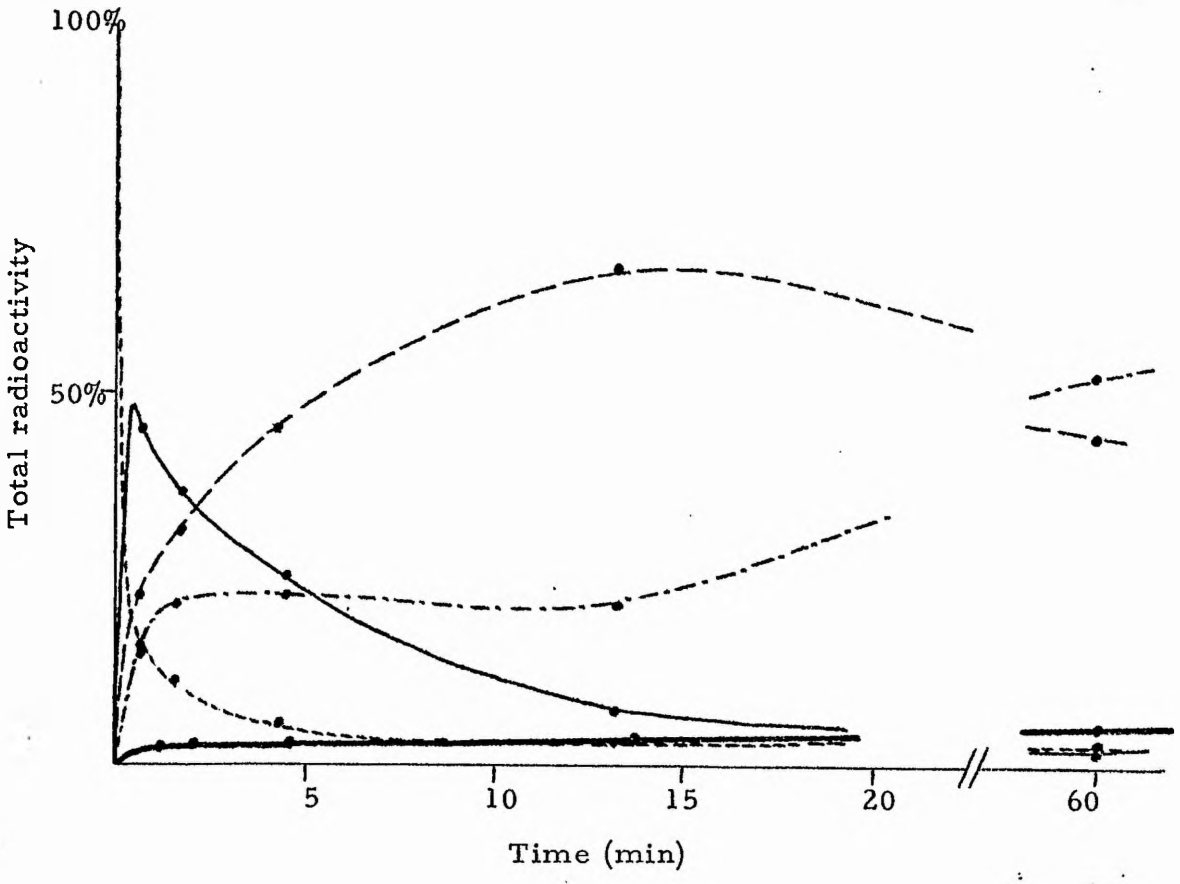
The lag period in Δ9 desaturation over the first two minutes was puzzling. The earliest explanation offered was a "pulse effect". Endogenous stearate and palmitate would bind to protein at sites which favoured Δ9 desaturation or activation, and had to be removed before [1-^{14}C] stearic acid could enter the pathway. This hypothesis

had two implications. Firstly endogenous saturated acids could not be regarded as mirroring the behaviour of exogenous [1-^{14}C] stearate. Secondly, the hypothesis would require a specific metabolic pool to handle saturated acids or acyl-CoA thiolesters for Δ9 desaturation, with no rapid equilibrium between free substrate and that bound to the pool.

The time-course study for the Δ6 desaturation of [1-^{14}C] oleic acid gave results which threw some light on the lag period of Δ9 desaturation, so in order to discuss Δ9 desaturation further it is necessary to first present the data for the incubation of [1-^{14}C] oleic acid (Figure 14). Because of the low % conversion of 18:1(9c) to 18:2(6c9c) the ratio of substrate to product in each lipid class was not measured with respect to time. The % distribution of radioactivity between the lipid classes can be taken as the % distribution of [1-^{14}C] oleate. The major features of this experiment were the very rapid removal of [1-^{14}C] oleic acid, the very high concentration of [1-^{14}C] oleoyl-CoA after 1 minute and its slow subsequent decrease. Oleoyl-CoA was preferentially incorporated into NL, with a subsequent transfer to PL occurring in the later stages of the incubation. A similar behaviour was observed for oleate produced by the Δ9 desaturation of stearate (Figure 13).

Product inhibition may play an important role in causing the lag phase of Δ9 desaturation. If endogenous oleic and palmitoleic acids produced approximately 50% CoA thiolesters after 1 min of incubation, this represents an acyl-CoA product concentration of approximately 20 μM. Substrate inhibition of Δ9 desaturation in whole microsomes begins at a stearoyl-CoA concentration of about 10 μM, at a protein concentration of 1 mg ml^{-1} [22], and oleoyl-CoA is believed to be at least as effective an inhibitor of Δ9 desaturation [19]. Discarding the "pulse effect" theory and therefore assuming that endogenous palmitic and stearic acids mimicked the time-course of [1-^{14}C] stearic acid then the maximum combined palmitoyl-CoA plus stearoyl-CoA concentration was unlikely to rise above 5 μM, and so substrate inhibition would be insignificant.

Figure 14: Time Course for the Incubation of [1-^{14}C] Oleate with Rat Liver Microsomes



FFA
Acyl-CoA
NL
PL

The activity measured for each class is the sum for substrate, 18:1(9c), and product, 18:2(6c9c)

% Δ6 desaturation, measured by GLC

The slow rate of activation of stearic acid was unexpected, and requires explanation. It may also be a factor in causing the lag phase of Δ9 desaturation. Whatever way this problem is approached a resultant specificity of activation in favour of oleate must be invoked. For example, if the slow production of [1-^{14}C] stearoyl-CoA is attributed to endogenous oleoyl-CoA inhibition then there must have been a rapid activation of oleic acid in preference to stearic acid to set it up. If acyl-CoA hydrolysis is postulated to explain the slow activation of [1-^{14}C] stearic acid then it must be specific for 18:0-CoA, not 18:1(9c)-CoA. If the endogenous Δ9c monoenoic acids are activated first free CoASH will be withdrawn from the system. However this is not likely to be a limiting factor, as its initial concentration was 52 μM.

The time-course studies of the Δ6 desaturation of [1-^{14}C] 18:3(9c12c15c) in the presence and absence of an optimum concentration of BSA and for the Δ5 desaturation of [1-^{14}C] 20:3(8c11c14c)(Figures 15-17) were similar in several aspects, and showed a marked contrast to the studies of [1-^{14}C] 18:0 and 18:1(9c). The most striking feature was a rapid incorporation of the PUFA substrates into mainly PL, but also NL, over the first 1.5 min. This stopped abruptly after 1.5 min, but desaturation continued. The % desaturation at the end of the incubation (60 min) was approximately equal to (100%-% substrate in PL + NL after 1.5 min) ie. (100% - X%)

Substrate	% Desaturation	X%	100%-X%
20:3(8c11c14c)	65	42	58
18:3(9c12c15c)	39	62	38
18:3(9c12c15c) + BSA	57	38	62

At any given time the % substrate remaining as FFA and acyl-CoA (ie. the potential substrate) approximately equaled the % substrate still requiring to be desaturated in order to give the eventual % desaturation. In other words after the rapid initial uptake of substrate all the remaining substrate is Δ6 or Δ5 desaturated.

Figure 15: Time Course for the Desaturation of [1-^{14}C] α-Linolenic Acid

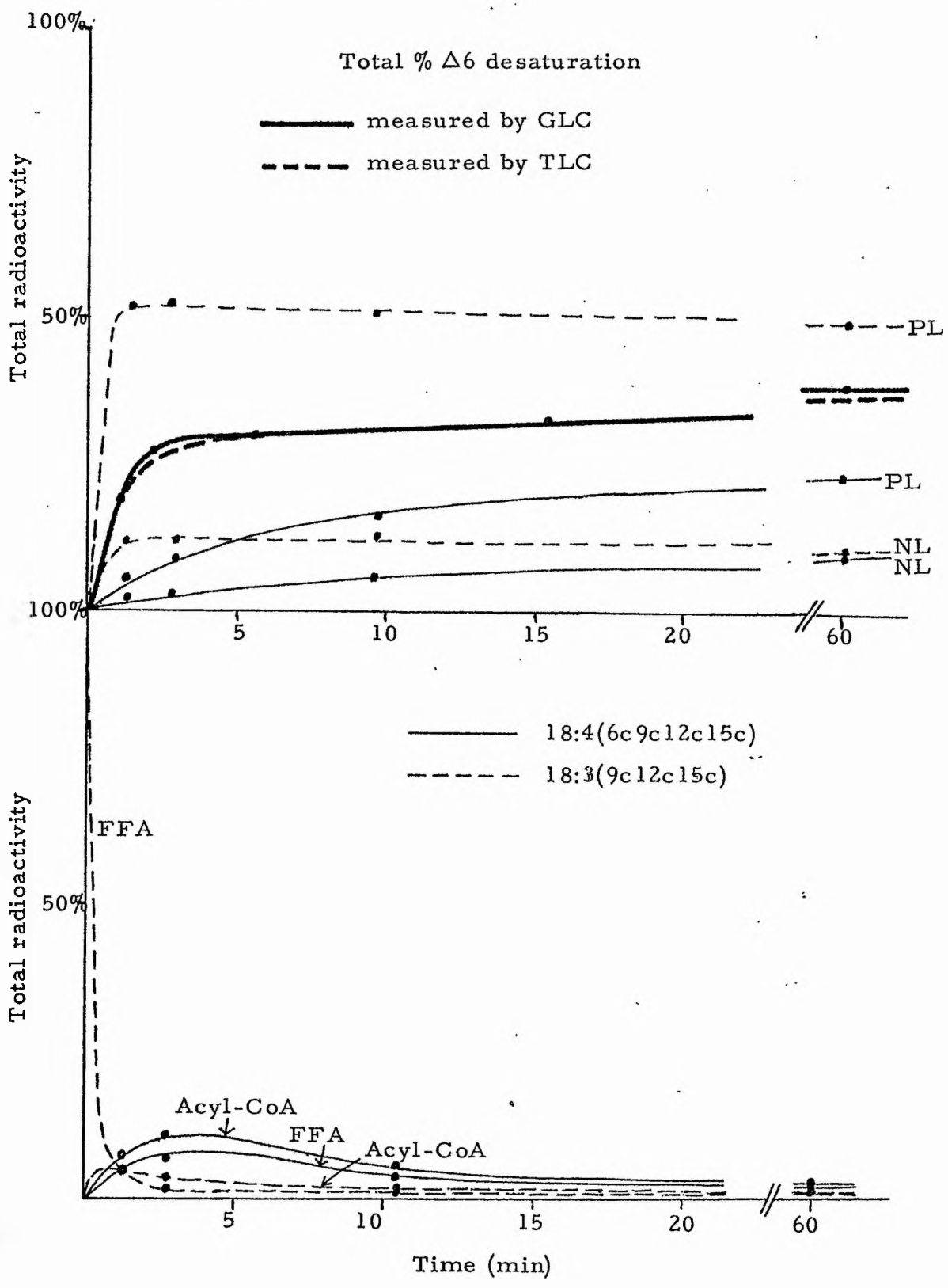


Figure 16: Time Course for the Δ5 Desaturation of

[1-^{14}C] 20:3(8c11c14c) Acid

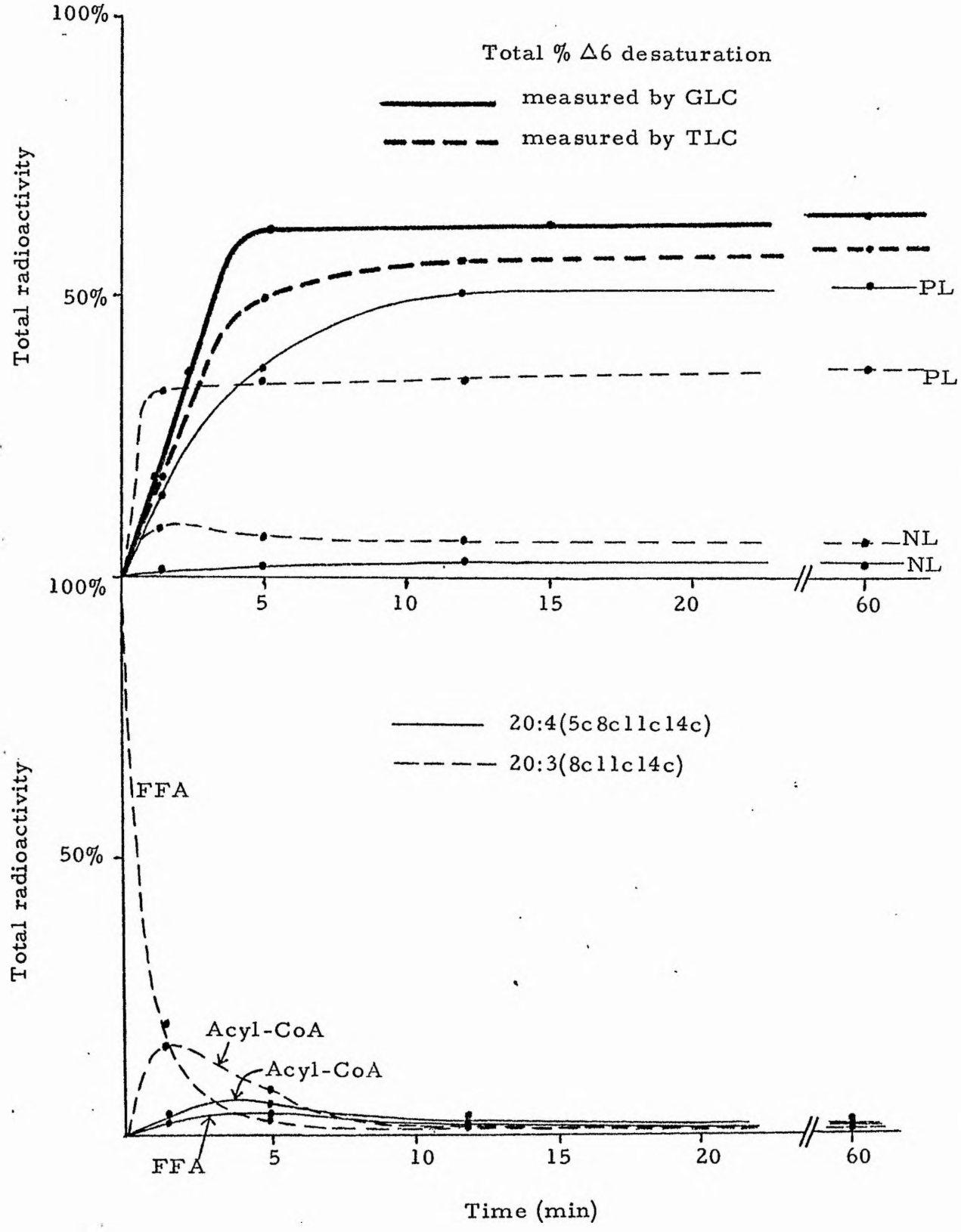
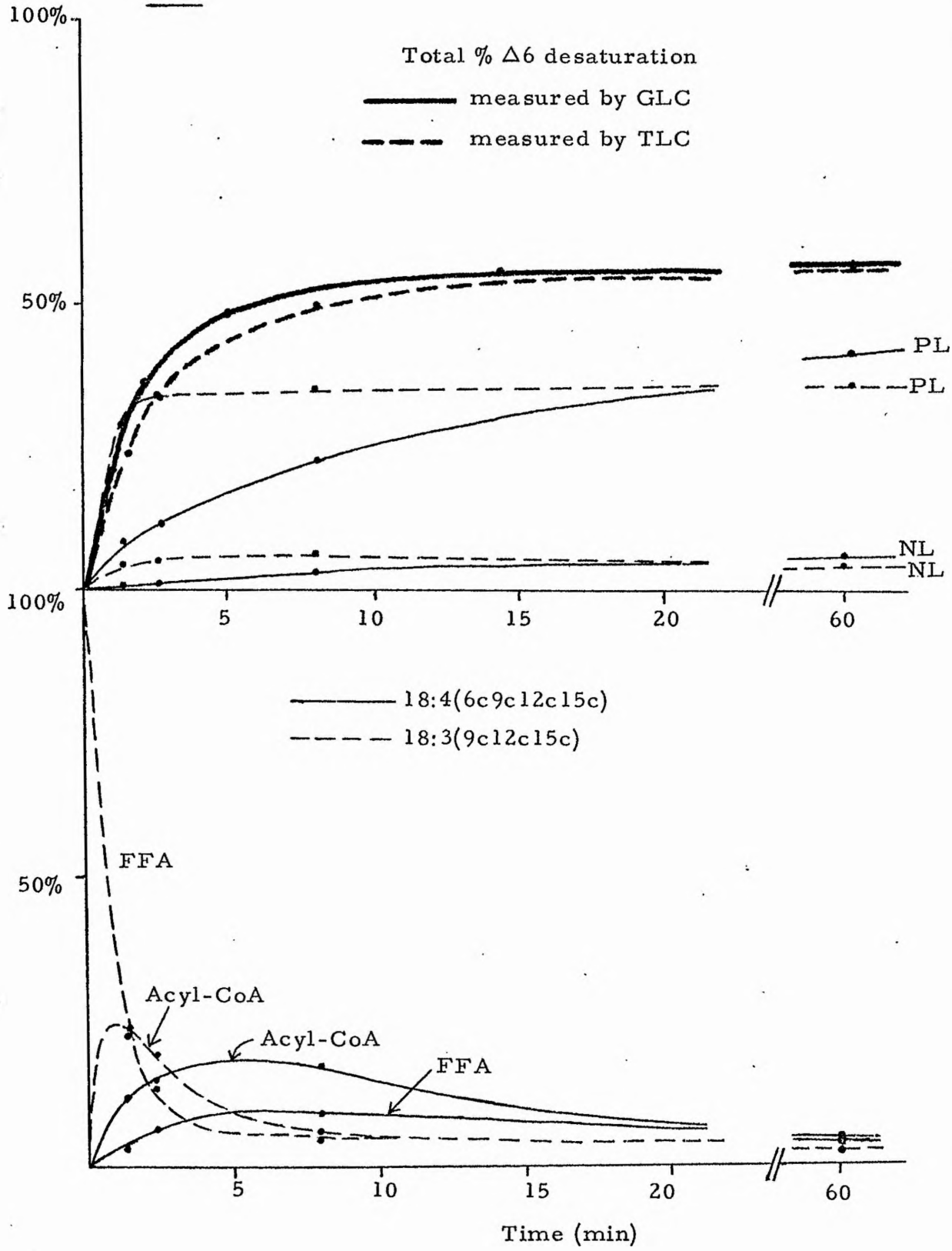


Figure 17: Time Course for the Desaturation of [1-^{14}C] α-Linolenic Acid in the Presence of a 2.8 mg ml^{-1} Concentration of BSA



The rate of Δ5 desaturation of [1-^{14}C] 20:3(8c11c14c) was linear over the first 3 min, and had a value of 0.65 nmole $min^{-1}mg^{-1}$ of microsomal protein (Figure 16). Sprecher measured the initial rate of Δ5 desaturation of 20:3(8c11c14c) by incubating the acid for 3 min with 5 mg of microsomal protein derived from the liver of rats raised on a fat free diet [18]. The velocity he obtained was 0.8 nmole $min^{-1}mg^{-1}$. These data suggest that the specific activity of Δ5 desaturation in whole microsomes measured in this study was near or at its maximum. The picture was not so clear for Δ6 desaturation. There were not enough points on the graph (Figure 15) to show decisively whether the rate of Δ6 desaturation over the first 1.5 min was linear or not. An initial rate of 0.7 nmole $min^{-1}mg^{-1}$ was estimated. Unfortunately no data is available on the V_{max} of 18:3(9c12c15c) desaturation, but Sprecher reported an initial rate of Δ6 desaturation of linoleic acid of 1.0 nmole $min^{-1}mg^{-1}$ [18]. Therefore it cannot be ascertained whether Δ6 desaturation in this study was for a short time operating close to its full capacity. Δ6 Desaturation showed a biphasic kinetic behaviour, with a slow, secondary Δ6 desaturation occurring over 3-60 min. This cannot be attributed entirely to potential α-linolenic substrate retained in the system, so it must be fueled by a slow turnover of substrate in lipid. This biphasic phenomenon was also noted by Jeffcoat for the desaturation of 75 nmole of [1-^{14}C] linoleoyl-CoA by unwashed microsomes [96]. Apart from the case of the Δ6 desaturation of α-linolenic acid in the absence of BSA (Figure 15) there is no need to invoke lipid turnover in order to explain the other kinetic studies (Figures 13, 14, 16 and 17). The % of substrate still requiring desaturation at any given time to give the eventual % desaturation approximately equals the potential substrate (ie. that remaining as FFA or acyl-CoA). No secondary phase was observed for the Δ5 desaturation of [1-^{14}C] 20:3(8c11c14c).

The time-course experiments for PUFA desaturation indicate a "pool" effect, because after the rapid incorporation of substrate into lipid there was an abrupt stop and the remaining substrate was

channeled through the desaturase. Clearly this "pool" did not operate by the simple expedient of a protein tightly binding the substrate, as acyl-CoA, and releasing it only to the desaturase. This is demonstrated by comparing the lipid incorporation profile of [1-^{14}C] 18:3(9c12c15c) in the presence and absence of cofactors for desaturation (Figure 18). In the absence of NADH and NADPH the lipid incorporation of [1-^{14}C] α-linolenate did not have the abrupt stop after 1.5 min, and approached completion (ie. 90% of activity was incorporated within 10 min). Therefore the binding of α-linolenate to a specific protein which acts as the "pool" (ie. protein bound substrate which is not in rapid equilibrium with free substrate, but which is only channeled to the desaturase) can be ruled out, except in the unlikely circumstance of such a protein requiring NADH or NADPH for activation.

A control on desaturation by the concentration of free substrate acyl-CoA is postulated. A balance between the rates of desaturation and lipid incorporation, based on the following criteria, is required:-

(1) The binding constant for PUFA CoA-thiolesters to the acyl-transferases is low, though these enzymes may have a high maximum velocity.

(2) The binding constant for PUFA CoA-thiolesters to the Δ6 or the Δ5 desaturase is much higher than to the acyl-transferases. The desaturases probably have a more limited capacity.

Therefore if the free acyl-CoA substrate concentration is low it will be effectively channeled to the desaturase. However above a certain concentration the desaturase will be operating at its maximum velocity, so at and above this concentration the substrate will be both desaturated and incorporated into lipids. An expanded time axis showing the projected kinetics over the first two minutes of the incubation of [1-^{14}C] PUFA is given in Figure 19. It is a notional scheme: the exact rates and concentrations shown should not be taken literally. The first point to note is that the [1-^{14}C] substrate acyl-CoA concentration measured is probably not the free concentration available to the competing enzymes. Most of it will probably be bound to protein, with a rapid equilibrium:

Figure 18: The Incorporation of [1-^{14}C] 18:3(9c12c15c) into Lipids in the Presence and Absence of Cofactors for Desaturation

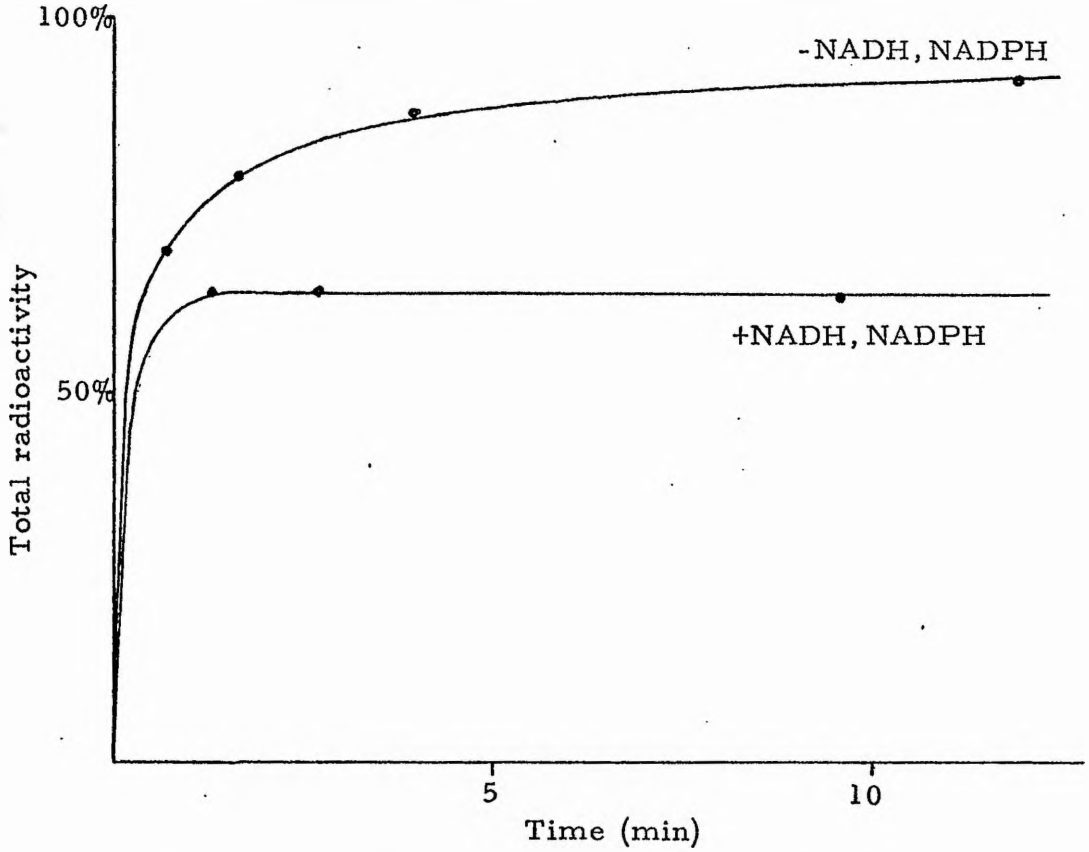
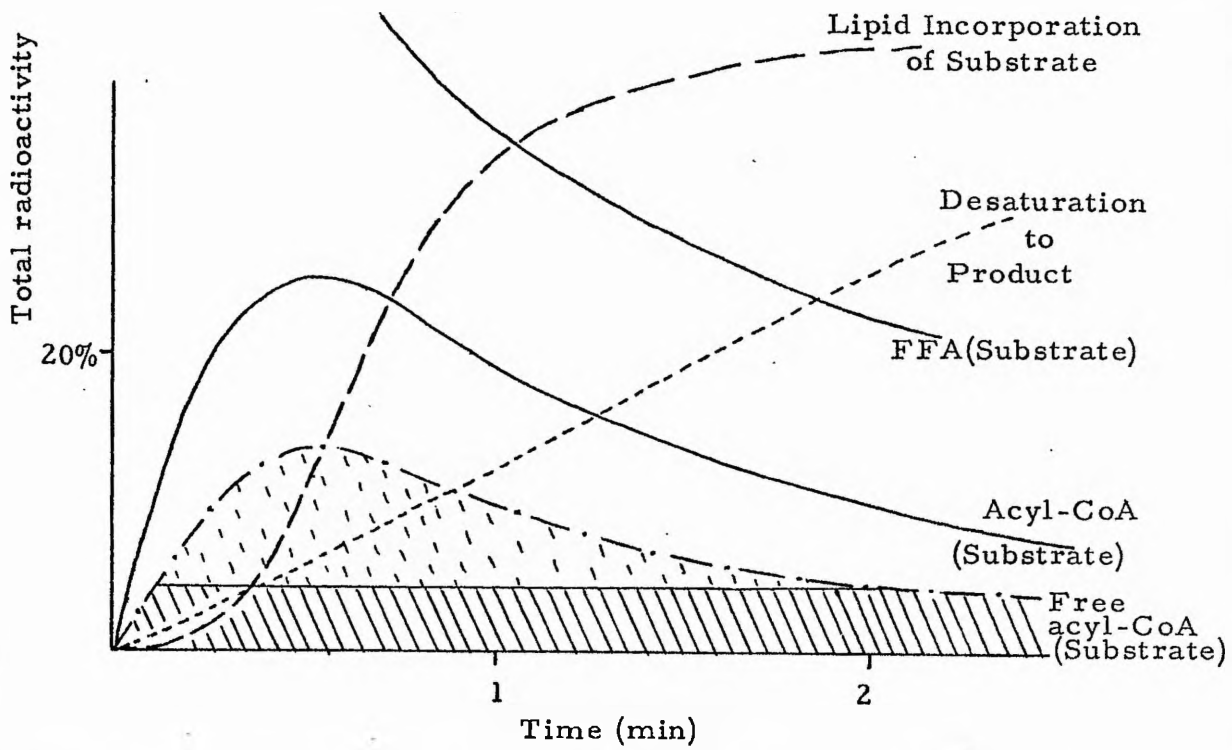


Figure 19: A Hypothetical Scheme for the Removal of PUFA Substrate During the First Two Minutes of Incubation



$$\text{Acyl-CoA} + \text{Protein} \rightleftharpoons \text{Acyl-CoA Protein}$$

The available acyl-CoA substrate concentration is represented in Figure 19 by —·—·—. The shaded area beneath this line represents the region of free substrate acyl-CoA concentration where it is channeled predominantly to the desaturase. The competing lipid incorporation is only effective in the dotted region of concentration. Therefore over the first two minutes the desaturase will be operating at its maximum capacity so that the rate of desaturation will be linear, whereas the competing lipid incorporation rate peaks by the first half minute and becomes insignificant by the end of the second minute. Desaturase specificity in a biological system operating in this manner is a function of the rate of desaturation and the binding constant of the substrate to this enzyme, the rate of lipid incorporation and the binding constant of the substrate to the acyl-transferases, and the concentration of free acyl-CoA substrate. Additional complications may arise in practice with this model. As product acyl-CoA is produced it may either decrease the rate of desaturation by causing a product inhibition or it may increase the extent of desaturation by being rapidly incorporated into lipids and hence partially blocking the lipid incorporation pathway for the substrate. Clearly, if the obligatory soluble protein required for Δ6 desaturation does transfer substrate directly to the desaturase then this will also result in a further complication.

It would have been interesting to have compared the Δ6 desaturations of [1-^{14}C] linoleic and α-linolenic acids. On the basis of the observations with other PUFA a rapid incorporation of linoleic acid would occur over 1.5 min, stopping abruptly at about 85% of the total radioactivity incorporated. More work is required on such enzyme kinetics in order to elaborate or refute this hypothesis, which is built on limited evidence. The kinetic studies where [1-^{14}C] PUFA were incubated needed more points over the 0.5-5 min period in order to draw more accurate curves. When the studies, shown in Figures 12 and 14-17, were undertaken the very

rapid nature of the reaction was not appreciated and the important 0-5 min period contained only two or three points. This was insufficient to give exact kinetic curves. Future work should include repeating the time-course experiments at increasing concentrations of PUFA or microsomes, and examining some of the substrates used in the specificity work reported in Sections 2:2:4 - 2:2:6.

The Δ6 desaturation of [1-^{14}C] oleate and [1-^{14}C] α-linolenate make an interesting comparison. The desaturation of oleate was slow, resulting in only 5.5% 18:2(6c9c) at the end of 1 hr (Figure 14). About 2% of the dienoic acid was produced within 1.5 min and the remaining 3.5% built-up slowly over the rest of the incubation period. At the end of the incubation there was a slight preference for [1-^{14}C] 18:2(6c9c) to be incorporated into PL (7% of the PL fraction) rather than NL (4% of the NL fraction). The rapid initial rate of Δ6 desaturation of 18:3(9c12c15c) is believed to indicate that the inhibition of Δ6 desaturation by endogenous oleoyl-CoA and palmitoleoyl-CoA was insignificant. The initial rate of Δ6 desaturation of [1-^{14}C] oleate was estimated to be of the order of 0.05 nmole min^{-1} mg^{-1} of microsomal protein, an average value for the 0-1.5 min period, and during this period the maximum concentration of [1-^{14}C] oleoyl-CoA reached was about 4 μM. Assuming endogenous FFA mirrored added [1-^{14}C] FFA in its kinetics, then a 24 μM acyl-CoA concentration would give an initial rate of Δ6 desaturation of Δ9c monoenoic acids of 0.25 nmole min^{-1}mg^{-1} of microsomal protein. A comparison of initial rates of desaturation and substrate acyl-CoA concentrations shows more dramatically the preference of the Δ6 desaturase for 18:2(9c12c) or 18:3(9c12c15c) over 18:1(9c) than a comparison of just initial rates or of % conversions. These results show that the Δ6 desaturase handles principally Δ9c12c and Δ9c12c15c PUFA and discriminates strongly against oleate. Oleic acid is a poor inhibitor of linoleic and α-linolenic acid desaturation whereas these PUFA are good inhibitors of oleate desaturation [76, 87]. In healthy, weanling rats the % Δ6 desaturation of oleate was reduced to an almost

	Estimated maximum acyl-CoA substrate concentration	Estimated initial rate (nmole $min^{-1}mg^{-1}$ of protein)
[1-^{14}C] oleate	4 μM	0.05
[1-^{14}C] oleate + endogenous monounsaturated FFA	24 μM	0.25
[1-^{14}C] α-linolenate	0.8 μM	0.7

Initial rate of desaturation of 150 nmoles of [1-^{14}C] acid, measured for a 3 min incubation [18]: 18:1(9c)	0.16
18:2(9c12c)	1.0
	% conversion
% conversion of 18:1(9c) to 18:2(6c9c)	5.5
% conversion of 18:3(9c12c15c) to 18:4(6c9c12c15c)	38

undetectable level ($\sim$1%) whereas the % Δ6 desaturations of linoleic and α-linolenic acids were comparable with those obtained for EFA deficient animals (Tables 1 and 2). Brenner noted an increase in Δ6 desaturase specific activity for rats raised on an EFA free diet, but the reason for this is not yet understood. [131, 132]. The almost complete absence of Δ6 desaturation of oleate in weanling rats may have been a consequence of the level of endogenous linoleic acid. In the EFA deficient animals this was very low; each incubation contained about 6 nmole of endogenous C_{18} dienoic acid (which was probably a mixture of Δ5c8c, Δ8c11c and Δ9c12c isomers) and therefore the incubation concentration of endogenous linoleic acid was 2.5 μM or less. Unfortunately this concentration was not measured for weanling rats. Perhaps the almost complete loss of oleate Δ6 desaturation in the weanling animals was a combination of a lower Δ6 desaturase specific activity (ie. less Δ6 desaturase protein per mg of microsomal protein) and much higher levels of competing endogenous linoleic acid.

The time-course experiment for α-linolenic acid Δ6 desaturation in the presence of an optimum concentration of defatted BSA

(2.8 mg ml^{-1}, 42 μM) is shown in Figure 17. When compared with the study where BSA was absent (Figure 15) the following differences were apparent:-

(1) The final % Δ6 desaturation of α-linolenic acid with BSA was 57%, without BSA 38%, a ratio of 1.46:1 .

(2) With BSA a reasonable rate of Δ6 desaturation extended over 5 min, not 2.5 min, and there were no distinct, biphasic kinetics.

(3) A rapid initial uptake of substrate occurred, which stopped abruptly after 2 min. With BSA only 39% of the substrate was incorporated into NL and PL during this initial phase, without BSA 62%.

(4) Substrate FFA was depleted more slowly, and much greater concentrations of acyl-CoA were present.

An important similarity between the two incubations was that the initial rate of Δ6 desaturation, measured over the first minute, was identical within experimental error. In the presence of BSA 4.0 nmole of [1-^{14}C] 18:4(6c9c12c15c) were produced in one minute, but when BSA was absent 3.6 nmole of [1-^{14}C] 18:4 were produced. (This assumes a 2.5 ml incubation containing 20 nmole of substrate.) Therefore BSA stimulation of % Δ6 desaturation could not be directly attributable to an increase in rate.

Albumin is well known as a FFA binding protein, for both saturated and unsaturated acids, and is believed to have a series of at least 8-12 binding sites with diminishing association constants [133, 134]. Although it has not been tested albumin is also believed to bind long-chain fatty CoA-thiolesters. The stimulating effect of BSA on Δ6 and Δ9 desaturation is well documented [22, 31, 135]. Jeffcoat et al considered that the stimulation of Δ9 desaturation of [1-^{14}C] stearoyl-CoA by BSA occurred via the binding of the substrate to BSA, reducing its free concentration to an optimum level and thereby removing substrate inhibition [22]. In a later paper the Colworth group elaborated this explanation by proposing also that BSA:stearoyl-CoA binding also protected the substrate from hydrolysis by the thioesterases, since in partially purified microsomal Δ9 desaturase preparations stimulation of specific activity did not

occur with the addition of BSA or other cytoplasmic stearoyl-CoA binding proteins. Stimulation of Δ9 desaturase activity was noticed in whole microsomes when stearoyl-CoA was the substrate: the enzyme preparation used had no fatty acid-CoA synthetase activity as the appropriate cofactors were absent [31].

The proposal that BSA binds to FFA and to acyl-CoA is in accord with the results of this study. The microsomal preparation used had a highly active thiokinase activity so that BSA stimulation of desaturation by protecting the acyl-CoA substrate from the thioesterase seemed unlikely. BSA binds α-linolenic acid, reducing its rate of removal (Figure 17). BSA can also be regarded as a substrate reservoir for [1-^{14}C] α-linolenoyl-CoA, releasing the substrate only slowly and thereby diminishing its free concentration. From the hypothesis presented earlier on the control of desaturation of PUFA by the preferential channeling of free substrate at low concentrations to desaturase and not to acyl-transferases, it follows that BSA:α-linolenoyl-CoA binding, which reduces free substrate concentration, will therefore increase desaturation.

Section 2:2:2 and Table 5 present the early results for the effect of defatted BSA on microsomal desaturation. The three PUFA substrates for Δ6 and Δ5 desaturation - 18:2(9c12c), 18:3(9c12c15c), 20:3(8c11c14c) - showed BSA stimulation. However Δ9 desaturation and the Δ6 desaturation of oleate were not affected by BSA. The lack of BSA stimulation of Δ9 desaturation reported here apparently contradicts the results of Howling, who used a similar enzyme preparation (FFA incubated with unwashed hen liver microsomes) [135]. He compared the Δ9 desaturation of FFA bound to BSA with that of FFA suspended in an unspecified concentration of aqueous Tween 20 detergent. The sensitivity of the microsomal lipogenic reactions to detergent has already been noted (Section 2:3:3 and Table 14) so he may have been comparing Δ9 desaturation of a BSA containing system with that of a partially detergent-inhibited system. The desaturations of 18:0, 18:1(5t) and 18:1(9c) were not BSA stimulated, though BSA probably still binds substrate FFA and acyl-CoA.

These are substrates where at low free acyl-CoA concentrations a preferential channeling towards desaturation is not believed to occur, so that a reduction of the free acyl-CoA substrate concentration will not significantly alter the eventual % desaturation.

2:3 Experimental

2:3:1 Incubations Using Rat Liver Microsomes

In these studies use was made of EFA deficient rats of the Colworth-Wistar strain, which had been fed for at least three months since weaning on a diet containing (by weight) 72% sucrose, 20% casein, 2% hardened coconut oil and the essential vitamins and salts. The animals weighed 240-320 g and showed the characteristic symptoms of EFA deficiency: scaly tails and feet, poor fur condition, stunted growth and an increase in the level of 20:3(5c8c11c) in liver lipids at the expense of arachidonate.

The rats were killed by cervical fracture, their livers quickly removed, weighed, and placed in a chilled buffer solution of 0.3M sucrose, 3 mM EDTA and 0.2M phosphate buffered at pH=7.3. All further manipulations were carried out on ice using chilled solutions. The livers were cut into small pieces with a pair of sharp-pointed scissors, rinsed several times with small volumes of buffer solution and homogenised in two volumes of buffer solution by a dozen strokes of a Jencon's Potter hand homogeniser. The homogenate was spun for 15 minutes at $4^{o}C$ at 1,500 g, the cell debris and the fat pad discarded, and the resulting supernatant spun for 30 minutes at $4^{o}C$ at 15,000 g on an MSE Superspeed 50 ultra-centrifuge. Leaving the more turbid lower layer of the mitochondrial supernatant and the mitochondrial pellet behind, the upper two-thirds of the mitochondrial supernatant was carefully pipetted out, and spun for 60 minutes at $4^{o}C$ at 100,000 g. The microsomes plus residual supernatant (approximately 1/10 of the total) were resuspended in buffer solution to give the volume originally spun at 100,000 g. This was the "enzyme preparation" used in the experiments.

Protein concentrations were estimated using the Biuret protein assay [136], with BSA as a calibrant. As sucrose interferes with the assay the optical densities measured for the protein solutions were compared with those for "blanks" containing identical volumes of buffer. 1 ml of enzyme preparation contained 8.6 ± 1.0 mg of protein.

The concentrations of cytoplasmic and microsomal protein were determined both via the difference between the mitochondrial and microsomal supernatant concentrations, giving a range of values from four separate enzyme preparations of 4.1-6.3 mg ml^{-1} for the microsomal protein and 3.3-3.8 mg ml^{-1} for the soluble protein, and once; more directly by measurement of microsomes washed twice with buffer solution (3.4 mg ml^{-1}) and of microsomal supernatant spun for a further 2 hours at 100,000 g (3.7 mg ml^{-1}). The two sets of data correlate well, and the slightly lower figure for the level of microsomal protein obtained from the more direct measurement may be explained by the removal of loosely bound microsomal protein after further washing and homogenisation.

Each incubation vial contained the following:-

(1) 0.1 ml of a 0.1 M aqueous magnesium chloride solution.

(2) 0.25 ml of a freshly made up cofactor solution containing 0.1 mg CoASH, 0.3 mg NADPH 0.6 mg NADH and 6.0 mg ATP.

(3) 1 ml of the enzyme preparation.

(4) A substrate solution containing 20 nmole of [1-^{14}C] fatty acid (specific activity 60 μCi μmole^{-1}, activity 1.2 μCi), made by sonicating the acid for 20 seconds in 2 drops of 10% (w/v) aqueous potassium carbonate solution plus 1.1 ml of distilled water. When fatty acid free BSA (Sigma Chemical Co. Ltd) was required in the incubation the substrate solution was made up containing BSA and was not sonicated.

The total incubation volume was 2.5 ml, and this gave the following concentrations in the incubation medium:-

[1-^{14}C] fatty acid	8 μM	NADPH	144 μM
ATP	4.35 mM	NADH	338 μM
CoASH	52 μM	sucrose	120 mM
Mg^{2+}	4.0 mM	EDTA	1.2 mM
HPO_4^{2-}	80 mM		
microsomal protein	1.6-2.5 mg/ml		
supernatant protein	1.3-1.5 mg/ml		

The reaction was started by the addition of the substrate

solution to the enzyme preparation containing all the necessary cofactors, and the incubation vial was gently agitated at 36-37°C in a water bath. The screw-capped vial used for the incubations had a dead space above the incubation medium of about 20 ml. Although molecular oxygen is an obligatory cofactor for desaturation it was not necessary to oxygenate the incubation medium (Table 12). This is not surprising since water in equilibrium with air at 30°C

TABLE 12: The Effect of Oxygen on the % Δ6 Desaturation of 18:3(9c12c15c)

	Δ6 Desaturation
Air	37%
Oxygen atmosphere	35%
Oxygen atmosphere and oxygen bubbled through the solutions for 30s.	34%

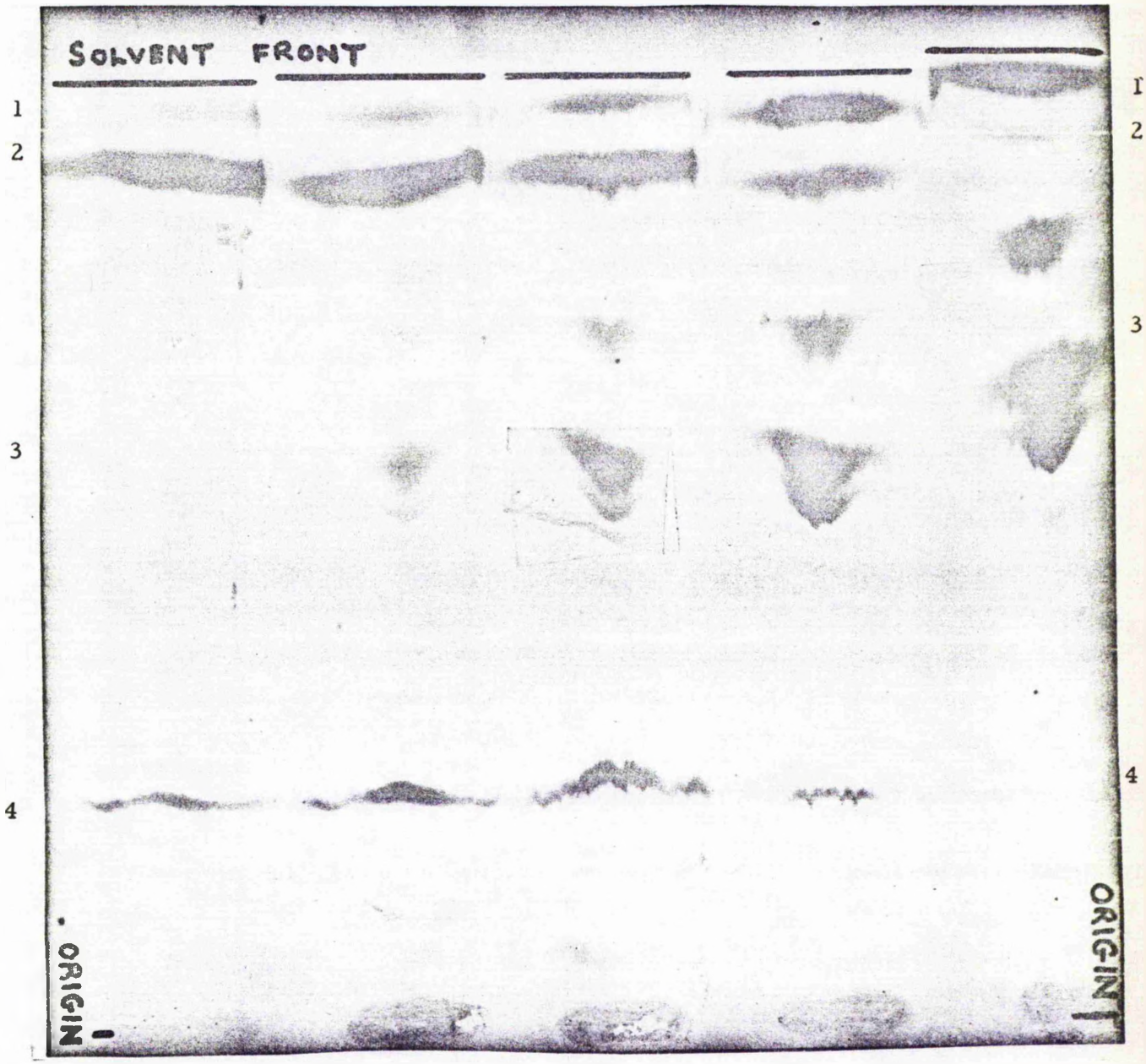
has a molecular oxygen concentration of 0.23 mM, while the stoichiometric concentration required for the desaturation of added ^{14}C plus endogenous fatty acids was about 0.05 mM.

