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A THESIS

entitled

SYNTHESES AND APPLICATIONS OF METHIONINES
SPECIFICALLY LABELLED WITH STABLE ISOTOPES

by

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Submitted in partial fulfilment of the requirements for the
degree of Doctor of Philosophy at the University of Warwick,
in the Department of Chemistry and Molecular Sciences.

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ABBREVIATIONSA

ATP	adenosyl triphosphate
ACC	1-aminocyclopropane-1-carboxylic acid
AR	analytical reagent grade
AVG	aminoethoxy vinyl glycine
aq	aqueous

B

BDH	British Drug Houses Ltd
bp	boiling point

C

cm	centimetres
°C	degrees Centigrade
c	concentration in grams/100 cm ³ solvent (in optical rotation measurements)
conc	concentrated

D

DMSO	dimethylsulphoxide
DNP	2,4-dinitrophenyl hydrazone
24 DNP	2,4-dinitrophenyl hydrazine
DEGS	diethylene glycol succinate polyester
D	deuterium
dia	diameter
DNA	deoxy ribonucleic acid
DSS	sodium 2,2-dimethyl-2-silapentane-5-sulphonate

E

equiv	equivalent
eqn	equation

G

g. l. c.	gas-liquid chromatography
g	grams

<u>H</u>	h	hours
<u>I</u>	i. r.	infra red
<u>K</u>	KMBA	4 methylthio-2-oxobutanoate (α -keto- γ -methylthio butyric acid)
<u>L</u>	l	litres
<u>M</u>	M	molar
	mol	mole(s)
	mmol	millimole(s)
	MHz	megahertz
	mM	millimolar
	Methional	3(methylthio)propinaldehyde
	min	minutes
	ms	mass spectrum
	m/e	mass/energy
	mm	millimetres
	mp	melting point
<u>N</u>	n. m. r.	nuclear magnetic resonance
<u>O</u>	ODC	orthinine decarboxylase
<u>P</u>	p. p. m.	parts per million
	p. s. i.	pounds per square inch
<u>R</u>	Rf	retardation factor
	Ref	Reference No

R (cont)

Rt Retention time

rac racemic

RNA ribo nucleic acid

S

SAM S-adenosyl methionine

TTMS tetramethyl silane (¹H n. m. r. internal standard)

t. l. c. thin layer chromatography

t_{1/2} half life

t. b. p. trigonal bi-pyramidal

TSS 3-(trimethylsilyl)-tetra^eteropropionic acid sodium saltU

u. v. ultra violet

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Finally I should like to thank my wife Lisabeth for her support and her patience during the time this work was being performed, and this thesis prepared.

DECLARATION

The work described herein was performed at the Department of Chemistry and Molecular Sciences, University of Warwick, England, and in part at the laboratories of BOC Prochem Limited, London, during the period 1976-1979, and was supported by an S.R.C. C.A.S.E. award. The work described is thought to be original except where due and proper acknowledgment is given for ideas and work previously published, or performed by others. The work described has not been submitted for any other degree previously.

ABSTRACT

The metabolic fate of the alkyl chain (C-1 to C-4) of methionine ($\text{CH}_3\text{SCH}_2\text{CH}_2\text{CH}(\text{NH}_2)\text{COOH}$) is a topic of current interest. A number of methionines specifically labelled with ^2H and ^{13}C have been synthesised, and used in metabolic studies of the metabolism of methionines alkyl chain.

After a general introduction to methionine metabolism, with emphasis on the alkyl chain, and to the techniques of isotopic labelling (chapter 1), chapter 2 outlines the materials and methods used in the project. In chapter 3 two syntheses of methyl labelled methionines are presented, one a modification of a literature procedure (providing ^{13}C or $^2\text{H}_3$ material) and the other a novel synthesis, providing (S)-[methyl- $^2\text{H}_3$] methionine via proton exchange in (1R,3S)dehydromethionine²⁴⁴.

Novel syntheses of rac-[3,4- $^{13}\text{C}_2$] methionine and rac-[3,4- $^2\text{H}_2$] stereospecifically labelled methionines (i.e. of known relative configuration at C-3 and C-4) are presented in chapter 4. The stereochemistry of the latter materials has been demonstrated by ^1H n.m.r. analysis of the dehydromethionines derived from them. Chapter 5 describes an examination of the conversion of 2-(methylthio)-[1- ^{13}C] ethanol into 1-chloro-2-(methylthio)ethane by a number of reagents, each of which produce a ca 1:1 mixture of C-1 and C-2 labelled material, due to neighbouring group participation by sulphur²⁰⁹. This demonstrates that a literature synthesis of rac-[3- ^{14}C] methionine actually produced a ca 1:1 mixture of C-3 and C-4 labelled materials^{209, 181}. Chapter 6 describes some chemistry of dehydromethionine, including the complete analysis of its 220 MHz ^1H n.m.r. spectrum (via the spectra of the ^2H labelled dehydromethionines obtained from the stereospecifically ^2H labelled methionines), and the preparation of (S)-methionine-(S)-sulphoxide from (1R,3S)dehydromethionine. Chapter 7 describes an examination, using the labelled methionines, of the biosynthesis of ethylene from methionine by microorganisms, and demonstrates the identity of a possible intermediate in this biosynthetic pathway as being 4-methylthio-2-oxobutanoate²⁴⁵. The results of a study of the biosynthesis of spermidine from methionine, using rac-[3,4- $^{13}\text{C}_2$] methionine are also reported²⁵⁷, and these demonstrate the precursorial role of C-3 and C-4 of methionine in the biosynthesis of this polyamine. A full bibliography follows chapter 7.