The pH of the incubation medium varied between 7.3 and 7.8 . The reaction was terminated after one hour by the addition of 10 ml of 5% (w/v) methanolic potassium hydroxide.

2:3:2 The Work-up and Analysis of the Incubation Products

Analysis of lipid classes was undertaken by the removal of 0.2 ml aliquots from the incubation and their addition to 0.25 ml of methanol to stop the reaction. The oxalate-silica gel TLC system used was that of Ullman and Radin [137], as modified by Holloway and Holloway [138]. The methanolic solution was applied to plates coated with a 0.5 mm thickness of 2%(w/w) potassium oxalate in silica gel H and freshly activated at 110°C for 30 minutes. The bands

Figure 20: Lipid Class Analysis By Oxalate-Silica Gel TLC



Aliquots taken at the above time intervals from the incubation of [1-^{14}C] stearic acid with rat liver microsomes (Table 13, p. 107)

1 - NL
2 - FFA
3 - PL
4 - Acyl-CoA

were left to dry thoroughly under nitrogen before development in a 45/35/10 (v/v/v) chloroform/methanol/water solvent system. The radioactive bands were located by autoradiography, scraped off and their activity counted. A typical autoradiogram is shown in Figure 20. The lipid classes were identified by a comparison of their R_f values with those reported by Holloway and Holloway [138], as in Table 13. Some standards were available to check the lipid class identifications. Stearic acid and stearoyl-CoA

TABLE 13: R_f Values for Lipid Classes

Lipid Class	Unlabelled Standards	Radioactive Bands Figure 20	Holloway and Holloway's data [138]
Triacylglycerols	0.99	Neutral Lipids	-
Diacylglycerols	0.97	0.97	-
Free fatty acid	0.89	0.90	0.82
Monoacylglycerols	0.86		-
		Phospholipids	
Phosphatidyl-ethanolamine	-	0.73, 0.65	0.6
Phosphatidyl-choline	-	0.54	0.5
Acyl-CoA	0.24	0.26	0.29

confirmed the FFA and acyl-CoA bands respectively; triacylglcyerols and diacylglycerols eluted essentially at the solvent front, and monoacylglycerols eluted just behind FFA. No phospholipid standards were available. Therefore incorporation of radioactivity into the following divisions was measured:-

Neutral lipids (sterol esters, triacylglycerols and diacylglycerols).

Free fatty acids.

Phospholipids (presumably mainly phosphatidyl-choline and -ethanolamine).

Acyl-CoA thiolesters.

A more detailed analysis of the lipid classes was not attempted.

The time study shown in Figure 20 is consistent with this assignment of the lipid class to the various radioactive bands, as with increasing time the levels of neutral lipids and phospholipids increase whereas the level of FFA diminishes. Acyl-CoA builds up and is later depleted.

The fatty products from the incubation were saponified by heating at 40°C overnight or by standing at room temperature for at least two days in 10 ml of 5% (w/v) methanolic potassium hydroxide. The mixture was added to water (20 ml), acidified with 5M aqueous sulphuric acid, and extracted with ether (3 x 5 ml). The ethereal extract was washed with water (2 x 20 ml), dried over anhydrous sodium sulphate, and the solvent blown off under nitrogen. A few drops of methanol were added, and the fatty acids esterified by the addition of an ethereal diazomethane solution. The methyl esters were stored at -8°C in small volumes of petrol, with BHT antioxidant. The recovery of radioactivity was about 70% (850 nCi). Approximately 10% of the activity was lost in the transfer of the sonicated substrate solution to the incubation vial and during the incubation period, and the remaining 20% in the subsequent work-up. The microsomal reaction was clean, as TLC showed up very little in the way of metabolites more or less polar than the fatty ester substrate or desaturated product. (However, see Section 2:3:3 on mitochondrial contamination.)

Where a novel substrate was tried, the product was identified by its ECL value on a 10% DEGS packed column and by its behaviour on Ag^+/TLC, and the additional double bond located by von Rudloff oxidative cleavage. The analysis of the product(s) usually followed the sequence given below:-

(1) RGLC (the sample was co-injected with cold standards) to give ECL values for the substrate and product and the % distribution of radioactivity (computed from peak area=peak height x retention distance).

(2) Ag^+TLC of a small sample ($\sim$ 50 nCi) to give R_f values and also the % distribution of activity.

(3) Sometimes a small sample (~ 150 nCi) was subjected to von Rudloff oxidative cleavage.

(4) The remainder of the sample was chromatographed by Ag^{+} TLC, the radioactive product bands removed and eluted with ether, and the methyl esters analysed by RGLC and then von Rudloff oxidative cleavage. In cases where the new double bond was inserted between an existing double bond and the methyl end reduction with hydrazine was employed prior to von Rudloff oxidative cleavage.

Where a control or a previously investigated substrate was incubated (2), (3) and (4) were often omitted.

The % desaturations quoted in this thesis are, unless specified otherwise, for a 60 minute incubation period according to the conditions described in Section 2:3:1.

$$\% \text{ Desaturation} = \frac{\text{Radioactivity of product}}{\text{Radioactivity of substrate + product}} \times 100$$

Where a sequence of desaturations occurred, the % desaturations given are for each individual step. For example, when the distribution of activity in the desaturation of stearic acid is stearate 50%, oleate 45% and diene, 18:2(6c9c), 5%, then Δ9 desaturation has occurred to an extent of 50% and Δ6 desaturation to an extent of 10%.

VON RUDLOFF OXIDATIVE CLEAVAGE [139, 140]

The sample of [1-^{14}C] fatty ester(s) was mixed with 1 ml of a solution of 3.9 mM K_2CO_3 in 3/2(v/v) t-BuOH/water plus 1 ml of an aqueous 3.3 mM $KMnO_4$, 102 mM $NaIO_4$ solution, and allowed to stand for one hour at room temperature. The mixture was acidified with a few drops of 5M aqueous sulphuric acid, excess oxidant destroyed by bubbling through sulphur dioxide gas until the solution turned pale green, water (3 ml) added and the product extracted into ether (3 x 2 ml). The organic layers were washed with water (2 x 2 ml), dried over anhydrous sodium sulphate, and the solvent blown off under nitrogen. A few drops of methanol were added and the carboxylic acids esterified with an ethereal diazomethane

solution. The ^{14}C methyl diesters produced were co-injected with cold diester standards on RGLC. Overoxidation was less than 1%.

PARTIAL REDUCTION WITH HYDRAZINE

The [1-^{14}C] fatty ester was dissolved in methanol (2 ml) and 5 mg of methyl linoleate added (to act as a carrier and as a reference with which to monitor the reduction by GLC). After the addition of 30 μl of glacial acetic acid and 100 μl of a 60% (w/w) hydrazine hydrate solution the mixture was heated at 55°C whilst oxygen slowly bubbled through it. The reduction was monitored by direct injection onto a 10% DEGS GLC column, and when methyl linoleate had been reduced to 20/55/25 diene/monoene/saturate distribution of products (ie. the maximum monoene formation, which occurred after 2-2.5 hours) the reaction was stopped by the addition of 2M aqueous hydrochloric acid (3 ml). Water (5 ml) was added, the product extracted into ether (3 x 3 ml), washed with water (3 x 3 ml), dried over anhydrous sodium sulphate and the solvent removed with nitrogen. For example 18:1(5t) gave 18:2(5t9c) as the only product of microsomal desaturation. After partial reduction of the diene a mixture containing [1-^{14}C] 18:0, 18:1(5t), 18:1(9c), and 18:2(5t9c) was obtained which could be separated by Ag^+TLC. The cis and trans monoene bands were analysed by von Rudloff oxidative cleavage, and the newly inserted double bond thus shown to be $\Delta 9$ cis. A direct von Rudloff analysis of the 18:2(5t9c) product would have only shown a ^{14}C-labelled C_5 diester fragment, not C_9.

A problem arose when a single substrate produced two or more isomers.

eg. 18:1(15c) ⟶ 18:2(6c15c) + 18:2(9c15c)

As in many cases RGLC or dual development Ag^+TLC would not separate the isomers quantitation of the relative amounts of each isomer was based solely on a von Rudloff analysis. This was not an exact quantification. The inaccuracy of the von Rudloff procedure was caused by the increasing loss of the short-chain diester fragments

as their chain length diminished. The loss was particularly acute with C_5 and C_6 diesters, and was presumably caused by their volatility and partial water solubility. However a correction could be applied to give a reasonable estimate of the real % distribution of radioactivity between the diester fragments. This correction was based on observing the relative amounts of C_5 and C_8 diesters for conversions of the type X:1(8c) ⟶ X:2(5c8c) and 20:3(8c11c14c) ⟶ 20:4(5c8c11c14c), of C_5 and C_9 diesters for conversions of the type X:1(9t) ⟶ X:2(5c9t), and of C_6 and C_9 diesters for conversions of the type X:2(9c12c) ⟶ X:3(6c9c12c) when the whole sample was subject to von Rudloff oxidation. The losses of C_8 and C_9 diesters were minimal, so that the loss of the C_5 and C_6 diesters could be calculated from the expected levels (ie. from the known % desaturation) minus the levels after von Rudloff oxidation.

In future work it may be useful to improve the techniques for the analysis of such isomer mixtures by developing a higher resolution Ag^+TLC system based on low temperature, multiple development as described by Morris et al [141] or by developing a von Rudloff analysis that is quantitative for short chain fragments. This could perhaps be achieved by recovering the oxidation fragments as their salts, placing them on the GLC column, acidifying, and chromatographing, or by adding C_5-C_{10} diacid monomethyl ester standards to the reaction mixture prior to the work-up and following their loss by GLC.

Good agreement (within 10%) was obtained for the % desaturation as measured by RGLC or by Ag^+TLC with liquid scintillation counting. However, with low % conversions the latter method proved more reliable for quantification. Very low conversions (1-2%) could only be detected after Ag^+TLC concentration of the product to give a recognisable peak on RGLC, and they could only be quantified by Ag^+TLC/liquid scintillation counting.

2:3:3 The Problems of Detergent Inhibition and Mitochondrial Contamination

DETERGENT INHIBITION

Preliminary work with rat liver microsomes involved the sonication of [1-^{14}C] free fatty acids in a detergent solution, in the hope of achieving complete solubilisation. Biosolv, a powerful non-ionic detergent was chosen. Although an inhibitory effect was present, it was small and therefore undetected in the early work where higher concentrations of protein were used in larger incubation volumes. Only later, when the enzyme preparation was "standardised" (ie. the incubation conditions described in Section 2:3:1 were used) did a marked inhibition occur. Here, unwashed microsomes were substituted for the mitochondrial supernatant, thus removing large amounts of soluble protein from the incubation medium, and a smaller volume of enzyme preparation (1 ml as opposed to 3 ml) was used, resulting in a smaller incubation volume, lower microsomal protein concentration and higher detergent concentration. Table 14 shows the % Δ6 desaturation of [1-^{14}C] 18:3(9c12c15c) and the incorporation of radioactivity into lipids for various incubation systems with and without detergent. These results indicate the danger of working with detergent in the medium when using microsomal enzymes. Even a mild, non-ionic detergent such as Triton X-100 had a noticeable effect. Such a practice should be avoided: sonication seems sufficient to solubilise the free fatty acids.

MITOCHONDRIAL CONTAMINATION

Care needs to be exercised when removing the mitochondrial supernatant after the 15,000 g centrifugation step. If the lowest layer is taken or the supernatant is decanted mitochondiral contamination will probably result. A 1,500 g spin was introduced prior to the 15,000 g spin in order to remove the large volume of cell debris from the liver homogenate. This resulted in a better

TABLE 14: % Δ6 Desaturation of α-Linolenic Acid and the Incorporation of Radioactivity into Lipids for Rat Liver Microsomal Systems With and Without Detergent

Detergent, Concentration in Incubation Medium	Protein Concentration	% Δ6 Desaturation	% Lipid Incorporation		
			NL	FFA	PL
-	1 ml }	36	21	12	67
0.29% Biosolv	1 ml } mitochondrial	7.5	2	36	63
0.07% Biosolv	1 ml } supernatant	26.5	-	-	-
0.22% Triton	1 ml }	30.5	2	32	66
0.1% Biosolv[1]	3 ml mitochondrial supernatant	26	3	23	74
-	1 ml } unwashed	38	26	9	65
0.25% Biosolv[2]	1 ml } microsomal pellet	<2	2	89	9

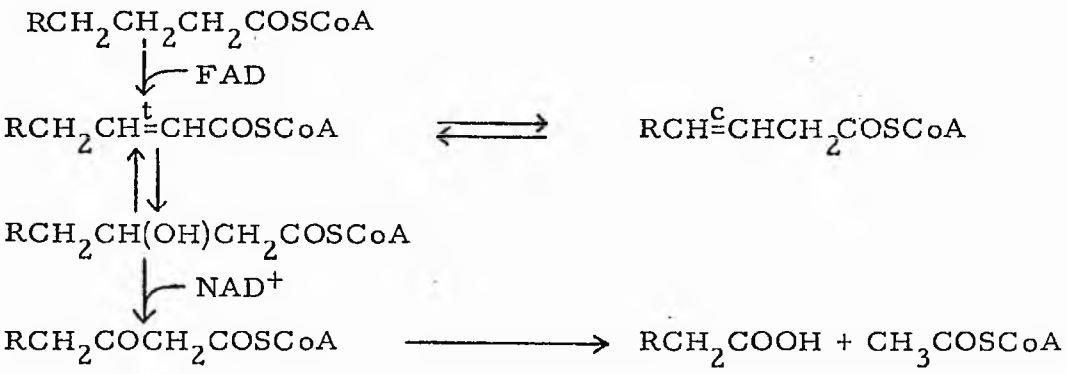
The experiment used liver from weanling rats (4-5 weeks old). Protein concentrations were not measured, but the enzyme preparation and amounts of cofactors were as described in Section 2:3:1

1. Marks the microsomal system used in preliminary work.
2. Marks the "standardised" system with detergent inhibition.

mitochondrial sedimentation in the 15,000 g spin (the effective centrifugation radius was greater) and the smaller, more compact pellet meant that it was easier to remove the bulk of the mitochondrial supernatant without disturbing the mitochondria.

Mitochondrial contamination resulted in several additional radioactive components being detected. A thorough investigation of these was not undertaken but preliminary evidence strongly suggested that they were products from a partial β-oxidation sequence. TLC (PE20) of [1-^{14}C] fatty methyl esters gave two major bands. The upper band at R_f = 0.63 corresponded to fatty esters without additional oxygen-containing functional groups. RGLC showed that this band contained mainly the substrate and the expected desaturated product, but also additional peaks with ECL values of 0.8, 1.6 and 2.4 greater than the substrate ECL value. The lower band had an R_f value (=0.18) identical to that of methyl 3-hydroxystearate. RGLC showed the major component(s) to have an ECL value of 5.7 to 6.0 greater than the substrate ECL value, and TMS derivatisation by co-injection with N,O-bis-trimethylsilyl acetamide reduced the ECL value of the major component(s) to only 1.1 to 1.3 units greater than the substrate's value. 3-OH and 3-OTMS methyl stearate standards had ECL values of 5.8 and 1.2 respectively greater than methyl stearate on the 10% DEGS column. This evidence, in conjunction with the use of 2-OH, 12-OH, and 4-oxo methyl stearate standards and literature data on the TLC and GLC properties of isomeric hydroxy esters [142-144] confirmed 3-OH esters as the major additional radioactive component.

The sequence of steps in β-oxidation is shown below.



The extra components from the TLC band at R_f = 0.63 may well be Δ2t and Δ3c isomers of the [1-^{14}C] substrate or product. This was not checked. The occurrence of high amounts of 3-hydroxy but not 3-oxo compounds could indicate either that a partial β-oxidation sequence occured, stopping after the formation of the 3-hydroxy compound, or that any 3-oxo compound formed rapidly lost ^{14}C-acetate.

2:3:4 Additional Experiments

HEN LIVER MICROSOMES

The liver of a laying hen was used instead of rat liver, but in all other respects the microsomal preparation and the incubation conditions were identical to those of rat liver (Section 2:3:1).

AN ESTIMATION OF THE LEVEL OF ENDOGENOUS FFA IN THE MICROSOMAL ENZYME PREPARATION

3 ml of the enzyme preparation were vigorously shaken with a standard of 100 nmole heptadecanoic acid in methanol plus 10 ml of a 2/1 (v/v) chloroform/methanol mixture. 10 ml of 0.7% saline were added, the mixture shaken again, allowed to settle and the chloroform layer run off. The solvent was removed at the rotary evaporator and the residue dried by azeotropic distillation with methanol. A portion was transesterified by refluxing for 2 hr in 2 ml of 20/10/1 (v/v/v) methanol/benzene/concentrated sulphuric acid, while the remainder was applied to a silica gel G TLC plate which was developed in 85/15/2 (v/v/v) petroleum (bp. 40-60°C)/diethyl ether/formic acid. A stearic acid standard was also run. The bands were detected under UV light after spraying the plate with a 0.02% methanolic 2',7'-dichlorofluorescen solution, and the free fatty acid band at R_f = 0.28 scraped off and eluted with ether then methanol. The acids were esterified with an ethereal diazomethane solution. GLC of the methyl esters from total lipids or free fatty acids was done on a 10% DEGS column at 175°C. The 17:0 standard was used to calculate the levels of free fatty acids present in the microsomal preparation.

THE COLD-STORAGE OF LIVER MICROSOMES

The unwashed microsomal pellet, stored at -20°C for several days, thawed and resuspended in phosphate buffer, gave at first sight an enzyme preparation which was perfectly usable. Measuring % Δ6 desaturation with α-linolenic acid as substrate, and lipid incorporation, allowed the following comparison to be made:-

	Fresh microsomes	Stored microsomes
% Δ6 Desaturation	35%	39.5%
Radioactivity in NL	18%	3%
FFA	1%	13%
PL	79%	80%
Acyl-CoA	2%	4%

However, a time-course experiment performed with [1-^{14}C] linoleic acid using stored microsomes indicated that although after 60 minutes the % Δ6 desaturation and lipid incorporation pattern resembled that for fresh microsomes, the rates for lipid incorporation or desaturation altered markedly and were very much slower than expected. Therefore the use of stored microsomes is not recommended.

TIME COURSE STUDIES

For the time course studies the incubations were performed as already described (Section 2:3:1) but on three times the scale. Aliquots were removed for lipid class and fatty acid analysis after approximately 1, 2, 5, 15 and 60 minutes. 1 ml aliquots were taken for saponification and subsequent RGLC analysis. The reaction was stopped by addition to 3-4 ml of 5% (w/v) methanolic potassium hydroxide. (For the following manipulations see Section 2:3:2.) For lipid analysis 0.3 ml aliquots were removed and added to 0.5 ml of methanol. 50-100 nCi was applied to an oxalate plate and chromatographed as described in Section 2:3:2 in order to give the % distribution of radioactivity among the lipid classes. The remainder

of the aliquot was also chromatographed on an oxalate plate, but the bands were scraped off and refluxed with 3 ml of 20/10/1 methanol/benzene/concentrated sulphuric acid transmethylation reagent for 2 hours. The distribution of radioactivity between the substrate and product was measured for each lipid class by Ag^{+} TLC of the methyl esters followed by liquid scintillation counting.

Section 3. ^{13}C Nuclear Magnetic Resonance Spectroscopy of Unsaturated Fatty Acids

3:1 Introduction

NMR measurements of nuclei such as ^{19}F, ^{31}P and particularly ^{1}H have been widely used in chemistry since the 1950's, but the routine use of ^{13}C nuclei is a recent innovation following the development of commercially available CMR spectrometers. The low sensitivity of natural abundance ^{13}C, caused by a smaller gyromagnetic ratio than for the proton and by an isotopic abundance of only 1.1%, delayed the development of suitable spectrometers, but this problem has now been overcome by the use of magnets of greater field strength and homogeneity and by pulse-Fourier transform techniques coupled with computer averaging of transients. Broad band proton decoupling simplifies the CMR spectrum by causing each resonance to be represented by a singlet and enhances the sensitivity of the signal.

The major advantage of CMR spectroscopy over PMR spectroscopy is that for the former chemical shifts are observed over a much wider range of 200 ppm, as opposed to 10 ppm (with both ^{13}C and ^{1}H line widths approximately equal to 1 Hz), so that much smaller changes in the chemical environment can be measured. The measurement of effects at carbon atoms themselves, rather than by inference from the chemical shifts of neighbouring protons is beneficial to chemists, especially when the carbon atoms in question are not bonded directly to hydrogen atoms.

Methods of assigning ^{13}C chemical shifts to individual carbon atoms are varied, and well reviewed by Gray [146]. However, it is worth mentioning them with respect to fatty acids, where the major difficulty is in coping with the large number of almost identical methylene carbon resonances. Except in specific instances, often in connection with more unusual π-systems, hetero-atoms, or branch points, ^{13}C-^{1}H chemical shift cross correlation, specific proton decoupling,

off-resonance and complete decoupling experiments are not helpful. Spin-lattice relaxation time (τ_1) measurements on individual carbon atoms are not useful in structural assignments, but are valuable in the study of the mobility of lipid membranes [147]. τ_1 diminishes as carbon atom motion becomes more restricted. Labelling by deuteration or ^{13}C enrichment gives conclusive proof of a correct assignment. A per-deuterated carbon has a large τ_1 and hence a very weak or absent signal, while a partially deuterated carbon signal shows up as a multiplet as a result of $^{13}C-^{2}D$ coupling. Carbon atoms α or β to a $C-^{2}D$ bond will have their ^{13}C chemical shifts displaced fractionally upfield and broadened by long range $^{13}C-^{2}D$ coupling (ie. second and third atom isotope effects). Tulloch and Mazurek assigned the separate ^{13}C shifts of methyl stearate, oleate and petroselinate by means of second and third atom isotope effects in the spectra of specifically deuterated esters [170]. Useful ^{13}C enrichment can be achieved by labelling with as little as 2% isotopic abundance ^{13}C. Labelling with ^{2}D or ^{13}C to check the assignment of fatty acid shifts may not always be a suitable procedure, as a considerable amount of synthetic chemistry may be necessary for a limited return of information. However, the approach is very useful in mechanistic and biosynthetic studies. Cronan and Batchelor [148] grew an E. coli mutant in the presence of [1-^{13}C] acetate and isolated the fatty acids. The expected chemical shifts of the odd-numbered carbon atoms of palmitoleic acid were intensified, helping to confirm the assignments. Lanthanide shift reagents have been used successfully to assign signals in PMR and CMR fatty acid spectra. For example, using PMR spectroscopy with $Eu(fod)_3$ Wineburg and Swern located the $\Delta 6$ double bond in methyl petroselinate and placed the double bond of oleic acid at or beyond the $\Delta 9$ position [149], while Batchelor et al used $Eu(fod)_3$ and $Pr(fod)_3$ in conjunction with CMR spectroscopy to confirm the C(5) and C(8) allylic and the C(6) and C(7) olefinic assignments of methyl petroselinate [150]. Quantification of ^{13}C signal intensities is not particularly reliable, especially for

carbon atoms not bonded directly to hydrogen. This is due to differences in spin-lattice relaxation times and to the nuclear Overhauser enhancement. However, the problem can be solved by paramagnetic doping, with for example chromium acetylacetonate (this shortens τ_1 of all the ^{13}C nuclei to less than one second) and/or Overhauser suppression (a Fourier transform experiment is performed with greater pulse delays and gated decoupling). Shoolery used these techniques to estimate the composition of mixtures of methyl esters and of seed oils, and the results compared favourably with GLC analysis [151]. Finally, perhaps the most commonly quoted method in the literature for the assignment of ^{13}C chemical shifts is from a comparison of a series of model compounds. The data is usually expressed in terms of a regression analysis [152-155]. As this is the principal approach used in this work it is discussed in detail in Section 3:2:1.

The chemical shift difference between a carbon atom in environments 1 and 2, δ^{12}, can be expressed as a sum of terms, given by Equations 1 and 2 [156].

$$\delta^{12} = \delta_{el} + \delta_A + \delta_{intermolecular} \qquad \text{Eq. 1}$$

$\delta_{intermolecular}$ represents mainly solvent effects, which are small unless a marked change in conformation of the solute occurs.

δ_{el} is the term for the local electronic distribution about the carbon nucleus.

δ_A is the term for the neighbour-anisotropy effect. It depends on the distance and orientation of the anisotropic group to the carbon atom, but not on the nature of the carbon atom.

δ_{el} and δ_A are the terms that change with substitution on the molecule, the former being the most significant.

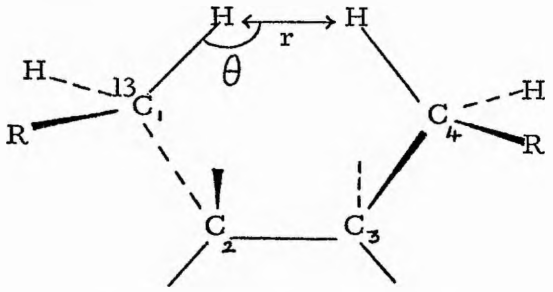
$$\delta_{el} = \delta_E + \delta_{FE} + \delta_S \qquad \text{Eq. 2}$$

δ_E represents the local electronic distribution caused by the bonding framework (ie. hybridisation of the carbon atom, inductive and mesomeric effects).

δ_{FE} is an electric field term, caused by through space interaction. δ_S represents steric interactions.

From these considerations it is apparent that an understanding of chemical shifts at a molecular level is complex, since a substituent change could alter all the terms discussed above. One useful generalisation is that the chemical shift moves upfield with an increase in electron density around the ^{13}C nucleus.

Some headway has been made in the rationalisation of ^{13}C shifts. When H was replaced by CH_3 in linear hydrocarbons Grant and Paul measured downfield α- and β-substituent shifts and an upfield γ-shift [153]. To explain the upfield γ-shift Grant and Cheney proposed a non-bonded hydrogen-hydrogen steric compression between carbon atoms 1 and 4 in a gauche conformation, which polarised the ^{13}C-H bond [157].



The steric perturbation at ^{13}C was dependent on the force constant of the hydrogen-hydrogen interaction along the ^{13}C-H bond, and therefore a greater upfield shift would result if r was smaller and $\cos\theta$ tended to unity. The magnitude of the γ-shift depended on which were the most favoured relative orientations of the hydrogen atoms on C(1) and C(4), and since no perturbation results from a trans rotamer it also depended on the relative population of trans and gauche conformers. It has since been recognised that for hetero-atom substituents the α- and β-shifts are also downfield and the γ-shift upfield [154, 158, 159]. The magnitude of the α-substituent shift is determined mainly by the δ_E term and the γ-substituent shift by the δ_S term.

A small, downfield δ-substituent shift of 0.3 ppm was also observed for n-alkanes [153]. Batchelor et al explained this by proposing an increase in the population of the trans conformer brought

about by any δ-substituent large enough to preclude g+g-conformers (Figure 21) [150]. The γ-shift at C(1) resulted from a gauche conformation about the C(2)-C(3) bond. Introducing C(5), a δ-substituent to C(1), then gauche conformers became possible about the C(3)-C(4) bond. However, of the two possible conformers when both C(2)-C(3) and C(3)-C(4) bond rotamers existed in the gauche form one was forbidden on the grounds of orbital overlap. A consequence of this steric interaction between C(1) and C(5) was that the trans population of the C(2)-C(3) bond increased slightly, thus diminishing the upfield γ-substituent shift felt at C(1).

Electric field effects are of crucial importance for an understanding of the CMR spectroscopy of unsaturated molecules. A point charge, permanent dipole or an induced dipole has an associated electric field which causes bond polarisation and therefore changes in ^{13}C chemical shifts. This bond polarisation is represented by a linear and a squared dependence on the electric field vector, $\vec{E}_Z$, along the bond in question, but the squared term is only significant at very short distances from the dipole and so is normally neglected. The polarisation of charge, δq, at a carbon atom, which causes a change in the chemical shift term δ_{FE} is thus a linear electric field effect, and can be represented by Equation 3 [150]

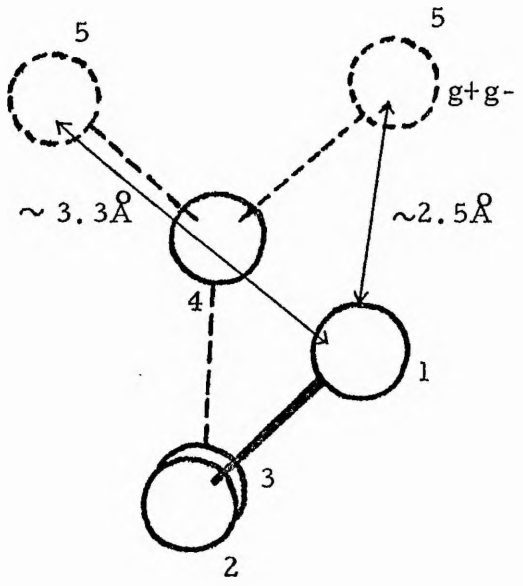
$$\delta q = \sum_{\substack{\text{all bonds} \\ \text{to the carbon} \\ \text{atom}}} \frac{b_Z . \vec{E}_Z}{e . l_Z} \qquad \text{Eq. 3}$$

where e is the unit electronic charge, l_Z is the bond length, b_Z is the longitudinal bond polarisability and $\vec{E}_Z$ is the electric field vector along the bond. δ_{FE} is related to δq by the term (δ/e), which is the change in chemical shift per unit electronic charge. (δ/e) is dependent on the nature of the carbon atom.

The permanent dipole of the carboxylate head-group affects π-bonds in the alkyl chain by increasing the electron density on the carbon atom nearest the head-group and diminishing that of the more

Figure 21: The δ-Substituent Shift in Hydrocarbons

$$-{}^{13}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2-$$
$$\quad\ 1 \quad 2 \quad 3 \quad 4 \quad 5$$



represents a carbon atom.

The hydrogen atoms are not shown.

The introduction of C(5) (the δ-substituent) to give a gauche conformation about the C(3)-C(4) bond leads to two possible conformers where both the C(2)-C(3) and the C(3)-C(4) bonds have a gauche conformation. The one designated g+g- is forbidden on the grounds of orbital overlap.

distant carbon. Since the longitudinal bond polarisability of acetylenic and olefinic bonds is much greater than for C-C or C-H single bonds, Equation 3 reduces to a single term, so the difference in chemical shift between the n and (n+1) carbon atoms of a π-bond, $\Delta\delta$ approximates to Equation 4.

$$\Delta\delta = 2.(\delta/e)\left(\frac{b_\pi}{e.l_\pi}\right)\vec{E}_\pi \qquad \text{Eq. 4}$$

Batchelor et al performed calculations to determine $\vec{E}_\pi$, which is dependent on the distance and orientation of the π-bond to the polar head-group, when treating the alkyl chain as a rigidly extended or a "random walk" model [160]. Comparing the calculated $\Delta\delta$ value with the observed value for olefinic unsaturation, the alkyl chain was shown to take up a conformation between these two extremes, with some degree of preference for trans conformers.

For saturated carbon atoms the linear electric field polarisation is much smaller, owing to the low longitudinal bond polarisabilities for C-H and C-C single bonds. The shifts caused by a carboxylic acid or ester head-group are upfield, and decrease in magnitude monotonically from the head-group [150].

The use of ^{13}C-^{1}H spin-spin coupling patterns as an aid to the elucidation of fatty acid structure appears very limited. One bond couplings (J_{CH}) are of the order of 120-300 Hz, their exact magnitude depending on the hybridisation of the ^{13}C atom and on substituent effects. Geminal (J_{CCH}) and vicinal (J_{CCCH}) coupling also occurs. ^{13}C-^{1}H coupling constants are given for ethane, ethylene and acetylene as examples [177]:-

	J_{CH}	J_{CCH}
C_2H_6	125 Hz	5 Hz
C_2H_4	156 Hz	2.5 Hz
C_2H_2	249 Hz	50 Hz

Off-resonance spectra give the multiplicity of the one-bond ^{13}C-^{1}H spin-spin coupling pattern of a ^{13}C resonance (ie. the number

of hydrogen atoms bonded to that carbon atom) without resorting to a fully undecoupled spectrum. However, the magnitude of J_{CH} and long range coupling information can only be obtained from an undecoupled spectrum. These data may be helpful in specific instances, but a lack of published data for comparison hinders their interpretation.

Finally, a review of the relevant sources of data for the interpretation of fatty acid CMR spectra, including those sources not mentioned explicitly so far in this introduction, is necessary. The CMR data for alkanes is based on the work of Grant and Paul [153], but Lindeman and Adams reported more extensive information on branched paraffins, effectively highlighting the problems of dealing with branched molecules [155]. Friedel and Retcofsky carried out some early studies on aliphatic alkenes, alkynes and allenes [161]. Their undecoupled spectra were useful in making some unequivocal assignments to unsaturated carbon atoms. More recent work on unsaturated systems was undertaken by Dorman et al on alkenes [154] and alkynes [152], by de Haan and de Ven on alkenes [163], and by Lippmaa et al on α,β -olefinic acids [162]. Extensive compilations of information are given in the texts of Levy and Nelson [175] and Stothers [176].

All the published work on CMR spectroscopy of fatty acids is post-1970. Stoffel et al produced data for saturated, mono- and polyenoic acids, phosphatidylcholines and spingosine bases [166], while Barton reported the spectra of the complete series of 1,2-dioctadec-cis-enoyl-sn-glycero-3-phosphorylcholines [165]. Bus and Frost discussed the spectra of the $\Delta 8$ to $\Delta 12$ cis and trans methyl octadecenoates [164]. None of these early studies attempted to unravel the more complex patterns of ^{13}C chemical shifts due to the bulk methylenes or to the olefinic carbons of PUFA, and indeed some of these early assignments are now believed to be incorrect. During the completion of this study Tulloch and Mazurek published a paper describing the complete assignment of the ^{13}C chemical

shifts of methyl stearate, oleate and petroselinate based on the spectra of specifically deuterated esters [170], assignments which agreed with our own [171, 172]. More recently still Bus et al presented data on the CMR spectroscopy of unsaturated acids [173, 174] which agreed with and complemented our own.

CMR spectroscopy has also been applied to lipid problems other than the structural elucidation of fatty acids. Prostaglandins were investigated [168], the mobility of membranes probed via ^{13}C spin-lattice relaxation time measurements [147], the fatty acid composition of intact seeds measured to assist in an efficient genetic breeding program of soybean [167], and successful attempts made to quantify fatty acid mixtures [151].

3:2 Results and Discussion

The purpose of this work was threefold:-

(1) To run CMR spectra of the extensive range of unsaturated fatty acids available in this laboratory in order to supplement and extend the literature data. Acetylenic compounds in particular have received scant attention, yet they are important as intermediates in the synthesis of unsaturated acids, and also occur naturally as in some fatty acids. This has now been remedied.

(2) To extract from this data a comprehensive set of shielding and deshielding parameters in order to give complete assignments to the spectra, and therefore to optimise the structural identification of unsaturated fatty acids by ^{13}CMR spectroscopy.

(3) To indulge in some speculative rationalisation of the results in the light of present ideas on the theory of ^{13}C chemical shifts.

3:2:1 The Assignment of ^{13}C Chemical Shifts by the Use of Shielding Parameters

Since fatty acids are linear molecules, their ^{13}C chemical shifts can be formulated by Equation 5, where δ_c is the shift of the carbon atom under consideration, δ_c^o is the shift of the class of carbon atom to which c belongs (eg. sp_3 methylene, sp_2 cis olefinic) when influenced only by the extended methylene chain, and $_\alpha\Delta^c(X)$ is the

$$\delta_c = \delta_c^o + \sum_{X,Y\ldots} {}_\alpha\Delta^c(X) \qquad \text{Eq. 5}$$

shielding parameter. X, Y... are the functional groups that alter the chemical shift value of carbon c from δ_c^o. Their positions relative to carbon c, which determine the value of the shielding parameters, are denoted by the appropriate subscript, $\alpha, \beta, \gamma, \delta, \epsilon \ldots$ Frost and Gunstone successfully applied this approach to the PMR chemical shifts of methylene groups in fatty acids [169]. It is simpler than the more conventional regression analysis, first used

by Grant and Paul for linear alkanes [153], where each carbon atom or functional group contributes a term to δ_c. δ_c would thus be expressed by Equation 6, where A_o is a constant, A_α is the parameter associated with a carbon atom or functional group at a position $\alpha, \beta, \gamma, \delta, \epsilon \ldots$ from carbon c, and n_α is the number of such carbon atoms or functional groups.

$$\delta_c = A_o + \sum_{\alpha,\beta\ldots} n_\alpha . A_\alpha \qquad \text{Eq. 6}$$

Care must be taken in the averaging processes for the regression analysis and the substituent shielding parameter approaches if small but meaningful and diagnostic differences in chemical shifts are not to be lost.

Both approaches are fundamentally similar, in that their use to predict chemical shifts depends on the additivity of shielding and deshielding influences. Our approach is easier to handle for the rapid calculation of fatty acid CMR spectra, but is not so flexible and gives less insight into the theoretical aspects of ^{13}C chemical shifts. In order to clarify most of the points made so far, consider C(3) in hexanoic acid. Its chemical shift can be written as Equation 7 or 8, and it is apparent that the two sets of symbols are related. When writing the shielding parameter for methylene carbon atoms the superscript CH_2 is normally omitted, and the symbolism becomes $_\alpha\Delta(X)$, not $_\alpha\Delta^{CH_2}(X)$.

$$\underset{\beta}{COOH}\text{-}\underset{\alpha}{CH_2}\text{-}\underset{\alpha}{^{13}CH_2}\text{-}\underset{\beta}{CH_2}\text{-}\underset{\gamma}{CH_2}\text{-}CH_3$$

$$\delta C(3) = \delta^{o}_{CH_2} + {}_\beta\Delta(COOH) + {}_\gamma\Delta(CH_3) \qquad \text{Eq. 7}$$

$$\delta C(3) = A_o + 2A_\alpha + A_\beta + A_\gamma + A(COOH)_\beta \qquad \text{Eq. 8}$$

$$\delta^{o}_{CH_2} = A_o + 2(A_\alpha + A_\beta + A_\gamma + A_\delta)$$

$${}_\beta\Delta(COOH) = A(COOH)_\beta - (A_\gamma + A_\delta + A_\beta)$$

$${}_\gamma\Delta(CH_3) = -A_\delta$$

Molecules containing a branched carbon framework require a more complex system of parameters in order to be adequately described [155]. However, branched-chain fatty acids were outside the scope of this work.

The assignment of ^{13}C chemical shifts to individual carbon atoms was based on four criteria:-

(1) Agreement with literature values, especially where conclusive evidence, such as ^{13}C enrichment or ^{13}C-^{1}H coupling studies, have been performed.

(2) A consideration of the number of signals and the peak heights. The use of a 1000 Hz scan over the 0-50 ppm region of the spectrum was particularly helpful in this context, as the number of signals resolved was usually increased.

(3) The spectra of molecules which contain similar structures were compared, and a common set of signals could be identified, thus characterising the structure.

(4) The main line of approach was the additivity of substituent shielding and deshielding parameters. CMR spectra were assigned by inspection, and the consistency of the shielding parameters for a given functional group was shown to hold, so validating the initial assumption of additivity. The parameters were later refined as additional data accumulated. As an example consider the methylene carbon atoms of octanoic and decanoic acids (Figure 22). The reference point, $\delta^{o}_{CH_2}$, is a methylene group at the centre of a long, linear hydrocarbon chain, unperturbed by either the carboxylic acid head-group or the terminal methyl group. From the CMR spectra of long chain n-alkanoic acids ($\geqslant$ 14:0) $\delta^{o}_{CH_2} = 29.81$. Figure 22 sets out the correct assignment for octanoic and decanoic acids, together with the relevant parameters that make contributions to each methylene carbon atom chemical shift. By inspection, it can be seen that the influence of the terminal methyl group does not extend beyond the γ-carbon and the influence of the carboxylic acid beyond the ϵ-carbon. Solving equation 5 for some of the parameters shows that their values are consistent, and therefore those assignments are

Figure 22: The Assignments for Octanoic and Decanoic Acids with Values of some Shielding Parameters Derived from their ^{13}C Chemical Shifts

COOH– CH_2	CH_2	CH_2	CH_2	CH_2	CH_2	CH_3		
34.30	24.87	29.22	29.11	31.84	22.76			Chemical shift
			γ	β	α	$\Delta(CH_3)$	}	Shielding
α	β	γ	δ	ϵ		$\Delta(COOH)$	}	parameters[1]

$$_{\delta}\Delta(COOH) + {}_{\gamma}\Delta(CH_3) = 29.11 - 29.81^{2} = -0.70$$

$$_{\epsilon}\Delta(COOH) + {}_{\beta}\Delta(CH_3) = 31.84 - 29.81 = +2.03$$

COOH– CH_2	CH_2	CH_2	CH_2	CH_2	CH_2	CH_2	CH_2	CH_2	CH_3
34.28	24.84	29.24	29.41	29.56	29.41	32.04	22.80		Chemical shift
					γ	β	α	$\Delta(CH_3)$	
α	β	γ	δ	ϵ				$\Delta(COOH)$	
								Shielding parameters[1]	

$$_{\delta}\Delta(COOH) = 29.41\text{-}29.81 = -0.40$$

$$_{\gamma}\Delta(CH_3) = 29.41\text{-}29.81 = -0.40$$

$$_{\epsilon}\Delta(COOH) = 29.56\text{-}29.81 = -0.25$$

$$_{\beta}\Delta(CH_3) = 32.04\text{-}29.81 = +2.23$$

1. The positional subscripts are shown beneath the appropriate carbon.
2. 29.81 ppm is the value for an unperturbed methylene carbon shift in an alkanoic acid.

likely to be correct. Using these values, the chemical shifts of other alkanoic acids can be predicted and then the data from additional spectra used to refine the substituent shielding parameters.

The assumption that shielding influences are additive breaks down when two functional groups are separated by one or two methylene groups. For the addition rule to hold each functional group must behave independently of the other, but this is not always the case if they are too close. However, although the shielding influences derived for isolated functional groups cannot be used to predict the chemical shifts accurately when two or more functional groups are near neighbours, they often give a reasonable approximation. For example, the shielding parameter for a cis-allylic methylene carbon, $_{\alpha}\Delta(c)$, is -2.5 ppm, which would give a predicted chemical shift for the c,c-diallylic methylene carbon of 29.8 + 2(-2.5) = 24.8 ppm. The observed value was 25.8 ppm.

3:2:2 The Reproducibility of ^{13}C Chemical Shifts, and Solvent Effects

The first question requiring an answer is with what resolution and to what accuracy can ^{13}C chemical shifts be reproduced. This is important because small differences in shifts are diagnostic in the interpretation of fatty acid CMR spectra.

The best resolution attainable with the Varian CFT-20 spectrometer was found to be of the order of 0.1 ppm for a 0-200 ppm (4000 Hz) scan. A considerable improvement was achieved using a 0-50 ppm (1000 Hz) scan, when experiments showed the C(5) and C(6) carbons of dodecane ($\Delta\delta$ = 0.03 ppm) and the two propargylic carbons of octadec-9-ynoic acid ($\Delta\delta$ = 0.02 ppm) to be resolved. It should be noted that the above considerations are for two adjacent signals only: obviously when many signals occur over a limited chemical shift range, such as the methylene "fingerprint" region of 28.5-30 ppm, the resolution may diminish markedly.

The 200 ppm and 50 ppm scans for the same sample gave an average variation in chemical shift of $\pm$0.015 ppm, counting only distinct, single ^{13}C resonances. However, octadec-8-ynoic and

stearic acids, and methyl palmitate and stearate were each run twice, different samples being made up each time, and a standard deviation of $\pm$0.055 ppm was obtained for chemical shift values. The variations occurred over a 0-0.10 ppm range and were not due to an aberrant locking of the spectrometer onto the tetramethylsilane signal as the discrepancies did not have a constant value (Table 15, 18:1(8a) acid). They had the nature of a solvent effect.

Changes in the nature of the medium surrounding the fatty acid or ester molecule, which produced solvents shifts, could arise from two sources. The first involves a simple change in the type of solvent used. Table 15 shows the solvent shifts for methyl oleate in $CDCl_3$ and in a 3:1 (v/v) mixture of CCl_4:$CDCl_3$. They were non-uniform, with the greatest variation being associated with the polar head-group. The terminal methyl showed the smallest solvent shift. This is a general feature, since the terminal methyl is more akin to the methyl groups of the tetramethylsilane internal reference than are other carbons of the fatty molecule. Hence its solvent shift is more completely compensated. The second source of solvent shifts occurred as a consequence of using concentrated ($\sim$1M) sample solutions, which was expedient in order to cut out long periods of scanning. Table 15 shows the solvent shifts obtained when comparing methyl eicosa-cis-8,cis-11,cis-14-trienoate spectra taken at 0.14M and 2M concentrations: values for intermediate concentrations are not reported, but followed the trends indicated. The shift differences were a result of three possible effects, which might be acting in opposite directions:-

(i) A change in [-COOMe] in the medium from 0.14M to 2M.

(ii) A change in [-CH:CH-] in the medium from 0.42M to 6M.

(iii) A change in the ratio of hydrocarbon:$CDCl_3$ (w/v) in the medium of approximately 1:20 to 2:1.

When increasing the concentration of 20:3(8c11c14c) ester, the overall shift for methylene carbons was downfield, but with the exception of (i) the constituent effects were not investigated. Two lines of evidence suggested that an increase in -COOMe concentration would give an

TABLE 15: Changes in Chemical Shift Values caused by Solvent Effects

Sample	Carbon Atom: Differences in C(1)	C(2)	C(3)	C(4)	C(5)	C(6)	C(7)	C(8)	C(9)
18:1(8a) acid Difference in chemical shift between two different samples	-.01	-.01	+.04	+.04	+.04	+.05	+.03	-.05	-.09
Methyl oleate $\delta_{CDCl_3} - \delta_{3:1\ CCl_4:CDCl_3}$	+.92	+.28	+.17	+.11	+.11	+.11	+.13	+.14	+.19
20:3(8c11c14c) Methyl ester, in $CDCl_3$ $\delta_{2M} - \delta_{0.14M}$	-.75	-.09	+.10	+.13	+.16	+.18	+.13	-.13	+.16
C_{16} Dimethyl dicarboxylate $\delta_{predicted} - \delta_{obs.}$	0	+.06	+.07	+.10	+.14	+.11	+.10	+.10	

1. Unsaturated carbon atoms are underlined.

Chemical Shift[1]										
C(10)	C(11)	C(12)	C(13)	C(14)	C(15)	C(16)	C(17)	C(18)	C(19)	C(20)
+.03	+.06	+.05	+.06	+.06	+.06	+.05	+.04	+.02		
+.18	+.14	+.20	+.12	+.12	+.12	+.13	+.12	+.05		
+.10	+.01	-.03	+.10	+.17	-.17	+.13	+.11	+.15	+.14	+.04

upfield shift for methylenes. Firstly $\delta^{o}_{CH_2}$ had a smaller value for methyl esters than for hydrocarbons. Secondly, using the shielding parameters for CH_3- and -COOMe, derived from methyl alkanoates, to predict the CMR spectra of 1M solutions of $C_{10,12,14,16}$ dimethyl dicarboxylates, it was apparent that the observed shifts for methylene carbons were consistently 0.1 ppm upfield from the predicted shifts, and this was attributed to a 2M -COOMe concentration in the medium (Table 15).

The main points that arose from an analysis of the reproducibility of the CMR data were:-

(1) Working in a concentration range of 0.1 to 1.0M, and with a 1/1 - 2/1 $CDCl_3$/2 mmole of sample ratio whenever possible, would result in small solvent shifts, of the order of 0.1 ppm or less. It would have been preferable to work at lower concentrations ($\sim$ 0.1M) to effectively remove these shifts, but that was not practical.

(2) For methylene carbons the differences between the absolute ^{13}C chemical shift values remained more constant than the absolute values themselves. Therefore the solvent effects could be minimised by choosing the appropriate values of $\delta^{o}_{CH_2}$ for hydrocarbons, acids and esters. These values, shown below, were averaged from the bulk

$\delta^{o}_{CH_2}$	- (hydrocarbon)	29.90 ppm
	- (carboxylic acid)	29.81 ppm
	- (methyl ester)	29.85 ppm

methylene chemical shift signal for C_{14} or longer molecules, such that the differences were probably due to 1M -COOMe or -COOH in the medium rather than long range shielding influences.

(3) The terminal methyl group was less susceptible to variations in shift caused by solvent effects.

(4) Unsaturated carbons and the α-methylene carbon adjacent to the carboxylic head group had solvent shifts which were more variable both in magnitude and direction.

(5) The study aimed at predicting chemical shifts using substituent shielding parameters to an accuracy of $\pm$ 0.05 ppm, but the averaged data in the succeeding sections is quoted to the nearest 0.01 ppm. Only deviations of $\pm$ 0.10 ppm or greater were regarded as statistically significant, and hence of diagnostic value.

3:2:3 ^{13}C Chemical Shift Data for Saturated and Monounsaturated Fatty Acids and their Methyl Esters

The averaging process used to compile mean ^{13}C chemical shift values and shielding parameters, for this and subsequent sections, did not include signals which contained more than two or three carbon atoms (where resolution would be reduced) or signals where significant deviations from the general pattern occurred.

The chemical shifts of long chain alkanoic acids, methyl alkanoates and alkanes are presented in Table 16, together with the sets of shielding parameters for -COOH, -COOMe and $-CH_3$ groups on methylene carbon atoms. It is noteworthy that the unperturbed methylene signal, $\delta^{o}_{CH_2}$, differed slightly for each group of molecules. Carboxylic acids could be readily distinguished from their methyl esters. Both had a weak C(1) signal owing to the absence of directly bonded hydrogen atoms. The acid signal, at approximately 180 ppm, had about one third of the peak height of a typical methylene signal, whereas the ester signal, occurring at 174 ppm, was very weak and sometimes masked by the baseline noise. However, the methyl ester was always identifiable by its methoxy carbon shift at 51 ppm. The shielding parameters for acids and esters were very similar. The magnitudes of the ζ and η- parameters were rather uncertain, as they were based on a limited amount of information from shorter chain molecules (C_8-C_{12}). The averaged values were less than would be predicted assuming a logarithmic decrease from the δ and ϵ-parameters [ie. assuming that the linear electric field term is the only contributing factor to the chemical shift at and beyond C(5)], but the ζ and η-parameters were real as indicated by the differences in chemical shift of allylic or propargylic carbons for $\Delta 8$ and $\Delta 9$ unsaturation.

Table 17 gives unsaturated carbon atom shifts and the sets of shielding parameters for isolated olefinic and acetylenic centres. The parameters for terminal unsaturation are calculated against $\delta^{o}_{CH_2}$, not the corresponding carbon shift in an alkane. The principal features that distinguished cis and trans olefins and acetylenes were the following:-

TABLE 16: Averaged Chemical Shift Values and Shielding Parameters

Carbon atom	C(1)	C(2)	C(3)	C(4)	C(5)	C(6)
Positional subscript for shielding parameter		α	β	γ	δ	ϵ
Alkanes						
Alkanoic acids	180.61 $\triangle$(COOH)	34.25 +4.44	24.81 -5.00	29.23 -0.58	29.40 -0.41	29.57[2] -0.24[2]
Methyl alkanoates	174.06[1] $\triangle$(COOMe)	34.17 +4.32	25.11 -4.74	29.35 -0.50	29.48 -0.37	29.64 -0.21[3]

For each class of compound, the upper line gives the observed chemical shift (in ppm) for that particular carbon atom shown above, and the lower line shows the value of the substituent shielding parameter whose positional subscript is shown above.

1. Also a methoxy carbon shift at 51.27 ppm
2. Estimated values for ${}_{\zeta}\triangle$(COOH) = -0.09, ${}_{\eta}\triangle$(COOH) = -0.05
3. Estimated values for ${}_{\zeta}\triangle$(COOMe) = -0.08, ${}_{\eta}\triangle$(COOMe) = -0.04

for Long Chain Alkanes, Alkanoic Acids and Methyl Alkanoates

C(n)	C(ω-4)	C(ω-3)	C(ω-2)	C(ω-1)	C(ω)
$\delta^{o}_{CH_2}$	δ	γ	β	α	
29.90	29.87 -0.03	29.56 -0.34	32.14 +2.24	22.85 -7.05	14.14 $\triangle(CH_3)$
29.81		29.50 -0.31	32.07 +2.26	22.80 -7.01	14.12 $\triangle(CH_3)$
29.85		29.54 -0.31	32.11 +2.25	22.83 -7.02	14.13 $\triangle(CH_3)$

TABLE 17: Shielding Parameters and Unsaturated Carbon Atom ^{13}C Shifts for Isolated Unsaturated Systems

Unsaturation	Chemical shift of unsaturated carbons	Shielding Parameters	α	β	γ	δ	ϵ	ζ
H_2C:CH-	114.15(ω) 139.15 (ω-1)	$\Delta(H_2C{:}CH\text{-})$	+4.09	-0.71	-0.53	-0.17		
-CH:CH- cis	129.90	$\Delta_{(c)}$	-2.51	0	-0.43	-0.17		
		$\Delta_{CH_3(c)}$	-1.40	+0.27	-0.36	-0.14		
-CH:CH- trans	130.40	$\Delta_{(t)}$	+2.87	-0.07	-0.54	-0.17		
		$\Delta_{CH_3(t)}$	+3.74	-0.11	-0.49	-0.17		
HC:C-	68.16(ω) 84.58 (ω-1)	$\Delta(HC{:}C\text{-})$	-11.35	-1.23	-1.03	-0.63	-0.24	-0.17
-C:C-	80.19	$\Delta_{(a)}$	-10.95	-0.53	-0.86	-0.50	-0.13	-0.09
		$\Delta_{CH_3(a)}$	-10.73	+0.30	-0.64	-0.49	-0.08	

(1) The α-methylene carbon had a characteristic value, if unperturbed, of:

27.3 ppm for cis-olefins

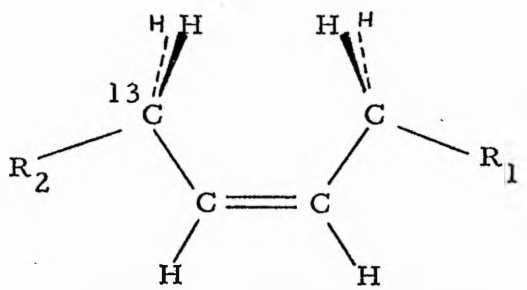
32.7 ppm for trans-olefins

33.9 ppm for terminal olefins

18.8 ppm for acetylenes.

(2) sp Carbon shifts occurred in the 70-90 ppm region of the spectrum, and were of approximately one third the peak height of a typical methylene signal. Olefinic sp_2 carbons resonated in the 120-140 ppm region, but the upfield shift of 0.5 ppm for cis-olefins compared to trans-olefins was not always an immediate diagnostic tool in the appraisal of a CMR spectrum, since large and complex splittings of the isolated olefinic signal could occur.

The cis-allylic carbon shift was 5.38 ppm upfield from the trans-allylic carbon shift. This is caused by a strong upfield γ-steric effect in the former, where the favoured conformation for the cis-allylic methylenes, according to both Batchelor et al [150] and de Haan and de Ven [163] is:-



(I)

The vinylic α-methylene carbon had a shift 1.22 ppm downfield from that for a trans-allylic carbon. Since neither can have a γ-steric effect contributing to their ^{13}C chemical shifts, this extra downfield shift probably resulted from the polarisation of the vinyl double bond. Both terminal double and triple bonds are polarised in the same direction, as shown by the sp_2 and sp shifts quoted in Table 17 (a partial negative charge resides on the terminal carbon, and a partial positive charge on the penultimate carbon), but the propargylic methylene to a terminal triple bond exhibits a 0.40 ppm upfield shift compared to that for a mid-chain triple bond. Therefore a simple explanation of the α-methylene shifts of terminal unsaturation in terms of charge polarisation is not adequate.

Using the shielding parameters obtained for mid-chain π-systems, deviations from the predicted values occurred in the methylene group chemical shifts as the π-system approached either the carboxyl or the methyl end. In order to correct calculations Table 18 lists these deviations for $\Delta 3$- $\Delta 6$ and $\omega 2$- $\omega 5$ unsaturation. The deviations were generally quite small, rarely being greater than ± 0.6 ppm. Some attempts were made to explain those at the methyl end by invoking preferred conformers [150, 163], but those at the carboxyl end will be more difficult to rationalise since δ_S, δ_A and δ_{FE} terms could all change markedly.

Batchelor et al [150] proposed a δ-shift across a cis-double bond which is similar in magnitude and direction to that suggested by Grant and Paul for alkanes [153]. Replacing R_1=H by R_1=CH_3- or -CH_2- when R_2 = alkyl favours conformer (I) above, resulting in less steric compression of the hydrogen atoms and hence a downfield shift of 0.25 ppm on the allylic carbon indicated. Obviously, no comparable effect will occur across a trans-double bond. The δ-shift across a cis-double bond can be seen from the results in Table 18. The allylic ^{13}C shift in going from an $\omega 2$ to an $\omega 3$ trans-olefin moves upfield by 0.25 ppm, and this may be caused by changes in δ_E, δ_{FE}, or δ_A terms. The corresponding shift difference for $\omega 2$ and $\omega 3$ cis-olefins is zero, so a steric contribution of +0.25 ppm must be operative.

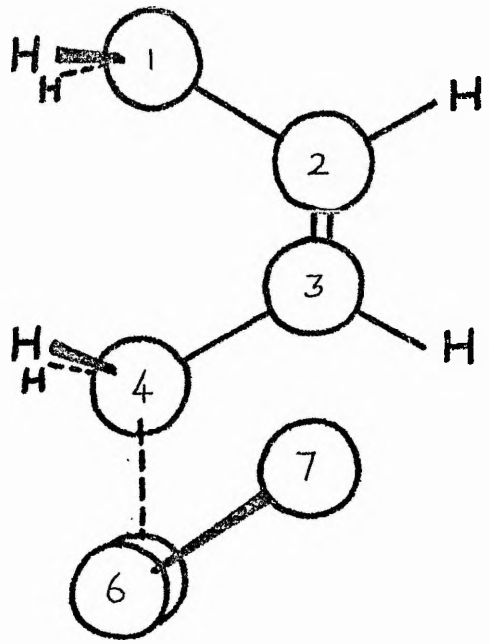
The δ-cis-allylic shift, also proposed by Batchelor et al [150], deserves mention as it will be used to explain the diallylic carbon shift of the methylene-interrupted c,c- diene system. An upfield shift of 0.31 ppm was observed at the α-methylene to a cis-olefin if there was a carbon atom on the same side of the double bond in the δ-position. This shielding effect is believed to occur as a result of the removal of severe g+g- interactions between the δ-methylene or methyl group and the nearer olefinic hydrogen, as shown by Figure 23. For a trans-olefin no δ-allylic shift was observed because the double bond does not have such a preferred conformation. From Table 18, comparing the results of the $\omega 3$ and $\omega 5$ cis, $\omega 3$ cis and trans, and $\omega 4$ cis and trans olefinic pairs shows an δ-allylic shift of -0.31 ppm

TABLE 18: Deviations in the Expected ^{13}C Shift Values for Methylene Carbons when an Unsaturated Centre is Close to the Methyl or Carboxyl End

Position and nature of unsaturation[1]	The deviation, expressed as $\delta_{expt} - \delta_{predicted}$ [2-4]
Δ3	COO-CH_2—C.C—CH_2— CH_2— CH_2-
c	+1.05 +0.60 -0.23
a	+2.67 +0.36 -0.09 -0.10
Δ4	COO-CH_2— CH_2— C.C-CH_2— CH_2-
c	+0.10 +0.35 +0.25
a	+0.40 +0.74 +0.12 -0.14
Δ5	COO-CH_2— CH_2— CH_2— C.C-CH_2-
c	-0.25 -0.08 -0.18 +0.11
t	-0.26 -0.17 -0.19 +0.07
a	-0.38 -0.11
Δ6	COO-CH_2-CH_2-CH_2— CH_2— C.C-
a	-0.15 +0.07
ω2	CH_3C.C-CH_2— CH_2— CH_2— CH_2-
c	-0.11 -0.30 -0.07 -0.08
t	+0.25 -0.07 -0.06
a	+0.25 -0.09
ω3	CH_3-CH_2-C.C-CH_2-
c	+0.31 -0.09
a	+0.63
ω4	CH_3-CH_2— CH_2-C.C-
c	+0.16 +0.16
t	+0.09 -0.15
a	+0.40 -0.25
ω5	CH_3-CH_2—CH_2— CH_2-C.C-
a	+0.08 -0.13

1. Where either c, t or a is absent this indicates that there were no significant deviations for that particular isomer, except for Δ3t and Δ4t isomers, which were not available.
2. -C.C- denotes a double or triple bond.
3. A blank denotes a deviation $\leqslant \pm 0.05$ ppm, which is within experimental error.
4. $\delta_{predicted}$ is calculated from the data in Tables 16 and 17, with the shielding influence of CH_3- or COO- taken as operating through the unsaturated centres.

Figure 23: The δ-Allylic Shift of a cis-Olefin



 - Carbon atom.

The cis-double bond is drawn in its preferred conformation with respect to the allylic hydrogen atoms, and the C(3) to C(7) part of the molecule is shown as the g+g- conformer. If C(3) was a methylene group, there would be a strong steric repulsion between the hydrogen atoms and those of the C(7) methylene group, precluding the g+g- conformer. However, because C(3) is sp_2, and owing to the favoured conformation about the cis double bond, its hydrogen atom is further removed from C(7) and so the δ-downfield shift for C(4) is partly lost.

for cis-olefins.

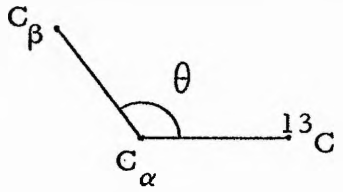
Less explanation of the shielding of adjacent methylenes by a triple bond has been offered in the literature [152]. The anisotropic shielding effect of a triple bond is an often cited phenomenon, but the electric field polarisation of adjacent methylene groups, caused by a triple or a double bond, has not been evaluated. Since an isolated π-bond has no permanent dipole, this contribution may be zero. The ^{13}C chemical shifts of methylene carbons in the α-position relative to a double or triple bond can be rationalised in terms of the α,β and γ regression parameters derived by Grant and Paul for linear alkanes [153] if certain assumptions are made. Considering that Grant and Paul's parameters have a value essentially independent of the hybridisation of the carbon atom causing the effect, but are dependent on geometry, then the ^{13}C shift of a trans-allylic carbon can be expressed by Equation 9, where on steric grounds A_γ and A_δ contributions cannot exist (See also Equation 6).

$$\delta_{\text{trans allylic}} \simeq \delta^{o}_{CH_3} + A_\alpha + A_\beta \qquad \text{Eq. 9}$$

$$\simeq 14.12 + 9.12 + 9.38$$

$$\simeq 32.62 \text{ ppm (Observed value 32.77 ppm)}$$

For a propargylic carbon, the regression analysis parameter A_α is present, and once again, on steric grounds, the A_γ and A_δ terms are absent. The A_β parameter has not yet received an adequate explanation, but if it has an angular dependance such that at $\theta = 180^{o}$ it has zero value, then the chemical shift of the propargylic carbon



can be expressed by Equation 10.

$$\delta_{\text{propargylic}} \simeq \delta^{o}_{CH_3} + A_\alpha \qquad \text{Eq. 10}$$

$$\simeq 23.24 \text{ ppm (Observed value 18.8 ppm)}$$

Therefore the propargylic methylene is expected to have a ^{13}C chemical

shift considerably upfield from a bulk methylene. Adding to the predicted value of 23.24 ppm is probably a strong anisotropic shielding effect such that the recorded shift of 18.8 ppm is in accord with the prediction.

A δ-propargylic shift of -0.63 ppm is proposed, on similar lines of evidence as presented for the δ-cis-allylic shift. However, since the sp carbon in question has no hydrogen atom, it is to be expected that the severity of the g+g- interaction (Figure 23: replace cis-olefinic C(3) by acetylenic) will be further reduced, leading to a δ-propargylic upfield shift larger than the δ-cis-allylic upfield shift. This effect will also contribute to the upfield propargyl shift discussed in the previous paragraph.

Table 19 gives the shielding parameters, obtained by different research workers, for the effect of a cis double bond on the methylene chain. There is very good agreement, and since different methods of assignment were used in some of the studies this corroborates the shielding parameter approach to CMR spectral interpretation as well as demonstrating the considerable reproducibility of the technique. For the three studies where deuterochloroform was the solvent the absolute differences in the value of the parameters had a maximum of 0.12 ppm, but the relative differences (eg. $_{\alpha}\Delta(c)-_{\beta}\Delta(c)$) were much less, the maximum being 0.04 ppm (Section 3:2:2).

The ^{13}C chemical shifts of carbon-carbon π-bonds were very diagnostic, both in identifying the type and extent of unsaturation and in locating the position(s) of unsaturation in fatty acids. Shift values and peak heights for isolated unsaturation have already been mentioned. For a mono-alkenoic or -alkynoic acid (or ester) two sp_2 or sp carbon signals were observed if the π-bond was in the $\Delta 2$- $\Delta 10$ or the $\omega 2$- $\omega 4$ positions. The effect of the methyl end and of the carboxyl end relative to the chemical shift of an unperturbed cis- or trans-olefinic or acetylenic bond is presented in Tables 20 and 21 respectively. These effects are additive. For example, the predicted ^{13}C chemical shifts of C(8) and C(9) in 11:1(8c) [$\omega 3$] acid are given by Equations 11 and 12, together with the observed values.

TABLE 19: A Comparison of Shielding Parameters for a 1,2-Dialkyl-*cis*-Olefin Obtained by Different Workers

Origin	Reference	△(c)				Measurement of spectra	
		α	β	γ	δ	Solvent	Ratio sample:solvent (w/v)
This work		-2.51	0	-0.43	-0.17	$CDCl_3$	1:2
Batchelor *et al*	150	-2.45	+0.10	-0.40	-0.10	$CHCl_3$	1:3
Tulloch and Mazurek	170	-2.42	+0.11	-0.31	-0.09	$CDCl_3$	1:6
Dorman *et al* (*cis*-3-octene)	154	-2.6	0	-0.4	-0.1	cyclo-hexane	unknown
Bus *et al*	173	-2.45	+0.05	-0.35	-0.10	$CDCl_3$	1:12-1:6

TABLE 20: The Effect of the Methyl End on the ^{13}C Chemical Shifts of Unsaturated Carbons

Position of unsaturation ω m	Cis-olefinic		Trans-olefinic		Acetylenic	
	C(ωm)	C(ω(m+1))	C(ωm)	C(ω(m+1))	C(ωm)	C(ω(m+1))
ω 4	-0.25	+0.23	-0.25	+0.21	-0.15	+0.22
ω 3	+1.63	-0.53	+1.50	-0.90	+1.35	-0.61
ω 2	-6.33	+1.01	-5.92	+1.39	-4.94	-0.83
ω 1 [1]	-16.25	+8.75	-16.25	+8.75	-12.05	+4.49

1. The parameters for the vinylic system, where a cis and trans nomenclature does not apply, are measured against the value for an isolated trans double bond of 130.40 ppm.

C(8) δ = 129.90-0.25-0.53 = 129.12 ppm Eq. 11

δ_{obs} = 129.11 ppm

C(9) δ = 129.90+0.25+1.63 = 131.78 ppm Eq. 12

δ_{obs} = 131.78 ppm

The splitting of the isolated signal in ω 1 to ω 4 unsaturation followed a similar pattern for cis- and trans-olefins and acetylenes, suggesting a dominant, underlying mechanism common to all three, with additional, smaller, differential effects superimposed. An interesting feature is that when using a regression analysis approach on the ω (m) unsaturated carbon, the parameters are very similar to those obtained by Grant and Paul for n-alkanes [153] (Table 22). This points to the α, β, γ, and δ effects used to explain the ^{13}C chemical shifts of n-alkanes also making the major contribution to the shift of the ω (m) unsaturated carbon atom. Generally, the ω(m+1) carbon appeared to be polarised in the opposite direction with a value

TABLE 21: The Effect of the Carboxylic Acid and Methyl Ester Groups on the ^{13}C Chemical Shifts of Unsaturated Carbons

Position of unsaturation Δn	Carboxylic acids						Methyl esters[1]			
	a		t		c		c		t	
	C(n)	C(n+1)	C(n)	C(n+1)	C(n)	C(n+1)	C(n)	C(n+1)	C(n)	C(n+1)
Δ 2	-7.44	+12.46	-		-		-10.49	+20.79	2.	
Δ 3	-9.50	+4.40	-		-9.85	+4.27	-8.90	+3.50		
Δ 4	-2.44	+1.28	-		-2.84	+2.05	-2.37	+1.64		
Δ 5	-1.57	+1.34	-1.68	+1.58	-1.70	+1.53	-1.45	+1.31		
Δ 6	-0.83	+0.63	-0.91	+0.73	-0.88	+0.72	-0.72	+0.58		
Δ 7	-0.45	+0.35	-0.51	+0.41	-0.44	+0.41	-0.41	+0.32	-0.38	+0.35
Δ 8	-0.28	+0.21	-0.27	+0.27	-0.25	+0.25	-0.22	+0.23	-0.19	+0.24
Δ 9	-0.15	+0.15	-0.17	+0.14	-0.11	+0.15	-0.12	+0.12		
Δ10	-0.05	+0.12	-0.09	+0.07	-0.07	+0.03	-			
Δ11			-0.05	+0.03	-0.01	+0.06				

1. No data are available for methyl alkynoates from this study, but Bus et al have published data [174].
2. According to Bus et al [174] the values of Δ^t (COOMe) for Δ2t are C(n) -9.40, C(n+1) +19.45, and for Δ 3t are C(n) -8.90, C(n+1) +4.60. Beyond the Δ 3 position Δ^t(COOMe) has values similar to Δ^c(COOMe).

TABLE 22: Regression Analysis Parameters for $\omega 1$- $\omega 4$ Unsaturation

	Regression Parmaeter			
	A_α	A_β	A_γ	A_δ
n-alkane	+8.70	+9.27	-2.57	+0.3
ω(m) carbon, cis-olefin	+9.92	+7.96	-1.88	+0.25
ω(m) carbon, trans-olefin	+10.33	+7.42	-1.75	+0.25
ω(m) carbon, acetylene	+7.11	+6.29	-1.50	+0.22

smaller in magnitude. The terminal carbon for $\omega 1$ double and triple bonds had a large partial negative charge, which was to be expected since their protons are more acidic than usual.

Figures 24 and 25 depict graphically the changes in ^{13}C chemical shift of π-bonds caused by the polar head group. At or beyond the $\Delta 5$ position the shielding parameters lie on a smooth curve, decreasing in magnitude monotonically with increasing distance from the carboxylic acid or ester group. From data for cis and trans $\Delta 8$- $\Delta 12$ methyl octadecenoates Bus and Frost [164] noted the relationship for the shift difference between π-bond carbons, $\Delta\delta$, as $\Delta\delta = 2^n$ Hz, while Bat chelor et al explained the effect in terms of a linear, electric-field polarisation of the π-bond. Although a triple bond has a greater longitudinal polarisability than a double bond such that a larger $\Delta\delta$ might be expected, the (δ/e) term (Equation 4, Section 3:1) for a sp carbon is correspondingly smaller than for a sp_2 carbon, so that for a given position $\Delta\delta$ is more or less identical for double and triple bonds [150]. The splitting $\Delta\delta$ is not quite symmetrical about the signal for an isolated unsaturated centre, and is marginally smaller for methyl esters when compared with their carboxylic acids, presumably owing to the slightly smaller permanent dipole of -COOMe.

Departures from this pattern occurred for $\Delta 2$- $\Delta 4$ unsaturation (Table 21, Figures 24 and 25). Mesomeric effects

Figure 24: The Alteration in Chemical Shift Value of Unsaturated Carbon Atoms caused by the Carboxylate Head Group

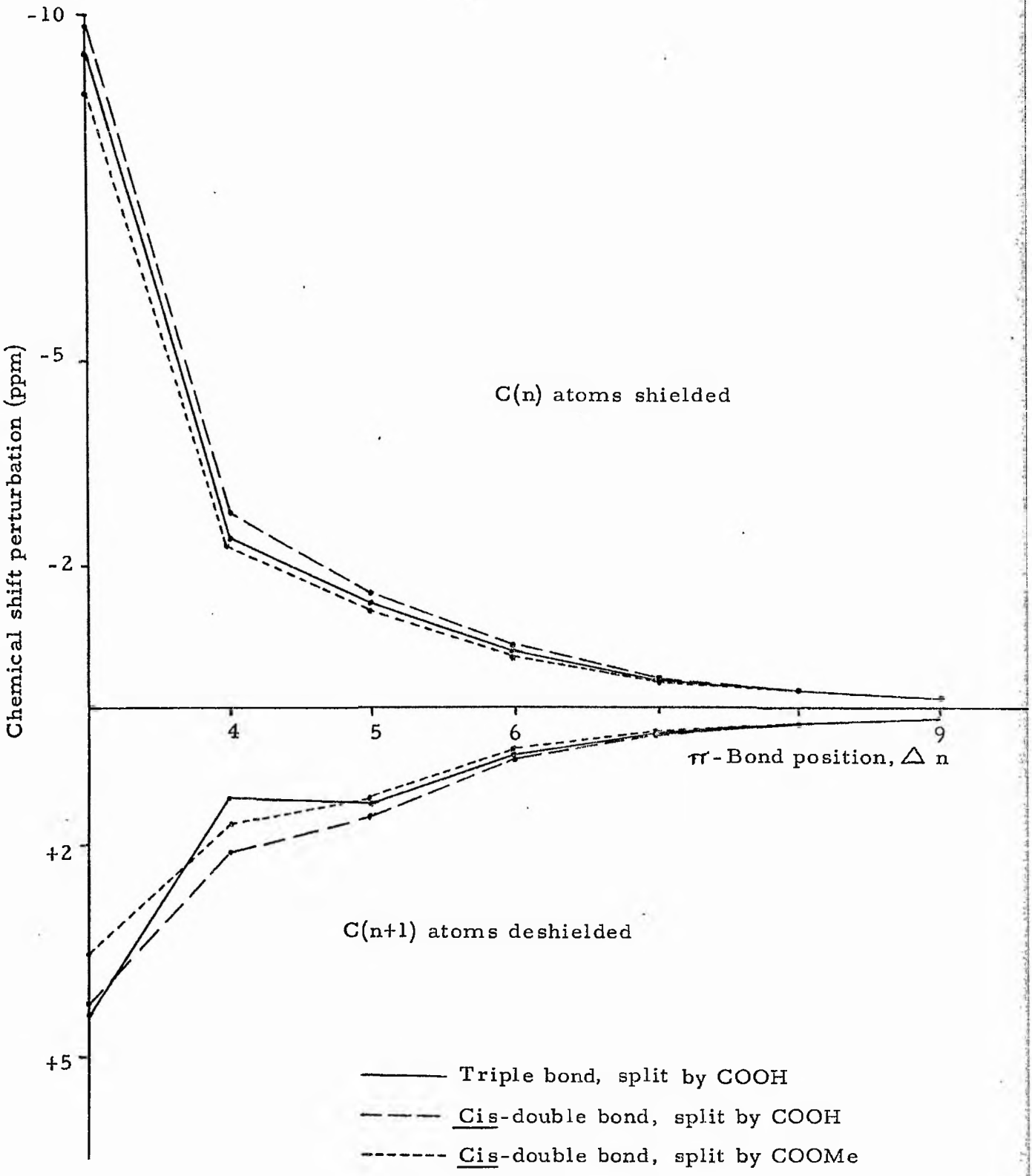
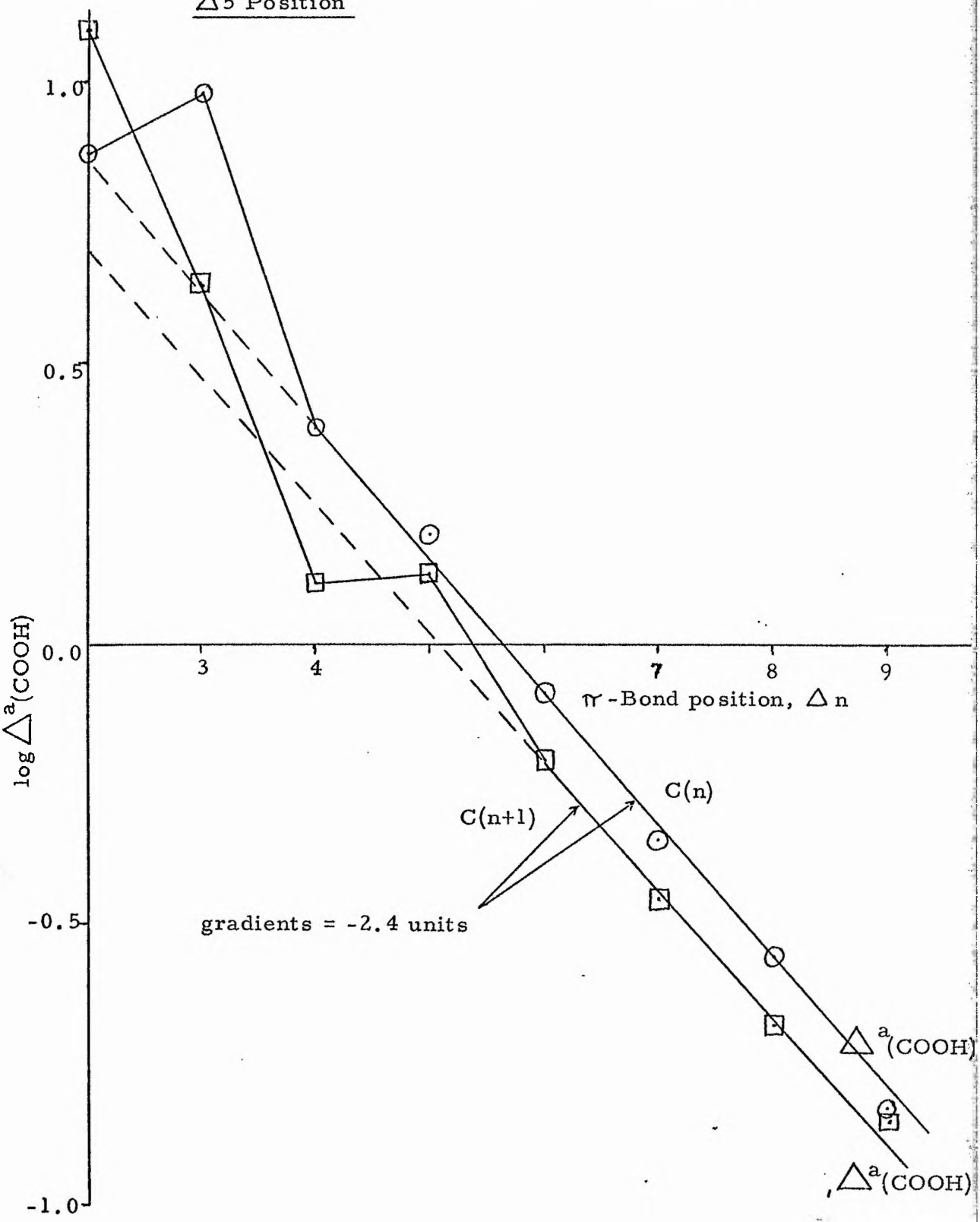


Figure 25: A Plot of Log Δ^a(COOH) Versus Triple Bond Position In Order to Show the Linear Relationship Beyond the Δ5 Position



could explain the large downfield shifts of C(3) in $\Delta 2$ conjugated unsaturation. The other major deviation was the large upfield shift of C(3) in $\Delta 3$ π-bonds. Possible explanations included a strong γ-steric interaction with the carboxyl group oxygen atoms and the onset of the importance of the squared term in the electric field polarisation of the π-bond.

Carboxylate ^{13}C chemical shifts were more variable in reproducibility than most other ^{13}C chemical shifts, and were not very useful or reliable in locating the position of unsaturation beyond the $\Delta 5$ position (Table 23).

TABLE 23: ^{13}C Chemical Shifts of Carboxylic Carbons Perturbed by Neighbouring Unsaturation

Position of unsaturation	Acetylenic acids	Trans-olefinic acids	Cis-olefinic acids	Cis-olefinic methyl esters	
				$-^{13}CO.O$	$-O^{13}CH_3$
none	180.61	180.61	180.61	174.06	51.27
$\Delta 5$	180.0 (180.17-179.76)	180.58	180.38 (180.15-180.50)	173.81	51.25
$\Delta 4$	178.74 (178.71-178.78)	-	180.02 (180.04-180.0)	173.10	51.21
$\Delta 3$	175.6 (175.51-175.69)	-	178.55 (178.48-178.68)	172.08	51.56
$\Delta 2$	158.44	-	-	166.53	50.71

- Data not available.

The bracketed figures indicate the range of chemical shift values, where appropriate.

3:2:4 Cyclopropene Fatty Acid Methyl Esters

From a limited amount of data (methyl 6,7-methyleneoctadec-6-enoate and methyl 9,10-methylenenonadec-9-enoate) it was possible to observe the characteristic ^{13}C chemical shifts for a mid-chain

unperturbed cyclopropene ring system. The unsaturated carbon atoms had a weak signal, of approximately a quarter of the peak height of a typical methylene signal, at 109.55 ppm, while the methylene bridge carbon resonated at 7.45 ppm. A tentative estimation of the shielding parameters for methylenes adjacent to the cyclopropene ring is given below.

△(cyclopropenyl)	α	β	γ	δ
	-3.73	-2.38	-0.3	-0.25

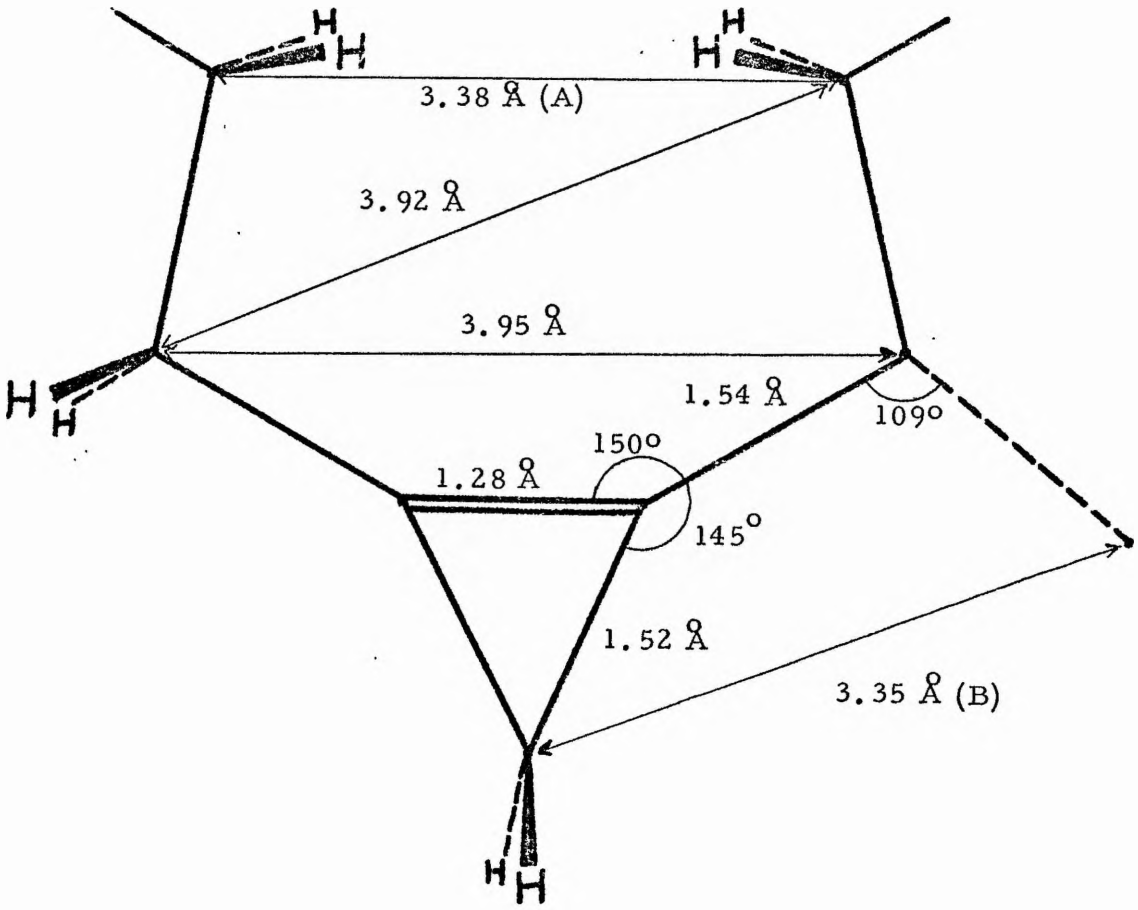
In particular, the α and β parameters may require inversion, but at least both show a marked shielding influence.

The chemical shift of the π-carbons lay almost midway between those for olefinic sp_2 and acetylenic sp carbon atoms, indicating an electronic structure somewhere between the two. This intermediacy is also apparent in the geometry of the cyclopropenyl "double" bond, which has a bond length of 1.28 Å and a bond angle of 150°, compared to the values of 1.34 Å and 120° for a double bond and 1.20 Å and 180° for a triple bond. The π-carbon chemical shift was split by the polar headgroup, presumably in the same manner as discussed previously. The $\Delta\delta$ value for methyl 6,7-methyleneoctadec-6-enoate was 1.32 ppm, which was close to that for a $\Delta 6$ cis-olefin ($\Delta\delta$ = 1.30 ppm) or a $\Delta 6$ triple bond ($\Delta\delta$ = 1.40 ppm, [174]) split by the methyl ester head group.

The methylene bridge carbon had the upfield shift characteristic of small, strained rings. The corresponding PMR chemical shift occurred at 0.76-0.78 ppm [180, 181], and Batchelor et al reported the ^{13}C chemical shift of the methylene bridge in the cis-cyclopropane ring as 11.06 ppm [150].

The α and β shielding parameters are both upfield. Considering the α-methylene group first, the γ-steric interaction occurring across a cis-olefin will be wholly removed (Figure 26), so that the cyclopropenyl α-methylene shift is expected to lie midway between the trans-allylic (32.7 ppm) and propargylic (18.8 ppm) ^{13}C chemical shifts. The observed value was 25.8 ppm. The β-methylene carbon had a larger upfield shift than hitherto encountered in olefins and acetylenes.

Figure 26: The Geometry of the Cyclopropene Ring System



1" = 1 Å

The corresponding distance between the allylic carbon atoms of a cis olefin is 2.88 Å, and between the 1,4-carbons in a gauche conformation in an n-alkane is 3.0 Å.

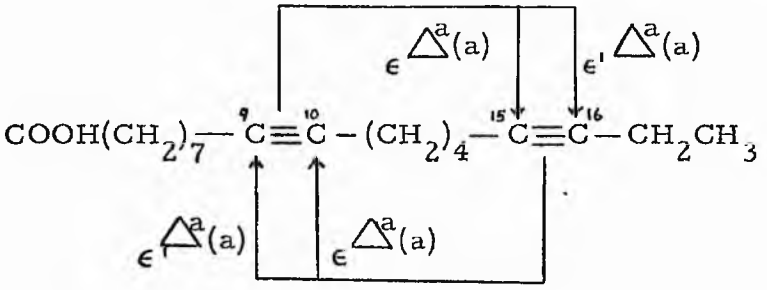
The drawing shows all carbon-carbon bonds in one plane.

It is proposed that this is due to an ϵ-shielding effect (Figure 26, A), arising between the steric interactions of both β-methylene groups as a consequence of the geometry of the cyclopropene ring. Figure 26 shows the most favoured conformation for this effect, and that a γ-steric upfield contribution due to non-bonded hydrogen-hydrogen repulsions between the methylene bridge and the β-methylene (B) can be ruled out on account of the moderate minimum carbon-carbon distance coupled with the fact that the hydrogen atoms of the methylene bridge can never be orientated to give a large steric repulsion.

3:2:5 ^{13}C Chemical Shifts of π-Carbon Atoms in Non-Conjugated, Polyunsaturated Systems

Additional shielding parameters are required, alongside those already mentioned in Section 3:2:3 for monounsaturated acids, in order to describe PUFA CMR spectra. These parameters were extensively evaluated for c,c-dienoic, all-c-polyenoic and diynoic acids and esters, and incomplete information was also obtained for t,t-dienoic acids and diunsaturated acids of mixed functionality. Conjugated unsaturation was not examined, but was believed not to be so readily interpretable by a shielding parameter approach (Section 3:2:6).

The simplest case to consider is a diunsaturated acid where two π-bonds of the same type (c, t or a) are separated by n methylene groups. 18:2(9a15a), where n = 4, is the example to be discussed. It is apparent that there may be an interaction between the triple bonds



such that the effect of triple bond Δ15a on C(9) and of Δ9a on C(16) can be expressed by a shielding parameter, $\epsilon'\Delta^{a}(a)$, and the effect of Δ15a on C(10) and of Δ9a on C(15) by $\epsilon\Delta^{a}(a)$. The shielding

parameter nomenclature has been given in Section 3:2:1, Equation 5, and the dash on the positional subscript denotes the effect on the π-carbon further removed from the perturbing π-bond. This dash nomenclature is routinely used in this section. The Δ9a bond also feels a perturbation from the carboxylic acid group and the Δ15a bond (ω3) a perturbation from the methyl end. If the additivity of shielding and deshielding effects for ^{13}C chemical shifts is assumed, the $\Delta^a(a)$ parameters can be calculated from the knowledge of the experimental sp carbon atom shifts [171], the shift of an unperturbed sp carbon (Table 17: δ^o_a = 80.19 ppm) and the appropriate $\Delta^a(COOH)$ and $\Delta^a(CH_3)$ parameters (Tables 20 and 21). The observed chemical shifts (79.16, 79.94, 80.36 and 81.85 ppm) are assigned to C(9), C(10), C(15) and C(16) to give the most consistent values for the $\Delta^a(a)$ parameters.

$$\delta C(9) = 80.36 \text{ ppm} = 80.19 \text{ ppm} + {}_{\epsilon'}\Delta^a(a) + {}_{\Delta 9}\Delta^a(COOH)$$
$$\therefore\ {}_{\epsilon'}\Delta^a(a) = 80.36 - 80.19 + 0.15 = +0.32 \text{ ppm}$$

$$\delta C(10) = 79.94 \text{ ppm} = 80.19 \text{ ppm} + {}_{\epsilon}\Delta^a(a) + {}_{\Delta 9'}\Delta^a(COOH)$$
$$\therefore\ {}_{\epsilon}\Delta^a(a) = 79.94 - 80.19 - 0.15 = -0.40 \text{ ppm}$$

$$\delta C(15) = 79.16 \text{ ppm} = 80.19 \text{ ppm} + {}_{\epsilon}\Delta^a(a) + {}_{\omega 3'}\Delta^a(CH_3)$$
$$\therefore\ {}_{\epsilon}\Delta^a(a) = 79.16 - 80.19 + 0.61 = -0.42 \text{ ppm}$$

$$\delta C(16) = 81.85 \text{ ppm} = 80.19 \text{ ppm} + {}_{\epsilon'}\Delta^a(a) + {}_{\omega 3}\Delta^a(CH_3)$$
$$\therefore\ {}_{\epsilon'}\Delta^a(a) = 81.85 - 80.19 - 1.35 = +0.31 \text{ ppm}$$

The parameters ${}_{\epsilon'}\Delta^a(a)$ and ${}_{\epsilon}\Delta^a(a)$ show good internal agreement, vindicating both the initial assumption and the correctness of the assignment. Average values for π-carbon: π-bond shielding parameters are presented in Table 24 for c,c-dienes (n=1-5), diynes (n=1-6) and t,t-dienes (n=1, 3).