PUBLICATIONS

Part of the work described in this thesis has been published, and a further part is being prepared for publication as follows:

1. D. C. Billington and B. T. Golding, "A ^{13}C labelling study of the reaction between 2-(methylthio)ethanol and carbon tetrachloride-phosphines", J. C. S. Chem. Comm., 1978, 208
2. D. C. Billington and B. T. Golding, "Proton exchange in dehydromethione; synthesis of (S)-[methyl- $^2\text{H}_3$] methionine", Tetrahedron Letters, 1979, 2937
3. D. C. Billington, B. T. Golding, and S. B. Primrose, "Biosynthesis of ethylene from methionine", Biochem. J., 1979, 182, 827
4. M. P. Atkins, D. C. Billington, B. T. Golding, M. D. Johnson, I. K. Nassereddin, and P. J. Sellars, "Mechanistic studies using ^{13}C labels", Paper presented at the (Prochem) Symposium on stable isotopes, 27 September 1979, at the University of Warwick.
5. D. C. Billington, B. T. Golding and I. K. Nassereddin, "Biosynthesis of spermidine from L-methionine in E. Coli", J. C. S. Chem. Comm., in the press
6. D. C. Billington, B. T. Golding and I. M. Lochart, "Syntheses of methionine specifically labelled with stable isotopes", in preparation, October 1979
7. D. C. Billington and B. T. Golding, "Some chemistry of dehydromethionine", in preparation, October 1979

ERRATA

<u>PAGE</u>	<u>LINE</u>	<u>DELETE</u>	<u>INSERT</u>
	Throughout	SPERMIDENE	SPERMIDINE
6	23	ORTHININE	ORNITHINE
7	6	""	""
10	12	""	""
48	6	ERGOSTOL	ERGOSTEROL
52	18	<u>STEREOSPECIFIC LABELLING</u>	
			<u>STEREOSPECIFIC LABELLING</u>
65	4	HETROCYCLE	HETEROCYCLE
""	6	""	""
81	7	""	""
99	22	HETROGENEOUS	HETEROGENEOUS
	FIG 5-B-5	SCHNIDER	SNYDER

This thesis is dedicated to my
parents, without whom none of
this would have been possible.

Thanks Mum and Dad.

David

'The time has come', the walrus said,

'To talk of many things:

Of shoes- and ships - and sealing wax -

Of cabbages - and kings -

And why the sea is boiling hot -

And whether pigs have wings.'

Lewis Carroll

CHAPTER 1

INTRODUCTION

1-A GENERAL

Much of the biochemistry of methionine has been elucidated since its discovery in 1922. However some aspects of its biochemical function, particularly the biosynthetic utilisation of its alkyl chain (C-1 to C-4) are still being revealed. In metabolic studies, tracer experiments using isotopically labelled compounds can provide valuable information. These tracer studies use enrichment of a given isotope (stable or radioactive) above natural abundance, to provide a label by which a given molecule can be distinguished from otherwise identical other molecules. The advent of sensitive detection techniques has resulted in stable isotopes being used more frequently in these tracer studies, in parallel with the more widely used radio isotopes. This is especially so for isotopes of carbon, where detailed information may be obtained directly from the ^{13}C n. m. r. spectrum of a ^{13}C labelled compound, without the need for the prolonged degradation procedures associated with ^{14}C labelling.

This thesis concerns the synthesis of a number of specifically labelled methionines containing the stable isotopes ^2H and/or ^{13}C . These methionines are of potential value in studies of the metabolism of methionine, for example in the examination of the metabolic fate of its alkyl chain. Some preliminary results of metabolic studies using the labelled methionines are presented.

A summary of current ideas on the metabolism of methionine is presented in section 1 - B, including a detailed treatment of the aspect of

methionine metabolism which was chosen for study, vis the metabolic fate of the alkyl chain. Section 1 - C describes the rationale and methodology of isotope labelling experiments, and compares and contrasts the use of radioactive and stable isotopes. Some of the recent applications of stable isotopes are summarised in section 1 - D. The project itself is outlined in section 1 - E.

1-B THE BIOCHEMISTRY OF METHIONINE

1-B-1 GENERAL

The isolation of a new sulphur containing amino acid from the hydrolysis products of caesin was reported in 1922¹. In 1928 its structure was demonstrated to be $\text{CH}_3\text{SCH}_2\text{CH}_2\text{CH}(\text{NH}_2)\text{COOH}$, and the new amino acid was named methionine². Dietary methionine has been shown to be necessary for growth in a number of organisms³. It is regarded as an essential dietary amino acid in man⁴, being one of only two forms of sulphur which can be assimilated by vertebrates⁵. Evidence for the biosynthesis of methionine in humans, involving the recycling of methionine sulphur, and the de novo biosynthesis of methyl groups has been presented⁶. The biosynthesis of methionine is closely interlinked with that of the other sulphur containing amino acids, including cysteine and homocysteine³. Methionine (1) is biosynthesised by methylation of homocysteine (2), which arises from cysteine (3), via cystathione (4) (Fig 1-B-1). The methyl donor in this methylation is methyl tetrahydrofolate. In vertebrates the sulphur moiety used in this biosynthesis comes from dietary methionine, or cysteine, whereas bacteria and plants can synthesise cysteine from inorganic sulphate. One of the primary biochemical functions of methionine is as a donor of methyl groups. The homocysteine by-product of this reaction is then used for the biosynthesis of more methionine (Fig 1-B-1).

Methionine is one of the common amino acids found in polypeptides and proteins⁷. Recently it has been demonstrated to be a constituent of the polypeptides classed as enkephalins and endorphins^{8,9}. These polypeptides

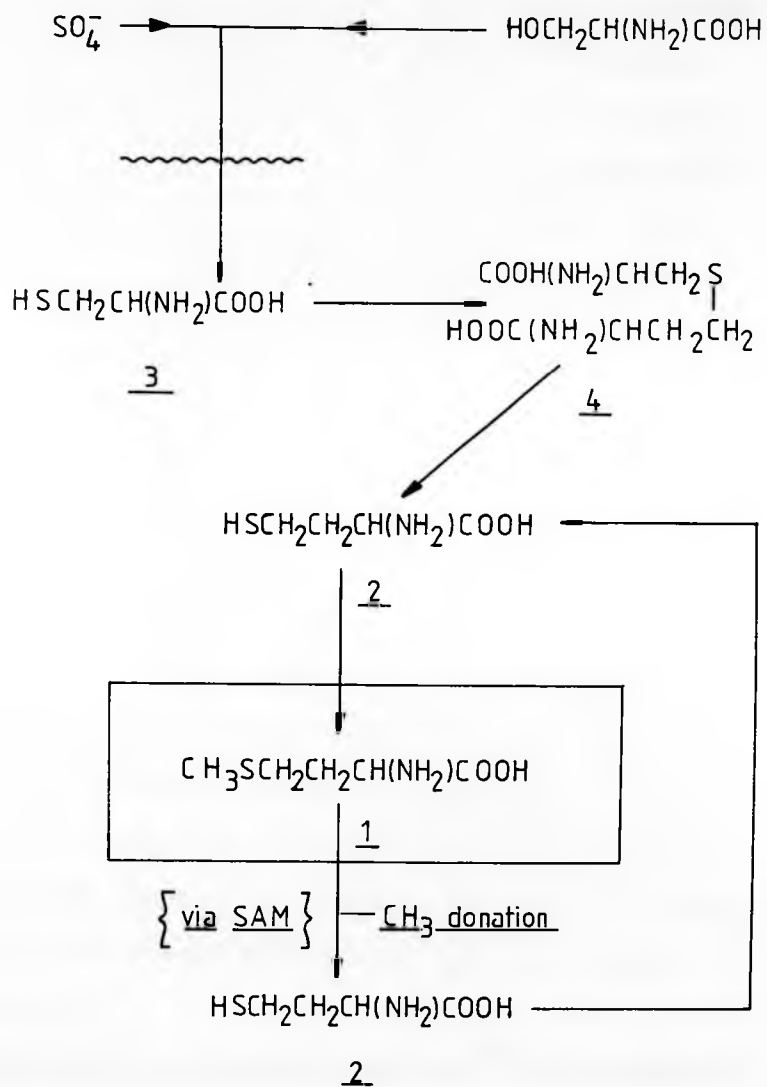


FIG 1-B-1

THE BIOSYNTHESIS OF METHIONINE FROM CYSTEINE VIA CYSTATHIONE. THE BIOSYNTHESIS OF CYSTEINE FROM INORGANIC SULPHATE IS ONLY OBSERVED IN BACTERIA

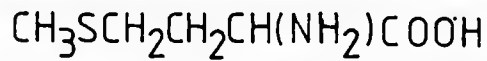
show opiate-like activity, and can be isolated from many animals including man^{8, 10}, and they are believed to be natural analgesics¹¹. Besides its incorporation into peptides, methionine is used as a source of molecular fragments in the biosynthesis of other compounds. The S-methyl group⁶, and the alkyl chain¹² of methionine are both used in this way. The intact molecule also plays a role in the initiation of protein biosynthesis¹³.