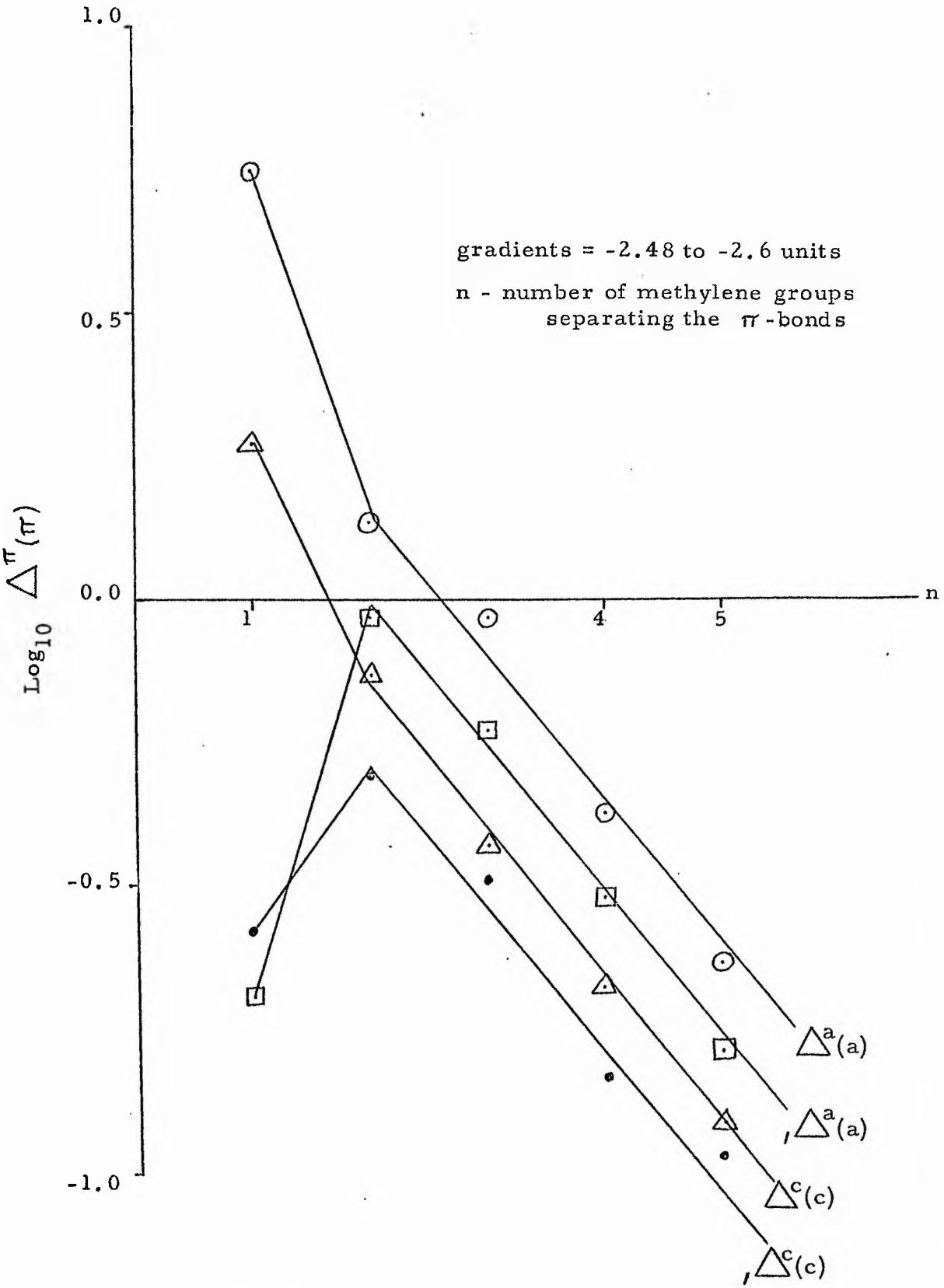
The π-π interactions are believed to be caused by a fluctuating dipole-induced dipole interaction, with the induced polarisation of charge at the π-bond depending linearly on the electric field vector at that bond, when $n \geqslant 2$. Figure 27 shows that the graph of $\log_{10} \Delta^{\pi}(\pi)$ versus n is linear for $n \geqslant 2$, with a gradient equal

TABLE 24: π-Carbon: π-Bond Shielding Parameters

Positional Subscript, (n)	Diynes			c,c-Dienes			t,t-Dienes		
	$\Delta^a(a)$	$,\Delta^a(a)$	No.[1]	$\Delta^c(c)$	$,\Delta^c(c)$	No.[1]	$\Delta^t(t)$	$,\Delta^t(t)$	No.[1]
β (n=1)	-5.52	+0.20	4	-1.87	+0.26	6	-1.75	+0.67	1
γ (n=2)	-1.35	+0.92	2	-0.73	+0.48	2			
δ (n=3)	-0.92	+0.58	2	-0.37	+0.32	2	-0.32	+0.28	1
ϵ (n=4)	-0.42	+0.30	3	-0.21	+0.14	2			
ζ (n=5)	-0.23	+0.16	1	-0.12	+0.11	1			
η (n=6)	-0.09	+0.13	1						

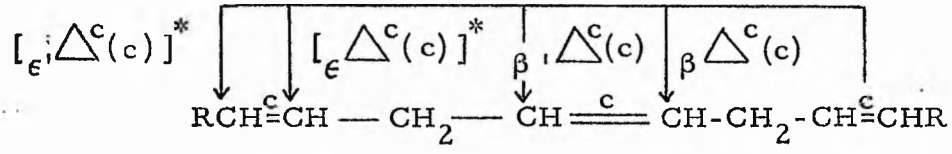
1. No. indicates the number of examples (ie. fatty acids) averaged to give the shielding parameters. Each fatty acid must give two values for each parameter.

Figure 27: A Plot of Log $\triangle^a(a)$ or Log $\triangle^c(c)$ Versus Distance Between The Two π-Bonds (n)



to that for other plots of log $\triangle$ versus distance where a linear electric field polarisation is thought to occur (ie. Figure 25, log $\triangle^{a}$(COOH) against $\triangle$). Because of the linearity at $n \geqslant 2$ steric effects must either be absent or self-cancelling. This is confirmed by the fact that the ${}_{\delta}\triangle^{t}(t)$ and ${}_{\delta}\triangle^{c}(c)$ parameters (n=3) are identical within experimental error. When n=1 large deviations occur from the relationship $\delta q \propto \vec{E}_z \propto r^{-x}$, where δq is the charge polarisation at the π-carbon atom, $\vec{E}_z$ is the electric field vector at that carbon and r is the distance from the dipole generating the electric field to the π-carbon. The mechanisms causing these deviations could be several: steric repulsions, hyperconjugation and the importance of the squared electric field term in the induced polarisation.

Data on tri- and tetraunsaturated acids or esters were only obtained for all-cis, methylene-interrupted polyenes. The c,c,c-triene, when unperturbed by carboxyl or methyl end influences, will show three π-carbon resonances. In order to describe these the ${}_{\beta}\triangle^{c}(c)$ and ${}_{\beta'}\triangle^{c}(c)$ shielding parameters evaluated from the c,c-dienoate CMR spectra were used, and two additional parameters were introduced - $[{}_{\epsilon}\triangle^{c}(c)]^{*}$ and $[{}_{\epsilon'}\triangle^{c}(c)]^{*}$. The asterisk denotes that these parameters may differ from ${}_{\epsilon}\triangle^{c}(c)$ and ${}_{\epsilon'}\triangle^{c}(c)$ obtained from the c,c-dienoate spectra, because the π-π interaction when n=4 will now be operating through $-CH_2CH{=}^{c}CHCH_2-$ and not $-(CH_2)_4-$. 129.90 ppm is the ^{13}C chemical shift of an isolated



$$\delta C(1) = \delta C(8) = 129.90 + {}_{\beta'}\triangle^{c}(c) + [{}_{\epsilon'}\triangle^{c}(c)]^{*}$$

$$\delta C(2) = \delta C(7) = 129.90 + {}_{\beta}\triangle^{c}(c) + [{}_{\epsilon}\triangle^{c}(c)]^{*}$$

$$\delta C(4) = \delta C(5) = 129.90 + {}_{\beta}\triangle^{c}(c) + {}_{\beta'}\triangle^{c}(c) .$$

cis-double bond.

Only $\omega 3$ and $\omega 6$ trienes were examined. The values for the additional $[{}_{\epsilon}\triangle^{c}(c)]^{*}$ and $[{}_{\epsilon'}\triangle^{c}(c)]^{*}$ parameters are shown

below, averaged and calculated separately for the $\omega 3$ and $\omega 6$ PUFA. The data indicated a small but statistically significant difference

	$_{\epsilon}\Delta^{c}(c)$ ppm		$_{\epsilon'}\Delta^{c}(c)$ ppm	
Dienes	-0.21		+0.14	
Trienes (average)	-0.27	$[\ ]^{*}$	+0.19	$[\ ]^{*}$
$\omega 3$ Trienes	-0.21		+0.10	
$\omega 6$ Trienes	-0.29		+0.22	

between $\omega 3$ and $\omega 6$ unsaturation, the cause of which was unknown. As the $_{\epsilon}\Delta^{c}(c)$ or $_{\epsilon'}\Delta^{c}(c)$ parameters calculated for the dienes and the trienes were similar, and since such parameters are a consequence of a through space interaction, it is inferred that the relative orientation of, and average distance between the π-bonds in question is not markedly altered by $-(CH_2)_4-$ or $-CH_2CH{=}CHCH_2-$ systems separating them.

The olefinic ^{13}C chemical shifts for 20:4(5c8c11c14c) methyl ester and 22:4(7c10c13c16c) acid were interpreted satisfactorily in terms of the four shielding parameters $_{\beta}\Delta^{c}(c)$, $_{\beta'}\Delta^{c}(c)$, $[_{\epsilon}\Delta^{c}(c)]^{*}$ and $[_{\epsilon'}\Delta^{c}(c)]^{*}$, the latter two values being derived from the $\omega 6$ trienes alone. The agreement between calculated and experimental values was within 0.15 ppm. A case could be made for shielding parameters describing the influence on each other of the outer, cis-double bonds in the tetraene system ie.

	Average value	Range of values
$[_{\theta}\Delta^{c}(c)]^{**}$	-0.11 ppm	-0.09 to -0.14 ppm
$[_{\theta'}\Delta^{c}(c)]^{**}$	+0.09 ppm	+0.04 to +0.13 ppm

The double asterisk indicates that the effect is transmitted through the two, inner, cis-double bonds. The uncertainty over the reality of these parameters was caused by the CMR spectra being run on dilute solutions of the acid or ester (0.2-0.3M). Solute effects could not be ruled out, as measurements to determine $\Delta^{c}(c)$ were almost invariably based on approximately 1M solutions. The values of these

parameters are at the limit of statistical significance discussed in Section 3:2:2. The magnitude of the $\Delta^{c}(c)$ parameters measured for c,c-dienes was low by n=5 (Table 24), and effectively zero at n=7, so if $[\ _{\theta}\Delta^{c}(c)\ ]^{**}$ and $[\ _{\theta'}\Delta^{c}(c)\ ]^{**}$ are real (n=7) the average, through-space distance between the outer π-bonds in the all-c-tetraene must be shorter than if separated by seven methylene groups, and perhaps the π-bonds in the tetraene also have a more favourable relative orientation.

Table 25 lists the shielding parameters required to interpret all the possible mixed, diunsaturated methylene-interrupted systems (c,t; c,a; a,t) and for completeness also includes data from Table 24 (c,c; t,t; a,a).

TABLE 25: The Shielding Parameters for Unsaturated Carbons in Methylene-Interrupted Diunsaturated Systems

Parameter	Value	Parameter	Value	Number of fatty acids examined to give the averaged values
$_{\beta}\Delta^{c}(c)$	-1.87	$_{\beta'}\Delta^{c}(c)$	+0.26	6
$_{\beta}\Delta^{t}(c)$	-1.96	$_{\beta'}\Delta^{t}(c)$	+0.40	3
$_{\beta}\Delta^{a}(c)$	-1.81	$_{\beta'}\Delta^{a}(c)$	-0.19	4
$_{\beta}\Delta^{c}(t)$	-2.07	$_{\beta'}\Delta^{c}(t)$	+0.51	3
$_{\beta}\Delta^{t}(t)$	-1.75	$_{\beta'}\Delta^{t}(t)$	+0.67	1
$_{\beta}\Delta^{a}(t)$	-2.57	$_{\beta'}\Delta^{a}(t)$	+1.77	1
$_{\beta}\Delta^{c}(a)$	-4.69	$_{\beta'}\Delta^{c}(a)$	+1.31	4
$_{\beta}\Delta^{t}(a)$	-5.43	$_{\beta'}\Delta^{t}(a)$	+1.31	1
$_{\beta}\Delta^{a}(a)$	-5.52	$_{\beta'}\Delta^{a}(a)$	+0.20	4

In order to make an unambiguous assignment to mixed function, diunsaturated acids or esters, two fatty acids or esters need to be examined, one of which must have either methyl or carboxyl end

perturbation affecting its unsaturation. These criteria were not fulfilled for the trans-en-yne, so its assignment was tentative as it was based on analogy with the cis-en-yne system.

The general pattern shown by Table 25 is the shielding of the inner π-carbon atoms of the diunsaturated system and the deshielding of the outer π-carbon atoms. The differences between cis and trans isomers show that configuration and conformation influence the interactions between π-bonds separated by a single methylene group. The only systems where unrestricted rotation about carbon-carbon single bonds in the unsaturated region can occur are a,a and a,t. For the remainder conformational preferences will alter the average relative orientation of the π-bonds, changing the δ_A and δ_{FE} terms (Section 3:1, Equations 1 and 2) while steric repulsions between olefinic hydrogens and other hydrogens will change the δ_S contribution.

3:2:6 ^{13}C Chemical Shifts of the π-Carbon Atoms in Methyl 14,15-Dehydrocrepenynate: An Attempted Assignment from an Undecoupled Spectrum

Methyl crepenynate, 18:2(9c12a), and methyl 14,15-dehydrocrepenynate, 18:3(9c12a14c), were isolated from the seed oil of Afzelia bipindensis [178] by a sequence involving the removal of the saturates as their urea-adducts, $AgNO_3$-silica column chromatography of the remaining unsaturated methyl esters, and Ag^+TLC of the crepenynate and 14,15-dehydrocrepenynate rich fraction (10% $AgNO_3$, dual development, PE12, 50 mg or less of material applied to each preparative plate). The structures of crepenynate and 14,15-dehydrocrepenynate were reported previously [179] and were confirmed here by GLC and Ag^+TLC behaviour and PMR spectroscopy. Their CMR spectra were not, however, reported in the earlier work.

Fatty Ester	Ag^+TLC - R_f value	ECL (20% DEGS, 190°C)
18:1(9c)	0.60	18.6
18:3(9c12a14c)	0.54	22.6
18:2(9c12a)	0.48	21.8
18:2(9c12c)	0.35	19.1

18:3(9c12a14c) was the only conjugated unsaturated system examined in this study, apart from $\Delta 2$ unsaturation, and its spectrum could not be readily interpreted. The conjugated enyne could not be treated using the shielding parameters derived from isolated double and triple bonds, owing to π-electron delocalisation and different steric and conformational restraints. Although molecular polarisability can often be enhanced by conjugation the electric field polarisation produced by the carboxylate group is likely to have a smaller effect on conjugated than non-conjugated unsaturation, when measured by ^{13}C chemical shift differences, as in conjugated systems the polarisation is increased by further separation of the charge and not by an increase in the magnitude of the charge [150]. Comparing the correctly assigned 18:2(9c12a) CMR spectrum with that for 18:3(9c12a14c), and making the assumption that Δ12a and Δ12a14c unsaturation had, to a first approximation, similar effects on the Δ9c double bond then the following partial assignment for 14,15-dehydrocrepenynate was reached:-

	18:2(9c12a)	18:3(9c12a14c)
C(9)	131.14 ppm	131.54 ppm
C(10)	125.28 ppm	124.60 ppm
C(12)	78.40 ppm	} 77.24 ppm, 92.37 ppm
C(13)	80.06 ppm	
C(14)	-	} 109.75 ppm, 142.28 ppm
C(15)	-	

No CMR study on the -CH=CH.C≡C- system or data on its charge distribution were known. A charge distribution would have been helpful in checking the eventual assignment as an increase in electronic charge at a carbon atom results in an upfield shift of its

^{13}C resonance.

An attempt to resolve the C(12, 13) and C(14, 15) assignments was made by running the undecoupled CMR spectrum of 18:3(9c12a14c) in order to analyse the long range ^{13}C-^{1}H couplings (Figure 28). The olefinic carbons showed one-bond ^{13}C-^{1}H coupling, giving doublets with J_{CH}=152-165 Hz. This compared with the value of J_{CH}=156 Hz for ethylene [177]. The acetylenic carbons showed no one-bond ^{13}C-^{1}H coupling. The multiplets produced by geminal ($J_{^{13}CCH}$) and vicinal ($J_{^{13}CCCH}$) spin-spin coupling were expected to provide sufficient information to make a full assignment to the π-carbons. The multiplets for C(9), C(10) and C(15) should be similar, as each will contain geminal coupling to the adjacent olefinic hydrogen and to the allylic methylene hydrogens, to give a doublet splitting superimposed upon a triplet. C(14), the remaining olefinic carbon would only show geminal coupling to the adjacent olefinic hydrogen. Of the acetylenic carbons C(12) has geminal coupling to the propargylic methylene hydrogens only whereas C(13) has geminal coupling to the C(14) olefinic hydrogen. The multiplets may be complicated by vicinal ^{13}C-^{1}H coupling.

(i) C(14) and C(15). The triplet shown by the C(10) resonance at 124.60 ppm was similar to that of the resonance at 109.76 ppm, which was therefore taken to be C(15). The triplet was presumably a consequence of allylic coupling, $J_{C(10)C(11)H}=J_{C(15)C(16)H}$=6.5 Hz. The olefinic geminal coupling was very small, and this coincided with the value of J_{CCH}=2.5 Hz measured on ethylene [177]. The C(9) signal at 131.54 ppm should also show a triplet pattern, but the multiplets were considerably distorted. By a process of elimination the C(14) resonance was 142.28 ppm, but no distinct doublet was observed so $J_{C(14)C(15)H}$ was small.

(ii) C(12) and C(13). The dominant splitting of the 92.37 ppm resonance was a triplet. It was therefore C(12), with $J_{C(12)C(11)H}$=11.5 Hz. The fine double structure was probably attributable to vicinal coupling through the triple bond (ie. $J_{C(12)CC(14)H}$) rather than through the propargylic methylene

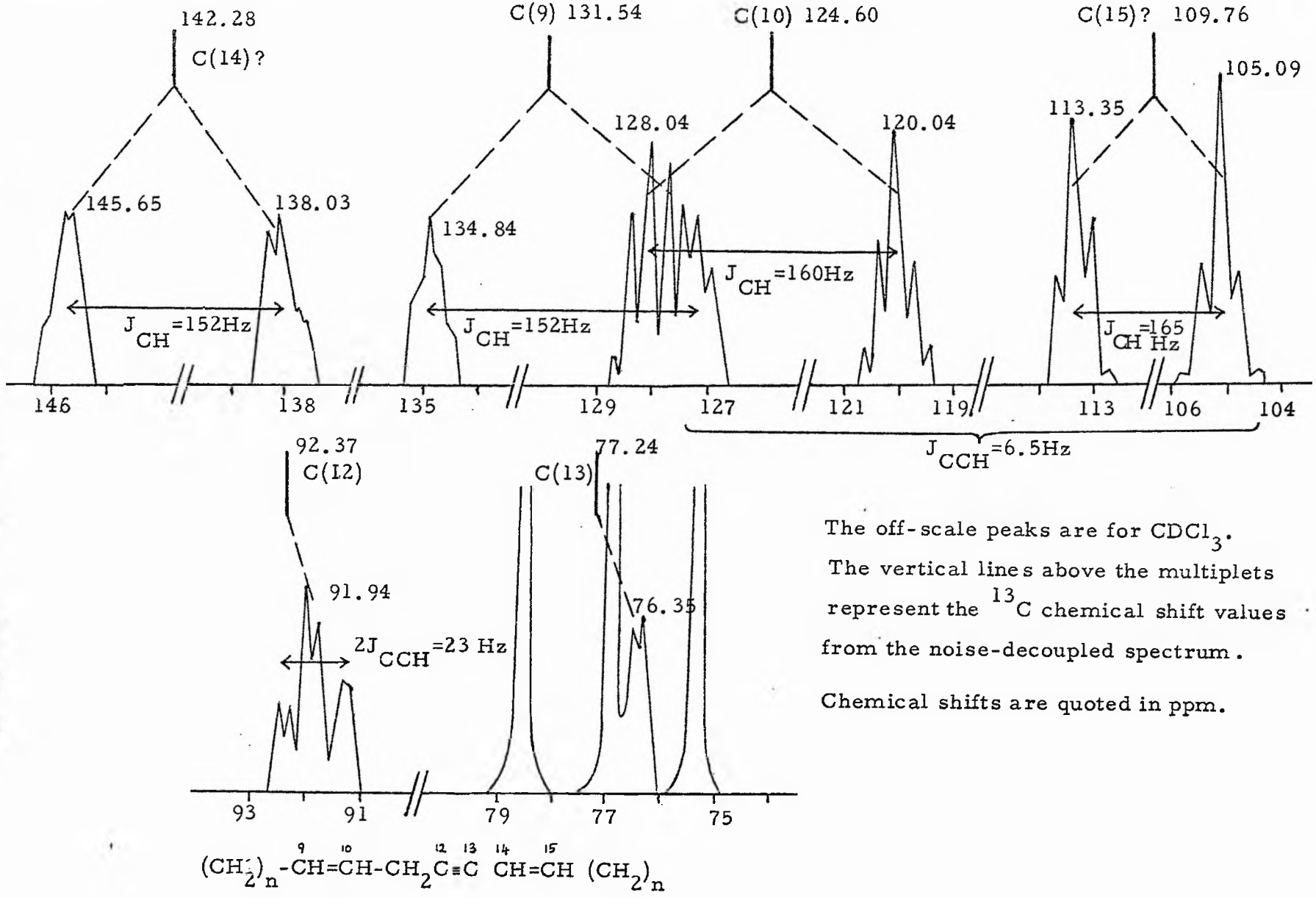


Figure 28: Undecoupled Olefinic and Acetylenic ^{13}C Signals for Methyl 14,15-Dehydrocrepenynate

The off-scale peaks are for $CDCl_3$.

The vertical lines above the multiplets represent the ^{13}C chemical shift values from the noise-decoupled spectrum.

Chemical shifts are quoted in ppm.

(ie. $J_{C(12)CC(10)H}$). Hence the resonance at 77.24 ppm was C(13). It was not clear whether this multiplet was a fine doublet or if the $CDCl_3$ solvent peaks masked a broad doublet, with $J_{C(13)C(14)H} \simeq 10$Hz, containing a fine doublet pattern.

The assignment of δ C(12)=92.37 ppm, δ C(13)=77.24 ppm, δ C(14)=142.28 ppm and δ C(15)=109.76 ppm was tentative, and requires further corroboration. Simpler molecules need to be examined to obtain more of the appropriate ^{13}C-^{1}H spin-spin coupling constants, and additional examples of simple, conjugated unsaturated systems should be studied.

3:2:7 Methylene ^{13}C Chemical Shifts in PUFA

When π-bonds are separated by a single methylene group (n=1) the sum of the appropriate shielding parameters gives a calculated ^{13}C chemical shift to that methylene carbon that is slightly upfield from the observed value (Table 26). The observed shifts for the diallylic methylenes in all-cis-trienes and tetraenes are the same as those for c,c-dienes. Batchelor et al explained the discrepancy between the

TABLE 26. Methylene ^{13}C Chemical Shifts for Methylene-Interrupted Diunsaturated Systems.

Unsaturation	δ_{expt} [1]	δ_{cal} [1]	$\delta_{expt} - \delta_{cal}$	Corrected δ_{cal} [2]
c,c	25.75	24.81	+0.94	25.43
c,t	30.55	30.19	+0.36	30.50
t,t	35.68	35.57	+0.11	35.57
a,a	9.73	7.93	+1.8	9.19
a,c	17.25	16.37	+0.88	17.41
a,t	22.07	21.75	+0.32	22.38

1. Values, in ppm, are averaged for acids and methyl esters.
2. See the text for explanation.

observed and calculated values for the c,c-diallylic methylene by the absence of the δ-cis-allylic shift [150] (Section 3:2:3 and Figure 23). A cis-allylic carbon feels three influences; that of the

cis double bond and the other cis-allylic methylene, the alkyl chain, and the deviation in the usual behaviour of the alkyl chain caused by the cis double bond (ie. the upfield δ-cis-allylic shift of 0.31 ppm). A methylene carbon adjacent to two cis double bonds does not possess the two δ-cis-allylic shifts inherent in the parameters, so these should be subtracted.

ie. Corrected $\delta_{cal} = 29.83 + 2(-2.51) - 2(-0.31) = 25.43$ ppm.

It is believed that there is no δ-trans-allylic shift, but there is a δ-propargylic shift of -0.63 ppm. Applying these δ-shift corrections to the calculated ^{13}C chemical shifts gives a better set of predicted values (Table 26), but they are still not exact so this analysis of the deviation from additivity of the shielding parameters is not a full explanation.

When two π-bonds are separated by two or more methylene groups significant deviations from the predicted ^{13}C chemical shifts occur only for n=2. For c,c-dienes ($\delta_{expt} - \delta_{cal}$) is +0.16 ppm and for diynes ($\delta_{expt} - \delta_{cal}$) is +1.27 ppm. No data were available for a t,t-diene where n=2.

The influence of polyunsaturation on the neighbouring methylene chain is, to within 0.1 ppm, the same as the influence of the outermost π-bond, providing $n > 2$. When n=1 or 2, the influence of an olefinic bond, through another π-bond, on the alkyl chain is negligible ($\leqslant 0.1$ ppm). However, for a triple bond corrections must be applied when n=1 or 2, and these were evaluated approximately (an extensive range of PUFA was not available). The corrections are given below as ($\delta_{expt} - \delta_{calculated}$), where the methylene ^{13}C chemical shifts have been calculated for the shielding effect of the "external" π-bond alone.

$C\equiv CCH_2CH_2C\equiv C-CH_2-CH_2-$

-0.15 ppm

$C\equiv CCH_2C\equiv C-CH_2-CH_2-$

-0.35 ppm

$C\equiv CCH_2CH\overset{c}{=}CH-CH_2-CH_2-$

-0.10 ppm -0.35 ppm

$C\equiv CCH_2CH\overset{t}{=}CH-CH_2-CH_2-$

-0.35 ppm -0.30 ppm

3:2:8 Examples of the Use of Shielding Parameters to Predict the CMR Spectra of Unsaturated Acids or Esters

The aim of this study was the ability to interpret fully and to predict the CMR spectra of novel unsaturated acids and methyl esters. Thus it is fitting to conclude Section 3:2 by working through an example, treating it as an unknown. The spectrum of the unknown is first analysed to deduce its probable structure(s) and then the full CMR spectrum of the probable acid(s) or ester(s) is calculated to compare with the recorded spectrum. The example was chosen from the data of Bus et al for three reasons [173, 174]. It had not been examined in the author's laboratory; the spectrum was obtained using a $CDCl_3$ solution; and not all the shielding parameters needed to calculate the spectrum were available from this study, so some estimates would have to be made.

The problem spectrum contains the following resonances, all of approximately equal peak height except where indicated:-
14.10, 22.70, 24.85, 25.85, 27.35, 27.45, 29.50, 31.70, 32.05, 32.70, 33.40, 51.30, 128.05, 128.60, 129.40, 129.60, 130.25, 131.00 and 173.95 (weak) ppm.

Firstly, these signals should be associated with the ^{13}C chemical shift regions where distinct types of carbon resonances are known to occur.

ie. Methyl ester 173.95 ppm, 51.30 ppm

C(2)	33.40 ppm	C(3)	24.85 ppm
C(ω)	14.10 ppm	C(ω-1)	22.70 ppm
C(ω-2)	31.70 ppm or 32.05 ppm		
Olefinic	128.05-131.00 ppm		
Trans-allylic	31.70 ppm or 32.05 ppm, 32.70 ppm		
Cis-allylic	27.35 ppm, 27.45 ppm		
Cis,cis-diallylic	25.85 ppm		
Methylene "envelope"	29.50 ppm		

There are nineteen signals, none of which has a peak height indicating two or more unresolved resonances, and since the compound is a methyl ester and there are six olefinic resonances, it is an 18:3 PUFA. The $-(CH_2)_m\overset{c}{CH}{=}CHCH_2\overset{c}{CH}{=}CH(CH_2)_m-$ structure, where

$m \geqslant 2$ is proposed on the basis of the two c-allylic and one c,c-diallylic methylene resonances, and an isolated t-double bond must also be present. For the C(2), C(3), C(ω), C(ω-1) and C(ω-2) signals not to be greatly perturbed limits the unsaturation to within Δ5 to ω5. Therefore possible structures are:-

Δ5c8c12t, Δ5c8c13t, Δ6c9c13t, Δ5t9c12c, Δ5t10c13c and Δ6t10c13c.

The second step is to examine more closely the ^{13}C chemical shift values of the highly distinctive resonances for C(2), C(3), C(ω), C(ω-1) and C(ω-2). This will enable the unsaturation nearest the carboxyl and the methyl ends to be located, either exactly or to within certain limits of position. Hence the unsaturation in question will be cis and trans double bonds, and the necessary data has already been published [172]. The information required is -

	C(2)	C(3)	C(4)	C(5)	
Δ5c	33.6	24.7	26.5		
Δ6c	34.1	24.4	29.2	26.9	
Δ5t*	33.39	24.87	32.08		
Δ6t*	33.98	24.52	29.19	32.29	
	C(ω)	C(ω-1)	C(ω-2)	C(ω-3)	C(ω-4)
ω5t*	13.97	22.30	32.00	32.40	
ω6t*	14.08	22.65	31.53	29.47	32.69
ω5c	14.0	22.4	32.0	27.0	
ω6c	14.1	22.6	31.6	29.4	27.3

* Corrected for methyl esters from the data available on 18:1 acids.

Inspection of these figures shows that the probable structure is 18:3(5t 9c12c).

Once a probable structure has been deduced the third stage is the calculation of the CMR spectrum for this molecule and its comparison with the experimental spectrum. The calculation is systematised as follows:-

(1) The full structure of the acid molecule is written down and each carbon chemical shift is described in terms of the appropriate shielding parameters. The asterisk indicates a shielding or deshielding

$$COOMeCH_2CH_2CH_2CH\overset{t}{=}CHCH_2CH_2CH\overset{c}{=}CHCH_2CH\overset{c}{=}CHCH_2CH_2CH_2CH_2CH_3$$

$$\delta(\text{-OMe}) = 51.27 + {}_{\Delta 5}\underline{\Delta^{OMe}(t)}$$
$$\delta C(1) = 174.06 + {}_{\Delta 5}\underline{\Delta^{COOMe}(t)}$$
$$\delta C(2) = 29.85 + {}_{\alpha}\Delta(COOMe) + {}_{\gamma}\Delta(t)$$
$$\delta C(3) = 29.85 + {}_{\beta}\Delta(COOMe) + {}_{\beta}\Delta(t)$$
$$\delta C(4) = 29.85 + {}_{\gamma}\Delta(COOMe) + {}_{\alpha}\Delta(t)$$
$$\delta C(5) = 130.40 + {}_{\Delta 5}\underline{\Delta^{t}(COOMe)} + {}_{\gamma'}\underline{\Delta^{t}(c)} + [{}_{\zeta'}\underline{\Delta^{t}(c)}]^{*}$$
$$\delta C(6) = 130.40 + {}_{\Delta 5'}\underline{\Delta^{t}(COOMe)} + {}_{\gamma}\underline{\Delta^{t}(c)} + [{}_{\zeta}\underline{\Delta^{t}(c)}]^{*}$$
$$\delta C(7) = 29.85 + [{}_{\zeta}\underline{\Delta(COOMe)}]^{*} + {}_{\alpha}\Delta(t) + {}_{\beta}\Delta(c)$$
$$\delta C(8) = 29.85 + [{}_{\eta}\underline{\Delta(COOMe)}]^{*} + {}_{\beta}\Delta(t) + {}_{\alpha}\Delta(c)$$
$$\delta C(9) = 129.90 + [{}_{\Delta 9}\underline{\Delta^{c}(COOMe)}]^{*} + {}_{\beta'}\underline{\Delta^{c}(c)} + {}_{\gamma}\underline{\Delta^{c}(t)}$$
$$\delta C(10) = 129.90 + [{}_{\Delta 9'}\underline{\Delta^{c}(COOMe)}]^{*} + {}_{\beta}\Delta^{c}(c) + {}_{\gamma'}\underline{\Delta^{c}(t)}$$
$$\delta C(11) = 29.85 + 2.\ {}_{\alpha}\Delta(c)$$
$$\delta C(12) = 129.90 + {}_{\beta}\Delta^{c}(c) + [{}_{\zeta}\underline{\Delta^{c}(t)}]^{*}$$
$$\delta C(13) = 129.90 + {}_{\beta'}\underline{\Delta^{c}(c)} + [{}_{\zeta'}\underline{\Delta^{c}(t)}]^{*}$$
$$\delta C(14) = 29.85 + {}_{\alpha}\Delta(c)$$
$$\delta C(15) = 29.85 + {}_{\gamma}\Delta(CH_3) + {}_{\beta}\Delta(c)$$
$$\delta C(16) = 29.85 + {}_{\beta}\Delta(CH_3) + {}_{\gamma}\Delta(c)$$
$$\delta C(17) = 29.85 + {}_{\alpha}\Delta(CH_3) + {}_{\delta}\Delta(c)$$
$$\delta C(18) = 14.13$$

influence through a π-bond, and the figures on the extreme left of the right hand side of the equations represent the unperturbed chemical shifts for the type of carbon atom in question.

(2) The values for these parameters should be looked up in Tables 16-21, 24 and 25 [171, 172]. Some may not be available - those underlined - and reasoned estimates should be made wherever possible. Shielding perturbations caused primarily by linear, electric field polarisations are taken to be similar for cis and trans double bonds when the effect is transmitted through at least two

methylene groups.

ie.

$$_{\gamma}\Delta^{t}(c) \simeq {}_{\gamma}\Delta^{c}(t) \simeq {}_{\gamma}\Delta^{c}(c)$$

$$_{\gamma'}\Delta^{t}(c) \simeq {}_{\gamma'}\Delta^{c}(t) \simeq {}_{\gamma'}\Delta^{c}(c)$$

$$_{\Delta5}\Delta^{t}(COOMe) \simeq {}_{\Delta5}\Delta^{c}(COOMe)$$

$$_{\Delta5'}\Delta^{t}(COOMe) \simeq {}_{\Delta5'}\Delta^{c}(COOMe)$$

$$_{\Delta5}\Delta^{OMe}(t) \simeq {}_{\Delta5}\Delta^{OMe}(c)$$

$$_{\Delta5}\Delta^{COOMe}(t) \simeq {}_{\Delta5}\Delta^{COOMe}(c)$$

The values of the underlined parameters superscripted with an asterisk are small ($\leqslant 0.2$ ppm), and the effect of the intermediate π -bond on the transmission of the shielding or deshielding effect is taken as minimal. ie.

$$[_{\beta}\Delta(COOMe)]^{*} \simeq {}_{\gamma}\Delta(COOMe)$$

$$[_{\gamma}\Delta(COOMe)]^{*} \simeq {}_{\eta}\Delta(COOMe)$$

$$[_{\zeta}\Delta^{t}(c)]^{*} \simeq [_{\zeta}\Delta^{c}(t)]^{*} \simeq {}_{\zeta}\Delta^{c}(c)$$

$$[_{\zeta'}\Delta^{t}(c)]^{*} \simeq [_{\zeta'}\Delta^{c}(t)]^{*} \simeq {}_{\zeta'}\Delta^{c}(c)$$

(3) The additivity of the shielding parameters is known to break down under certain circumstances. The probable structure should be examined to see where such deviations are likely, and the appropriate corrections be made from Tables 14 and 22. For 18:3(5t9c12c) the carbon atoms requiring a correction to their calculated ^{13}C chemical shifts are C(2)-C(4), and C(7) (Table 18; Δ5t unsaturation) and C(11) (Table 26; c,c-diallylic-methylene).

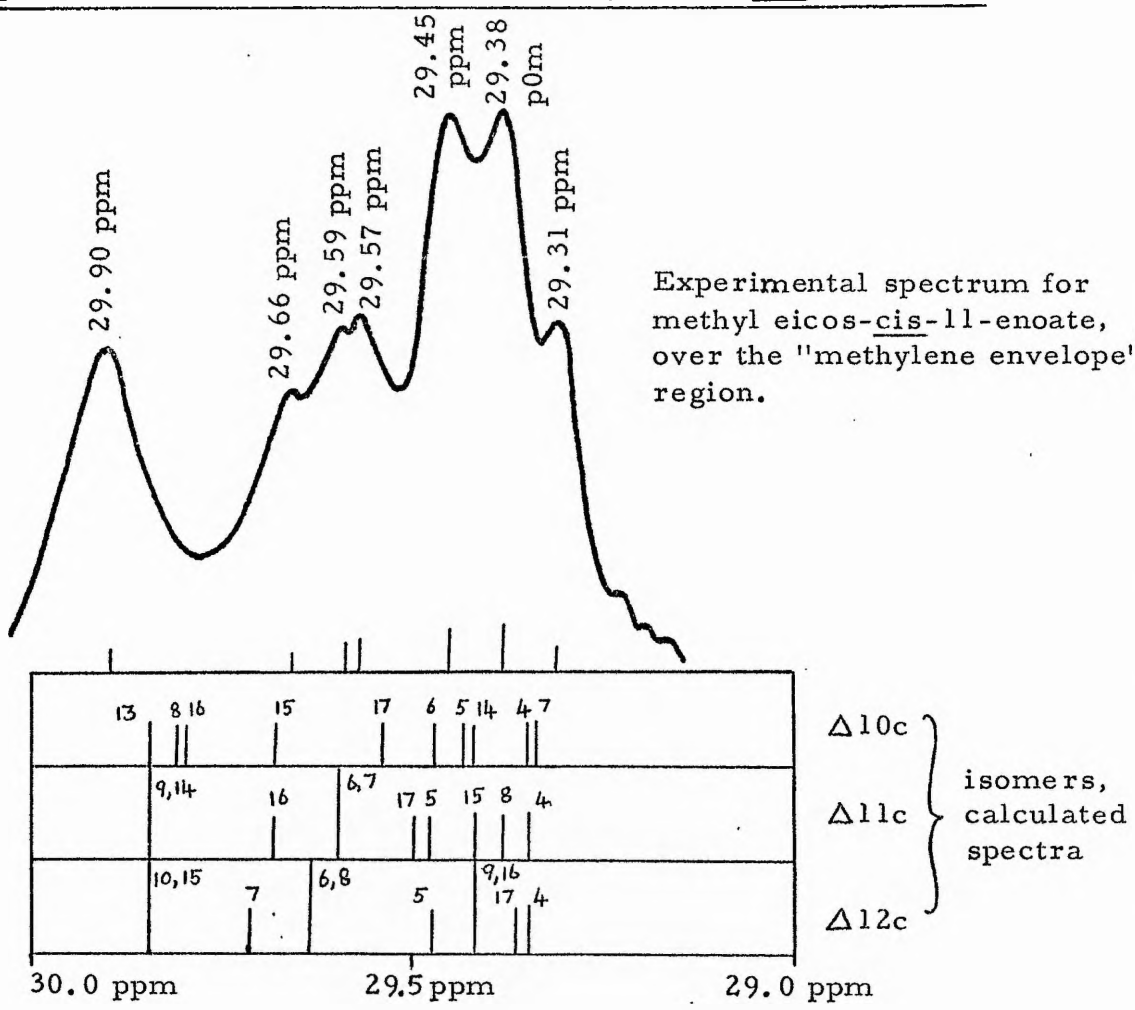
(4) The CMR spectrum can now be calculated and compared with the observed spectrum. The corrections for the deviations from additivity are underlined. As there is close agreement between the calculated and observed spectra the fatty ester has the structure 18:3(5t9c12c).

		$\delta_{calculated}$ (ppm)	$\delta_{observed}$ (ppm) [173, 174]
δ(-OMe)	= 51.27-0.02	= 51.25	51.30
δC(1)	= 174.06-0.25	= 173.81	173.95
δC(2)	= 29.85+4.32-0.54-0.26	= 33.37	33.40
δC(3)	= 29.85-4.74-0.07-0.17	= 24.87	24.85
δC(4)	= 29.85-0.50+2.87-0.19	= 32.03	32.05
δC(5)	= 130.40-1.46+0.48+0.11	= 129.53	129.60
δC(6)	= 130.40+1.31-0.72-0.12	= 130.88	131.00
δC(7)	= 29.85-0.08+2.87+0.0+0.07	= 32.71	32.70
δC(8)	= 29.85-0.04-0.07-2.51	= 27.23	27.35
δC(9)	= 129.90-0.12+0.26-0.73	= 129.31	129.40
δC(10)	= 129.90+0.12-1.87+0.48	= 128.63	128.60
δC(11)	= 29.85-2x2.51+1.0	= 25.83	25.85
δC(12)	= 129.90-1.87-0.12	= 127.91	128.05
δC(13)	= 129.90+0.26+0.11	= 130.27	130.25
δC(14)	= 29.85-2.51	= 27.34	27.45
δC(15)	= 29.85-0.31+0.0	= 29.54	29.50
δC(16)	= 29.85+2.25-0.43	= 31.67	31.70
δC(17)	= 29.85-7.02-0.17	= 22.66	22.70
δC(18)	= 14.13	= 14.13	14.10

The above example shows that a complete identification of unsaturated fatty acid structure is possible from a CMR spectrum alone. This is more likely for PUFA. Long-chain monounsaturated acids may pose a greater problem in determining the chain length and in positioning the unsaturation if located near the centre of a C_{18} or greater acid. ECL values used in conjunction with CMR data should eliminate the former difficulty. The shape of the "methylene envelope" region (28-30 ppm) is a useful comparative criterion.

The CMR spectrum of 20:1(11c) methyl ester is given in Figure 29 [171]. The olefinic (129.88 ppm) and cis-allylic methylene (27.33 ppm) resonances indicate a cis double bond at or beyond $\Delta 10$ and $\omega 6$. A consideration of the highly distinctive methylene resonances (C(2), C(3), C(ω), C(ω-1) and C(ω-2)) shows the cis-double bond to be at or beyond $\Delta 10$ and $\omega 8$. Since the

Figure 29: The CMR Spectrum of Methyl Eicos-cis-11-enoate



Observed total spectrum (the values bracketed are peak heights):-
173.93 (7), 129.88 (42), 51.22 (28), 34.11 (45), 32.05 (42), 29.90 (68), 29.66 (64), 29.59 (75), 29.57 (77), 29.45 (119), 29.38 (120), 29.31 (75), 27.33 (80), 25.06 (44), 22.79 (45) and 14.11 (37).

number of carbon atoms cannot be accurately evaluated from the spectrum, possible structures are:-

18:1 (10c), ω8.

19:1 (10c), ω9, 19:1 (11c), ω8.

20:1 (10c), ω10, 20:1 (11c), ω9, 20:1 (12c), ω8 etc.

If the ECL value for this ester was available the C_{20} monoene structure would be revealed, but it is unlikely that an ECL value obtained on a capillary GLC column would conclusively differentiate between ω8, ω9 and ω10 isomers. The "methylene envelope" resonances, calculated for the three 20:1 isomers, are presented in Figure 29 together with the observed pattern. That for 20:1(11c) fits most closely. In some cases the "methylene envelope" fingerprint can be completely distinctive and so be used to positively identify an isomer from several possibilities, but here it is only a useful guide and the Δ11 position should be checked by an oxidative-degradative procedure or by derivatisation and mass spectroscopy.

The interpretation of CMR spectra of even simple fatty acid mixtures is likely to be fraught with difficulty. The pattern of the ^{13}C resonances will be complex, their unravelling tedious, and information could be lost by overlap of the signals. Simplifications may be made if some of the components in the mixture are known and the appropriate spectra subtracted from the whole. Thus the technique is envisaged as primarily one for the investigation of isolated compounds (ie. $>$ 90% purity). Another limitation of the method, at present, is the minimum sample size of about 50 mg required by most CMR spectrometers to give an adequate spectrum of a C_{18} acid.

3:3 Experimental

Natural abundance ^{13}C NMR spectra were obtained with a Varian CFT-20 spectrometer in the Fourier transform mode at 20 MHz, with proton noise decoupling (1 KHz band width). For most spectra data-length was 8192 points, spectral width 4000 Hz (0-200 ppm), acquisition time 1.023s and pulse width 7 μs. The sample was spun at 20 rps, at room temperature, and the spectrometer's computer printed out peak height and chemical shift (ppm downfield from tetramethylsilane) data.

The samples were dissolved in deuterochloroform to give a total volume of about 2 ml, with 3% (v/v) tetramethylsilane added as the internal standard. In order to run spectra in a reasonable time 1M solutions were used whenever possible. This entailed dissolving 700 mg of a C_{18} fatty acid or methyl ester in 1.3 ml of $CDCl_3$, and a good 0-200 ppm spectrum could then be obtained in one or two hours from 4000 to 8000 transients. Where necessary resolution over the 0-50 ppm range could be improved by scanning the sample overnight using a 1000 Hz spectral width, and when only small quantities of material were available (50-200 mg) the 0-200 ppm spectrum was also obtained by overnight scanning.

The chemical shift data treated in Section 3:2 were collected from the wide range of compounds available to us. Most of the assignments have already been reported [171, 172] and are not reproduced here except for those few not included in these two papers (Appendix 2). However, the conclusions based on all these observations will be discussed.

Section 4: Synthesis

4:1 Introduction

Although syntheses of saturated acids have been known for a long time, routes to unsaturated acids and in particular PUFA are a fairly modern development, given impetus by the rapid expansion of lipid biochemistry since the 1940's. Linoleic acid was first prepared in 1950 by Raphael and Sondheimer, who used the following coupling reaction [182] as the basis of the synthesis:

$$BrMgC{:}C(CH_2)_6Cl \xrightarrow{CH_3(CH_2)_4C{:}CCH_2OMes} CH_3(CH_2)_4C{:}CCH_2C{:}C(CH_2)_6Cl$$

Methods of preparation of unsaturated acids fall into two broad categories: the isolation and subsequent chemical modification of natural acids, and total synthesis from units of shorter chain length. The former method is somewhat limited in the range of novel acids that it can supply, but it is often more convenient if an accessible source of the natural starting material is available. An exhaustive review of unsaturated fatty acid synthesis is beyond the scope of this introduction, and such reviews may be found elsewhere [13, 14, 183, 184]. However, reactions of relevance to this program of work do require discussion and, in particular, these include the synthesis of cis and trans double bonds and the disubstituted cyclcopropene ring, the use of acetylenic intermediates, and the introduction of the ^{14}C label as the final synthetic step.

Existing acids can be modified by chain-extension or chain-shortening. Condensation of alkyl bromides or their Grignard derivatives, or acyl halides, to alicyclic molecules can be used to add on at least four carbon atoms to the chain, but often these methods are not easily applied to unsaturated acids [13]. Labelling of the carboxyl end has generally been limited to the addition of C_1 or C_2 units. The most common method is the preparation of a [1-^{14}C] nitrile

by the reaction of sodium or potassium ^{14}C-cyanide with an alkyl chloride [114, 188] or an alkyl mesylate [185-187]. Subsequent methanolysis of the [1-^{14}C] nitrile gives the [1-^{14}C] methyl ester.

$$\left.\begin{array}{l} RCH{:}CH(CH_2)_nCl \xrightarrow[120\text{-}130^oC]{K^{14}CN,\ DMSO} \\ RCH{:}CH(CH_2)_nOMes \xrightarrow[80\text{-}90^oC]{K^{14}CN,\ DMSO} \end{array}\right\} RCH{:}CH(CH_2)_n{}^{14}CN \xrightarrow{HCl/MeOH} RCH{:}CH(CH_2)_n{}^{14}COOMe$$

Although good radiochemical yields have generally been reported for the labelling reaction with K^{14}CN, Brett [74] and Howling [135] recorded low radiochemical yields (50% or less) when reacting K^{14}CN (ex Amersham) with alkyl bromides or tosylates in DMSO at 90°C. These authors attributed this to an artifact in the radiolabelling process rather than the formation of unexpected ^{14}C-labelled by-products such as amides, imides, isocyanates, isonitriles or free fatty acids and their salts. Another C_1 chain-extension procedure which has been used to introduce a ^{14}C-carboxyl label is the condensation of $^{14}CO_2$ with the Grignard derivative of a polyenoic bromide [189, 190]. The synthesis of [2-^{14}C] unsaturated acids has been carried out by condensing unsaturated iodides with [2-^{14}C] diethyl malonate, followed by hydrolysis of the diester then decarboxylation [34, 193].

Milder conditions for the chain-extension of alkyl chlorides with sodium cyanide are reported using a biphasic system with a phase transfer catalyst such as hexadecyltributylphosphonium bromide [194]. This has not yet been tried as a method for radio-labelling.

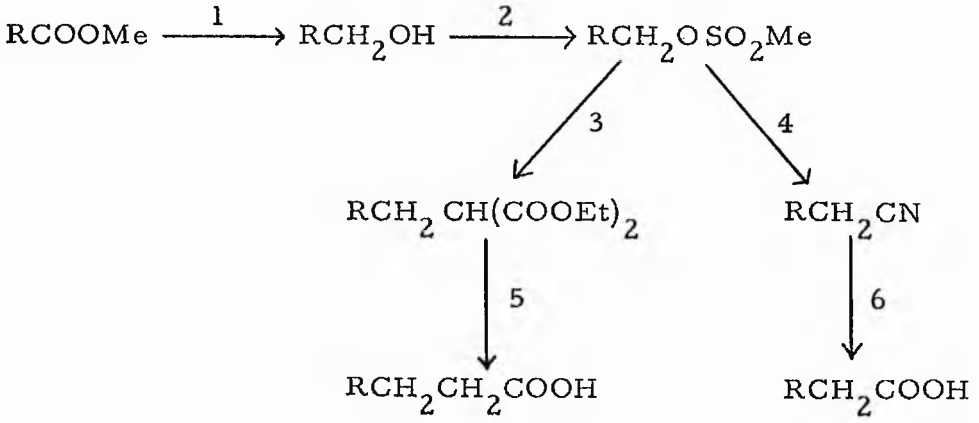
$$RCl + NaCN \xrightarrow[105^oC,\ 2\ hr]{H_2O/decane,\ (C_{16}H_{33})Bu_3P^+Br^-} RCN$$

Kunau recommended the use of mesylates over tosylates, chlorides, bromides and iodides as leaving groups [183]. Mesylates can be readily prepared from primary alcohols by several methods:-

$RCH_2OH \xrightarrow{1,\ 2 \text{ or } 3} RCH_2OMes$

1. $Et_2\overset{+}{N}MeOMes\ FSO_3^-$, MeCN, DMAA, 0°C [195]
2. MesCl, pyridine, 20°C [196]
3. MesCl, Et_3N, CH_2Cl_2, 20°C [197].

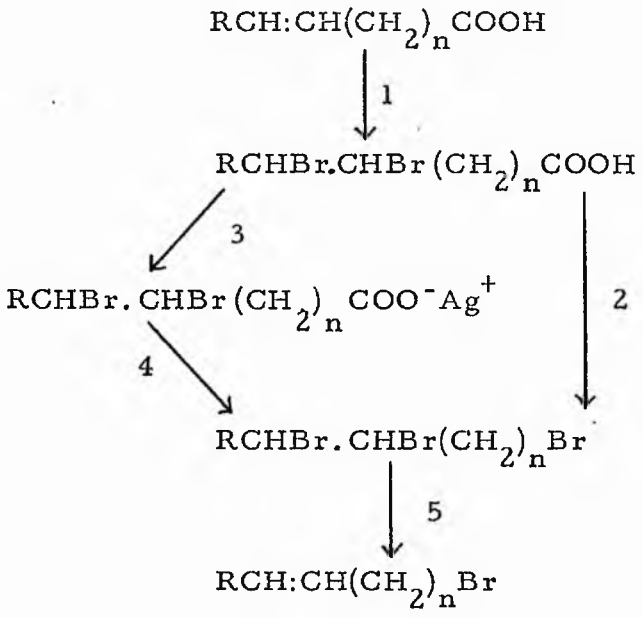
Spener and Mangold reported a high yield "malonic ester synthesis" via alkyl mesylates suitable for polyunsaturated molecules [198]. Therefore the most convenient routes for C_1 and C_2 chain-extension of unsaturated fatty acids are:-



1. $LiAlH_4$, dry ether
2. MesCl or $Et_2\overset{+}{N}MeOMes\ FSO_3^-$
3. $Na^+\ ^-CH(COOEt)_2$, dry xylene, 110°C
4. KCN, dry DMSO, 80-90°C
5. Hydrolysis, then decarboxylation by heating at 120-160°C under nitrogen.
6. Base hydrolysis, or methanolysis/hydrolysis

A C_1 chain-extension rarely used in fatty acid chemistry is the Arndt-Eistert synthesis [199, 200]. The acyl chloride is converted to the diazoketone, which undergoes a Wolff rearrangement with loss of nitrogen to give the homologous product. C_2 chain-extension has been achieved by the treatment of a Grignard reagent, prepared from an alkyl bromide [201] or a terminal alkyne [202], with ethylene oxide.

The chain-shortening of unsaturated fatty acids is a more difficult task than their chain-lengthening. The Hunsdiecker silver salt decarboxylation [189, 203] and its Cristol and Firth modification using mercuric oxide [204] are frequently used. Double bonds must first be protected by bromination. Owing to the bromination-



1. Br_2, CCl_4
2. HgO, Br_2, reflux in CCl_4 (Cristol and Firth degradation)
3. Ammonical $AgNO_3$, THF } (Hunsdiecker degradation)
4. Br_2, reflux in CCl_4 }
5. Zn, reflux in EtOH

debromination steps the overall yield for the above sequence is low for PUFA, and a direct synthesis of the nor-bromide, -chloride, -iodide or -alcohol is to be preferred. Such a step would greatly facilitate the synthesis of [1-^{14}C] PUFA from natural acids. A promising preliminary report indicated that anodic decarboxylation might be a suitable method [205, 206]. Moderate yields of nor-alcohols from oleic and linoleic acids were reported (44% and 21% respectively) in a single step electrolysis, though α- and γ-linolenic acids did not give the desired products.

$$\text{RCOOH} \xrightarrow[\text{Dinh-Nguyen cell}]{\text{Electrolysis in NaOH/aqueous MeOH}} \text{ROH}$$

Another promising possibility for the decarboxylation of unsaturated acids was a photochemical method [207]. The acyl-hypoiodites of saturated, oxo- and acetoxy-fatty acids, generated *in situ*, were photolytically decomposed to their corresponding *nor*-iodides. When cholesteryl acetate was treated with the Bu^tOI generated reagents

$$\text{RCOOH} \xrightarrow{1 \text{ or } 2} \text{RI}$$

1. hν, $K^+Bu^tO^-$, I_2 in benzene
2. hν, $Pb(OAc)_4$, I_2, reflux in benzene or CCl_4.

no reaction occurred, and the double bond remained intact.

Alkenoic and alkynoic acids were prepared by anodic synthesis [209, 210], a subject reviewed by Weedon [209]. Recently the method was used to elongate PUFA by two or four methylene groups,

$$\begin{array}{l} \text{RCOOH} + \text{COOH}(\text{CH}_2)_n\text{COOMe} \\ \qquad\qquad \Big\downarrow \text{ MeOH/NaOMe, Kolbe electrolysis} \\ \text{R.R} + \text{R}(\text{CH}_2)_n\text{COOMe} + \text{COOMe}(\text{CH}_2)_{2n}\text{COOMe} \end{array}$$

but when unsaturation approached the carboxylic acid group ($\Delta 2$- $\Delta 6$) the synthesis became less suitable [205]. Yields from Kolbe electrolysis will always be low because of the statistical nature of the coupling of radicals at the anode.

The Wittig reaction has been known as a route to olefins for many years [211]. The drawback to its use in fatty acid chemistry was that in non-polar solvents only *trans* olefins were formed, but in 1963 Bergelson and Shemyakin showed that with polar, aprotic solvents or Lewis bases *cis* olefins are the major product [212, 213].

$$RCH_2Br \xrightarrow{PPh_3} RCH_2\overset{+}{P}Ph_3\ Br^- \xrightarrow{base} RCH{=}PPh_3$$

$$\xrightarrow[DMF\ or\ I^-]{R'CHO} RCH\overset{cis}{=}CHR'$$

The facts that the entire reaction sequence has to be carried out with olefinic intermediates, and that each double bond is introduced by a separate step render this method less flexible as a route to PUFA than the acetylenic approach. Nevertheless, the Wittig reaction was successfully employed for syntheses of specific PUFA that could not easily be obtained using the acetylenic approach [214, 215].

Although the total synthesis of unsaturated acids from smaller building blocks has been achieved by the Kolbe half-ester electrolysis or the Wittig reaction, acetylenic coupling remains the most flexible and most used route. It was made possible by the stereospecific partial reduction of triple bonds to cis double bonds. The preparation of monounsaturated acids was reviewed by Barve [216] and of methylene-interrupted cis-polyenoic acids by Kunau [183] and Osbond [184].

For the synthesis of monounsaturated acids two basic coupling reactions are applicable.

$$\left.\begin{array}{l} RC{:}CH + X(CH_2)_nY \\ RX + HC{:}C(CH_2)_nY \end{array}\right\} \xrightarrow{base} RC{:}C(CH_2)_nY$$

The choice between the two will depend on their relative yields and on the availability or ease of synthesis of the starting materials. The coupling reaction involves the use of base to produce an acetylide, which then undergoes a nucleophilic displacement reaction with RX or $X(CH_2)_nY$. The leaving group X is usually a halide, and Y is a functional group which will eventually be converted to the carboxylate group.

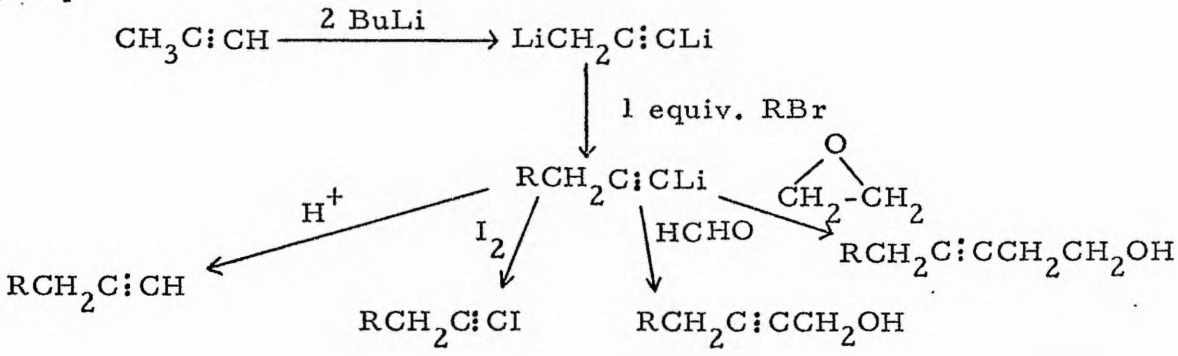
1-Alkynes were often prepared by the reaction of sodium or lithium acetylide, in liquid ammonia, on alkyl bromides [216, 217], but yields diminish with longer chain lengths. More recent preparations of 1-alkynes were given by Smith and Beumel [218],

$$RX \xrightarrow[DMSO\ (X=Br, Cl)]{LiC{:}CH.\ Ethylene\ diamine\ complex} RC{:}CH$$

and by Meijer and Vermeer [219].

$$RMgBr \xrightarrow[dry\ ether]{CuBr,\ H_2C{:}C{:}CH(OMe)} RCH_2C{:}CH$$

These result in higher yields of the long chain 1-alkynes, but have not yet been applied to fatty acid chemistry. A flexible synthetic route via dilithiopropyne has also still to be used in lipochemistry [222].



The coupling of lithium or sodium salts of 1-alkynes with α, ω-bifunctional alkanes has been achieved using iodochlorides [216, 217, 223], 1-chloro-12-(2-tetrahydropyranyloxy)dodecane [224], ω-bromoalkan-1-ols [225], ω-bromocarboxylic acids [226-228] and their N, N-dimethylamides [229]. For condensation reactions in liquid ammonia Ames stated a preference for lithioalkynes over sodioalkynes, and noted that at long chain lengths the yields were low [227, 228]. Coupling $CH_3(CH_2)_aC{:}CH$ with $Br(CH_2)_bCOOH$ gave acceptable yields when $a \leqslant 6$, $b \leqslant 10$ and with $I(CH_2)_cCl$ when $a \leqslant 9$. More recently n-butyl lithium was employed as base and HMPA as solvent [226].

For the condensation of alkyl bromides with $HC{:}C(CH_2)_nY$ Y has been the hydroxyl [230], chloro [216, 217], N, N-dimethyl-carboxyamide [229] and carboxylate [226] groups respectively.

Ames was unable to couple alkyl bromides with ω-acetylenic acids using lithamide in liquid ammonia [227] but Gilman and Holland were successful using n-butyl lithium in HMPA [226]. Kunau recommended the use of HMPA, dioxan or DMF as solvent rather than liquid ammonia for the coupling of long chain alkyl bromides with the lithium salts of propargyl derivatives ($LiC{:}CCH_2OX$; X = Li, Me or thp) in order to avoid solubility problems [231]. However the yields for the reaction

$$CH_3(CH_2)_aBr + LiC{:}C(CH_2)_bOLi \xrightarrow[THF]{\text{liquid ammonia}} CH_3(CH_2)_aC{:}C(CH_2)_bOH$$

seem quite reasonable (>70%) when $a \leqslant 12$ and b = 1-4 [225, 228].

The use of mesylates or tosylates over bromides as leaving groups in these condensation reactions has not been tested.

In a series of elegant papers Kunau described the optimisation of the acetylenic approach to the synthesis of the cis, cis-1,4-diene system for PUFA [232-235]. The central part of the synthesis, described for tetraenoic, pentaenoic and hexaenoic acids, was the coupling of propargyl halides with the diGrignard derivatives of ω, (ω-3)-alkadiynoic acids. Propargyl iodides were preferred over bromides [235].

$$R(C{:}CCH_2)_mI + BrMgC{:}CCH_2C{:}C(CH_2)_nCOOMgBr$$

$$\downarrow \quad m=2\text{-}4 \quad Cu^+, THF \text{ then } H^+, H_2O$$

$$R(C{:}CCH_2)_{m+2}(CH_2)_{n-1}COOH$$

The ω,(ω-3)-alkadiynoic acids were prepared by the following route [232]:-

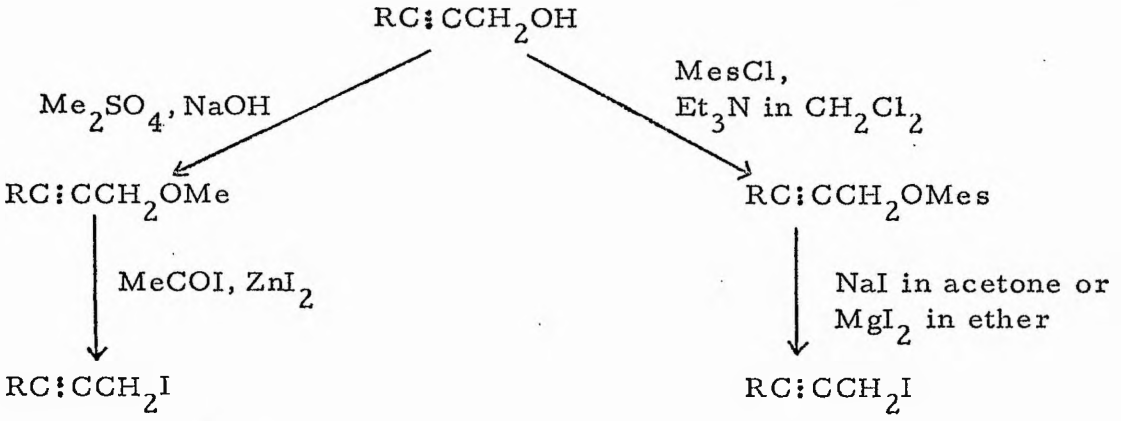
$$HC{:}C(CH_2)_nOH \xrightarrow{\text{DHP}} HC{:}C(CH_2)_nOthp$$

$$\xrightarrow[HC{:}CCH_2OTos]{EtMgBr;\ then\ Cu^+,} HC{:}CCH_2C{:}C(CH_2)_nOthp \xrightarrow[2.\ CrO_3 \text{ in acetone}]{1.\ H^+/MeOH}$$

$$HC{:}CCH_2C{:}C(CH_2)_{n-1}COOH$$

Two methods for the synthesis of propargyl iodides were developed [233, 234], which could also be applied to the synthesis of propargyl

bromides and which were preferable to the use of phosphorus tribromide to synthesise polyynoic propargyl bromides.



Other methods of synthesis of all-cis methylene-interrupted PUFA using a variety of permutations on the acetylenic approach will not be reviewed here [38, 92, 114, 117, 184, 185, 236-238]. The acetylenic pathways can be used alone or in conjunction with other methods to synthesise non-methylene-interrupted and mixed-unsaturation PUFA, but this will not be discussed here either [214, 215, 228, 237, 239-241].

The use of acetylenic intermediates in the synthesis of olefinic acids was made possible by the stereospecific partial reduction of triple bonds to cis double bonds. Catalytic hydrogenation is almost invariably used, with Lindlar catalyst, a 5-10% suspension of palladium on calcium carbonate, poisoned by lead and quinoline [242, 243]. A variety of solvents can be used. The hydrogenation stops at the olefinic stage and is highly cis stereospecific. However, small amounts of saturated and trans olefinic compounds are formed, and the amount of quinoline should be carefully controlled, especially when hydrogenating polyynoic compounds, which need to be very pure [183, 184, 235, 244]. An early chemical method for the reduction of acetylenes to cis-olefins was the use of a $Zn/HCl/TiCl_4$ reagent system in boiling aqueous acetic acid [245]. More recent chemical methods are limited and little information is available regarding their degree of stereospecificity, by-products and general applicability

[183, 184]. However, partial reduction via hydroboration with disiamylborane seems a promising method [246, 247].

The stereospecific partial reduction of triple bonds to trans double bonds is usually accomplished chemically, by the use of sodium in liquid ammonia [248]. Long chain monoynoic acids required lithium in liquid ammonia, at 10-12 atmospheres pressure in an autoclave [202, 216]. Diynoic acids, where the triple bonds were separated by more than one methylene group, could also be reduced to the corresponding t,t-dienoic acids by lithium in liquid ammonia [120, 237], but the method was not successful for the reduction of methylene-interrupted or conjugated diynoic acids.

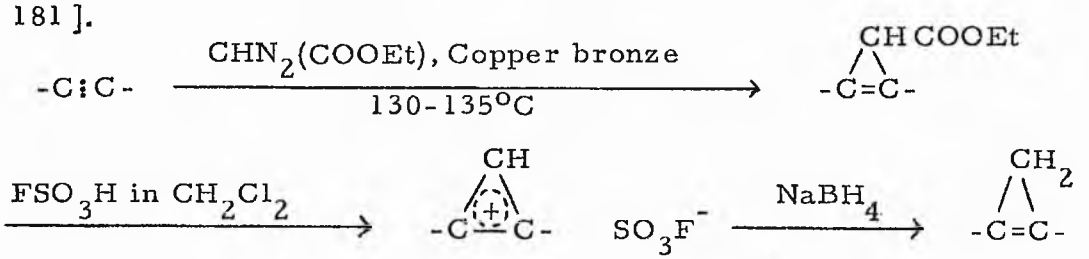
The interconversion of cis and trans olefins can be achieved by either radical stereomutation or a sequence of stereospecific chemical reactions. Many reagents have been used for radical isomerisation, but NO_2, generated from mixtures of aqueous sodium nitrite and nitric acid, has been the most popular. Gunstone and Ismail showed that the method of heating with selenium was accompanied by extensive double bond migration, whereas a method involving irradiation by ultra-violet light in the presence of diphenyl sulphide was not [249]. NO_2 radical isomerisation is also free from double bond migration, and was successfully employed to convert methylene-interrupted c,c-dienes to their t,t-isomers [237, 250]. NO_2 [135] and thiophenol [151] radical isomerisations were applied to produce micro-quantities of trans-olefinic ^{14}C-labelled fatty acids. The problem of radical promoted isomerisation is that the reaction does not go to completion but an equilibrium cis:trans ratio of about 1:3 is eventually reached. Using NO_2, yields are decreased by polar nitrogenous by-products, though these can be readily removed by chromatography.

The chemical interconversion of cis and trans olefins is a much more controlled process, but requires several steps of manipulation. The route usually employed is via the vicinal diol [252, 253].

$$RCH{=}^{c}CHR' \xrightarrow{1,2,3} \underset{\text{threo}}{RCH(OH)CH(OH)R'} \xrightarrow[\text{or } 5]{4} RCH{=}^{t}CHR'$$

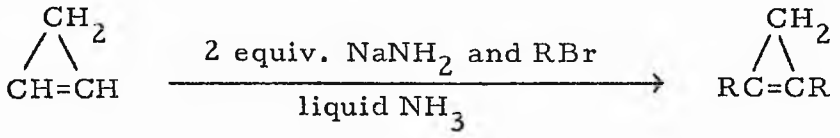
1. Peracid 2. HOAc 3. NaOMe 4. MesCl, then NaI/Zn in DMF
5. HBr/HOAc/H_2SO_4, then reflux in Zn/ethanol

The isolation of naturally occurring cyclopropene fatty acids has led to attempts to synthesise these compounds. Early efforts involving the addition of methylene carbene, generated *in situ* from a Zn/Cu couple and diiodomethane or diazomethane and Cu^+ salts, across the triple bond of stearolic acid were unsuccessful [254, 255]. Gensler *et al* first developed a suitable synthesis [255]. It was achieved by the addition of carbethoxy carbene, generated *in situ* from ethyl diazoacetate, across the triple bond of stearolic acid, followed by a five step hydrolysis/decarbonylation /reduction procedure, giving an overall yield of 30%. This sequence was later simplified [256], and the preparation requiring the fewest steps and of the highest overall yield (60-65%) was reported by Williams and Sgoutas [180, 181].



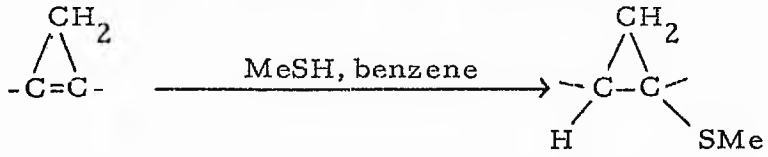
Cyclopropene fatty acids have been ^{14}C-labelled by chain extension of the appropriate chloride [257] or tosylate [74] with $K^{14}CN$. Gensler *et al* also reported the synthesis of methyl malvalate with ^{14}C in positions other than the carboxyl group [257].

The direct alkylation of cyclopropene was recently reported, but this route has yet to be applied to cyclopropene fatty acid synthesis [258].

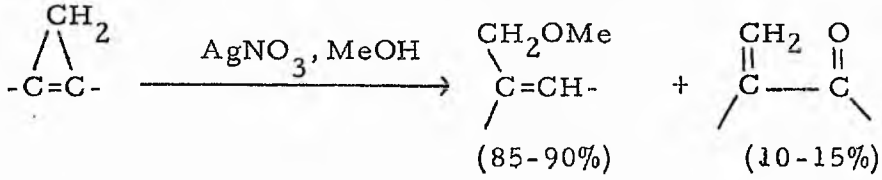


The cyclopropene ring is a highly reactive centre, and in particular is acid labile. Reactions performed on cyclopropene fatty acids should be done under as mild conditions as possible, at neutral or alkaline pH, and such compounds should be stored as their methyl esters or as urea-adducts. Direct GLC analysis of cyclopropene fatty esters is impossible because they decompose on the column, so derivatisation is required. Two methods often applied

are those of Raju and Reiser [259] and Schneider et al [260]. The former workers used mercapto derivatives



while the latter used the keto and ether derivatives from the reaction of the cyclopropene ring with saturated methanolic silver nitrate.



4:2 Results and Discussion

4:2:1 General Pathways

^{14}C-labelled acids were required for the biochemical experiments in preference to T-labelled acids. RGLC was the principal form of analysis, and ^{14}C could be counted to approaching 100% efficiency by the flow proportional counter of the instrument, whereas T (being a weaker energy β^- emitter) was less readily detected. Autoradiography and the Panax radio-TLC scanner were also less sensitive to T. ^{14}C-labelling at the carboxyl end was necessary in order to make von Rudloff oxidative identification of the position of the double bond inserted by the desaturase a straightforward procedure without recourse to prior partial reduction with hydrazine.

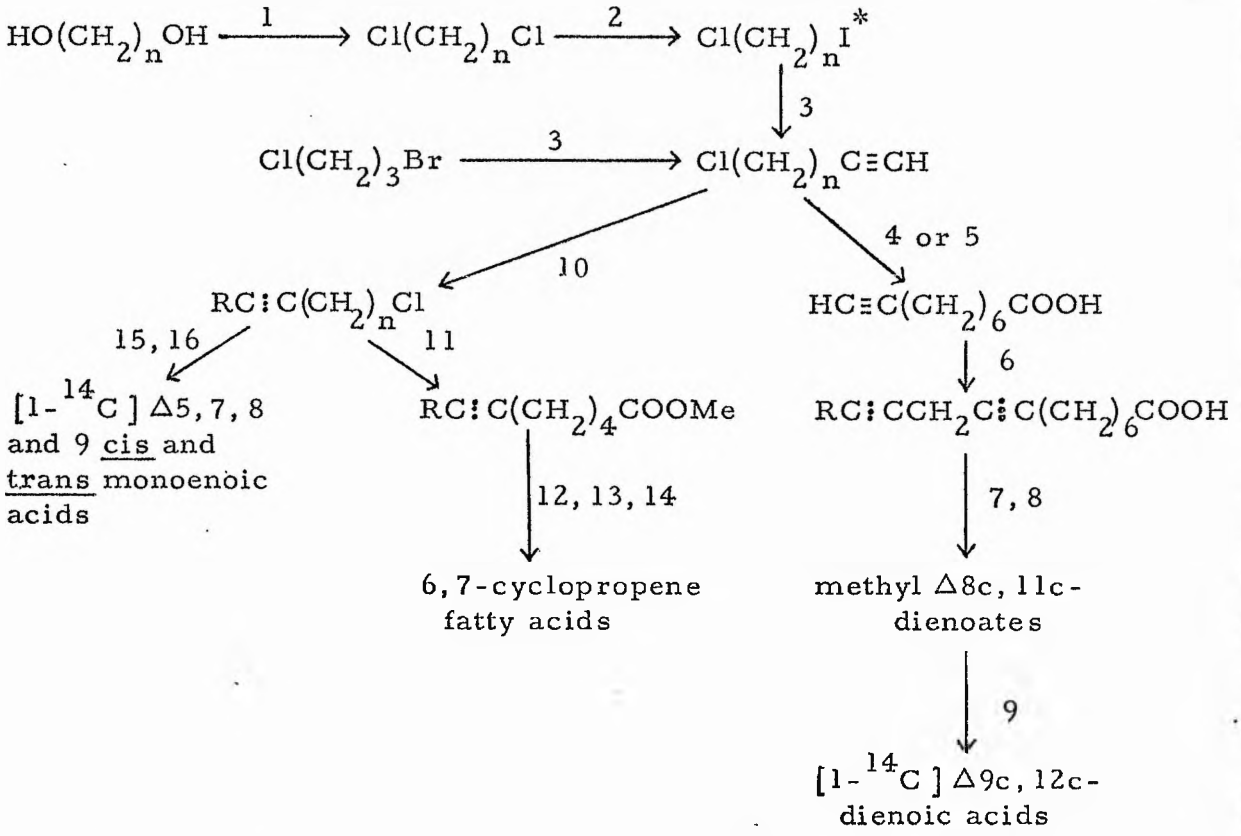
$$RCH{:}CH(CH_2)_n{}^{14}COOH \xrightarrow{\text{Desaturation}} RCH{:}CH(CH_2)_p CH{:}CH(CH_2)_q{}^{14}COOH \quad (p+q+2)=n$$

$$\xrightarrow[\text{2. Esterification}]{\text{1. } KMnO_4/KIO4} COOMe(CH_2)_q{}^{14}COOMe \quad \text{(Identified by RGLC)}$$

The requirement for fatty acids of high specific activity (30-60 μCi μmole^{-1}) necessitated the introduction of the ^{14}C label as the last stage of the synthesis. Of the carboxyl-end labelling procedures outlined in Section 4:1 chain extension with $K^{14}CN$ was chosen as it could be performed on mesylates, tosylates and halides and was most readily adapted to a micro-scale. High specific activity [1-^{14}C] or [2-^{14}C] diethyl malonate was not available from Amersham.

The routes used for the total synthesis of the unsaturated and cyclopropene fatty acids (Figure 30) were chosen to minimise the number of reactions performed. The key intermediates — α-chloro-ω-alkynes —were prepared by coupling sodium acetylide with α-chloro-ω-iodo(bromo)alkanes in liquid ammonia. These compounds could be alkylated to give 1-chloroalk-n-ynes (n=4-7) or converted to ω-ynoic acids such as non-8-ynoic acid used in the synthesis of 8,11-diynoic acids.

Figure 30: The General Scheme for the Total Synthesis of [1-^{14}C] Unsaturated and Cyclopropene Fatty Acids.



1. Excess $SOCl_2$, dry pyridine, n=5, 6
2. 1 mole NaI, dry acetone, n=5, 6
3. NaC:CH, liquid ammonia, n=3-6
4. KCN chain extension and base hydrolysis, n=6
5. Diethyl malonate chain extension, saponification, and thermal decarboxylation at 120-160°C, n=5
6. 2 mole EtMgBr, dry THF, $RC{:}CCH_2I$, Cu^I; followed by acid hydrolysis
7. H_2, Lindlar catalyst
8. H^+/MeOH esterification
9. $K^{14}CN$ chain extension of the corresponding mesylate
10. $NaNH_2$, liquid ammonia, RBr, n=3-6
11. KCN, DMSO, 130°C, followed by 25% (w/w) anhydrous HCl/MeOH
12. 2 mole N_2.CH.COOEt, Cu bronze, 130-135°C
13. Excess 1/1 (v/v)CH_2Cl_2/HSO_3F, then excess methanolic $NaBH_4$
14. KOH/MeOH hydrolysis
15. H_2, Lindlar catalyst, n=3, 5, 6
16. Chain extensions with KCN or $K^{14}CN$ and cis-trans isomerisation with oxides of nitrogen

* $I(CH_2)_4Cl$ was available from previous synthetic programs.

The 1-chloroalk-n-ynes [n=4, 6, 7] were partially hydrogenated to the corresponding 1-chloroalk-cis-n-enes using Lindlar catalyst. Small portions of the latter were first isomerised to cis-trans mixtures using oxides of nitrogen and then chain elongated with $K^{14}CN$. The geometric isomers were separated by Ag^{+}TLC of the [1-^{14}C] methyl esters. This gave the following [1-^{14}C] acids used in the biochemical study:- 16:1(5t), 17:1(5t), 18:1(5t), 18:1(5c), 16:1 (7c), 16:1 (7t), 16:1(8c) and 16:1(8t). The 1-chloroalk-7c/t-enes were also chain extended using unlabelled KCN prior to further chain extension with $K^{14}CN$ to give:- 11:1(9c), 13:1(9c), 13:1(9t), 15:1(9c), 16:1(9c), 17:1(9c) and 17:1(9t) [1-^{14}C] acids.

The 1-chloroalk-5-ynes were chain elongated with cold KCN to give the methyl alk-6-ynoates, which were used in the synthesis of the 6,7-cyclopropene C_{12} and C_{18} fatty acids according to the method of Williams and Sgoutas [180, 181]. Much lower overall yields for the three step cyclopropene ring synthesis were obtained (6.5-15%) than were quoted in the literature (~50%).

The diynoic acids were prepared by the method of Kunau [183, 231-235]. Propargyl alcohol was alkylated by the reaction of its dilithium salt with alkyl bromide (or methyl iodide) in liquid ammonia/THF, and the alk-2-yn-1-ol produced was converted to the 1-iodoalk-2-yne by mesylation [197] followed by the reaction of the propargyl mesylate with sodium iodide in acetone [234]. The coupling of the 1-iodoalk-2-ynes (C_4-C_{12}) with the diGrignard derivative of non-8-ynoic acid in THF in the presence of Cu^{I} proceeded with high yields (63-80.5%). Rather than Kunau's recommendation of a 2:1 molar ratio of alkynoic acid:propargyl iodide in the coupling reaction an approximately 1:1 ratio was used, and the crude product was cleaned up by argentation column chromatography. Although the product was reasonably pure ($>$ 90%) as judged by GLC and its PMR and CMR spectra, and was free from polar materials as judged by TLC it proved very difficult to hydrogenate satisfactorily. Repeated low temperature crystallisation from methanol or aqueous methanol was required

before a controlled partial hydrogenation to give the c,c-dienoic acid was achieved. Chain extension with $K^{14}CN$ gave the following [1-^{14}C] dienoic acids which were used in the biochemical studies: 14:2(9c12c), 16:2(9c12c), 20:2(9c12c) and 22:2(9c12c).

The following [1-^{14}C] acids were obtained from Amersham: 16:0, 18:0, 18:1(9c), 18:2(9c12c) and 18:3(9c12c15c). Other [1-^{14}C] acids were prepared by cis-trans isomerisation or by chain extension of existing compounds. Several C_{18} monoenoic acids were decarboxylated to their nor-alcohols by anodic decarboxylation, and then chain extended with $K^{14}CN$. Figure 31 summarises these manipulations.

The radiochemical yields for the overall reaction

$$RX \xrightarrow[X=Cl,OMes]{1,2,3} R^{14}COOH$$

1. $K^{14}CN$, DMSO
2. HCl, MeOH, followed by Ag^+TLC
3. KOH, aqueous MeOH

were low (5-30%), for a procedure which gives high yields when performed on a larger scale with cold KCN. The first step, the nucleophilic displacement of X^- by ^{14}C-cyanide, resulted in a radiochemical yield of less than 50%. A considerable proportion of the radioactivity occurred as a polar material (as judged by TLC) of unknown composition. These findings were in accord with the observations of Brett [74] and Howling [135] (Section 4:1). Ag^+TLC gave variable radiochemical recoveries (30-75%). For the first step the sealing of the reaction mixture in a glass tube with a small dead space, after thoroughly purging with nitrogen, gave higher radiochemical yields than simply heating in a stoppered quickfit test-tube.

Acceptable yields ($>$ 30%) were obtained for the coupling of alkyl bromides with α-chloroalk-ω-ynes in liquid ammonia with sodamide base only if the alkyl bromide chain length was $\leqslant C_9$. This results from solubility problems already noted by other researchers using liquid ammonia as solvent [225, 227, 228, 231]. Side reactions occurring in the $Fe(NO_3)_3/NaNH_2/NH_{3(l)}$ system included (Section

Figure 31: The Synthesis of [1-^{14}C] Unsaturated Acids from Existing Long Chain Compounds

(i) 18:3(6c9c12c) $\xrightarrow{1}$ 19:3(7c10c13c) $\xrightarrow{2}$ [1-^{14}C]20:3(8c11c14c)

18:3(6c9c12c) $\xrightarrow{2}$ [1-^{14}C]19:3(7c10c13c)

20:4(5c8c11c14c) $\xrightarrow{1}$ 21:4(6c9c12c15c) $\xrightarrow{2}$ [1-^{14}C]22:4(7c10c13c16c)*

18:2(9c12c) $\xrightarrow{1}$ 19:2(10c13c) $\xrightarrow{2}$ [1-^{14}C]20:2(11c14c)*

18:1(7t) $\xrightarrow{1}$ 19:1(8t) $\xrightarrow{2}$ [1-^{14}C]20:1(9t)

18:1(6c) $\xrightarrow{1}$ 19:1(7c) $\xrightarrow{2}$ [1-^{14}C]20:1(8c)

(ii) 1-chlorohexadec-11-yne $\xrightarrow{6}$ [1-^{14}C]17:1(12a)

$\downarrow$ 3,4

1-chlorohexadec-11c/t-ene $\xrightarrow{6}$ [1-^{14}C]17:1(12c) and 17:1(12t)

$\downarrow$ 5

17:1(12c/t) $\xrightarrow{2}$ [1-^{14}C]18:1(13c) and 18:1(13t)

(iii) [1-^{14}C]18:1(9c) $\xrightarrow{4}$ [1-^{14}C]18:1(9t)

[1-^{14}C]18:2(9c12c) $\xrightarrow{4}$ [1-^{14}C]18:2(9t12t)

and [1-^{14}C]18:2(9t12c + 9c12t)

(iv) 18:1(c/t) acid $\xrightarrow{7}$ C_{17} nor-alcohol $\xrightarrow{2}$

[1-^{14}C]18:1 Δ8c, 8t, 10c, 10t, 11c, 11t, 14c, 14t, 15c and 15t

1. Chain extension with KCN, via the mesylate
2. Chain extension with K^{14}CN, via the mesylate
3. Partial reduction, H_2/Lindlar catalyst
4. Cis-trans isomerisation with oxides of nitrogen
5. Chain extension with KCN, via the chloride
6. Chain extension with K^{14}CN, via the chloride
7. Anodic decarboxylation

* 20:4(5c8c11c14c) contained a 10% 18:2(9c12c) impurity. The diene was separated from the tetraene after the chain extensions (1, 2) by Ag^+TLC

4:3:8):-

$$ClCH_2CH_2R \longrightarrow H_2C{:}CHR$$

$$RX \xrightarrow[X=Cl,Br]{NO_3^-} RONO_2$$

4:2:2 Photochemical Decarboxylation of Oleic Acid

Photochemical decarboxylation had not, prior to this work, been tested as a method for the direct chain shortening by one carbon atom of unsaturated fatty acids. However the method held some promise as Barton et al noted that the double bond in cholesteryl acetate was unaffected by the t-butyl hypoiodite reagent [207]. The reagents used for photodecarboxylation are believed to generate the acyl hypoiodite in situ, which then decomposes photochemically to give carbon dioxide and the nor-iodide.

$$RCOOI \xrightarrow{h\nu} RI + CO_2$$

It is not known whether this proceeds directly or via a free radical chain mechanism.

$$RCOOI \xrightarrow{h\nu} RCO_2^{\cdot} + I^{\cdot} \qquad \text{Initiation}$$

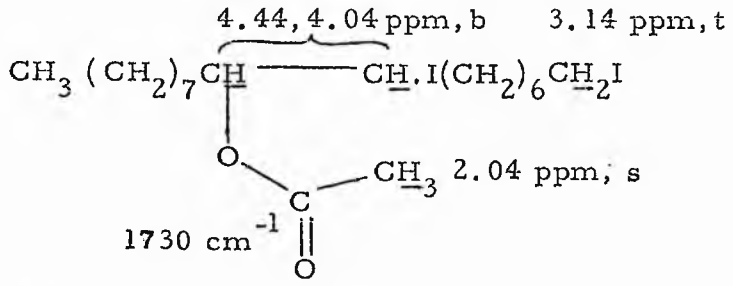
$$RCO_2^{\cdot} \longrightarrow R^{\cdot} + CO_2$$
$$R^{\cdot} + RCOOI \longrightarrow RCO_2^{\cdot} + RI \qquad \text{Propagation}$$

Using lead tetraacetate and iodine to generate oleoyl hypoiodite is believed to involve the following equilibria [207]:-

$$Pb(OAc)_4 + I_2 \rightleftharpoons Pb(OAc)_3I + AcOI$$
$$Pb(OAc)_4 + RCOOH \rightleftharpoons Pb(OAc)_3OCOR + HOAc$$
$$Pb(OAc)_3OCOR + I_2 \rightleftharpoons Pb(OAc)_3I + RCOOI$$
$$RCOOH + AcOI \rightleftharpoons RCOOI + HOAc$$

Acetyl hypoiodite is stable relative to other acyl hypoiodites and does not undergo extensive decarboxylation. However, the olefinic centre proved to be susceptible to attack by acetyl hypoiodite. The major product (Section 4:3:16) was identified as 9(10)-acetoxy-1,10(9)-diiodoheptadecane by its IR and PMR spectra and its TLC behaviour. The

significant proton resonances and IR absorptions are shown below:-



The crude product was fairly pure as judged by TLC and PMR spectroscopy, but labile to hydrolysis. Elemental analysis of the major component (42.8% C, 7.1% H) was not very close to the theoretical value for 9(10)-acetoxy-1, 10(9)-diiodoheptadecane (39.2% C, 6.8% H), but this might be due to the difficult of obtaining it in a high purity. Certainly the low %C in the molecular composition indicated that the presence of two iodine atoms per molecule was very likely.

Tabulated PMR data [220] confirmed the assignment of $-CH_2I$ and $CH_3CO.O-$ protons and indicated that the methine protons of -CHI- and -CH(OAc)- would resonate at about 4.0 ppm. However an unequivocal assignment of the 4.04 ppm and 4.44 ppm signals to the -CHI.CH(OAc)- system could not be made.

An experiment using insufficient quantities of $Pb(OAc)_4$ and I_2 showed that addition of acetyl hypoiodite across the double bond occurred in preference to photodecarboxylation. The crude product was essentially a mixture of 9(10)-acetoxy-10(9)-iodostearic acid and 9(10)-acetoxy-1, 10(9)-diiodoheptadecane.

The method could not therefore be used to directly decarboxylate olefinic fatty acids. However, on standing 9(10)-acetoxy-1, 10(9)-diiodoheptadecane slowly reverted to the desired product, 1-iodo-heptadec-cis-8-ene (oleoyl nor-iodide). Its structure was confirmed by PMR and IR spectroscopy and by its GLC retention time.

$$RCH^{c}{=}CH(CH_2)_7COOH \xrightarrow[h\nu]{Pb(OAc)_4,\ I_2} RCH(OAc)CHI(CH_2)_7I$$

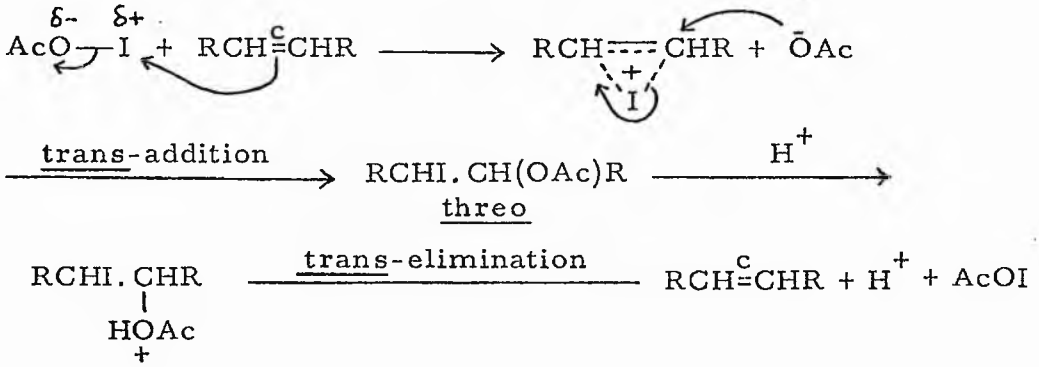
$$\xrightarrow{\text{standing}} RCH^{c}{=}CH(CH_2)_7I$$

The cis-double bond was presumably regenerated by a stereospecific "deiodoacetoxylation", without double bond migration. ω-Iodoalkanes and methyl alkanoates were used as GLC standards to predict the ECL values on a 20% DEGS column at 180°C of oleoyl nor-iodide and the expected methyl 8-iodooctanoate from the von Rudloff analysis.

	ECL(predicted)	ECL(observed)
$CH_3(CH_2)_7CH^{c}{=}CH(CH_2)_7I$	18.85	18.70
$COOMe(CH_2)_7I$	17.95	17.8

Although the yield for the photochemical step was high the yield for the deiodoacetoxylation was low (~5%). An attempt was made to investigate and improve the deiodoacetoxylation step, so as to render photodecarboxylation a suitable method for chain shortening of unsaturated acids. In a simple experiment 9(10)-acetoxy-1,10(9)-diiodoheptadecane was subjected to a variety of conditions. Methyl palmitate was added as an internal standard to monitor the reaction by GLC. The results (Section 4:3:16, Figure 33) indicated an acid-catalysed rather than a free radical reaction, as at 70°C p-toluene-sulphonic acid speeded up the reaction whereas a radical initiator had little effect. Although GLC analysis by comparison with the methyl palmitate internal standard pointed to good yields for the acid-catalysed reaction this was misleading. It appeared that the standard was also consumed during the deiodoacetoxylation, as oleoyl nor-iodide was never recovered in a greater than 10% yield. The use of solvents or higher temperatures did not improve the reaction.