1-B-2 METHIONINE AS A METHYL DONOR

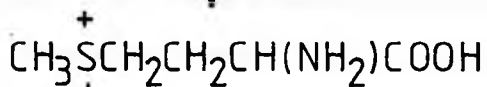
In 1939¹⁴ the function of methionine as a methyl donor in the trans-methylation reaction ($\text{donor} - \text{CH}_3 + \text{acceptor} \rightarrow \text{acceptor} - \text{CH}_3 + \text{donor}$) was inferred. This was confirmed in 1953¹⁵ when S-adenosyl methionine (SAM) was identified as the methyl donor involved in biological transmethylation. SAM (5) is biosynthesised from methionine and ATP⁶, (Fig 1-B-2), and owes its reactivity to the presence of the tricoordinate sulphonium group. At first SAM was considered to be a modifying agent for small molecules only. Further studies⁶ have shown however that this activated form of methionine may donate its methyl group to a wide variety of acceptors, and also donate the methionine alkyl chain, or an adenosyl residue, to suitable acceptors.

Many hundreds of compounds are now known¹⁶ which contain methyl groups, or C-1 units¹⁷ derived from methionine, via SAM. These include alcohols, amino acids, terpenoids and sterols, aromatic and heterocyclic compounds. Reviews on the scope of transmethylation in the biosynthesis of compounds of these types are available^{16, 16a, 16b}. In addition to its

FIG 1-B-2



1

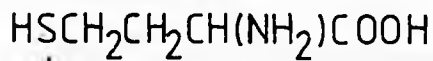


Ad

5

Acceptor

CH₃-Acceptor



Ad

FIG. 1-B-2

Methionine as a methyl donor. Methionine (1) is activated by S adenylation to give SAM (5) which may then donate a methyl group to a suitable acceptor.

biosynthetic role in transmethylation reactions, many macromolecules are now known which, after primary biosynthesis, are methylated in reactions involving SAM⁶. The reasons for these methylation reactions, and the effects that methylation has on the structure and function of the macromolecule in question are largely unknown. The methylation of nucleic acids (DNA, tRNA, mRNA), proteins, polysaccharides, and lipids has been demonstrated. In all of these reactions the methyl donor is SAM, and the hypothesis advanced by Cantoni¹⁸, "that SAM is the methyl donor in all methyl transfer reactions, except those of methionine biosynthesis", still holds true.

When foreign (e.g. viral) DNA enters a bacterial cell it may be degraded or it may survive and replicate. The methylation of DNA involving SAM has been shown to play an important part in this process¹⁹. The specific endonuclease which degrades foreign DNA has been demonstrated to differentiate between foreign and native DNA by the degree of methylation of the macromolecules in question. Thus it cleaves the foreign non-methylated DNA rather than the native methylated DNA. The methylation of tRNA by SAM was discovered by Borek²⁰, and has since been extensively studied²¹. The degree and pattern of methylation has been shown to be different in tRNA obtained from different organisms, and in tRNA from the same organism under different physiological conditions. No firm evidence exists as to the significance of these methylations with regard to the structure and function of tRNA. The methylation of intact proteins has been observed²², and enzymes capable of methylating lysine, arginine, histidine, and carboxyl

residues have been isolated²³. The timing of these reactions in relation to the biosynthesis of the proteins concerned, and the effects of these methylations, have not yet been established²⁴. The degree of methylation of the unsaturated fatty acid components of E. coli membranes has been observed to affect the transport of nutrients across these membranes²⁵. In particular, specific nutrient transport defects may be corrected by addition of SAM, which results in methylation of the membrane in question.

1-B-3 THE BIOCHEMICAL UTILISATION OF THE ALKYL CHAIN OF METHIONINE

1. Methionine and the biosynthesis of spermidene and spermine

a) Introduction

In 1958²⁶ methionine was identified as a precursor of the polyamines spermidene (6) and spermine (7). These polyamines are biosynthesised from putrescine (8) and methionine (1), by way of SAM (Fig. 1-B-3)²⁷. The polyamines (6), (7) and (8) are found in a wide variety of bacteria, yeasts, plants, and animals, and may be present in all living systems. During the last 30 years the biosynthesis of these polyamines has been elucidated, and many biological functions have been proposed for them. The literature on these compounds is extensive, and the discussion here is limited. For further information see the reviews by Tabor and Tabor^{12, 28, 29} (over 700 references).

b) Biosynthesis of the polyamines (6), (7) and (8)

The pathway involved in the biosynthesis of the polyamines (6), (7), and (8) from ornithine (9), or arginine (10) and methionine (Fig. 1-B-3) was

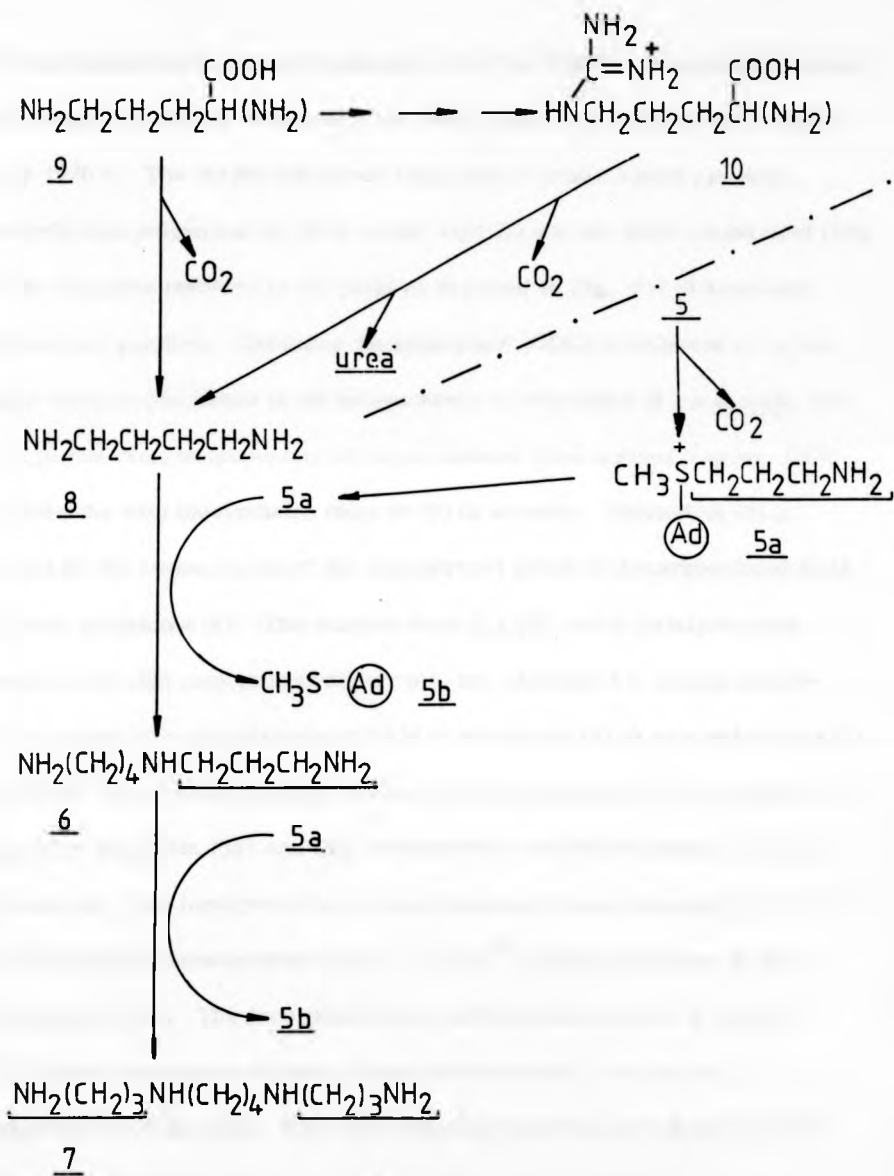


FIG 1-B-3

THE BIOSYNTHESIS OF SPERMIDENE (6) AND SPERMINE (7) FROM PUTRESCINE (8) AND METHIONINE, VIA SAM (5) AND DECARBOXYLATED SAM (5a)

first demonstrated in microorganisms in the late 1950's. Animals and plants were shown to possess essentially the same pathway in the late 1960's and early 1970's. The major difference being that animal tissues can only biosynthesise polyamine (8) from amino acid (9) and not from amino acid (10). All the enzymes involved in the pathway depicted in Fig. 1-B-3 have been isolated and purified. Ornithine decarboxylase (ODC) is believed to be the major enzyme concerned in the biosynthesis of polyamine (8) in E. coli, and as arginine decarboxylase has not been isolated from animal tissues, ODC provides the only biosynthetic route to (8) in animals. Polyamine (6) is formed by the condensation of the aminopropyl group of decarboxylated SAM (5a) with polyamine (8). The enzyme from E. coli which catalyses this reaction will also catalyse, at higher pH, the addition of a second aminopropyl group from decarboxylated SAM to polyamine (6) to give polyamine (7). In animals two distinct enzymes exist, one which catalyses the formation of polyamine (6), from (5a) and (8), and the other that of polyamine (7), from (5a) and (6). The incorporation of intact diamine (8) into the polyamines (6) and (7) has been demonstrated using ^{14}C and ^{15}N double labelled (8) and precursors of (8). The incorporation of radioactivity from C-2 labelled ^{14}C (1) into polyamines (6) and (7) has been reported. As yet the incorporation of the intact alkyl chain of amino acid (1) into (6) and (7) has not been demonstrated.

c) The role of the polyamines (6), (7) and (8) in vivo

There is substantial evidence that the polyamines perform an essential role in living systems²⁸. They have been shown to be intimately

concerned with the response of cells to growth stimuli, to stimulate many of the reactions involved in protein synthesis, to interact physically with nucleic acids, and to affect membrane stability. They are also the possible precursors of a number of alkaloids.