Since both the addition and elimination of acetyl hypoiodite were highly stereospecific and the latter was acid catalysed the following mechanism is tentatively advanced.

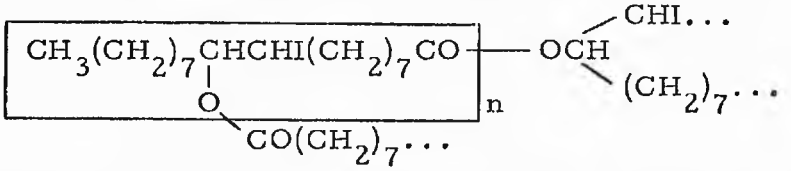


The problem of deiodoacetoxylation, at least according to this mechanism, is the removal of acetyl hypoiodite. Presumably regenerated AcOI is destroyed by other reactions, possibly polymeric in nature. When 9(10)-acetoxy-1,10(9)-diiodoheptadecane was heated at 70°C or 80°C, the rate of production of oleoyl *nor*-iodide with time followed the sigmoidal pattern characteristic of autocatalysis (Figure 33). It is believed that some acetic acid was generated as the deiodoacetoxylation proceeded, causing the autocatalysis. Under vacuum, when acetic acid is removed, no reaction occurred at 70°C.

The t-butylhypoiodite reagent is formally assigned the structure Bu^tOI, though its true molecular composition is likely to be considerably more complex, depending on the ratio of $KOBu^t$ and I_2 used to generate it. Oligomeric species and I^{III} are likely to be present [261]. Oleoyl hypoiodite is generated by the reaction

$$Bu^tOI + RCOOH \longrightarrow Bu^tOH + RCOOI$$

The major product of the photolysis reaction of oleic acid with t-butylhypoiodite reagent was a polymer of the general formula:



This formula is consistent with the PMR and IR spectra, and also with the product and the mechanism of the $Pb(OAc)_4/I_2$ decarboxylation of oleic acid. It highlights the fact that acyl hypoiodites preferentially add across double bonds rather than undergoing radical decarboxylation. The reason why Barton *et al* reported that the double bond in cholesteryl acetate was insensitive to t-butylhypoiodite photodecarboxylation was that there was no free carboxylic acid group present to generate acyl hypoiodite [207].

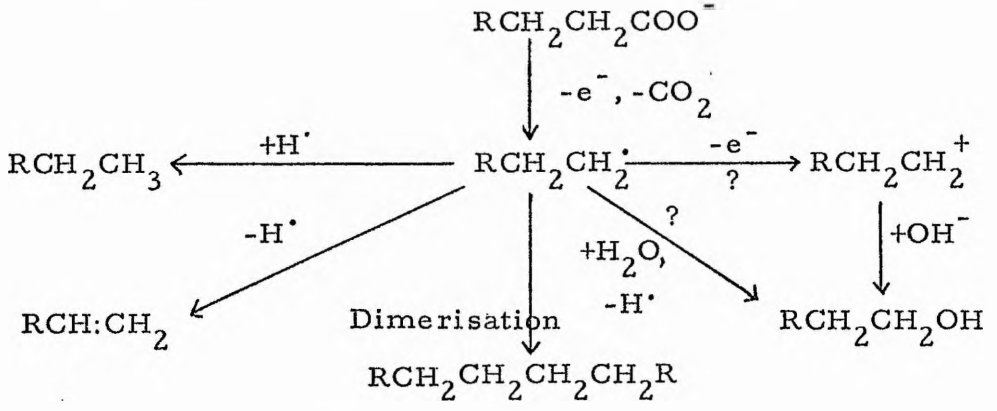
Summarising, photochemical decarboxylation *via* acyl hypoiodites is not recommended as a procedure for the direct decarboxylation of unsaturated fatty acids. Acyl hypoiodites add across the double bond in preference to radical decarboxylation, and although the acyl hypoiodite can be eliminated stereospecifically

to regenerate the double bond (without migration) as yet this process gives highly unsatisfactory yields of the required nor-iodide.

4:2:3 Anodic Decarboxylation

Anodic decarboxylation of oleic acid using the method of Vedanayagam et al [205, 206] did not give the expected yield of oleoyl nor-alcohol (about 40%) but one of 18%. The low yield could not be attributed to extensive involvement of the double bond in forming byproducts as lauric acid gave undecan-1-ol in only a 23% yield. The major hydrocarbon product for both electrolyses was the dimeric species (C_{22} or C_{34}) but smaller amounts of C_{11} or C_{17} hydrocarbons were also generated. The "methyl ether" band from TLC was more complex than reported previously [205] but did not constitute a major fraction of the product. The two major components in this band were not separated or identified, but the double bond seemed intact and the PMR spectrum showed that simple $-CH_2OMe$ (3.22, s, 3H and 3.26, t, 2H) or -CH(OMe)- (3.20, s, 3H and 3.0, qu, 1H) structures were absent. The nor-alcohol was the major product from the more polar material. It is probably produced via a carbonium ion rather than via a free radical [205].

Therefore present knowledge on the product-forming processes occurring at the anode during electrolysis can be summed up as follows:



The electrolysis using the Dinh-Nguyen cell was unsatisfactory on several counts.

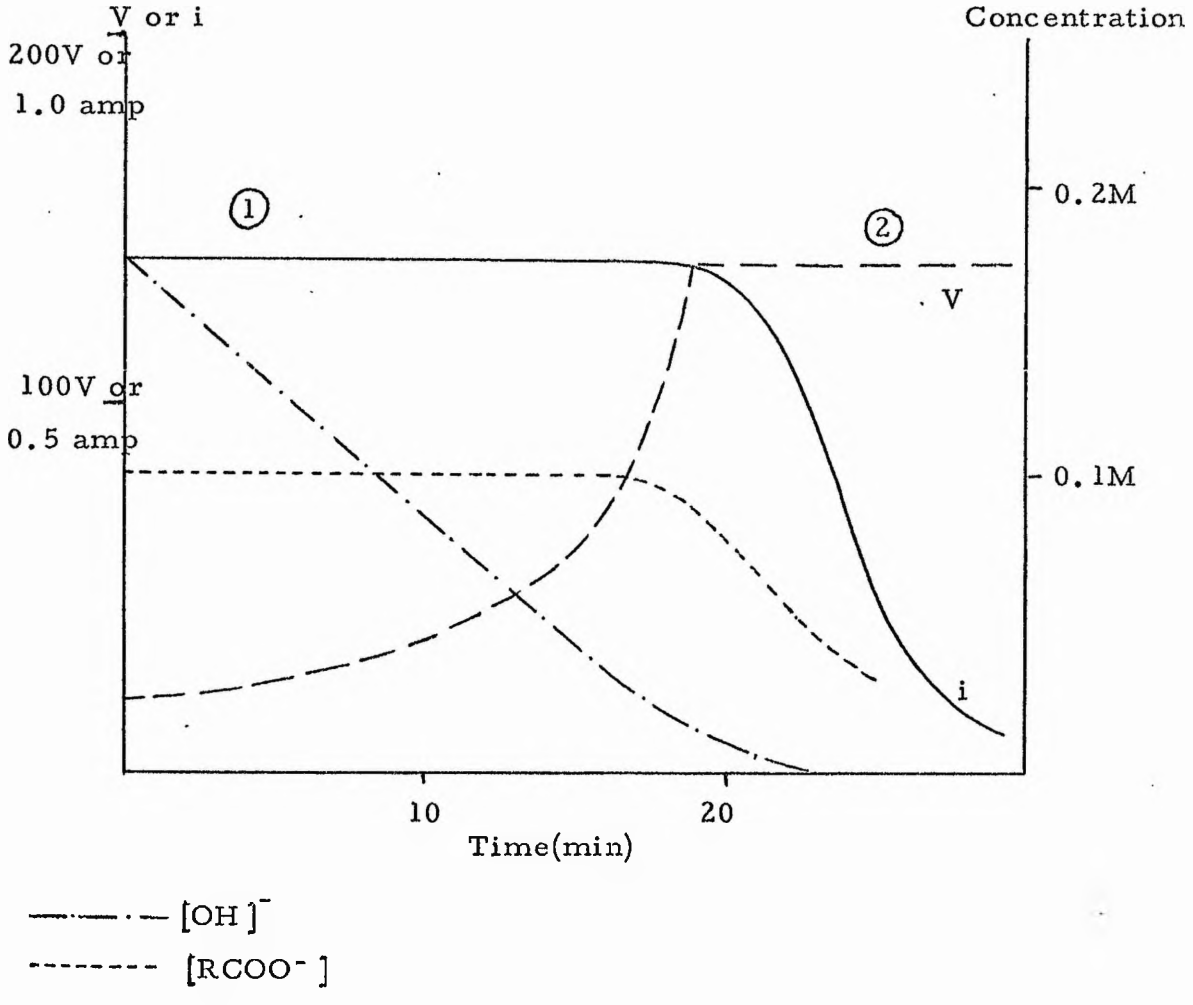
(i) Low yields ($\leqslant$ 25%) were obtained.

(ii) Although the total electrolysis time for 1 g of fatty acid was

about 5 hr considerable periods (4-24 hr) were required for the current to recover before electrolysis could proceed (Section 4:3:17). This was thought to be due to the slow release of sodium in amalgam with the mercury cathode to regenerate base. The rapid fall in current across the cell coincided with the number of moles of electrons passed through the cell in order to remove the major current carrying species $Na^{+}OH^{-}$, while addition of more NaOH resulted in the recovery of the current.

(iii) The variables current (i), voltage (V) and [NaOH] could not be kept constant during the electrolysis. Their behaviour is shown schematically in Figure 32.

Figure 32: Current, Voltage and Ion Concentrations During Electrolysis Using the Dinh-Nguyen Cell.



(iv) The voltage across the anode-electrolyte interface was unknown and could not be controlled.

To overcome drawbacks (ii) and (iii) an electrolysis cell consisting of a water jacket, magnetic stirrer and two platinum electrodes (1 cm^2, 1 cm apart) was tried. Oleic acid (2 mmole) in 5% aqueous methanol (40 ml) was electrolysed in the presence of NaOH (4.0 or 0.4 mmole) for 2 hr. The polarity of the cell was reversed at 5-10 min intervals. In the presence of excess base for neutralisation a current of 0.7 amp was maintained at 20-25 V (ie. Region (1) in Figure 32), and no noticeable electrolysis of the carboxylate anion occurred. In the presence of excess oleic acid a current of 0.1-0.2 amp could be maintinaed at 140 V (ie. Region (2) in Figure 32). Presumably the high voltage was required to counter the low specific conductivity of sodium oleate in 5% aqueous methanol, giving a large voltage drop across the electrolyte but not effectively altering the electrode interfacial potential. The 2 hr electrolysis resulted in a 60% consumption of oleic acid, as judged by GLC after esterification of an aliquot. The electrolytic efficiency was ~10%. The product, oleoyl nor-alcohol, could not however be satisfactorily cleaned up by TLC, as judged by IR and PMR spectroscopy, as compared to the product from electrolysis using the Dinh-Nguyen cell.

The suggestions that carbon electrodes favoured the production of carbonium ions and that nor-alcohol formation proceeded via a carbonium ion [205] prompted an experiment where the platinum electrodes were replaced by graphite rods but the other variables were identical with the experiment just described. A complex product mixture was obtained, with no oleoyl nor-alcohol.

Further work on anodic decarboxylation using the cell with two platinum electrodes was not undertaken in order to maximise the yield of oleoyl nor-alcohol, owing to the difficulty of obtaining pure nor-alcohol. The Dinh-Nguyen cell was used to produce the monoenoic nor-alcohols required for the ^{14}C-carboxyl labelling.

4:3 Experimental

4:3:1 Solvents

Solvents were of reagent grade. For chromatography petroleum (bp 40-60°C) and diethyl ether were redistilled before use, and all radiochemical manipulations were carried out using Analar grade solvents.

Pyridine was dried by refluxing with potassium hydroxide pellets for 2 hr before distillation and the dry pyridine stored over potassium hydroxide.

Acetone was dried by refluxing with anhydrous calcium sulphate (60 g/l) for 3 hr with vigorous mechanical stirring prior to distillation.

Diethyl ether and THF were given a preliminary drying by standing over sodium wire (one pellet of sodium/l). For use in the Grignard condensations and the coupling reactions in liquid ammonia these ethers were further dried by refluxing for 2 hr with lithium aluminium hydride (1g/l) and then distilled.

DMSO was dried by refluxing with calcium hydride (30g/l) for 2 hr before distillation and the dry DMSO was stored over calcium hydride.

Ethanol was dried as described by Vogel (method 1) [262].

Dichloromethane was dried by refluxing with phosphorus pentoxide (30 g/l) for 3 hr prior to distillation.

Benzene was dried by azeotropic distillation and stored over sodium wire.

4:3:2 Spectroscopy

IR spectra were recorded on a Perkin Elmer 257 grating spectrophotometer. Unless stated to the contrary samples were run as films between sodium chloride discs.

Mass spectra were recorded with direct-probe insertion of samples into the source of an AEI MS902 mass spectrometer. The source pressure was 2×10^{-7} torr, source temperature about 200°C, and the ionisation voltage 70 eV.

PMR spectra were recorded at 100 MHz on a Varian HA100 instrument using 10-15% solutions in carbon tetrachloride with 3% tetramethylsilane as the internal standard. Chemical shift values are given in ppm downfield from tetramethylsilane. The bulk methylene (δ=1.25-1.3 ppm) and terminal methyl resonances (δ=0.88-0.90 ppm) are omitted from the experimental data unless deviations from the above ranges occurred. Integrals gave good correlation with the number of protons for a particular resonance.

4:3:3 Chromatography

Analytical TLC was carried out on 20 cm glass plates coated with a 0.1 mm layer (wet thickness) of silica gel G. Where a high resolution separation was not required (often for monitoring column chromatography) microscope slides, coated by dipping in a silica gel G-chloroform slurry and dried in air, were used. For separations on a preparative scale 20 cm glass plates coated with a 1.0 mm layer (wet thickness) of silica gel G were used. After their preparation analytical TLC plates were activated at 110-120^{o}C for one hour and preparative plates for two hours. Plates stored for more than several days were reactivated for a further 30 min prior to use. Argentation TLC used 5-10% silver nitrate in silica gel G (w/w).

Mixtures of petroleum (bp 40-60^{o}C) and diethyl ether were normally used as developing solvents for TLC. Abbreviations such as PE|8 indicate mixtures of petrol in ether in a ratio of 92:8 (v/v). Double development was commonly used with preparative TLC.

The components on an analytical TLC plate were detected by spraying with a 10% (w/v) ethanolic solution of phosphomolybdic acid then heating at 120^{o}C for 5 minutes. When long-chain saturated compounds were analysed heating on a hot plate ensured more rapid and complete charring. Preparative TLC plates were sprayed with a 0.1% (w/v) ethanolic solution of 2',7'-dichlorofluorescein and viewed under ultra-violet light. The bands were marked, scraped off and eluted with diethyl ether, which was then blown off with nitrogen.

Column chromatography was used for purification of more than 1 g of material. The column was packed, in the presence of solvent,

with Sorbsil (a silica preparation) or 15% silver nitrate in Sorbsil (w/w) such that the packing (dry) weighed 10-20 times that of the material to be purified. Silver nitrate and Sorbsil were slurried in distilled water and activated at 120°C for 24 hr. The components were eluted with progressively more polar mixtures of diethyl ether in petroleum (bp 40-60°C) and the fractions analysed by microscale-TLC and/or GLC.

GLC was carried out with a Pye 104 chromatograph fitted with a flame ionisation detector. Packed stainless steel or glass columns (1.52m x 4.75 mm id) were used and unless otherwise specified these contained a 20% (w/w) DEGS stationary phase coated on a Chromosorb W (80-100 mesh) support. The carrier gas, nitrogen, was maintained at a flow rate of 60-70 ml min^{-1} and the oven temperature was 120-190°C, although very occasionally a temperature of 200°C was used. Quantification of the peaks was based on peak height x retention distance, and is reported as such (ie. "GLC purity" or "GLC composition"). Saturated straight chain methyl esters were used as external or internal standards to determine ECL values. Apparent inconsistencies in ECL values reported in the text are due either to different operating temperatures or to the deterioration of the polar liquid phase with prolonged use. ECL values were used to identify compounds.

4:3:4 Radioactivity (^{14}C) Detection and Measurement

The radioactivity of ^{14}C containing samples was measured by liquid scintillation counting using a Philips Liquid Scintillation Analyser. NE 260 (14 ml) was the scintillation medium, and the maximum activity counted was about 30 nCi.

For TLC work using ^{14}C-labelled materials radioactive bands on the TLC plate were located by autoradiography or by the use of a Panax Thin Layer Scanner (RTLS-1). Autoradiography gave better resolution and was more frequently used. The TLC plate was spotted with radioactive ink markers and left in direct contact with a 8" x 10" Kodak X-ray film for an exposure time that depended on the amount

of activity applied to the plate. As a rough guide 1 μCi applied per cm required an exposure time of 1 hr whereas a 50 nCi cm^{-1} loading was usually left for 36 to 48 hours. The X-ray film was developed in Kodak DX-80R Replenisher diluted with 4 volumes of distilled water or in Kodak D-19 Developer, rinsed in a 3% (v/v) aqueous acetic acid stop bath, and fixed with Kodak Rapid Fixer solutions. From the autoradiograph the required bands could be located exactly and were scraped off. The material could either be recovered by elution with ether (for fatty acids or their methyl esters) or the activity counted directly after shaking up the silica with 14 ml of NE 260 scintillation medium in a scintillation vial.

For radio-GLC analysis an instrument built at the Colworth laboratory was used. The Pye 104 oven unit contai ned a 9 ft glass column packed with 10% DEGS stationary phase on 80-120 mesh celite. Argon, at a flow rate of 50-60 ml min^{-1}, was the carrier gas, and the mass and radioactivity detection was essentially that as described by James and Piper [265]. A combustion tube containing powdered copper (II) oxide and then iron filings converted organic material from the column to carbon dioxide and hydrogen. Subsequently a bleed of 5% carbon dioxide was introduced into the carrier gas. Radioactivity was measured in a flow proportional counter, mass was measured in a katharometer, and the count rate and mass responses displayed on two separate chart recorders with their chart speeds synchronised. Samples of activity up to 50 nCi were co-injected with cold, saturated fatty acid methyl ester standards. However, owing to the problem of matching the two traces, ECL values were accurate only to ± 0.1 units.

4:3:5 Esterification

For small scale methylation the fatty acid ($\leqslant$50 mg) was refluxed for 30 min with 2% methanolic sulphuric acid (2 ml). The cooled reaction mixture was poured into 5% brine (6 ml) and extracted with ether (3 x 3 ml). The ether extracts were pooled, washed with

5% brine (2 x 5 ml) and dried. This procedure was scaled up where necessary (eg. 2 g of fatty acid were refluxed for 40 min with 2% methanolic sulphuric acid (30 ml) etc.).

For radiochemical work micro-scale methylation was achieved by dissolving the acid in a few drops of methanol and adding an ethereal diazomethane solution until a yellow colour persisted.

4:3:6 The Synthesis of α-Chloroalk-ω-ynes

$$HO(CH_2)_nOH \longrightarrow Cl(CH_2)_nCl \longrightarrow Cl(CH_2)_nI$$

$$\longrightarrow Cl(CH_2)_nC{:}CH$$

This scheme was followed exactly for n=5 and 6, but 1-chloro-4-iodobutane (n=4) was available from previous synthetic programmes in this laboratory, and 1-bromo-3-chloropropane (n=3, Koch-Light Ltd) was used in the coupling reaction with sodium acetylide.

α,ω-DICHLOROALKANES [182, 216, 217]

Thionyl chloride (8 mole) was added dropwise to a stirred mixture of α,ω-alkanediol (2 mole) and dry pyridine (24 ml) at 5°C. The resulting mixture was stirred at room temperature for 2 hr and then refluxed for 2 hr. Excess thionyl chloride was destroyed by the careful addition of ice-cold water, more water added (1 l) and the product extracted into ether (3 x 400 ml) and washed with water (1 l), saturated aqueous sodium bicarbonate (1 l) and 5% brine (1 l). The crude product was purified by vacuum distillation over a 20 cm column packed with glass helices.

n=5. 578 g (5.6 mole) of pentane-1,5-diol gave 691 g (4.9 mole) of 1,5-dichloropentane. Yield = 87%, bp = 78-80°C/22 mm (lit. bp = 76-78°C/21 mm [266]), GLC purity ⩾ 99%.

n=6. 500 g (4.24 mole) of hexane-1,6-diol gave 539 g (3.5 mole) of 1,6-dichlorohexane. Yield = 82%, bp = 99-100°C/24 mm (lit. bp = 99°C/28 mm [182]), GLC purity ⩾ 99%.

α-CHLORO-ω-IODOALKANES [182, 216, 217]

Sodium iodide (1.4 mole) in dry acetone (1 l) was slowly added to a stirred solution of α,ω-dichloroalkane (1.4 mole) in dry acetone (250 ml), and the mixture refluxed with vigorous mechanical stirring

for 4 hr. The sodium chloride precipitate was filtered off, the bulk of the acetone removed at the rotary evaporator and water (1 l) added. The product was extracted into ether (3 x 300 ml) and washed with water (2 x 500 ml). The iodochloride was separated from the dichloride and diiodide by vacuum distillation using a 0.6 m Fenske column and a Perkin triangle system.

n=5. 736 g (5.22 mole) of 1,5-dichloropentane gave 212 g of recovered material (1.5 mole, 28.5%) and 500 g (2.06 mole) of 1-chloro-5-iodopentane. Yield = 39.5%, bp = 62-65°C/2 mm (lit. bp = 94-98°C/8 mm [217]), GLC purity = 96%.

n=6. 640 g (4.13 mole) of 1,6-dichlorohexane gave 146 g of recovered material (0.95 mole, 22%) and 512 g (1.97 mole) of 1-chloro-6-iodohexane. Yield = 47.5%, bp = 82-86°C/1.5 mm (lit. bp = 73-74°C/0.7 mm [182]), GLC purity = 95%.

Both iodochlorides were produced in batches, with intermediate fractions from one distillation pooled and added to the next distillation. The higher yield for 1-chloro-6-iodohexane is explained by the recycling of some recovered 1,6-dichlorohexane from the early distillations into the iodination reaction.

α-CHLOROALK-ω-YNES [216, 217]

1 l of anhydrous liquid ammonia was distilled into a 2 l three-necked flask fitted with a magnetic stirrer and a cardice condenser. The apparatus was kept under a slight pressure of dry nitrogen and the outlet to the atmosphere was protected by a Dreschel bottle filled with potassium hydroxide pellets. 1-1.5 g of hydrated ferric nitrate crystals were added, stirring commenced, and sodium acetylide prepared by the titration method [267]. Acetylene (passed through a cardice-cooled trap to condense out acetone, two Dreschel bottles filled with concentrated sulphuric acid and one of potassium hydroxide pellets to dry the gas) was bubbled through the liquid ammonia at the rate of 4-5 bubbles a second while small pieces of sodium (25.3 g, 1.1 mole in total) were added. Each piece of sodium was added after the blue colour produced by the preceding piece had disappeared. The flow of acetylene was stopped about half an hour after the blue colour produced by the last piece had disappeared.

A solution of the α-chloro-ω-iodoalkane (1 mole) in dry ether (250 ml) was added dropwise to the sodium acetylide suspension, and the mixture refluxed while stirring for 4 hr. The ammonia was allowed to evaporate overnight, the product filtered through a glass wool plug, extracted into ether (3 x 300 ml) after the addition of water, washed with water (2 x 500 ml) and dried. The crude product was purified by distillation or vacuum distillation over a 20 cm column packed with helices.

Data for the four syntheses (n=3-6) are given in Table 27.

4:3:7 The Preparation of 1-Bromoalkanes

Most of the 1-bromoalkanes required for coupling with acetylenic molecules were available but a few had to be prepared from the corresponding alkan-1-ols. Concentrated sulphuric acid (20 ml) was added slowly to 47 % aqueous hydrobromic acid (100 ml), followed by the alkan-1-ol (0.5 mole) and a further 15 ml of concentrated sulphuric acid. The mixture was refluxed for 3.5 hr, cooled and carefully poured into water (500 ml). The product was extracted into ether (3 x 200 ml), washed with water (500 ml), saturated aqueous sodium bicarbonate (500 ml) and water (500 ml), dried and distilled under vacuum over a short Vigreux column.

28.8 g (0.28 mole) of hexan-1-ol gave 41 g (0.25 mole) of 1-bromohexane. Yield = 88%, bp = 52-54°C/18 mm (lit. bp = 88°C/90 mm [268]), GLC purity $\geqslant$ 99%.

36.4 g (0.31 mole) of heptan-1-ol gave 44.8 g (0.25 mole) of 1-bromoheptane. Yield = 79%, bp = 72-74°C/20 mm (lit. bp = 71°C/19 mm [268]), GLC purity $\geqslant$ 99%.

39.2 g (0.27 mole) of nonan-1-ol gave 47 g (0.22 mole) of 1-bromononane. Yield = 81%, bp = 107-109°C/20 mm (lit. bp = 98°C/11 mm [271]), GLC purity = 97%.

100 g (0.58 mole) of undecan-1-ol gave 128 g (0.53 mole) of 1-bromoundecane. Yield = 90%, bp = 147-149°C/26 mm (lit. bp = 134-137°C/18 mm [217]), GLC purity = 97%.

TABLE 27. IR, NMR, GLC, bp and Yield Data for the Synthesis of α-Chloroalk-ω-ynes

Product	8-chlorooct-1-yne	7-chlorohept-1-yne	6-chlorohex-1-yne	5-chloropent-1-yne
Starting material	1-chloro-6-iodo-hexane 2.02 mole	1-chloro-5-iodo-pentane 2.06 mole	1-chloro-4-iodo-butane 0.83 mole	1-bromo-3-chloro-propane 1.60 mole
Product recovery (moles) Yield	1.49 mole 74%	1.67 mole 81%	0.52 mole 62%	0.67 mole 42%
Product bp(°C) lit. bp (°C)	44.5-48/2mm 34/2mm [217] 73-76/10 mm [182]	72-75/25mm 78-79/28 mm [217]	54-56/26mm 47-48/17 mm [269]	106-110 110-112 [217]
GLC purity ECL (20% DEGS, 135°C)	96.5% 9.55	95% 8.55	97% 7.35	94% 6.0
IR spectrum	Terminal acetylene gave C-H and C≡C stretching frequencies at 3320 cm^{-1} (s) and 2135 cm^{-1} (s) respectively			
PMR spectrum[1]				
$\underline{H}C{:}C\text{-}CH_2\text{-}$	1.78, t, 1H	1.81, t, 1H	1.83, t, 1H	1.85, t, 1H
$HC{:}C\text{-}C\underline{H}_2\text{-}$	2.15, m, 2H	2.17, m, 2H	2.21, sx, 2H	2.34, sx, 2H
$\text{-}C\underline{H}_2Cl$	3.47, t, 2H	3.49, t, 2H	3.52, t, 2H	3.60, t, 2H
$\text{-}(CH_2)_n\text{-}$	1.64 and 1.75, b, 8H	1.6-1.8, b, 6H	1.6-2.0, b, 4H	1.95, qu, 2H

1. ^1H-^1H spin-spin coupling constant $J_{HC{:}CCH_2\text{-}} = 2.5$ Hz

4:3:8 The Synthesis of 1-Chloroalk-n-ynes (n=4-7) and of Heptadec-4-yn-1-ol by Condensation Reactions in Liquid Ammonia

C_8-C_{17} 1-substituted alk-n-ynes (n=4-7) were prepared, with one exception, by the coupling of 1-bromoalkanes with the sodio derivative of α-chloroalk-ω-ynes. The exception was the synthesis of heptadec-4-yn-1-ol by the coupling of 1-bromododecane with the dilithio derivative of pent-4-yn-1-ol.

Anhydrous liquid ammonia (250 ml) was distilled into a 500 ml, three-necked flask fitted with a cardice condenser and a magnetic stirrer and kept under a slight pressure of dry nitrogen. 0.3-0.4 g of hydrated ferric nitrate was added, followed by small pieces of sodium (0.23 mole in total) to give a stirred suspension of sodamide. Each piece was added after the blue colouration caused by the preceding piece had disappeared. A solution of the α-chloroalk-ω-yne (0.20 mole) in dry ether (50 ml) was added dropwise while stirring and the mixture allowed to reflux for 1.5 hr. 1-Bromoalkane (0.23 mole) in dry ether (50 ml) was added dropwise and refluxing continued for at least 5 hr. The ammonia was allowed to evaporate overnight, the residue diluted with water (500 ml), filtered through a glass wool plug, and the product extracted into ether (3 x 100 ml), washed with water (2 x 200ml) and dried.

Purification of the crude product, $CH_3(CH_2)_mC{:}C(CH_2)_nCl$, was usually accomplished by vacuum distillation over a short (10 cm) Vigreux column, but when m=0 $AgNO_3$-silica column chromatography was used prior to distillation to remove unreacted α-chloroalk-ω-yne efficiently. For the longer chain 1-chloroalkynes prepared ($m+n \geqslant 13$), where yields were low, silica column chromatography was used either on its own or after distillation to upgrade the product.

The coupling of 1-bromododecane with pent-4-yn-1-ol was done according to Ames et al [228] using the experimental procedure detailed above with the following modifications:-

(1) A lithamide suspension was prepared from 1.1 g lithium (0.16 mole) in 200 ml of liquid ammonia plus 0.2 g of hydrated ferric nitrate.

(2) Dry THF replaced ether as the solvent for pent-4-yn-1-ol (0.07 mole) and 1-bromododecane (0.07 mole).

(3) The residue was acidified with dilute aqueous hydrochloric acid prior to the work-up.

(4) The crude product was purified by chromatography. A column packed first with 200 g of silica, then 70 g of a 2:5 (w/w) $AgNO_3$:silica mixture was employed.

Yield, purity, bp, ECL and PMR spectroscopic data for all the syntheses are presented in Table 28, along with the method of purification of the crude product. GLC analysis of the latter showed that the low % yields were co-incident with low % conversions, thus indicating insolubility problems for long chain alkyl compounds in a liquid ammonia reaction medium.

Although no systematic study of the condensation reaction byproducts was attempted, on several occasions some were identified. The early fractions from the distillation of 1-chlorohexadec-7-yne gave an enrichment of hexadec-1-en-7-yne. Its structure was deduced from the following evidence:-

(1) Its TLC behaviour showed it to be less polar than 1-chlorohexadec-7-yne, while it had an ECL value of approximately 10.0 (20% DEGS, 180°C).

(2) Its IR spectrum , run as a 2% CCl_4 solution, showed the characteristic absorptions of $H_2C{:}CH-$ at 3085 cm^{-1}, 1640 cm^{-1} and 920 cm^{-1}.

(3) The characteristic olefinic proton resonances of a terminal double bond were observed in its PMR spectrum [169].

During the column chromatography of heptadec-4-yn-1-ol a 0.5 g fraction of an unknown compound (GLC purity = 97%) was obtained. It was characterised as dodecyl nitrate thus:-

(1) TLC showed it to be less polar than an alcohol but more polar than an alkyl halide. On a 20% DEGS column at 190°C it had an ECL value of 13.7 .

(2) A Lassaigne sodium fusion test indicated the presence of nitrogen.

TABLE 28. 1-Chloroalk-n-ynes (n=4-7) and Heptadec-4-yn-1-ol:

Product	Starting Materials	Method of purification bp (°C)
1-chlorononon-7-yne	8-chlorooct-1-yne (0.19 mole) methyl iodide (0.23 mole)	$AgNO_3$-silica column; distillation 60-62/0.5 mm
1-chloroundec-7-yne	8-chlorooct-1-yne (0.19 mole) 1-bromopropane (0.23 mole)	Distillation 94-98/2 mm
1-chlorotridec-7-yne	8-chlorooct-1-yne (0.14 mole) 1-bromopentane (0.15 mole)	Distillation 112-115/2 mm
1-chlorotetradec-7-yne	8-chlorooct-1-yne (0.145 mole) 1-bromohexane (0.18 mole)	Distillation 116-119/0.5 mm
1-chloropentadec-7-yne	8-chlorooct-1-yne (0.145 mole) 1-bromoheptane (0.18 mole)	Distillation 127-129/0.5 mm
1-chlorohexadec-7-yne	8-chlorooct-1-yne (0.145 mole) 1-bromooctane (0.21 mole)	Distillation; silica column 136-138/0.5 mm
1-chlorooct-6-yne	7-chlorohept-1-yne (0.19 mole) methyl iodide (0.24 mole)	$AgNO_3$-silica column; 56-59/3 mm distillation
1-chlorononon-6-yne	7-chlorohept-1-yne (0.19 mole) ethyl bromide (0.24 mole)	Distillation 61-64/1 mm
1-chloroundec-6-yne	7-chlorohept-1-yne (0.145 mole) 1-bromobutane (0.17 mole)	Distillation 80-84/1 mm
1-chlorotridec-6-yne	7-chlorohept-1-yne (0.12 mole) 1-bromohexane (0.15 mole)	Distillation 108-111/1.5 mm
1-chlorotetradec-6-yne	7-chlorohept-1-yne (0.12 mole) 1-bromoheptane (0.15 mole)	Distillation 105-108/0.5 mm
1-chloropentadec-6-yne	7-chlorohept-1-yne (0.14 mole) 1-bromooctane (0.17 mole)	Distillation 118-120/0.5 mm
1-chloroundec-5-yne	6-chlorohex-1-yne (0.15 mole) 1-bromopentane (0.17 mole)	Distillation 91-93/1.5 mm
1-chloroheptadec-5-yne	6-chlorohex-1-yne(0.15 mole) 1-bromoundecane (0.17 mole)	Distillation; silica column 158-161/2 mm
1-chloropentadec-4-yne	5-chloropent-1-yne (0.135 mole) 1-bromodecane (0.15 mole)	Distillation; silica column 160-162/3.5 mm
1-chlorohexadec-4-yne	5-chloropent-1-yne (0.13 mole) 1-bromoundecane (0.14 mole)	Silica column -
Heptadec-4-yn-1-ol	pent-4-yn-1-ol (0.07 mole) 1-bromododecane (0.08 mole)	$AgNO_3$-silica column -

1. % Yield is calculated against moles of α-substituted alk-ω-yne.

2. 20% DEGS, 180°C

% Yield[1]	% GLC purity[2] ECL	PMR Spectrum[3] $CH_3(CH_2)_aCH_2C{:}CCH_2(CH_2)_bCH_2CH_2X$				
45	95 10.9	1.73,t	-		2.07,m	1.77,m 3.46,t
44	97 11.8	0.96,Dt	2.08,m		"	"
49	97 13.8	0.90,Dt	2.09,m		"	"
62	98 14.7	0.89,Dt	"		"	"
58	96 15.7	"	"		"	"
31	97 16.7	"	"		"	"
43	98 9.9	1.72,t	-		2.09,m	1.78,m 3.47,t
57	98 10.2	1.09,t	2.13,m		2.07,m	" "
71	98 11.9	0.91,Dt	2.10,m		"	"
60	95 13.8	0.89,Dt	"		"	"
52	93 14.8	"	"		"	"
50	94 15.7	"	"		"	"
47	97 11.6	0.90,Dt	2.0 ,m		2.15,m	~1.8,m 3.50,t
20	94 17.5	0.89,Dt	"		"	" "
12	96 15.2	0.89,Dt	2.09,m		2,30,m	1.89,qu 3.58,t
5	95 16.2	"	"		"	" "
28	89 20.9	"		~2.15,m		1.7,m 3.72,b[4]

3. Only the distinctive resonances, from the protons underlined, are quoted. Integrals were consistent with the structure, and are not given.

4. Hydroxyl proton resonance 4.50, b, 1H.

(3) Its IR spectrum, obtained as a 0.5% carbon tetrachloride solution, showed very strong, sharp absorptions at 1635 cm^{-1} and 1280 cm^{-1}, indicating the presence of an alkyl nitrate R-O.NO_2 [264].

(4) Its mass spectrum displayed the dominant fragmentation pattern of a long n-alkyl chain, with no molecular ion peak but additional mass peaks at m/e = 46, 76, 105. These presumably corresponded to NO_2^+, $CH_2NO_3^+$ and $C_3H_7NO_3^+$ respectively.

(5) A general structure $CH_3(CH_2)_{8-10}CH_2CH_2X$, where X had a strong deshielding influence and no protons, could be deduced from its PMR spectrum, which showed the following signals:- 0.89, Dt, 3H; 1.27, b, 16-20H; 1.72, m, 2H; 4.40, t, 2H. Other nitrate ester by-products were noted in some of the condensation reactions.

4:3:9 Partial Hydrogenation of Monoacetylenes using Lindlar Catalyst

Quinoline poison was required for the stereospecific partial hydrogenation of alkynes to cis-olefins, otherwise appreciable amounts (up to 25%) of the trans-olefin could be formed. The % trans content was determined by IR spectroscopy using 1-2% (w/v) carbon disulphide solutions of known molarity and sodium chloride cells of path length 1.0 mm. The optical density of trans-olefin absorption at 970 cm^{-1} was compared with a calibration graph obtained from standard mixtures of methyl oleate and elaidate [271].

The hydrogenations were performed on a 0.5-6 g scale, with an acid extraction to remove quinoline. No other purification was necessary. In a typical experiment 1.6 g of chloroalkyne, 0.2 g of Lindlar catalyst and 0.15 g of quinoline were shaken in methanol (50 ml) in an atmosphere of hydrogen. A rapid uptake of hydrogen occurred over 5 min, which stopped as the theoretical volume for partial hydrogenation was reached. The resulting suspension was filtered, with washings, through two layers of filter paper, and the bulk of the methanol removed. The residue was taken up in ether (80 ml), washed with 1 M aqueous hydrochloric acid (2 x 50 ml), water (50 ml), saturated aqueous sodium bicarbonate (50 ml) and water (50 ml), and dried.

The ratio of Lindlar catalyst to alkyne was about 1:10 and the ratio of catalyst to quinoline was kept between 1:1 and 3:2. The alkene was analysed by GLC and often by PMR spectroscopy (Table 29). IR spectroscopy showed the trans content of the alkene never to exceed 5%. Recoveries of the longer chain length compounds ($\geq C_{11}$) were greater than 85%, and of the shorter chain length compounds (C_8, C_9) between 55 and 70%.

4:3:10 Cis-Trans Isomerisation with Oxides of Nitrogen

0.5-1.0 g of cis-chloroalkene in ether (20 ml) was shaken with 2M aqueous sodium nitrite (15 ml) and 6M aqueous nitric acid (10 ml) for 2 hr. The mixture was transferred to a separating funnel and the ether layer washed with water (30 ml), saturated aqueous sodium bicarbonate (30 ml) and water (30 ml). The product was purified by preparative TLC, and the weight of chloroalkene recovered lay in the range 49-72%.

A control experiment using 1-chloropentadec-cis-6-ene, where the reaction was monitored by Ag^+TLC during 4 hr, indicated that 2 hr would be a suitable time for obtaining an approximately equal cis:trans olefin ratio and for minimising the formation of polar byproducts. Von Rudloff oxidative cleavage of the cis- and trans-chloroalk-6-ene products indicated no double bond migration [272].

4:3:11 The Preparation of Non-8-ynoic Acid by Chain Extension of α-Chloroalk-ω-ynes

A stock of non-8-ynoic acid was needed for condensation with 1-iodoalk-2-ynes to give alka-8, 11-diynoic acids (Section 4:3:13). This material was prepared by chain extension of 8-chlorooct-1-yne with potassium cyanide or by chain extension of 7-chlorohept-1-yne with diethyl malonate.

8-Chlorooct-1-yne (48 g, 0.3 mole) and potassium cyanide, 23 g, 0.35 mole) in dry DMSO (200 ml) were vigorously stirred at 120-130°C for 4 hr. On cooling, the mixture was added to 5% brine (1 l), and the product extracted into ether (4 x 100 ml) and washed with 5% brine

TABLE 29. Data for the Partial Hydrogenation of Monoacetylenic Compounds

Product	% GLC purity[1] ECL	PMR spectrum[2] $CH_3(CH_2)_aCH_2CH^{c}{=}CHCH_2(CH_2)_6CH_2CH_2X$					
1-chlorononcis-7-ene	98 8.3	1.58,d	-	5.32,m	2.03,m	1.75,Dqu	3.45,t
1-chloroundec-cis-7-ene	96 9.9	0.91,Dt	2.01,m	5.29,m	2.01,m	"	"
1-chlorotridec-cis-7-ene	88 11.7	0.89,Dt	2.0,m	5.28,m	2.0,m	"	"
1-chlorotetradec-cis-7-ene	99 12.7	"	"	"	"	"	"
1-chloropentadec-cis-7-ene	98 13.7	"	"	"	"	"	"
1-chlorohexadec-cis-7-ene	96 14.7	"	"	"	"	"	"
1-chlorooct-cis-6-ene	98 ~7.0	1.58,d	-	5.32,m	2.03,m	"	"
1-chlorononcis-6-ene	95 8.0	0.95,t	2.0,m	5.27,m	2.0,m	"	"
1-chloroundec-cis-6-ene	99 10.1	0.90,Dt	"	"	"	"	"
1-chlorotridec-cis-6-ene	97 12.0	0.89,Dt	"	"	"	"	"
1-chlorotetradec-cis-6-ene	96 12.8						
1-chloropentadec-cis-6-ene	95 13.7						

continued on next page

1-chloropentadec-cis-4-ene	96	13.6	0.89, Dt	~2.0, m	5.28, 5.36 m	~2.18, m	1.79, qu	3.46, t
1-chlorohexadec-cis-4-ene	97	14.6	"	"	"	"	"	"
heptadec-cis-4-en-1-ol	90	19.2						
1-chlorohexadec-cis-11-ene[3]	94	14.9						

TABLE 29 (cont)

1. 20% DEGS, 180°C
2. Only the distinctive resonances, from the protons underlined, are quoted. Integrals were consistent with the structure, and are not given.
3. 16-Chlorohexadec-5-yne was available from previous synthetic programmes in this laboratory.

(3 x 250 ml). The residue (containing 95% nitrile as indicated by GLC) was hydrolysed by refluxing in a 5% aqueous ethanolic 1M potassium hydroxide solution (750 ml) for 3 hr. The bulk of the ethanol was distilled off, the mixture added to 2M aqueous hydrochloric acid (1.2 l) and the product extracted into ether (3 x 150 ml) and washed with water (2 x 500 ml).

Sodium (11.5 g, 0.5 mole) was dissolved in dry ethanol (700 ml) in a 2 l, three-necked flask fitted with a reflux condenser and a mechanical stirrer and kept under a slight pressure of dry nitrogen. Redistilled diethyl malonate (88g, 0.55 mole) was added dropwise while stirring and then the mixture refluxed for 1 hr. On cooling, sodium iodide (75 g, 0.5 mole) was added, followed by 7-chlorohept-1-yne (65.5 g, 0.5 mole) in dry ethanol (100 ml). The mixture was refluxed for 18 hr, the bulk of the ethanol distilled off, and water (1.5 l) added. The product, $HC{:}C(CH_2)_5CH(COOEt)_2$, was extracted into ether (3 x 200 ml) and washed with 5% brine (2 x 500 ml). The diester was saponified by refluxing in 5% aqueous ethanolic 1M potassium hydroxide solution (1 l.) for 2.5 hr, and decarboxylation was achieved by heating the neat dicarboxylic acid in an atmosphere of dry nitrogen at a modest partial vacuum. The temperature was raised from 120°C to 160°C over 1 hr and held at 160°C for a further 1 hr.

The non-8-ynoic acid residues from both chain extensions were pooled and purified by vacuum distillation over a 20 cm Vigreux column. 96 g (0.62 mole) of non-8-ynoic acid was collected, giving a combined, overall yield of 77.5%. GLC purity was 97%, and ECL = 12.6 (20% DEGS, 150°C) as measured after esterification of an aliquot. bp = 154-157°C/17 mm (lit. bp = 128°C/4 mm [270]). The PMR spectrum of non-8-ynoic acid gave the following signals:- 1.46, b, 8H ($-(C\underline{H}_2)_4-$); 1.78, t, 1H ($\underline{H}C{:}C-$); 2.15, D sx, 2H ($HC{:}CC\underline{H}_2-$); 2.32, t, 2H ($-C\underline{H}_2COOH$) and 11.7, s, 1H ($-COO\underline{H}$).

4:3:12 The Preparation of 1-Iodoalk-2-ynes

$$RBr + LiC{\equiv}CCH_2OLi \longrightarrow RC{\equiv}CCH_2OH \longrightarrow$$

$$RC{\equiv}CCH_2OMes \longrightarrow RC{\equiv}CCH_2I$$

ALK-2-YN-1-OLS

A 2l, three-necked flask, fitted with a mechanical stirrer and a cardice condenser and maintained under an atmosphere of dry nitrogen, was filled with anhydrous liquid ammonia (1 l). Hydrated ferric nitrate (0.3 g) was added and stirring commenced. After 15 min small pieces of lithium metal (1.05 mole) were added to give a lithamide suspension and 1 hr later a solution of propargyl alcohol (0.5 mole) in dry THF (50 ml) was added dropwise. After a further hour the alkyl bromide (0.3 mole) in dry THF (150 ml) was added slowly and the mixture refluxed for 5 hr while stirring. The ammonia was allowed to evaporate overnight, water added (1 l) and the product extracted into ether (3 x 200 ml) and washed with 5% brine (3 x 500 ml). It was purified by distillation through a short Vigreux column. Table 30 gives the yield, bp, GLC behaviour and purity, and IR and PMR spectra of the alk-2-yn-1-ols.

1-IODOALK-2-YNES

The method of Crossland and Servis was used for the preparation of the reactive propargyl mesylate [197], and the iodination was carried out according to Wallat and Kunau [234].