The role played by the polyamines in the response of cells to growth stimuli can be inferred from the remarkable characteristics of the enzyme ODC. This enzyme has been described in many tissues, and many species. It provides the only route to the diamine (8) in animal tissues. In quiescent tissues the level of enzyme activity is low, but much higher levels are observed in tissues which are growing rapidly, or engaged in protein synthesis. In tissues subjected to a wide variety of growth stimuli a peak in ODC activity occurs ca 4h after application of the stimulus, and this corresponds with a peak in protein synthesis. Increases in all the enzymes involved in polyamine synthesis, as well as in the levels of the polyamines themselves, are also observed. This rapid change in ODC level implies an essential role in the induction of new protein synthesis, i.e. growth.

ODC has a very fast turnover rate, its $t_{\frac{1}{2}}$ being only ca 10 min in living tissues, and this means its concentration can change very rapidly indeed. Most enzymes have $t_{\frac{1}{2}}$'s of ca 24h, and enzymes normally considered to have fast turnover rates have $t_{\frac{1}{2}}$'s of ca 1h. The very short half life of ODC means that if the rate of protein synthesis increases by a factor of 4, the concentration of ODC doubles in 10 min, compared to an enzyme with $t_{\frac{1}{2}}$ ca 24h, whose concentration would only rise ca 1% in this time. This is the cell's way of ensuring a fast reaction to growth stimuli. Other enzymes in

the biosynthetic route to polyamines (6) and (7) also have short half lives (e.g. for SAM decarboxylase, $t_{\frac{1}{2}} = 1h$), but not of the same order as ODC.

The exact role played by the polyamines in the initiation of protein synthesis is hard to assess, owing to their wide spectrum of stimulatory effects. The marked increase in the rate of protein synthesis that occurs when polyamines are added to living tissue is therefore not well understood. Recent interest has surrounded the observation that polyamine levels are abnormal in patients suffering from cancer. Changes in polyamine levels associated with changes in the rate of tumour growth, or tumour cell death, have been described. Thus polyamine concentrations in physiological fluids provide a monitor of tumour growth kinetics.

The polyamines (6), (7) and (8) have a high affinity for nucleic acids in solution. Both DNA and RNA are stabilised against denaturation and shearing in the presence of polyamines, and this has been interpreted to indicate the binding of polyamines to nucleic acids. Attempts to demonstrate that polyamines are bound to nucleic acids in vivo have however met with little success. Only DNA isolated from bacteriophages has been shown to be bound to polyamines in vivo.

A stabilising effect has also been observed when membranes or mitochondria from E. coli are subjected to osmotic stress in vitro, in the presence of polyamines. This has been attributed to the binding of polyamines to the membranes concerned, but no in vivo binding has been observed.

Recent studies have implicated the polyamines as precursors of a number of alkaloids. Maytenin (11) for example, was demonstrated in 1973³⁰

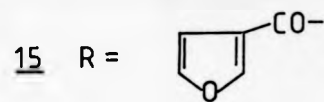
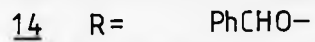
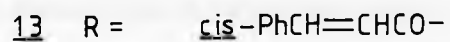
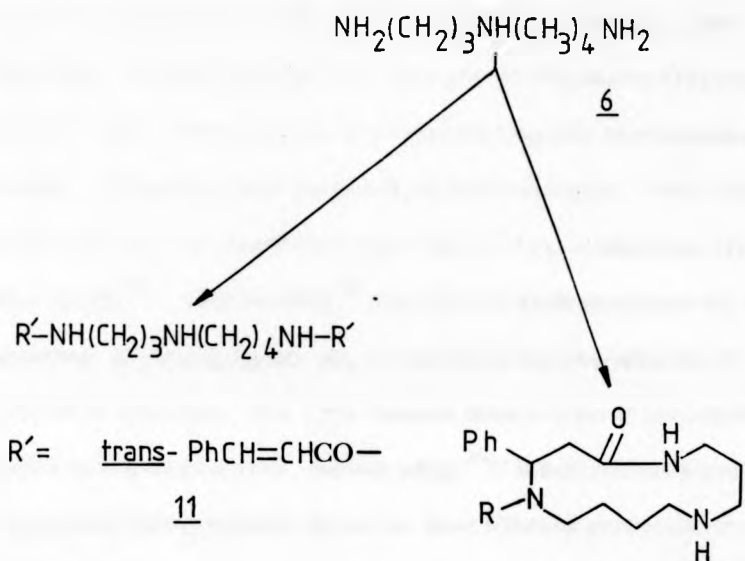


FIG 1-B-4

ALKALOIDS WHICH ARE BELIEVED TO BE DERIVED FROM SPERMIDENE AND CINNAMOYL
 PRECURSORIAL UNITS

to be the di-trans-cinnamoyl amide of the terminal amine groups in spermidene. The following year the structure of celacinnine (12) was reported³¹ (Fig. 1-B-4), and its 13 membered ring was interpreted as reflecting spermidene and cinnamoyl precursorial units. This 13 membered ring also occurs in the alkaloids cellalocinnine (13), celabenzene (14) and celafurine (15)³¹. Very recently³² evidence has been presented for the involvement of the polyamines (6), (7) and (8) in the biosynthesis of pyrrolizidine alkaloids. The most common base portion of pyrrolizidine alkaloids is retronecine (16). Studies using ¹⁴C labelled polyamines (6), (7) and (8) showed that polyamine (6) is the most efficient precursor of the retronecine portion of retrorsine (17), followed by (7), (8) and ¹⁴C labelled orthinine. The position of ¹⁴C labelling was determined by degradation, and the results implied the involvement of a symmetrical intermediate of the type shown, (18) in Fig. 1-B-5.

As a final observation on the biochemistry of the polyamines, almost all the polyamine (6) present in stationary phase E. coli cells is covalently bound to glutathione. On dilution into fresh medium this polyamine is rapidly released. This observation is interesting as both polyamine (6), and glutathione are present in high concentrations in E. coli cells, and the function of neither is well understood.

2. The biosynthesis of ethylene from methionine

a) Introduction

Ethylene has been shown to be a natural plant hormone, exhibiting remarkable effects on the growth and maturation of plants. The role of

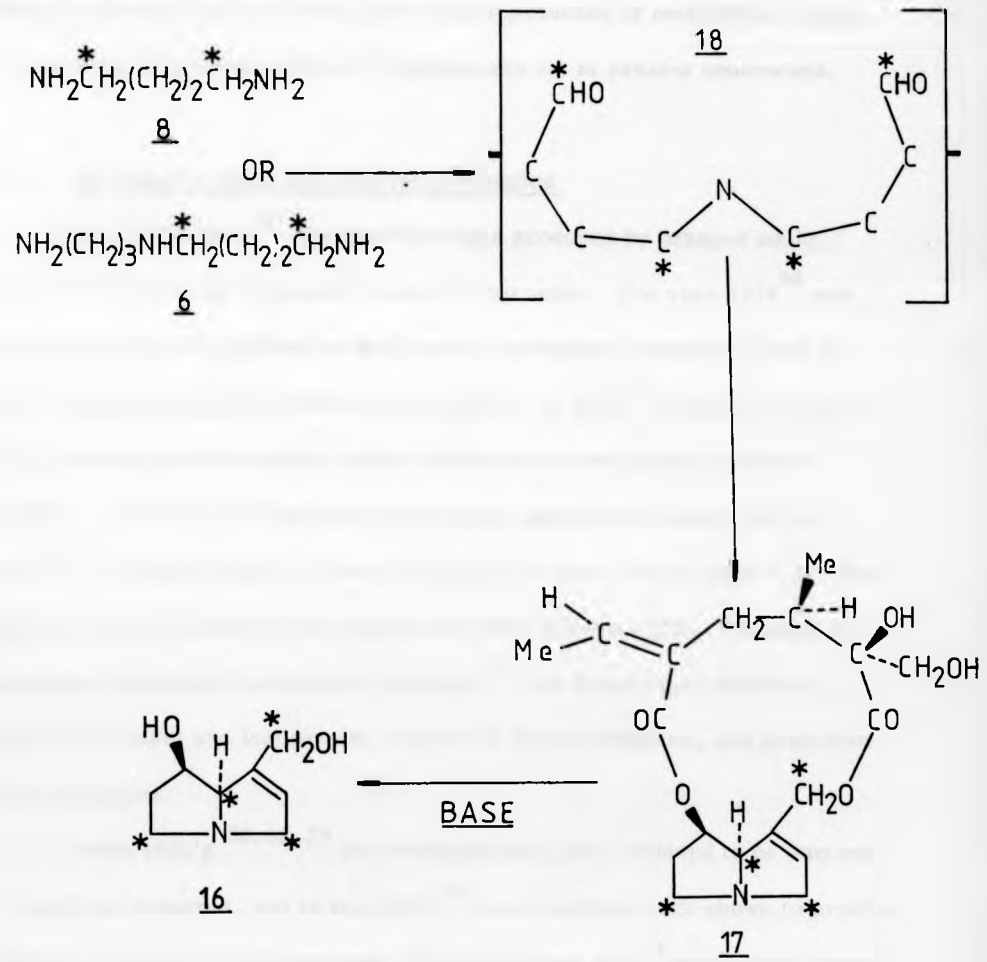


FIG 1-B-5

THE BIOSYNTHESIS OF RETROSCINE (17) FROM SPERMIDENE (6) OR PUTRESCINE (8), AS ELUCIDATED BY ¹⁴C LABELLING STUDIES

methionine as the precursor of the ethylene produced by plants was established in the late 1960's. Recently many microorganisms have been shown to produce ethylene when grown in the presence of methionine. Many facets of the natural production of ethylene are not at present understood.