A solution of the alk-2-yn-1-ol (0.15 mole) and a 50% molar excess of redistilled triethylamine (0.225 mole) in dry methylene chloride (500 ml) was stirred mechanically in a 2 l, three-necked flask fitted with a pressure-equalising dropping-funnel and maintained under an atmosphere of dry nitrogen at 0 to -10°C by an ice-salt bath. A 10% molar excess of fresh methanesulphonyl chloride (0.165 mole) was added dropwise over 15 min and the mixture stirred for a further 20 minutes prior to a rapid extraction using chilled solutions and glassware. The reaction mixture was transferred to a 2 l separating funnel with the aid of more methylene chloride and washed with water (500 ml), 10% aqueous HCl (500ml), 5% brine (500 ml) and saturated

TABLE 30. The Synthesis and Properties of Alk-2-yn-1-ols

Product	Starting materials	% GLC purity[1] ECL	% Yield
But-2-yn-1-ol	Propargyl alcohol (0.6 mole) Methyl iodide (0.4 mole)	94.5 9.4	41[3]
Hex-2-yn-1-ol	Propargyl alcohol (0.5 mole) 1-Bromopropane (0.5 mole)	94 10.6	68.5
Dec-2-yn-1-ol	Propargyl alcohol (0.5 mole) 1-Bromoheptane (0.33 mole)	99 14.7	77
Dodec-2-yn-1-ol	Propargyl alcohol (0.4 mole) 1-Bromononane (0.23 mole)	94 16.7	81

1. 20% DEGS, 160°C
2. Integrals were consistent with the structure, and are not given
3. The low yield is presumably due to the volatility and/or water-solubility of the C_4 compound.

bp (°C)	IR Spectrum	PMR Spectrum[2] $\underline{H}O-C\underline{H}_2-C\equiv C-C\underline{H}_2-(C\underline{H}_2)_n-C\underline{H}_3$				
137-141, lit. [268] 143	3350 cm^{-1}(b) 2230 cm^{-1}(s)	3.4, b	4.11, q	-	-	1.82, t
167-9, lit. [268] 100/53mm	3350 cm^{-1}(b) 2220 cm^{-1}(m) 2280 cm^{-1}(m)	3.2, b	4.19, b	2.19, m	1.55, sx	1.00, t
137-140/ 26mm	"	2.9, b	4.14, b	2.16, m	1.30, b	0.89, Dt
90-94/ 0.3mm	"	2.7, b	4.12, b	2.16, m	1.28, b	0.89, Dt

aqueous sodium bicarbonate (500 ml). After drying the propargyl mesylate was checked by IR spectroscopy. The hydroxyl absorption at 3350 cm^{-1}(b) was absent, while the characteristic mesylate O=S=O and S-CH_3 stretching frequencies at 1350 cm^{-1}(s) and 1170 cm^{-1}(s) were observed.

The iodination was then accomplished without delay in a 2 l, three-necked flask fitted with a pressure-equalising dropping funnel and a mechanical stirrer and maintained under an atmosphere of dry nitrogen. The mesylate, dissolved in dry acetone (100 ml), was added dropwise over 30 min to a stirred solution of sodium iodide (0.6 mole) in dry acetone (600 ml) at room temperature. After a further hour of stirring the precipitate was filtered off, most of the acetone removed at the rotary evaporator, and all the residues added to 2% aqueous sulphuric acid (1.2 l). The propargyl iodide was extracted into petrol (3 x 200 ml), washed with 0.2 M aqueous sodium thiosulphate (500 ml), saturated aqueous sodium bicarbonate (500 ml) and 5% brine (500 ml), dried and the solvent removed at the rotary evaporator. Care must be taken over the removal of solvent from 1-iodobut-2-yne, as it is fairly volatile.

The structure and purity of the 1-iodoalk-2-ynes were checked by IR and PMR spectroscopy, and GLC (Table 31). The product was judged to be of sufficient purity to be used directly in the next stage of the synthesis. Storage as the neat liquid at 2°C for a year did not result in any marked deterioration of the propargyl iodide.

The maximum GLC operating temperature that could be used to analyse the propargyl iodides on a 20% DEGS column before the onset of decomposition to a component of lower retention time was 150°C.

4:3:13 The Synthesis of Alka-8, 11-diynoic Acids by the Condensation of 1-Iodoalk-2-ynes with the Di-Grignard Derivative of Non-8-ynoic Acid

$$HC{\equiv}C(CH_2)_6COOH \xrightarrow{2EtMgBr} BrMgC{\equiv}C(CH_2)_6COOMgBr$$

$$\xrightarrow[2.\ H^+/H_2O]{1.\ RC{\equiv}CCH_2I} RC{\equiv}CCH_2C{\equiv}C(CH_2)_6COOH$$

TABLE 31. The Synthesis and Properties of 1-Iodoalk-2-ynes

Product, % Yield	Starting material	% GLC purity[1] ECL
1-Iodobut-2-yne 45[3]	But-2-yn-1-ol (0.13 mole)	90 8.75
1-Iodohex-2-yne 75	Hex-2-yn-1-ol (0.19 mole)	94.5 9.4
1-Iododec-2-yne 84	Dec-2-yn-1-ol (0.23 mole)	97.5 13.5
1-Iodododec-2-yne 75	Dodec-2-yn-1-ol (0.16 mole)	91 15.5

1. 20% DEGS, 150°C

2. Integrals were consistent with the structure, and are not given

3. The low yield is presumably due to the volatility of the C_4 compound

The IR spectra showed characteristic absorptions at 2230 cm^{-1}(s), 1175 cm^{-1}(s), and 1140 cm^{-1}(s).

PMR spectrum[2] $ICH_2-C{:}C-CH_2-(CH_2)_n-CH_3$			
3.60, t	-	-	1.80, t
3.65, t	2.16, m	1.53, sx	0.99, t
3.65, t	2.18, m	1.3, b	0.89, Dt
3.66, t	2.18, m	1.3, b	0.89, Dt

The Grignard acetylenic-coupling reaction was essentially that as described by Kunau et al [235]. To 3.05 g of magnesium turnings (0.125 mole) in dry THF (50 ml) (in a 500 ml flask fitted with a magnetic stirrer, reflux condenser and a pressure-equalising dropping funnel and maintained under an atmosphere of dry nitrogen) was added a solution of redistilled ethyl bromide (0.12 mole) in dry THF (75 ml) at a sufficient rate to maintain refluxing by the heat generated by Grignard formation. After the addition refluxing was continued for 15 min, then the flask and its contents were cooled to $0^{o}C$ and a solution of non-8-ynoic acid (0.05 mole) in dry THF (50 ml) was added dropwise. Stirring was continued at room temperature for 3 hr. The flask was cooled to $0^{o}C$, 0.2 g of cuprous cyanide was added, and 15 min later the 1-iodoalk-2-yne (0.05 mole) in dry THF (50 ml) was added dropwise to the mixture (at $0^{o}C$). The reaction mixture was stirred overnight at room temperature, filtered, and most of the THF removed. The residues were added to 1M aqueous sulphuric acid (600 ml), and the product was extracted into ether (4 x 60 ml), washed with water (3 x 200 ml) and dried.

An aliquot of the residue was esterified for GLC analysis (Table 32). The results showed that the large molar excess (2/1) of ω-acetylenic acid to propargyl iodide used by Kunau et al [235] is not necessary to give almost complete reaction of the propargyl iodide.

The diynoic acid was purified by argentation column chromatography. The column was packed first with Sorbsil then a 15% silver nitrate-Sorbsil mixture (w/w). The total acid fraction collected off the column was judged greater than 90% pure by GLC, with almost complete removal of the starting materials. Individual fractions, with GLC purity equal to or greater than 95%, were used for PMR (Table 32) and CMR (Appendix 2) spectroscopy. The yields for the total acid fraction were of the order of 60-80% (Table 32).

The diynoic acids deteriorated rapidly on storage.

TABLE 32. Data from the Synthesis, GLC Behaviour and PMR Spectra of Alka-8,11-diynoic Acids

Product	13:2(8a11a)	15:2(8a11a)	19:2(8a11a)	21:2(8a11a)
Starting materials:				
Non-8-ynoic acid	0.05 mole	0.07 mole	0.05 mole	0.05 mole
1-Iodoalk-2-yne	0.04 mole	0.053 mole	0.055 mole	0.06 mole
Crude product composition (GLC)				
Diynoic acid	66%	56%	78%	68%
Non-8-ynoic acid	31%	40%	10%	10%
1-Iodoalk-2-yne	1.5%	2%	3%	13%
% Yield[1]	72	68	80.5	63
% GLC purity[2]	94	93	90	91
ECL	20.9	21.55	25.3	27.1
PMR spectrum[3,4]				
Propargylic H	C(7) 2.12, m C(13) 1.74, t	2.10, m	2.10, m	2.10, m
Dipropargylic H	2.95, sx	2.99, qu	2.99, qu	2.98, qu
Other distinctive H resonances		C(15) 0.98, Dt		

1. Based on product recovered from argentation column chromatography
2. 20% DEGS, 190°C, methyl esters
3. Integrals were consistent with the structure, and are not given
4. Methyl ester (3.60, s and 2.23, t) and carboxylic acid (2.31, t and ~, 11, b) resonances are not given

4:3:14 The Lindlar Partial Reduction of the Alka-8,11-diynoic Acids and the Subsequent $LiAlH_4$ Reduction of the Methyl Alka-*cis*-8, *cis*-11-dienoates

Considerable difficulty was encountered in the partial hydrogenation of the diynoic acids. Why the Lindlar catalyst was easily poisoned was not clear, since repeated column chromatography of the methyl diynoate failed to produce a material that would hydrogenate, even when used directly off the column and judged pure by micro-TLC. Column chromatographic purification of the acid was also unsuccessful. However repeated low temperature crystallisation (-20°C to -70°C) from methanol or aqueous methanol (2-20% H_2O) gave a diynoic acid that could be hydrogenated satisfactorily. It was not known whether autoxidation products were responsible for the poisoning of the Lindlar catalyst. The methylene-interrupted diyne system autoxidised very rapidly, and a faint yellow tint was observed both for the methyl diynoate solution directly off the column, which did not hydrogenate, and for the diynoic acid in methanol, from the crystallisation, which did hydrogenate.

A small scale hydrogenation of the 15:2(8a11a) acid using a ratio of Lindlar catalyst to quinoline of 3:1 gave over-hydrogenation. There was no abrupt stop in the uptake of hydrogen, the product composition as analysed by GLC was 1% 15:0, 70% monoene and 27% diene, and IR spectroscopy showed the presence of *trans* double bonds. Therefore a 1:1 ratio of catalyst to quinoline, which gave reliable hydrogenation behaviour, was always used.

The diynoic acid was recrystallised at low temperature and dissolved in methanol. A pilot hydrogenation was attempted first. When 0.25 g of diynoic acid (wet wt.), 0.1 g of Lindlar catalyst, and 0.1 g of redistilled quinoline in methanol (5 ml) showed a rapid uptake of hydrogen which stopped abruptly after 3-6 minutes, the rest of the diynoic acid was hydrogenated. A ratio of 4:1:1 (w/w/w) of diynoic acid (1-2.5 g wet wt.):Lindlar catalyst:quinoline in methanol (15-20 ml) was used. After hydrogenation the solution was filtered through two thicknesses of filter paper to remove the catalyst and a solution of concentrated sulphuric acid (1 ml) in methanol (10 ml)

added. The dienoic acid was esterified by refluxing for 40 min, the product added to water (300 ml), extracted into ether (3 x 40 ml) and washed with 1M aqueous hydrochloric acid (100 ml) and 5% brine (3 x 100 ml). The residue was examined by IR spectroscopy, which showed the absence of the trans-olefinic absorption at 970 cm^{-1}, and by GLC, which showed very little overhydrogenation to the monoene. The methyl alka-cis-8,cis-11-dienoates were purified by preparative Ag^{+}TLC (dual development, PE15). The recovery of material, based on the weight applied to the plates, was 60-75%, and its GLC purity was high (Table 33). ECL and PMR spectroscopic data are also given in Table 33, while the CMR spectra are to be found in Appendix 2.

Small quantities (250-500 mg) of the methyl dienoates were reduced to the corresponding alcohols using lithium aluminium hydride in dry ether (Section 4:3:18). Data for this step are given in Table 33, and in each case the amount of methyl ester left unreacted was $\leqslant$ 0.25% as judged by GLC.

4:3:15 Synthesis of Cyclopropenoid Fatty Acids from the Corresponding Methyl Alkynoates [180,181]

A 100 ml 3-necked flask, fitted with a magnetic stirrer and a condenser and maintained under a slight pressure of dry nitrogen, contained 0.01 mole of methyl alkynoate and 0.2 g of powdered copper bronze. The flask was placed in a preheated oil bath at 130-135°C and ethyl diazoacetate (3.4 g, 0.03 mole) added dropwise directly onto the heated, stirred reaction mixture over 90 min. Heating and stirring were continued for a further 15 min, and the flask then allowed to cool. Silica column chromatography of the product yielded a monoester fraction (ie. recovered methyl alkynoate) and a diester fraction (ie. the appropriate carbethoxycyclopropene methyl ester). The latter was analysed by IR and PMR spectroscopy (Table 34). The PMR spectrum of the cyclopropene diester showed it to be reasonably pure, though small additional signals at 4.18 ppm, q and 6.1 ppm, s were noted. These were not attributable to ethyl diazoacetate, and possibly arose from COOEtCH:CHCOOEt.

TABLE 33. Data for Methyl Alka-8c, 11c-dienoates and their Corresponding Alka-8c, 11c-dien-1-ols

Product	13:2(8c11c)	15:2(8c11c)	19:2(8c11c)	21:2(8c11c)
Wt. of methyl c,c-dienoate[1]	1.4g	1.0g	1.2g	0.6g
% GLC purity[2]	98	98	96.5	94
ECL	15.2	16.7	20.4	22.3
PMR spectrum[3,4]				
Allylic H	C(7) 2.04,m C(13) 1.61,d	2.02,m	2.03,m	2.02,m
Diallylic H	2.73,Dt	2.72,Dt	2.72,Dt	2.70,b
Olefinic H	5.29,m	5.29,m	5.28,m	5.28,m
Other distinctive H resonances		C(15) 0.91,Dt		
Wt. of c,c-dien-1-ol	0.25g	0.16g	0.4g	0.3g
% GLC purity[2]	97	97.5	95	94
ECL	17.05	18.35	22.15	24.05

1. After Ag^+ TLC purification
2. 20% DEGS, 180°C
3. Integrals were consistent with the structure, and are not given
4. Methyl ester (3.60, s and 2.23, t) resonances are not given

Decarboxylation was accomplished by slowly dropping 6 ml of a 1:1 (v/v) fluorosulphonic acid:dry methylene chloride solution (ie. approximately 0.05 mole HSO_3F) onto 0.005 mole of the cyclopropene diester at room temperature while stirring and under an atmosphere of dry nitrogen. After an additional 40 min stirring cold (-78°C), dry methylene chloride (20 ml) and 7 g of activated 4Å molecular sieve

TABLE 34. Methyl ω-(3'-Carbethoxy-2'-alkyl-1'-cyclopropenyl)-alkanoates[1]

1. Referred to in the text as the cyclopropene diesters. 2. The hydrogen resonance is underlined IR spectra, obtained with 0.5% CCl_4 solutions, showed characteristic absorptions at 1895 cm^{-1}(w), 1740 cm^{-1}(s) and 1720 cm^{-1}(s) [181]

	Methyl 5-(3'-carbethoxy-2'-pentyl-1'-cyclopropenyl)-pentanoate	Methyl 5-(3'-carbethoxy-2'-undecyl-1'-cyclopropenyl)-pentanoate	Methyl 8-(3'-carbethoxy-2'-nonyl-1'-cyclopropenyl)-octanoate
Starting material	12:1(6a) 15 mmole	18:1(6a) 9 mmole	19:1(9a) 10 mmole
Recovered methyl alkynoate (Yield)	5.7 mmole (38%)	1.2 mmole (13%)	2.6 mmole (26%)
Product (Yield)	6.0 mmole (40%)	5.7 mmole (63%)	5.1 mmole (51%)
PMR Spectrum[2]			
CH_3-$(CH_2)_n$	0.90, Dt, 3H	0.89, Dt, 3H	0.88, Dt, 3H
$(CH_2)_n$	1.3, b and 1.6, m, 10H	1.3, and 1.6, m, 22H	1.28, b, 24H
CH_2-C=C	2.41, Dt, 4H	2.4, Dt, 4H	2.38, t, 4H
CH_2-COOMe	2.26, t, 2H	2.25, t, 2H	2.22, t, 2H
-CH / C=C	1.93, s, 1H	1.93, s, 1H	1.92, s, 1H
$CO.OCH_2CH_3$	4.02, q, 2H	4.02, q, 2H	4.02, q, 2H
$CO.OCH_2CH_3$	1.21, t, 3H	1.21, t, 3H	1.2, t, 3H
$CO.OCH_3$	3.60, s, 3H	3.59, s, 3H	3.58, s, 3H

were added. The cold solution, designated as the cyclopropenium cation solution, was allowed to stand for 1 hr under nitrogen.

A 1.05M methanolic sodium borohydride solution was made by adding 30 ml of dry methanol, saturated with sodium hydroxide, to $NaBH_4$ (1.2 g, 0.031 mole) at -50 °C. The resulting solution was stirred for 10 min then allowed to stand for 10 min at -50°C. The cyclopropenium cation solution was added dropwise and the mixture stirred for 30 min at - 50°C prior to being allowed to warm up to room temperature. 5% Brine (250 ml) was added and the product extracted into petrol (3 x 50 ml). The organic phase was washed with 5% brine (2 x 100 ml), then with saturated, aqueous sodium bicarbonate (100 ml), and dried. The crude product was purified by silica column chromatography. Some cyclopropene diester was recovered and the monoester fraction, analysed by TLC (PE20), contained mainly the cyclopropene ester (R_f = 0.62) but also some methyl alkynoate (R_f = 0.57). Preparative TLC (PE12, dual development) of the monoester fraction resulted in a pure (95%) cyclopropene ester product as judged by TLC and by GLC of the adducts formed by reaction in saturated, methanolic silver nitrate (Table 35). The IR, PMR (Table 36) and CMR (Section 3:2:4 and Appendix 2) spectra of the cyclopropene esters were run.

The synthetic materials were stored neat as the methyl esters in sealed ampoules at -10°C. Prior to saponification they were once more purified by preparative TLC. Methyl sterculate (donated to Colworth House by Dr. A.R. Johnson, C.S.I.R.O. Division of Food Preservation, Ryde, N.S.W., Australia) was released from its urea adduct and also purified by preparative TLC. 10 mg of each methyl ester was saponified by standing in a 5% aqueous methanolic 1M KOH solution (2 ml) for 48 hr. A rapid extraction with chilled solutions followed. The reaction mixture was added to water (6 ml), carefully acidified with 0.1M aqueous sulphuric acid until pH=4-5 was reached, and the product was extracted into petrol (3 x 3 ml), and washed with water (2 x 3 ml). The cyclopropene acids were stored as dilute benzene solutions at -27°C. Storage for 2 weeks as FFA did not result in any appreciable deterioration of the sample, as judged by TLC after esterification of an aliquot with diazomethane.

TABLE 35. GLC Analysis of the Ether and Keto Derivatives of Cyclopropene Fatty Methyl Esters

Sample	GLC Analysis[1,2] Ether derivative ECL	%	Keto derivative ECL	%
Methyl sterculate[3]	22.1	86	24.3	11
Methyl 5-(2'-pentyl-1'-cyclopropenyl)-pentanoate	16.7	84	18.8	12
Methyl 5-(2'-undecyl-1'-cyclopropenyl)-pentanoate	22.1	83	24.15	10

1. 10% DEGS column at 185°C
2. The adducts were prepared by mixing the cyclopropene ester (1 mg) with anhydrous methanol (1 ml) saturated with silver nitrate, and warming at 30°C for 2 hr [260]. The mixture was poured into water (3 ml), and the adducts extracted into petrol, washed and dried.
3. An authentic sample of methyl sterculate was available to make a comparison with the synthetic materials.

TABLE 36. Methyl ω-(2'-Alkyl-1'-cyclopropenyl) alkanoates[1]

	Methyl 5-(2'-pentyl-1'-cyclopropenyl)-pentanoate	Methyl 5-(2'-undecyl-1'-cyclopropenyl)-pentanoate	Methyl 8-(2'-nonyl-1'-cyclopropenyl)-octanoate
Diester starting material	6.0 mmole	5.7 mmole	5.1 mmole
Recovered diester (Yield)	1.1 mmole (18%)	0.8 mmole (14%)	0.6 mmole (12%)
Product (Yield)	0.96 mmole (16%)	1.37 mmole (24%)	1.02 mmole (20%)
PMR Spectrum[2]			
$C\underline{H}_3(CH_2)_n$	0.9, Dt, 3H	0.88, Dt, 3H	0.88, Dt, 3H
$(C\underline{H}_2)n$	1.3, b and 1.6, m, 10H	1.3, b and 1.6, m, 22H	1.28, b, 24H
$\underline{CH}_2$ / \ C=C	0.73, s, 2H	0.73, s, 2H	0.72, s, 2H
$C\underline{H}_2COOMe$	2.25, t, 2H	2.24, t, 2H	2.22, t, 2H
$C\underline{H}_2$ C=C	2.38, Dt, 4H	2.37, Dt, 4H	2.36, t, 4H
$COOC\underline{H}_3$	3.60, s, 3H	3.59, s, 3H	3.58, s, 3H

1. Referred to in the text as the cyclopropene esters. 2. The hydrogen resonance is underlined. IR spectra, obtained with 0.5% CCl_4 solutions, showed characteristic absorptions at 1870 cm^{-1}(w) and 1740 cm^{-1}(s) [181]

LEAD TETRAACETATE PHOTODECARBOXYLATION

2.85 g (10 mmole) of oleic acid and 9g (20 mmole) of lead tetraacetate, recrystallised from acetic acid and stored under vacuum over KOH and P_2O_5, were refluxed for 10 min in dry benzene (100 ml). The apparatus was kept under a slight pressure of dry nitrogen, and illuminated by two 150W tungsten lamps. Small portions of iodine in dry benzene were added, while refluxing, over 3 hr, until a permanent red colour persisted. Approximately 4 g (16 mmole) of iodine in 100 ml of dry benzene were added in total. The reaction mixture was allowed to cool and was filtered under suction to remove lead salts. Ether (150 ml) was added, and the organic layer was washed with aqueous sodium thiosulphate (2 x 100 ml) then water (2 x 100 ml) and dried. 5 g of crude product were recovered. It was analysed by:-

(1) GLC (20% DEGS, 190°C). No components were detected, so the ECL value was greater than 25.

(2) IR spectroscopy (film). Strong absorptions at 1735 cm^{-1} and 1230 cm^{-1} were noted, with absorptions characteristic of cis (3000 cm^{-1}) and trans (970 cm^{-1}) olefins and carboxylic acids (1710 cm^{-1}) absent.

(3) PMR spectroscopy. The following signals were noted:- 4.44, b, 1H; 4.04, b, 1H; 3.14, t, 2H; 2.04, s, 3H; 1.3, b, 25-30H; 0.88, Dt, 3H.

(4) TLC (PE15). The major component had an R_f = 0.53, while other components present in moderate amounts had R_f values of 0.46 and 0.05. Methyl palmitate, run as a standard, gave an R_f value of 0.59.

Preparative TLC (PE08, dual development) of 0.5 g of the crude product gave the major component (A) at R_f = 0.55 in a reasonably pure state. IR and PMR spectra of A were essentially the same as for the crude product. Elemental analysis for C and H showed A to contain 42.8% C and 7.1%H by weight.

After repeated TLC, or column chromatography, A showed evidence of partial hydrolysis. A broad O-H absorption at 3350 cm^{-1}

appeared in the IR spectrum, while small additional resonances in the PMR spectrum, at 4.89 and 3.35-3.8 ppm were observed. On standing the crude product turned deep red after several days, and became black and tarry, with a distinct smell of acetic acid, after several weeks. TLC (PE10) of this residue indicated a less polar material B (R_f = 0.66) compared to A (R_f = 0.42) as well as more polar material developing. 500 mg of crude product, after standing for four weeks, was cleaned-up by preparative TLC (PE04, dual development) and 20 mg of B recovered. B had the following properties:-

(1) GLC (20% DEGS, 190°C). A component (85%) at ECL = 18.70 was detected.

(2) IR spectroscopy (film). A strong absorption at 3000 cm^{-1} was noted, with absorptions at 1735, 1710, 1230 and 970 cm^{-1} absent.

(3) PMR spectroscopy. The following signals were noted:-
5.27, Dt, 2H; 3.14, t, 2H; 2.0-1.8, b, 6H; 1.28, b, 18-22H; 0.88, Dt, 3H.

(4) GLC (20% DEGS, 170°C) of the methyl esters from the von Rudloff oxidative cleavage of B gave the following fragments:-

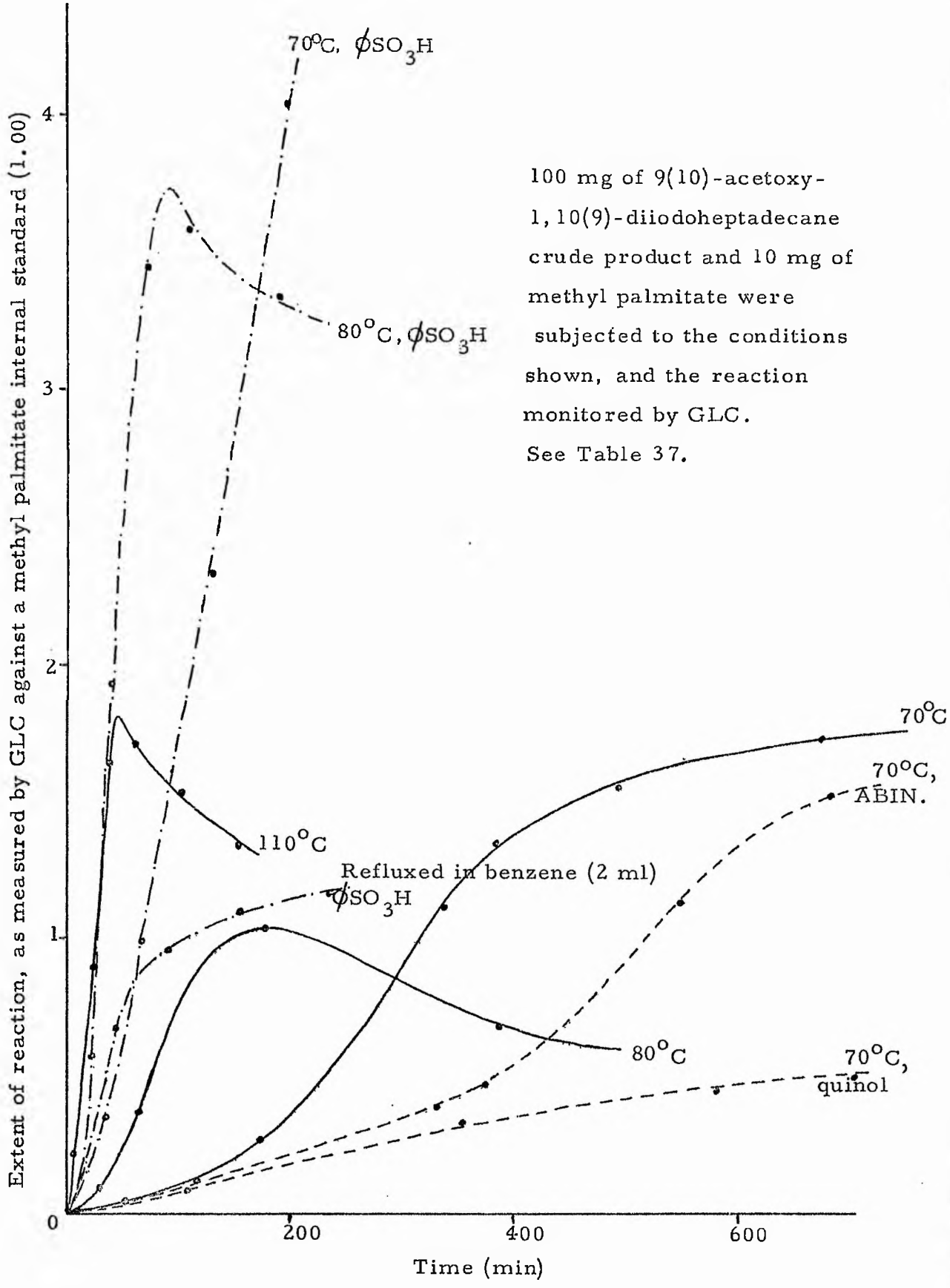
40% at ECL = 9.0

42% at ECL = 17.8

<2% at ECL = 8.0, 10.0, 16.8 or 18.8 .

Since the crude product was believed to be 9(10)-acetoxy-1,10(9)-diiodoheptadecane and B to be oleoyl nor-iodide (Section 4:2:2), the process of deiodoacetoxylation was examined more closely. 10 mg of methyl palmitate (an internal standard) and 100 mg of the crude product were subjected to various treatments, as described in Table 37 and Figure 33. The production of oleoyl nor-iodide was followed by GLC, by comparing the relative amount of oleoyl nor-iodide to methyl palmitate (1.00). The deiodoacetoxylation reaction employing p-toluenesulphonic acid at 70°C indicated a moderately high yield in comparison to the methyl palmitate internal standard, but preparative TLC resulted in only 9 mg of oleoyl nor-iodide being recovered (GLC purity 83%).

Figure 33. Deiodoacetoxylation of 9(10)-Acetoxy-1, 10(9)-Diiodo-heptadecane



100 mg of 9(10)-acetoxy-1, 10(9)-diiodoheptadecane crude product and 10 mg of methyl palmitate were subjected to the conditions shown, and the reaction monitored by GLC. See Table 37.

TABLE 37. Deiodoacetoxylation of 9(10)-Acetoxy-1,10(9)-Diiodo-heptadecane

Conditions employed[1]	Oleoyl nor-iodide formation, relative to methyl palmitate, after ( ) min or days	
Room temperature	0.22 (3 days)	0.60 (7 days)
Room temperature, dark	0.15 (3 days)	0.27 (6 days)
Room temperature, 0.1 ml pyridine	no reaction	
Room temperature, 0.1 ml acetic acid	0.12 (3 days)	0.24 (6 days)
Irradiate by two 150W tungsten lamps	0.30 (120 min)	0.58 (300 min)
70, 80, or 110°C	See Figure 33	
70°C, under vacuum	no reaction	
70°C, ABIN (1 mg)[2]	See Figure 33	
70°C, quinol (18 mg)[3]	See Figure 33	
70°C, ϕSO_3H (16 mg)[4]	See Figure 33	
80°C, ϕSO_3H (24 mg)[4]	See Figure 33	
Reflux in 2 ml toluene (108°C)	no reaction	
Reflux in 2 ml petroleum (105°C)	no reaction	
Reflux in 2 ml benzene, ϕSO_3H (35 mg) (80°C)[4]	See Figure 33	
2 ml DMSO, 80°C, ϕSO_3H (30 mg)[4]	no reaction	

1. 10 mg methyl palmitate, 100 mg crude product
2. ABIN— azo-bis-isobutylnitrile, a free radical initiator
3. Free radical scavanger
4. ϕSO_3H— p-toluenesulphonic acid

PHOTODECARBOXYLATION USING t-BUTYL HYPOIODITE

Completely anhydrous conditions were required for this reaction, and the t-butyl hypoiodite reagent solution needed minimum exposure to light. Commercial potassium t-butoxide was sublimed under vacuum before use, and an anhydrous iodine in benzene solution was obtained by azeotropic distillation of iodine with dry benzene. The generation of the t-butyl hypoiodite reagent was carried out in a dry box under an atmosphere of dry nitrogen.

1.9 g (17 mmole) of sublimed potassium t-butoxide was placed in a round-bottomed 100 ml flask wrapped in aluminium foil to exclude light and equipped with a magnetic stirrer. 20 ml of an anhydrous, saturated iodine in benzene solution (9.5 mmole I_2) and a further 30 ml of dry benzene were added, and the mixture stirred for 90 min. It was rapidly filtered under suction through a wide-necked sintered glass funnel and the filtrate, a pale orange-red solution designated as "t-butyl hypoiodite reagent", was quickly transferred to a pressure-equalising dropping-funnel also wrapped in aluminium foil to exclude light.

The photolysis vessel, maintained under a slight pressure of dry nitrogen, contained 1 g (3.54 mmole) of oleic acid in dry benzene (10 ml). The dropping funnel containing the t-butyl hypoiodite reagent was transferred from the dry box to the photolysis apparatus, and the solution added dropwise over 40 min while stirring and irradiating the mixture with two 150W tungsten lamps. Stirring and irradiation was continued for a further 50 min. The temperature of the mixture was not allowed to rise above 35°C. The mixture was poured into aqueous sodium thiosulphate (200 ml), ether (100 ml) added, and the organic layer washed with 5% brine (2 x 100 ml) and dried.

1.1 g of crude product was recovered, and analysed as follows:-

(1) GLC (20% DEGS, 190°C). No major component, up to an ECL value of 25, was observed.

(2) TLC (PE10). A plate, streaked from the origin to R_f = 0.6 but with the greatest intensity at the origin, was obtained.

(3) IR spectroscopy (film). A strong absorption at 1730 cm^{-1} was noted, with absorptions characteristic of cis (3000 cm^{-1}) and trans (970 cm^{-1}) olefins and carboxylic acids (3500-2500 cm^{-1}, 1710 cm^{-1}) absent.

(4) PMR spectroscopy. The following signals were noted:- 4.45, b, 1H; 4.02, b, 1H; 2.30, Dt, 2H; 1.25, b, 25-30H; 0.89, Dt, 3H.

On standing for several days IR spectroscopy showed a diminution in the ester carbonyl absorption (1730 cm^{-1}) and an increase in the carboxylic acid carbonyl (1705 cm^{-1}) and OH (3500-2500 cm^{-1}) absorptions for the crude product.

4:3:17 The Anodic Decarboxylation of Lauric And Monoenoic Acids

LAURIC ACID ANODIC DECARBOXYLATION [205]

Sodium hydroxide (0.4 g, 10 mmole) and lauric acid (1 g, 5 mmole) in methanol (35 ml) and water (1 ml) were subjected to electrolysis in a Dinh-Nguyen cell. A rotating platinum anode, of surface area 1 cm^2, was held about 1 cm away from the mercury cathode, and the cell was cooled by a water jacket. The current was held at 0.7A as the electrolysis proceeded by increasing the voltage across the cell from 15 to 140V. Electrolysis was stopped when the current fell to 0.1A (140V), and fairly long recovery times (4-24 hr) were required to allow the sodium in the mercury amalgam to regenerate base before electrolysis was continued. Aliquots were removed at hourly intervals, esterified by refluxing with 2% methanolic sulphuric acid, and analysed by GLC (20% DEGS, 140°C) (Table 38). After 250 min electrolysis time the mixture was poured

TABLE 38. Composition of the Reaction Mixture During the Anodic Decarboxylation of Lauric Acid

Component	ECL Value[1]	Time Intervals			
		66 min	114 min	172 min	250 min
lauric acid	12.0[2]	76%	59%	31%	11%
undecan-1-ol	12.6	8%	17.5%	31%	40%
docosane	13.9	10%	16.5%	26%	34%

1. 20% DEGS, 140°C

2. Analysed as its methyl ester

into water (200 ml), acidified with sulphuric acid and extracted with ether (3 x 30 ml). The organic layer was washed with water (2 x 100 ml) and dried. 700 mg of residue were recovered.

TLC showed two major products, hydrocarbon and alcohol. The crude product was purified by preparative TLC using the following procedure: the plate was first developed in PE03, and the hydrocarbon band running just behind the solvent front removed, then the remainder of the plate was developed twice with PE30 and the "methyl ether" and alcohol bands removed. The products were characterised as indicated in Table 39. 200 mg (23%) of nor-alcohol was recovered. It was contaminated by traces of lauric acid, as shown by the IR spectrum, since the tail of the alcohol band overlapped with the carboxylic acid band.

OLEIC ACID ANODIC DECARBOXYLATION [205]

The experiment was repeated as for lauric acid, but with 1 g (3.5 mmole) of oleic acid. The electrolysis time was 310 min, and 810 mg of crude product were recovered. The C_{34} hydrocarbon could not be detected on a 20% DEGS column so the products were analysed using both 20% DEGS and 1% SP-2300 columns. The residue contained the following major components:

5%	9.5	ECL, 20% DEGS, 180°C
9.5%	10.4	
4%	13.05	
4%	13.3	
9%	18.55*	
26%	19.3	
37%	27.8	ECL, 1% SP-2300, 230°C

* Methyl oleate after esterification of the crude product.

It was purified by preparative silica TLC, as described for the lauric acid anodic decarboxylation, and the hydrocarbon band was further purified by preparative Ag^+ TLC (PE03, dual development).

TABLE 39. Products from the Anodic Decarboxylation of Lauric Acid

Band	Wt. Recovered	GLC Analysis[1] ECL	% Purity	R_f[2] (PE05)	Characteristic IR Absorptions	PMR Spectrum
Hydrocarbon	240 mg White solid	3.7 4.4 13.9	4 4 90	0.76	none[3]	0.88, Dt, 6H 1.26, b, 40H
"Methyl Ether"	30 mg	6.9 7.1 7.8	29 40 13	two major components 0.43, 0.33	1740 cm^{-1}(m) 1260 cm^{-1}(s) 1090 cm^{-1}(s)	-
Alcohol	200 mg	12.6	90	0.05	3350 cm^{-1}(b, s) 1705 cm^{-1}(w) 3500-2500 cm^{-1}(w)	0.88, Dt, 3H 1.27, b, 18H 3.18, b, 1H 3.62, Dt, 2H

1. . 20% DEGS, 100°C

2. Methyl laurate had an R_f value of 0.27

3. IR spectrum obtained as a 1% solution in CS_2

The products were characterised in Table 40. The yield of oleoyl nor-alcohol, which had a GLC purity of 89% and which contained a trace of oleic acid, was 170 mg (18%).

OLEIC ACID ANODIC DECARBOXYLATION USING TWO PLATINUM ELECTRODES

Oleic acid (2 mmole) in 5% aqueous methanol (40 ml) was electrolysed in the presence of sodium hydroxide (4.0 mmole or 0.4 mmole) for 2 hr in a cell consisting of a water jacket, magnetic stirrer and two platinum electrodes (1 cm^2) placed 1 cm apart. The polarity of the cell was reversed every 5-10 min. In the presence of excess base for neutralisation a current of 0.7 amp was maintained at 20-25 V, while in the presence of excess oleic acid a current of 0.1-0.2 amp resulted from an applied voltage of 140 V. After 2 hr aliquots were removed for esterification then analysis by GLC. Electrolysis of the carboxylate anion occurred only in the case of insufficient base for neutralisation, and the product was cleaned up by preparative TLC, as described previously, to give oleoyl nor-alcohol.

The electrolysis using excess oleic acid was also performed with carbon electrodes (i = 0.2 amp, V = 140V).

THE PREPARATION OF NOR-ALCOHOLS FOR ^{14}C-CARBOXYL LABELLING

500 mg of a cis-trans mixture of a positional isomer of octadecenoic acid (Δ8, 10, 11, 14 and 15 isomers) was electrolysed in the Dinh-Nguyen cell for 4 hr as described previously, and the nor-alcohol recovered by preparative TLC (dual development, PE30). 45-75 mg of nor-alcohol were obtained, of GLC purity 85-90%. ECL values (20% DEGS, 185°C) for the nor-alcohols were:-

Δ7c/t	19.3	
Δ9c/t	19.3	
Δ10c/t	19.35	
Δ13c	19.6	Δ13t 19.35
Δ14c	19.8	Δ14t 19.5

TABLE 40. Products from the Anodic Decarboxylation of Oleic Acid

Band	Wt. Recovered	GLC Analysis[1,2] ECL	% Purity	R_f[3,4]	Characteristic IR Absorptions	PMR Spectrum
Alcohol	170 mg	19.3[1]	89	0.05[3]	3350 cm^{-1}(s) 3000 cm^{-1}(i) 3500-2500 cm^{-1}(w) 1705 cm^{-1}(w)	0.88, Dt, 3H 1.27, b, 20-24H 1.99, Dd, 4H 3.52, t, 2H 5.27, Dt, 2H
"Methyl Ether"	40 mg	12.9[1] 13.2[1]	39 42	0.45[3] 0.36[3]	3000 cm^{-1}(i) 2810 cm^{-1}(i) 1740 cm^{-1}(m) 1260 cm^{-1}(s) 1090 cm^{-1}(s)	Distinctive signals: 1.05, d, (J=6.5Hz), ~2H 1.98, Dd, 4H 3.13, s, ~1H 3.21, d(J=2Hz), ~3H 5.27, Dt, 2H
Hydrocarbon	240 mg			0.85[3]		
150 mg of hydrocarbon gave bands 5, 6, and 7 after preparative Ag^+TLC						
5	5 mg	9.5[1]	75	0.77[4]	3000 cm^{-1}(i)	
6	22 mg	10.3[1]	86	0.34[4]	3065 cm^{-1}(m) 3000 cm^{-1}(i) 1635 cm^{-1}(m) 985 cm^{-1}(m) 905 cm^{-1}(s)	0.88, Dt, 3H 1.27, b, 16-20H 2.0, b, 6H 5.26, Dt, 2H 4.8-5.0, m, 2H 5.6-5.8, m, 1H
7	70 mg	27.8[2]	87	0.65[4]	3000 cm^{-1}(i)	0.88, Dt, 6H 1.26, b, 46-50H 1.98, Dd, 8H 5.26, Dt, 4H

1. 20% DEGS, 180°C 2. 1% SP-2300, 230°C 3. PE05, silica, single development
4. PE03, 5% $AgNO_3$, dual development 5. Identified as $CH_3(CH_2)_7CH^{c}{=}CH(CH_2)_6CH_3$
6. Identified as $CH_3(CH_2)_7CH^{c}{=}CH(CH_2)_5CH{=}CH_2$ 7. Identified as $CH_3(CH_2)_7CH^{c}{=}CH(CH_2)_{14}CH^{c}{=}CH(CH_2)_7CH_3$

4:3:18 Chain Extensions using "Cold" Potassium Cyanide

In a typical experiment, starting with 1 mmole (~ 300 mg) of the methyl ester of a C_{18} fatty acid, the following manipulations were carried out:

$$RCOOMe \longrightarrow RCH_2OH \longrightarrow RCH_2OMes \longrightarrow RCH_2CN \longrightarrow RCH_2COOMe$$

A solution of the methyl ester in dry ether (10 ml) was added dropwise to a stirred suspension of lithium aluminium hydride (75 mg, 2 mmole). Stirring was continued for 10 min then water was carefully added to destroy excess $LiAlH_4$. 2M aqueous sulphuric acid (20 ml) was added and the ether layer washed with water (3 x 20 ml) and dried. Recovery of the material was in excess of 90%. The alcohol had an ECL value on a 20% DEGS column of ~1.7 greater than the methyl ester, and the reaction went to completion as judged by GLC ($\geqslant$99.5%).

Redistilled mesyl chloride (1.2 mmole) was added dropwise to a stirred solution of the alcohol (1 mmole), redistilled triethylamine (1.5 mmole) and dry methylene chloride (6 ml) at 0°C. Stirring was continued for 20 min at room temperature. The reaction mixture plus methylene chloride washings (~20 ml) were transferred to a separating funnel and washed with water (20 ml) followed by 3M aqueous hydrochloric acid (2 x 10 ml), water (20 ml), saturated aqueous sodium bicarbonate (20 ml) and water (20 ml), then dried. The reaction was quantitative. IR spectroscopy showed strong absorptions at 1170 cm^{-1} and 1350 cm^{-1} characteristic of the methanesulphonyl group but no hydroxyl absorption, while TLC indicated that the product was free from chloride.

The mesylate (1 mmole) was heated with KCN (130 mg, 2 mmole) in dry DMSO (10 ml) in a stoppered flask at 80-90°C for 3 hr, while stirring. After cooling the mixture was poured into 5% brine (40 ml) and extracted with ether (3 x 15 ml). The combined organic layers were washed with 5% brine (3 x 50 ml) and dried. The residual nitrile was mixed with a 20-25% (w/w) anhydrous HCl/methanol reagent (15 ml) and allowed to stand for 48 hr at room temperature. The mixture was poured into 5% brine (50 ml) and the product was extracted

into ether (3 x 15 ml), washed with 5% brine (50 ml) followed by water (2 x 50 ml) and dried. Recovery of material was greater than 80%, and the crude product was analysed by GLC to check that complete methanolysis of the nitrile had occurred. The product was cleaned up by preparative TLC (PE10) prior to further chemical manipulations.

Using chlorides instead of mesylates the same procedure for chain extension with potassium cyanide was adopted, with the exception that the chloride was heated in dry DMSO at 125-135°C for 3-5 hr.

The entire procedure was scaled up or down as required, and the compounds which were chain extended are given in Section 4:2:1.

The method of Crossland and Servis [197] for mesylation was preferred to that of Bauman and Mangold [196] because the former gave no chloride. The later method, where the alcohol was stirred for 5 hr at room temperature in dry pyridine with a 10-50% molar excess of mesyl chloride gave variable amounts of chloride together with the mesylate (as judged by TLC).

Caution was needed with the chain extension of acetylenic compounds. The methanolysis of 1-cyanoheptadec-5-yne did not go to completion after 48 hr so additional methanolic HCl reagent was added and the mixture left for a further 48 hr. This produced a noticeable proportion of methyl 6(7)-chlorooctadec-6-enoate in the product. GLC composition (20% DEGS, 190°C) was 79% at ECL = 20.6 (18:1(6a) methyl ester) and 19% at ECL = 22.4 while Ag^+TLC (developed in CH_2Cl_2) gave bands at R_f = 0.65 (18:1(6a) methyl ester) and at R_f = 0.81 (Methyl oleate had an R_f = 0.61 and methyl elaidate an R_f = 0.73). The upper band had an absorption at 1660 cm^{-1}(m) in its IR spectrum, and the following PMR spectrum - 0.88, Dt, 3H; 1.3, b, 18H; 2.22, m, 6H; 3.58, s, 3H and 5.38, t, 1H. These features are consistent with the proposed structure of the additional component.

4:3:19 Chain Extensions Using High Specific Activity Potassium ^{14}C-Cyanide

15-20 μmole of alkyl mesylate (prepared as described in Section 4:3:18) was heated with 5 μmole of K^{14}CN (specific activity = 60 μCi μmole^{-1}, 300 μCi) in dry DMSO (0.3 ml) in a sealed, small bore tube for 16 hr at 85^{o}C. The tube had a small dead volume ($\sim$ 0.5 ml) and its contents were thoroughly purged with nitrogen before sealing. When using an alkyl chloride instead of a mesylate the tube was heated for 16 hr at 125-135^{o}C. On cooling the ampoule was broken and the contents added to 5% brine (5 ml), and the product was extracted into petrol (3 x 3 ml) and washed with 5% brine (2 x 3 ml). Between 30-60% of the original radioactivity remained in the organic layer. Although TLC was not routinely used to clean up the [1-^{14}C] nitrile, on the few occasions where this step was adopted the radioactivity was approximately divided up as 40-60% nitrile, 10-30% of a more polar material and 20-40% of autoxidised material at the origin.

The [1-^{14}C] nitrile residue was allowed to stand in a 20% (w/w) anhydrous methanolic HCl reagent (3 ml) with 40 μl of water for 48 hr at room temperature. 5% brine (7 ml) was added and the product extracted into petrol (3 x 3 ml) and washed with 5% brine (2 x 3 ml). The [1-^{14}C] methyl ester was purified by dual development Ag^{+}TLC and the product(s) analysed by RGLC and its proximal double bond position checked by von Rudloff oxidative cleavage (Section 2:3:2). Some [1-^{14}C] methyl esters were also examined by GLC and their specific activity measured. Mass purity was high ($>$ 95%), specific activity was within $\pm$ 10 μCi μmole^{-1} of the expected figure of 60 μCi μmole^{-1}, and no double bond migration had occurred. Table 41 gives the radiochemical yields and purity, and ECL values of the [1-^{14}C] methyl esters prepared.

The [1-^{14}C] esters were saponified by refluxing in 5% aqueous methanolic 1M KOH (2 ml) for 1 hr. 5M aqueous sulphuric acid (1 ml) and 5% brine (6 ml) were added, and the product extracted into petrol (3 x 3 ml) and washed with 5% brine (2 x 3 ml). Recovery of radioactivity for the saponification was 86-98%. The [1-^{14}C] acid was stored at -27^{o}C as a dilute benzene solution. Storage over 6 months

TABLE 41. The Synthesis of [1-^{14}C] Unsaturated Methyl Esters

	Compound[1]	Starting material	Activity (μCi)	Radiochemical yield (%)[1]	%RGLC purity	ECL[2]	
Experiment A	11:1(9c)	mesylate	15	5	95	11.9[3]	
	13:1(9c)	"	5	6	98	13.3[3]	14.0
	13:1(9t)	"	13		98	13.3[3]	13.8
	15:1(9c)	"	31	10.5	97	15.2[3]	
	16:1(9c)	"	29	9.5	98	16.2[3]	
	17:1(9c)	"	11	7	98	17.2[3]	17.6
	17:1(9t)	"	10		97		17.6
Experiment B	16:1(5c)	chloride	15	12.5	97	16.5	
	16:1(5t)	"	22		97	16.5	
	17:1(5c)	"	11	10.5	96	17.5	
	17:1(5t)	"	20		98	17.5	
	18:1(5c)	mesylate	31	19	97	18.4	
	18:1(5t)	"	26		95	18.4	
	16:1(7c)	chloride	35	18	98	16.5	
	16:1(7t)	"	19		98	16.5	
	16:1(8c)	"	26	18.5	97	16.5	
	16:1(8t)	"	30		98	16.5	
	17:1(12c)	"	2	5	98	17.9	
	17:1(12t)	"	13		97	17.7	
	18:1(13c)	mesylate	9	12.5	96	18.95	
	18:1(13t)	"	29		94	18.8	
	20:2(11c14c)	"	2	5	98	21.4	
	22:4(7c10c13c16c)	"	13		98	24.7	
	19:3(7c10c13c)	"	37	12.5	98	21.0	
	20:3(8c11c14c)	"	26	8.5	96	21.9	
Experiment C	18:1(8c)	mesylate	33	17	95	18.5	
	18:1(8t)	"	18		98	18.5	
	18:1(10c)	"	6	8	95	18.75	
	18:1(10t)	"	18		93	18.65	
	18:1(11c)	"	12	13.5	94	18.7	
	18:1(11t)	"	29		95	18.6	
	17:1(12a)	chloride	12	4	97	19.7	
	18:1(14c)	mesylate	21	19.5	94	19.0	
	18:1(14t)	"	37		95	18.8	
	18:1(15c)	"	20	15	92	19.1	
	18:1(15t)	"	25		93	18.9	
	20:1(9t)	"	95	31.5	97	20.45	
	20:1(8c)	"	85	28.5	96	20.45	
	14:2(9c12c)	"	47	15.5	97	16.4	
	16:2(9c12c)	"	34	11.5	95	17.75	
	20:2(9c12c)	"	43	14.5	96	21.2	
	22:2(9c12c)	"	51	17	96	23.0	

1. The esters bracketed were prepared from a cis/trans mixture of the chloride or mesylate, and the isomers were separated by Ag^+TLC of the [1-^{14}C] methyl esters. The % radiochemical yields are therefore quoted for the isomeric pair, where appropriate.
2. 10% DEGS, 170-190°C
3. 10% DEGA, 190°C

resulted in little autoxidative or radiolytic alteration of the [1-^{14}C] acid (< 10%) as judged by TLC. However for more prolonged periods of storage the [1-^{14}C] acid was converted to its methyl ester and cleaned up by Ag^+TLC as a routine precaution.

The method quoted above for chain extension with K^{14}CN was employed in experiment C (Table 41). In earlier batches (experiments A and B), where, on average, lower radiochemical yields were noted, the chloride or mesylate was heated in dry DMSO (1 ml) in a quickfit test-tube for only 5 hr.

4:3:20 Cis-Trans Isomerisation of Small Masses of [1-^{14}C] Unsaturated Acids by Oxides of Nitrogen [135]

50 μCi of [1-^{14}C] oleic or linoleic acid (specific activity 60 μCiμmole^{-1}) was dissolved in dimethyl diethylene glycol (1 ml). 10 μl of 6M aqueous nitric acid and 15 μl of 2M aqueous sodium nitrite were added, the solutions well mixed and heated at 60°C for 2 hr in a stoppered quickfit test-tube. On cooling water (5 ml) was added, the mixture acidified with a few drops of 2M aqueous hydrochloric acid, and extracted with ether (3 x 3 ml). The combined organic layers were washed with water (3 x 3 ml) and dried by azeotropic distillation with methanol. The acids were dissolved in a few drops of methanol and esterified by the addition of an ethereal diazomethane solution. The cis and trans isomers were separated as the methyl esters by Ag^+TLC.

The isomerisation of [1-^{14}C] oleic acid gave:-

	μCi Recovered	R_f (Ag^+TLC, PE10, dual development)	ECL (180°C, 10% DEGS)
[1-^{14}C] 18:1(9t)	15.5	0.58	18.35
[1-^{14}C] 18:1(9c)	10.5	0.42	18.35

The total recovery of radioactivity was 52%.

The isomerisation of [1-^{14}C] linoleic acid gave:-

	μCi Recovered	R_f(Ag^+TLC, PE15, dual development)	ECL(180°C, 10% DEGS)
[1-^{14}C]18:2(9t12t)	10	0.70	18.85
[1-^{14}C]18:2(9t12c + 9c12t)	9.5	0.60	18.95
[1-^{14}C]18:2(9c12c)	2	0.35	19.0

The total recovery of radioactivity was 43%.

For both radical isomerisations double bond migration, as detected by von Rudloff oxidative cleavage, was minimal (< 2%).

References

1. A.L. Lehninger, "Biochemistry", Worth Publishers Inc., New York (1970)
2. M.I. Gurr and A.T. James, "Lipid Biochemistry: an Introduction", Chapman and Hall, London (1971)
3. C. Hitchcock, "Recent Advances in the Chemistry and Biochemistry of Plant Lipids", (ed. T. Galliard and E.I. Mercer), Academic Press, 1 (1975)
4. P. Crabbé, Chemistry in Britain, 11, 132 (1975)
5. F. Lynen, Biochem. J., 128, 1 (1972)
6. A.T. James, "The Metabolism of Unsaturated Acids by Animal Systems: an Attempted Rationalisation", Priv. comm. (1972)
7. A.T. James, P. Harris and J. Bezard, Eur. J. Biochem., 3, 318 (1968)
8. R. Reiser and P.K. Raju, Biochem. Biophys. Res. Comm., 17, 8 (1964)
9. A.R. Johnson, J.A. Pearson, F.S. Shenstone and A.C. Fogerty, Nature, 214, 1244 (1967)
10. P. Strittmatter, L. Spatz, D. Corcoran, M.J. Rogers, B. Setlow and R. Redline, Proc. Natl. Acad. Sci. USA, 71, 4565 (1974)
11. E.W. Hammond and C.H.S. Hitchcock, Unilever Report 75 1496, (1975)
12. M.I. Gurr, MTP International Review of Science, vol. 4 "Biochemistry of Lipids" (ed. T.W. Goodwin), Butterworths, London, 181 (1974)
13. F.D. Gunstone, "An Introduction to the Chemistry and Biochemistry of Fatty Acids and their Glycerides", Chapman and Hall, London (1967)
14. K.S. Markley, "Fatty Acids", part 3 (ed. K.S. Markley), Wiley, 1769 (1964).

15. H. Sprecher, Fed. Proc., 31, 1451 (1972)

16. F.D. Gunstone, "Recent Advances in the Chemistry and Biochemistry of Plant Lipids" (ed. T. Galliard and E.I. Mercer), Academic Press, 21 (1975)

17. R.R. Brenner, Molecular and Cellular Biochem., 3, 41 (1974)

18. H. Sprecher, "Interconversions of PUFA", private communication, (1975)

19. H.G. Enoch, A. Catala and P. Strittmatter, J. Biol. Chem., 251, 5095 (1976)

20. P.W. Holloway, R. Peluffo and S.J. Wakil, Biochem. Biophys. Res. Comm., 12, 300 (1963)

21. R.R. Brenner, R.O. Peluffo, A.M. Nervi and M.E. De Tomas, Biochim. Biophys. Acta, 176, 420 (1969)

22. R. Jeffcoat, P.R. Brawn and A.T. James, Biochim. Biophys. Acta, 431, 33 (1976)

23. W. Stoffel, Biochem. Biophys. Res. Comm., 6, 270 (1961)

24. Y.L. Marcel and G. Suzue, J. Biol. Chem., 247, 4433 (1972)

25. G. Suzue and Y.L. Marcel, Biochemistry, 11, 1704 (1972)

26. S.V. Pande, Biochim. Biophys. Acta, 270, 197 (1972)

27. K. Lippel, F.D. Gunstone and J.A. Barve, Lipids, 8, 119 (1973)

28. K. Lippel, D. Carpenter, F.D. Gunstone and I.A. Ismail, Lipids, 8, 124 (1973)

29. J.R. Paulsrud, S.E. Stewart, G. Graff and R.T. Holman, Lipids, 5, 611 (1970)

30. M. Nakagawa and M. Uchiyama, J. Biochem., 63, 684 (1968)

31. R. Jeffcoat, P.R. Brawn, R. Safford and A.T. James, Biochem. J., 161, 431 (1977)

32. D.H. Nugteren, Biochim. Biophys. Acta, 106, 280 (1965)

33. H. Sprecher, Biochim. Biophys. Acta, 360, 113 (1974)

34. W. Stoffel, Z. Physiol. Chem., 333, 71 (1963)

35. W. Stoffel and K.L. Ach, Z. Physiol. Chem., 337, 123 (1964)

36. W. Stoffel, Die Naturwissenschaften, 24, 623 (1966)

37. K. Christiansen, Y.L. Marcel, M.V. Gan, H. Mohrhauer and R.T. Holman, J. Biol. Chem., 243, 2969 (1968)

38. H. Sprecher, Biochim. Biophys. Acta, 144, 296 (1967)

39. S. Ayala, G. Gaspar, R.R. Brenner, R.O. Peluffo and W. Kunau, J. Lipid Res., 14, 296 (1973)

40. W. Stoffel, W. Ecker, H. Assad and H. Sprecher, Z. Physiol. Chem., 351, 1545 (1970)

41. W.H. Kunau and F. Bartnik, Eur. J. Biochem., 48, 311 (1974)

42. E.E. Hill and W.E.M. Lands, Biochim. Biophys. Acta, 152, 645 (1968)

43. R.C. Reitz, W.E.M. Lands, W.W. Christie and R.T. Holman, J. Biol. Chem., 243, 2241 (1968)

44. R.C. Reitz, M. El-Sheikh, W.E.M. Lands, I.A. Ismail and F.D. Gunstone, Biochim. Biophys. Acta, 176, 480 (1969)

45. P.W. Holloway and S.J. Wakil, J. Biol. Chem., 245, 1862 (1970)

46. P.W. Holloway, Biochemistry, 10, 1556 (1971)

47. C.T. Holloway and P.W. Holloway, Archives Biochem. Biophys., 167, 496 (1975)

48. N. Oshino, Y. Imai and R. Sato, J. Biochem., 69, 155 (1971)

49. T. Shimakata, K. Mihara and R. Sato, J. Biochem., 72, 1163 (1972)

50. N. Oshino, Y. Imai and R. Sato, Biochim. Biophys. Acta, 128, 13 (1966)

51. L. Spatz and P. Strittmatter, J. Biol. Chem., 248, 793 (1973)

52. J. Ozols, Biochemistry, 13, 426 (1974)

53. M.J. Rogers and P. Strittmatter, J. Biol. Chem., 248, 800 (1973)

54. A. Catala, A.M. Nervi, R.R. Brenner, J. Biol. Chem., 250, 7481 (1975)

55. R.E. Barden and W.W. Cleland, J. Biol. Chem., 244, 3677 (1969)

56. M. Sumper and H. Träuble, FEBS Lett., 30, 29 (1973)

57. M. Sumper, Eur. J. Biochem., 49, 469 (1974)

58. L.J. Morris, R.V. Harris, W. Kelly and A.T. James, Biochem. J., 109, 673 (1968)

59. A.R. Johnson and M.I. Gurr, Lipids, 6, 78 (1971)

60. G.J. Schroepfer and K. Bloch, J. Biol. Chem., 240, 54 (1965)

61. D. Brett, D. Howling, L.J. Morris and A.T. James, Archives Biochem. Biophys., 143, 535 (1971)

62. A.R. Johnson, A.C. Fogerty, J.A. Pearson, F.S. Shenstone and A.M. Bersten, Lipids, 4, 265 (1969)

63. H.W. Sprecher, H.J. Dutton, F.D. Gunstone, P.T. Sykes and R.T. Holman, Lipids, 2, 122 (1967)

64. M.I. Gurr, M.P. Robinson, A.T. James, L.J. Morris and D. Howling, Biochim. Biophys. Acta, 280, 419 (1972)

65. A.J. Fulco and J.F. Mead, J. Biol. Chem., 235, 3379 (1960)

66. A.C. Fogerty, A.R. Johnson and J.A. Pearson, Lipids, 7, 335 (1972)

67. R. Jeffcoat and M.R. Pollard, accepted for publication in Lipids (1977)

68. P.K. Raju and R. Reiser, Biochim. Biophys. Acta, 176, 48 (1969)

69. P.K. Raju and R. Reiser, J. Biol. Chem., 242, 379 (1967)

70. S.V. Pande and J.F. Mead, J. Biol. Chem., 245, 1856 (1970)

71. H.W. Kircher, J.Amer. Oil Chem. Soc., 41, 4 (1964)

72. R.L. Ory and A.M. Altschul, Biochem. Biophys. Res. Comm., 17, 12 (1964)

73. K. Wallenfels and H. Sund, Biochem. Z., 329, 17 (1957)

74. D. Brett, PhD Thesis (Loughborough University of Technology, 1972)

75. K. Bloch, Accounts Chem. Res., 2 , 193 (1969)

76. R.R. Brenner and R.O. Peluffo, J. Biol. Chem., 241, 5213 (1966)

77. R.R. Brenner, Lipids, 6, 567 (1971)

78. R. Ninno, M. Polero de Torrengo, J.C. Castuma and R.R. Brenner, Biochim. Biophys. Acta, 360, 124 (1974)

79. L.S.S. Guo and J.C. Alexander, Biochim. Biophys. Acta, 369, 411 (1974)

80. P. Lemarchal and M. Bornens, Bull. Soc. Chim. Biol., 50, 195 (1968)

81. H.W. Cook and M.W. Spence, Biochim. Biophys. Acta, 369, 129 (1974)

82. R.L. Anderson, C S. Fullmer and E.J. Hollenbach, J. Nutr., 105 , 393 (1975)

83. O.S. Privett, E.M. Stearns and E.C. Nickell, J. Nutr., 92, 303 (1967)

84. D. Sand, N. Sen and H. Schlenk, J. Amer. Oil Chem. Soc., 42, 511 (1965)

85. M.W. Spence, Lipids, 6, 831 (1971)

86. Y. Kishimoto and N.S. Radin, J. Lipid Res., 5, 98 (1964)

87. R.R. Brenner and R.O. Peluffo, Biochim. Biophys. Acta, 176, 471 (1969)

88. D. Ullman and H. Sprecher, Biochim. Biophys. Acta, 248, 186 (1971)

89. H. Sprecher and C.J. Lee, Biochim. Biophys. Acta, 388, 113 (1975)

90. H. Schlenk and D.M. Sand, Biochim. Biophys. Acta, 144, 305 (1967)

91. J.J. Rahm and R.T. Holman, J. Nutr., 84, 149 (1964)

92. E. Klenk, Adv. Lipid Res., 3, 1 (1965)

93. H. Schlenk, T. Gerson and D.M. Sand, Biochim. Biophys.Acta, 176, 740 (1969)

94. D. Ullman and H. Sprecher, Biochim. Biophys. Acta, 248, 61 (1971)

95. U.H. Do and H. Sprecher, Archives Biochem. Biophys., 171, 597 (1975)

96. R. Jeffcoat, private communication (1976)

97. C.E. Høy and G. Holmer, Lectures to the 13th ISF Congress (Marseilles, 1976)

98. F.A. Kummerow, J. Amer. Oil Chem. Soc., 51, 255 (1974)

99. W.G. Knipprath and J.F. Mead, J. Amer. Oil Chem. Soc., 41, 437 (1964)

100. Z. Selinger and R.T. Holman, Biochim. Biophys. Acta, 106, 56 (1965)

101. E.M. Stearns, J.A. Rysavy and O.S. Privett, J. Nutr., 93, 485 (1967)

102. T. Takatori, F.C. Phillips, H. Shimasaki and O.S. Privett, Lipids, 11, 272 (1976)

103. H.C. Chang, J. Janke, F. Pusch and R.T. Holman, Biochim. Biophys. Acta, 306, 21 (1973)

104. A.M. Nervi, A. Catala, R.R. Brenner and R.O. Peluffo, Lipids, 10, 348 (1975)

105. D.H. Nugteren, Biochim. Biophys. Acta, 106, 656 (1962)

106. D. Ullman and H. Sprecher, Biochim. Biophys. Acta, 248, 61 (1971)

107. H.W. Sprecher, Biochim. Biophys. Acta, 369, 34 (1974)

108. C.A. Inkpen, R.A. Harris and F.W. Quackenbush, J. Lipid Res., 10, 277 (1969)

109. M.E. De Tomas, O. Mercuri and R. Peluffo, Lipids, 10, 360 (1975)

110. R.O. Sinnhuber, J.D. Castell, D.J. Lee, Fed. Proc., 31, 1436 (1972)

111. J.J. Rahm and R.T. Holman, J. Lipid Res., 5, 169 (1964)

112. J.M. Owen, J.W. Adron and C.B. Cowey, Lipids, 10, 528 (1975)

113. A.T. James and H. Sprecher, private communication (1976)

114. J. Bundy and H. Sprecher, Biochim. Biophys. Acta, 239, 190 (1971)

115. R.B. Bridges and J.G. Coniglio, J. Biol. Chem., 245, 46 (1970)

116. R.R. Linko and H. Karinkanta, J. Amer. Oil Chem. Soc., 47, 42 (1970)

117. H. Sprecher, Biochim. Biophys. Acta, 152, 519 (1968)

118. G.R. Jamieson, "Topics in Lipid Chemistry", Vol. 1, Ch. 2 (ed. F.D. Gunstone), Logos Press (1969)

119. J.A. Barve, F.D. Gunstone, F.R. Jacobsberg and P. Winlow, Chem. Phys. Lipids, 8, 117 (1972)

120. M.S.F. Lie Ken Jie, PhD Thesis, University of St. Andrews (1968)

121. J.G. Batchelor, R.J. Cushley and J.H. Prestegard, J. Org. Chem., 39, 1698 (1974)

122. P. Brawn, private communication (1975)

123. D. Whittaker, "Stereochemistry and Mechanism", Clarendon Press, Oxford (1973)

124. W.W. Christie, J. Chromatography, 37, 27 (1968)
125. W.W. Christie, J. Chromatography, 34, 405 (1968)
126. S.W. Hall, private communication (1974)
127. R.K. Pullarkat, J. Maddow and H. Reha, Lipids, 11, 802 (1976)
128. C. Strouvé-Vallet and M. Pascaud, Biochimie, 53, 699 (1971)
129. S. Rous, Compt. Rend., 254, 3446 (1962)
130. R.G. Dupuis and P. Favarger, Helv. Physiol. Pharmacol. Acta, 21, 300 (1965)
131. J.C. Castuma, A. Catalá and R.R. Brenner, J. Lipid Res., 13, 783 (1972)
132. R.O. Peluffo, A.M. Nervi and R.R. Brenner, Biochim. Biophys. Acta, 441, 25 (1976)
133. D.S. Goodman, J. Amer. Chem. Soc., 80, 3892 (1958)
134. J.D. Ashbrook, A.A. Spector, E.C. Santos and J.E. Fletcher, J. Biol. Chem., 250, 2333 (1975)
135. D. Howling, PhD Thesis (Loughborough University of Technology, 1969)
136. D.N. Brindley and G. Hubscher, Biochim. Biophys. Acta, 106, 495 (1965)
137. M.D. Ullman and N.S. Radin, J. Lipid Res., 13, 422 (1972)
138. C.T. Holloway and P.W. Holloway, Lipids, 9, 196 (1974)
139. E. von Rudloff, Can. J. Chem., 34, 1413 (1956)
140. E. von Rudloff J.Amer. Oil Chem. Soc., 33, 126 (1956)
141. L.J. Morris, D.M. Wharry and E.W. Hammond, J. Chromatog., 31, 69 (1967)
142. A.P. Tulloch, J. Amer. Oil Chem. Soc., 41, 833 (1964)
143. J.S. O'Brien and G. Rouser, Anal. Biochem., 7, 288 (1964)
144. L.J. Morris, D.M. Wharry and E.W. Hammond, J. Chromatog., 33, 471 (1968)
145. M.I. Gurr and M.P. Robinson, Anal. Biochem., 47, 146 (1972)
146. G.A. Gray, Anal. Chem., 47, 546A (1975)
147. A.G. Lee, N.J.M. Birdsall and J.C. Metcalfe, Chem. Brit., 9, 116 (1973)

148. J.E. Cronan and J.G. Batchelor, Chem. Phys. Lipids, 11, 196 (1973)

149. J.P. Wineburg and D. Swern, J. Amer. Oil Chem. Soc., 49, 267 (1972)

150. J.G. Batchelor, R.J. Cushley and J.H. Prestegard, J. Org. Chem., 39, 1698 (1974)

151. J.N. Shoolery, Varian Application Notes, NMR-73-3 (1973) and NMR-75-3 (1975)

152. D.E. Dorman, M. Jautelat and J.D. Roberts, J. Org. Chem., 38, 1026 (1973)

153. D.M. Grant and E.G. Paul, J. Amer. Chem. Soc., 86, 2984 (1964)

154. D.E. Dorman, M. Jautelat and J.D. Roberts, J. Org. Chem., 36, 2757 (1971)

155. L.P. Lindeman and J.Q. Adams, Anal. Chem., 43, 1245 (1971)

156. G.E. Maciel, "Topics in ^{13}C NMR Spectroscopy", (ed. G.C. Levy), Wiley, 1, 53 (1974)

157. D.M. Grant and B.V. Cheney, J. Amer. Chem. Soc., 89, 5315 (1967)

158. N.K. Wilson and J.B. Stothers, "Topics in ^{13}C NMR Spectroscopy" (ed. G.C. Levy), Wiley, 1, 1 (1974)

159. R.S. Pregosin and E.W. Randall, "Determination of Organic Structure by Physical Methods", (ed. F.C. Nachod and J.J. Zuckerman), Academic Press, 4, 263 (1971)

160. J.G. Batchelor, J.H. Prestegard, R.J. Cushley and S.R. Lipsky, J. Amer. Chem. Soc., 95, 6358 (1973)

161. R.A. Friedel and H.L. Retcofsky, J. Amer. Chem. Soc., 85, 1300 (1963)

162. E. Lippmaa, T. Petik, K. Andersson and C. Rappe, Org. Magnetic Resonance, 2, 109 (1970)

163. J.W. de Haan and L.J.M. Van de Ven, Org. Magnetic Resonance, 5, 147 (1973)

164. J. Bus and D.J. Frost, Recl. Trav. Chim. Pays-Bas, 93, 213 (1974)

165. P.G. Barton, Chem. Phys. Lipids, 14, 336 (1975)

166. W. Stoffel, O. Zierenberg and B.D. Tunggal, Z. Physiol. Chem., 354, 1962 (1972)

167. J. Schaefer and E.O. Stejskal, J. Amer. Oil Chem. Soc., 57, 210 (1974)

168. G. Lukas, F. Piriou, S.D. Gero, D.A. Van Dorp, E.W. Hagaman and E. Wenkert, Tetrahedron Letters, 515 (1973)

169. D.J. Frost and F.D. Gunstone, Chem. Phys. Lipids, 15, 53 (1975)

170. A.P. Tulloch and M. Mazurek, Lipids, 11, 228 (1976)

171. F.D. Gunstone, M.R. Pollard, C.M. Scrimgeour, N.W. Gilman and B.C. Holland, Chem. Phys. Lipids, 17, 1 (1976)

172. F.D. Gunstone, M.R. Pollard, C.M. Scrimgeour and H.S. Vedanayagam, Chem. Phys. Lipids, 18, 115 (1977)

173. J. Bus, I. Sies and M.S.F. Lie Ken Jie, Chem. Phys. Lipids, 17, 501 (1976)

174. J. Bus, I. Sies and M.S.F. Lie Ken Jie, Chem. Phys. Lipids, 18, 130 (1977)

175. G.C. Levy and G.L. Nelson, "Carbon-13 Nuclear Magnetic Resonance for Organic Chemists", Wiley-Interscience, New York (1972)

176. J.B. Stothers, "Carbon-13 NMR Spectroscopy", Academic Press, New York (1972)

177. D. Graham and C.E. Holloway, Can. J. Chem., 41, 2114 (1963)

178. F.D. Gunstone, S.R. Steward, J.A. Cornelius and T.W.Hammonds, J. Sci. Fd. Agric., 23, 53 (1972)

179. F.D. Gunstone, D. Kilcast, R.G. Powell and G.M. Taylor, Chem. Comm., 295 (1967)

180. J.L. Williams and D.S. Sgoutas, J. Org. Chem., 36, 3064 (1971)

181. J.L. Williams and D.S. Sgoutas, Chem. Phys. Lipids, 9, 295 (1972)

182. R.A. Raphael and F. Sondheimer, J. Chem. Soc., 2100 (1950)

183. W.H. Kunau, Chem. Phys. Lipids, 11, 254 (1973)

184. J.M. Osbond, "Progress in the Chemistry of Fats and Other Lipids", (ed. R.T. Holman), Pergamon Press, 9, 119 (1966)

185. Y.L. Marcel and R.T. Holman, Chem. Phys. Lipids, 2, 173 (1968)

186. H. Sprecher, Lipids, 6, 889 (1971)

187. D. Howling, L.J. Morris, M.I. Gurr and A.T. James, Biochim. Biophys. Acta, 260, 10 (1972)

188. W. Stoffel, Ann. Chemie, 673, 26 (1964)

189. D.R. Howton, R.H. Davies and J.C. Nevenzel, J. Amer. Chem. Soc., 76, 4970 (1954)

190. D.A. van Dorp, R.K. Beerthuis, D.H. Nugteren and H. Vonkeman, Nature, 203, 839 (1964)

191. E.W. Horton, Chem. Soc. Rev., 4, 589 (1975)

192. R.T. Holman, "Progress in the Chemistry of Fats and Other Lipids", (ed. R.T. Holman), Pergamon Press, 9, 275 and 611 (1968)

193. E. Klenk and H. Mohrhauer, Z. Physiol. Chem., 320, 218 (1960)

194. C.M. Starks, J. Amer. Chem. Soc., 93, 195 (1971)

195. J.F. King and J.R. du Manoir, J. Amer. Chem. Soc., 97, 2566 (1975)

196. W.J. Baumann and H.K. Mangold, J. Org. Chem., 29, 3055 (1964)

197. R.K. Crossland and K.L. Servis, J. Org. Chem., 35, 3195 (1970)

198. F. Spener and H.K. Mangold, Chem. Phys. Lipids, 11, 215 (1973)

199. F. Arndt, B. Eistert and S. Amende, Ber., 61, 1949 (1928)

200. P. Karrer and H. Koening, Helv. Chim. Acta, 26, 619 (1943)

201. J. Casson and W.R. Winnans, J. Org. Chem., 15, 139 (1950)

202. J. Barve and F.D. Gunstone, Chem. Phys. Lipids, 7, 311 (1971)

203. J.C. Nevenzel and D.R. Howton, J. Org. Chem., 23, 933 (1958)

204. S.J. Cristol and W.C. Firth, J. Org. Chem., 26, 280 (1961)

205. H.S. Vedanayagam, PhD Thesis (St. Andrews University, 1976)

206. F.D. Gunstone, C.M. Scrimgeour and H.S. Vedanayagam, J. Chem. Soc. (Commun), 916 (1974)

207. D.H.R. Barton, H.P. Faro, E.P. Serebryakov and N.F. Woolsey, J. Chem. Soc., 2438 (1965)

208. B.C.L. Weedon,"Advances in Organic Chemistry (Methods and Results)", (ed. R.A. Raphael, E.C. Taylor and H. Wynberg), Interscience, New York, 1, 1 (1960)

209. D.G. Bounds, R.P. Linstead and B.C.L. Weedon, J. Chem. Soc., 448 (1954)

210. B.W. Baker, R.P. Linstead and B.C.L. Weedon, J. Chem. Soc., 2218 (1955)

211. G. Wittig, Experimentia, 12, 41 (1956)

212. L.D. Bergelson and M.M. Shemyakin, Angew. Chem. Internat. Ed., 3, 250 (1964)

213. S. Trippett, Quart. Rev., 17, 406 (1963)

214. R.W. Bradshaw, A.C. Day, E.R.H. Jones, C.B. Page, V. Thaller and R.A. Vere Hodge, J. Chem. Soc. (C), 1156 (1971)

215. R.K. Beerthuis, D.H. Nugteren, H.J.J. Pabon, A. Steenhoek and D.A. van Dorp, Rec. Trav. Chim., 90, 943 (1971)

216. J.A. Barve, PhD Thesis (St. Andrews University, 1970)

217. I.A.Ismail, PhD Thesis (St. Andrews University, 1966)

218. W.N. Smith and O.F. Beumel, Synthesis, 6, 441 (1974)

219. J. Meijer and P. Vermeer, Rec. Trav. Chim., 93, 183 (1974)

220. N.F. Chamberlain, "The Practice of NMR Spectroscopy; with Spectra-Structure Correlations for Hydrogen-1", Plenum Press, New York and London (1974)

221. R.C. Baker, R.L. Wykle, J.S. Lockmiller and F. Snyder, Arch. Biochem. Biophys., 177, 299 (1976)

222. F. Scheinmann, lecture given to the Lipid Chemistry Group, Chem. Soc. Meeting (Reading, 1975)

223. K.Ahmad and F.M. Strong, J. Amer. Chem. Soc., 70, 1699 (1948)

224. R. Klok, G.J.N. Egmond and H.J.J. Pabon, Recl. Trav. Chim., 93, 222 (1974)

225. D.E. Ames, A.N. Covell and T.G. Goodburn, J. Chem. Soc., 5889 (1963)

226. N.W. Gilman and B.C. Holland, Chem. Phys. Lipids, 13, 239 (1974)

227. D.E. Ames and A.N. Covell, J. Chem. Soc., 775 (1963)

228. D.E. Ames, A.N. Covell and T.G. Goodburn, J. Chem. Soc., 894 (1965)

229. D.E. Ames and P.J. Islip, J. Chem. Soc., 351 (1961)

230. J.L. Williams and D.S. Sgoutas, Chem. Phys. Lipids, 9, 295 (1972)

231. W.H. Kunau, lecture given to the Lipid Chemistry Group, Chem. Soc. Meeting (Reading, 1975)

232. W.H. Kunau, Chem. Phys. Lipids, 7, 101 (1971)

233. W.H. Kunau, Chem. Phys. Lipids, 7, 108 (1971)

234. S. Wallat and W.H. Kunau, Chem. Phys. Lipids, 13, 159 (1974)

235. W.H. Kunau, H. Lehmann and R. Gross, Z. Physiol. Chem., 352, 542 (1971)

236. H. Sprecher, Lipids, 3, 14 (1968)

237. F.D. Gunstone and M. Lie Ken Jie, Chem. Phys. Lipids, 4, 1 (1970)

238. R.K. Beerthuis, D.H. Nugteren, H.J.J. Pabon and D.A. Van Dorp, Rec. Trav. Chim., 87, 461 (1968)

239. C.H. Lam and M.S.F. Lie Ken Jie, J. Chromatog., 115, 559 (1975)

240. D.E. Ames and P.J. Islip, J. Chem. Soc., 4363, (1963)

241. D.E. Ames and S.H. Binns, J. Chem. Soc. (PI), 255 (1972)

242. H. Lindlar, Helv. Chim. Acta, 35, 340 (1952)

243. H. Lindlar and R. Rubins, "Organic Syntheses", Wiley Press, 46, 89 (1966)

244. L. Crombie, J. Chem. Soc., 3510 (1955)

245. G.M. Robinson and R. Robinson, J. Chem. Soc., 127, 175 (1925)

246. H.C. Brown and G. Zweifel, J. Amer. Chem. Soc., 81, 1512 (1959)

247. D.S. Sgoutas, H. Sandersand and E.M. Yang, J. Lipid Res., 10, 642 (1969)

248. D.R. Howton and R.H. Davies, J. Org. Chem., 16, 1405 (1951)

249. F.D. Gunstone and I.A. Ismail, Chem. Phys. Lipids, 1, 264 (1967)

250. R.D. Harlow, C. Litchfield and R. Reiser, J. Amer. Oil. Chem. Soc., 40, 505 (1963)

251. D.S. Sgoutas and F.A. Kummerow, Lipids, 4, 283 (1969)

252. E.D. Gunstone and F.R. Jacobsberg, Chem. Phys. Lipids, 9, 112 (1972)

253. D.E. Ames, A.N. Covell and T.G. Goodburn, J. Chem. Soc., 4373 (1965)

254. N.T. Castellucci and C.E. Griffin, J. Amer. Chem. Soc., 82, 4107 (1960)

255. W.J. Gensler, M.B. Floyd, R. Yanase and K.W.Pober, J. Amer. Chem. Soc., 92, 2472 (1970)

256. N.E. Pawlowski, D.J. Lee and R.O. Sinnhuber, J. Org. Chem., 37, 3245 (1972)

257. W.J. Gensler, D.M. Solomon, R. Yanase and K.W. Pober, Chem. Phys. Lipids, 6, 280 (1971)

258. A.J. Schipperijn and P. Smael, Rec. Trav. Chim., 92, 1159 (1973)

259. P.K. Raju and R. Reiser, Lipids, 1, 10 (1966)

260. E.L. Schneider, S.P. Loke and D.T. Hopkins, J. Amer. Oil Chem. Soc., 45, 585, (1968)

261. J.E. Rowe, PhD Thesis (Edmonton, Alberta) (1974)

262. A.I. Vogel, "A Textbook of Practical Organic Chemistry", Longmans (1948)

263. R.A. Raphael, "Acetylenic Compounds in Organic Synthesis", Butterworth Scientific Publications, 196 (1955)

264. J.C.D. Brand and G. Eglinton, "Applications of Spectroscopy to Organic Chemistry", Oldbourne Press (London), 111 (1965)

265. A.T. James and E.A. Piper, Anal. Chem., 35, 515 (1963)

266. R. Pummerer and M. Schonamsgruber, Ber., 72, 1834 (1939)

267. R.A. Raphael,"Acetylenic Compounds in Organic Synthesis", Butterworths Scientific Publications, 193 (1955)

268. "Dictionary of Organic Compounds", (ed. J.B. Thomson), Eyre and Spottiswoode Publishers Ltd

269. W.R. Taylor and F.M. Strong, J. Amer. Chem. Soc., 72, 4263 (1950)

270. J.H. Wotiz and E.S. Hudak, J. Org. Chem., 19, 1580 (1954)

271. Report of the Spectroscopy Committee, J. Amer. Oil Chem. Soc., 36, 627 (1959)

272. W.W. Christie, "Lipid Analysis", Pergamon Press, 137 (1973)

Appendix 1 : Incubations where Competitive or Sequential Desaturation Occurred

Experiment 1.	16:0 $\xrightarrow{77\%}$ 16:1(9c) $\xrightarrow{10.5\%}$ 16:2(6c 9c) 16:0 $\xrightarrow{\sim 1\%}$ 16:1(6c) 17:0 $\xrightarrow{65.5\%}$ 17:1(9c) $\xrightarrow{13\%}$ 17:2(6c 9c) 17:0 $\xrightarrow{\sim 1\%}$ 17:1(6c) 18:0 $\xrightarrow{55.5\%}$ 18:1(9c) $\xrightarrow{7\%}$ 18:2(6c 9c) 17:1(12t) $\xrightarrow{18\%}$ 17:2(9c 12t) $\xrightarrow{80\%}$ 17:3(6c 9c 12t) 17:1(12t) $\xrightarrow{15\%}$ 17:2(6c 12t) 18:1(13t) $\xrightarrow{60\%}$ 18:2(9c 13t) $\xrightarrow{35\%}$ 18:3(6c 9c 13t)
Experiment 2.	18:0 $\xrightarrow{55\%}$ 18:1(9c) $\xrightarrow{4\%}$ 18:2(6c 9c) 18:1(10t) $\xrightarrow{10\%}$ 18:2(6c 10t) 18:1(10t) $\xrightarrow{5.5\%}$ 18:2(5c 10t) 18:1(11c) $\xrightarrow{\sim 1\%}$ 18:2(6c 11c) 18:1(11c) $\xrightarrow{\sim 1\%}$ 18:2(5c 11c) 18:1(11t) $\xrightarrow{62\%}$ 18:2(9c 11t) $\xrightarrow{41\%}$ 18:3(6c 9c 11t) 18:1(11t) $\xrightarrow{5\%}$ 18:2(6c 11t) 17:1(12t) $\xrightarrow{14\%}$ 17:2(9c 12t) $\xrightarrow{85\%}$ 17:3(6c 9c 12t) 17:1(12t) $\xrightarrow{8\%}$ 17:2(6c 12t) 18:1(13c) $\xrightarrow{\sim 1\%}$ 18:2(9c 13c) 18:1(13c) $\xrightarrow{\sim 1\%}$ 18:2(6c 13c) 18:1(13t) $\xrightarrow{57.5\%}$ 18:2(9c 13t) $\xrightarrow{27\%}$ 18:3(6c 9c 13t) 18:1(13t) $\xrightarrow{1.5\%}$ 18:2(6c 13t) 18:1(14c) $\xrightarrow{6\%}$ 18:2(9c 14c) 18:1(14c) $\xrightarrow{3.5\%}$ 18:2(6c 14c) 18:1(14t) $\xrightarrow{41.5\%}$ 18:2(9c 14t) $\xrightarrow{5\%}$ 18:3(6c 9c 14t) 18:1(14t) $\xrightarrow{10\%}$ 18:2(5c 14t) 18:1(15c) $\xrightarrow{4\%}$ 18:2(9c 15c) 18:1(15c) $\xrightarrow{4.5\%}$ 18:2(6c 15c) 18:1(15t) $\xrightarrow{48.5\%}$ 18:2(9c 15t) $\xrightarrow{4\%}$ 18:3(6c 9c 15t) 18:1(15t) $\xrightarrow{10\%}$ 18:2(5c 15t)

Appendix 1 (cont)

Experiment 5.	17:0 $\xrightarrow{\text{sterculate}}$	17:1(9c)	7.5%
		17:1(6c)	8.5%
		17:1(5c)	3%
Experiment 6.	18:0 $\xrightarrow{\text{sterculate}}$	18:1(9c)	1.5%
		18:1(6c)	1.5%
		18:1(5c)	7%

No desaturation whatever was detected for the following substrates:- 11:1(9c), 13:1(9c), 13:1(9t), 16:1(7c), 18:1(5c), 18:1(10c), 19:3(7c10c13c) and 22:4(7c10c13c16c).

Appendix 2: ^{13}C Chemical Shifts[1]

Fatty acid or ester	^{13}C Chemical Shifts (ppm)[2]										
	OMe	C(1)	C(2)	C(3)	C(4)	C(5)	C(6)	C(7)	C(8)	C(9)	C(10)
Methyl diesters											
C_{10}	51.26	173.93	34.06	25.03	29.19	29.19					
C_{12}	51.30	174.06	34.11	25.03	29.24	29.30	29.44				
C_{14}	51.28	174.04	34.11	25.04	29.26	29.33	29.52	29.57			
C_{16}	51.27	174.06	34.11	25.04	29.25	29.34	29.53	29.68	29.68		
Cyclopropene esters											
6,7-cyclopropene C_{18}	51.38	-	33.93	24.79	27.02	25.75	108.88[3a]	110.20	26.12	27.48[3b]	29.54
9,10-cyclopropene C_{19}	51.37	-	34.11	24.98	29.15	29.15	29.15	27.40	26.06	109.55	109.55
13:2(8c11c) ester	51.36	174.18	34.12	25.02	29.16	28.99	29.56	27.23	130.03	128.09	25.41
15:2(8c11c) ester	51.45	174.45	34.16	25.02	29.14	28.99	29.54	27.24	129.96	128.26	25.76
19:2(8c11c) ester	51.29	174.00	34.11	25.05	29.25	29.09	29.41	27.30	129.91	128.30	25.78
21:2(8c11c) ester	51.31	174.03	34.09	25.00	29.17	28.99	29.54	27.24	129.91	128.26	25.74
13:2(8a11a) acid	-	180.28	34.07	24.61	28.56	28.56	28.56	18.69	80.28	74.71	9.65
15:2(8a11a) ester	51.35	174.06	34.05	24.95	28.66	28.56	28.66	18.72	80.13	74.89	9.70
19:2(8a11a) ester	51.42	174.25	34.06	24.95	28.70	28.70	28.70	18.76	80.08	74.92	9.72
21:2(8a11a) acid	-	180.38	34.11	24.67	28.65	28.52	28.65	18.73	80.13	74.90	9.73
21:2(8a11c) ester[4]	51.31	174.03	34.08	24.95	28.76	28.52	-	18.79	79.72	78.61	17.26
21:2(8c11a) ester[4]	51.31	174.03	34.08	25.00	-	-	-	27.08	130.95	125.45	17.26

1. Data already published [171, 172] are not included in this Appendix
2. ^{13}C chemical shifts of uncertain assignment are underlined
3. Methylene bridge signal at (a) 7.46 and (b) 7.41
4. The CMR spectrum of a mixture of 21:2(8a11c), 21:2(8c11a) and 21:2(8c11c) was run, and the distinctive signals picked out.

Appendix 2 (cont)

Fatty acid or ester	C(11)	C(12)	C(13)	C(14)	C(15)	C(16)	C(17)	C(18)	C(19)	C(20)	C(21)
Methyl diesters											
C_{10}											
C_{12}											
C_{14}											
C_{16}											
Cyclopropene esters											
6,7-cyclopropene C_{18}	29.54	29.74	29.74	29.74	29.54	32.04	22.77	14.11			
9,10-cyclopropene C_{19}	26.06	27.40	29.46	29.46	29.46	29.46	31.93	22.70	14.09		
13:2(8c11c) ester	129.06	123.90	12.72								
15:2(8c11c) ester	128.26	129.96	29.43	22.89	13.79						
19:2(8c11c) ester	128.06	130.18	27.30	29.83	29.41	29.25	32.03	22.80	14.13		
21:2(8c11c) ester	128.01	130.22	27.33	29.79	29.45	29.70	29.79	29.54	32.03	22.76	14.11
13:2(8a11a) acid	75.83	73.73	3.44								
15:2(8a11a) ester	74.89	80.13	20.82	22.34	13.50						
19:2(8a11a) ester	74.68	80.36	18.76	28.97	28.97	28.97	31.92	22.75	14.11		
21:2(8a11a) acid	74.61	80.49	18.81	28.97	28.97	29.30	29.65	29.40	32.02	22.78	14.11
21:2(8a11c) ester[4]	125.20	131.24	27.24	-	-	-	-	-	32.03	22.76	14.11
21:2(8c11a) ester[4]	78.34	80.03	18.86	-	-	-	-	-	32.03	22.76	14.11

Publications

"Fatty Acids. Part 48. ^{13}C Nuclear Magnetic Resonance Studies of Acetylenic Fatty Acids."
F.D. Gunstone, M.R. Pollard, C.M. Scrimgeour, N.W. Gilman and B.C. Holland, Chem. Phys. Lipids, 17, 1 (1976)

"Fatty Acids. Part 50. ^{13}C Nuclear Magnetic Resonance Studies of Olefinic Fatty Acids and Esters."
F.D. Gunstone, M.R. Pollard, C.M. Scrimgeour and H.S. Vedanayagam, Chem. Phys. Lipids, 18, 115 (1977)

"Studies on the Inhibition of the Desaturases by Cyclopropenoid Fatty Acids."
R. Jeffcoat and M.R. Pollard, accepted for publication, Lipids (1977)

Lectures

"^{13}C NMR Spectra of Unsaturated Fatty Acids"
M.R. Pollard, presented to the 8th Scottish Lipid Discussion Group, Aberdeen, April, 1976

"^{13}C NMR Study of Unsaturated Fatty Acids and Esters"
M.R. Pollard, F.D. Gunstone, C.M. Scrimgeour and H.S. Vedanayagam, presented to the 13th ISF Congress, Marseilles, September, 1976

"The Specificity of the Mammalian $\Delta 9$, $\Delta 6$ and $\Delta 5$ Desaturases"
M.R. Pollard, presented to the 9th Scottish Lipid Discussion Group, Glasgow, April, 1977