b) The history of natural ethylene production

In 1910 Cousins³³ reported that a gas produced by oranges could cause the degreening of bananas in mixed shipments. The year 1924³⁴ saw the identification of ethylene as the cause of premature ripening of fruit by gases from the incomplete burning of paraffin. In 1934³⁵ ethylene was shown to be a natural plant hormone, when it was proved that apples produced ethylene. Since then, it has been shown to be produced by many parts of plants³⁶, including stems, leaves, flowers, and roots, and to have a profound effect on many aspects of the regulation of their growth cycle. Amongst the properties attributed to ethylene's presence³⁷, are breaking of dormancy, inhibition of stem and leaf growth, control of flower induction, and premature or late ripening.

In the 1930's^{35,38,39} the production of a gas believed to be ethylene by fungi was reported, and in the 1940's⁴⁰ pure cultures were shown to produce this gas. However, it was not until 1951 that Young et al⁴¹ proved that microorganisms can produce ethylene, when they isolated ethylene from pure cultures of the fungus Penicillium digitatum. Since then, ethylene has been demonstrated as a natural product from many fungi³⁷, but until the early 1970's very few bacteria had been examined. This stimulated research in this area, and in

1976^{42, 43} Primrose reported ethylene producing bacteria to be "ubiquitous in the environment". Today, ethylene's role as a powerful plant growth hormone is recognised, and its production by higher plants, fungi, and bacteria is established.

c) Substrates for ethylene production

Two types of pathway can be identified for the production of ethylene by natural systems: those which utilise methionine as a precursor, and those which do not^{36, 42, 43, 44}.

In higher plants, the fungi Mucor hiemalis, Candida vartiovaariai and Trichosporon cutaneum, and all bacteria, the precursor of ethylene is methionine^{36, 42, 43, 44}. Other fungi have pathways capable of producing ethylene from other precursors. In P. digitatum the precursor is α -keto-glutarate, or glutamic acid, and ethylene production is stimulated by the addition of many compounds to culture media³⁷, including ethanol, ethionine, malonic acid, serine, and sugars. Other Penicillia can produce ethylene only in the presence of methionine however, and P. digitatum has been shown to produce ethylene from methionine. Recently⁴⁵ the production of ethylene by a strain of Fusarium oxysporum grown on glucose as the sole organic nutrient has been reported.

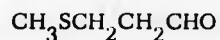
The discussion here will be limited to the production of ethylene from methionine.

d) The Biosynthesis of ethylene from methionine by plants

Owing to the profound effects ethylene has on the growth and development

of many plants, much of the early work on its biosynthesis was performed by plant physiologists. [More recently the biosynthesis of ethylene by microorganisms has also been studied.] During the period 1951 to 1974, many possible precursors of the ethylene produced by plants were suggested, including ethanol, ethionine, β -alanine, and methionine. The merits of these precursors, and the proposed pathways involved have been reviewed by Yang³⁶, and he concluded that from the available evidence methionine was the precursor of ethylene in higher plants. This view is a generally accepted fact today.

A model system capable of converting methionine into ethylene was described in 1965⁴⁶. In this copper (II) catalysed reaction, tracer experiments with ¹⁴C labelled methionines showed that C-3 and C-4 of methionine gave rise to ethylene, whilst C-1 gave rise to CO₂. The fate of C-2 and C-5 was not established, although it was suggested that C-5 became either methane thiol, or methane. In this model system, methional (19) was ca 6 times as reactive in the production of ethylene

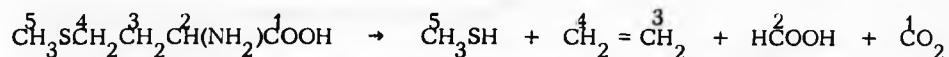


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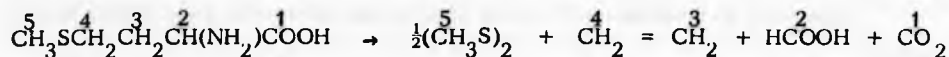
as methionine. However no evidence could be obtained in support of the intermediacy of methional in the reaction producing ethylene from methionine. The same authors published an examination of the production of ethylene from methionine by apple tissues⁴⁷. It was demonstrated that C-3 and C-4 of methionine became ethylene, as in the model system. No conversion of other possible substrates to ethylene was observed. The authors suggested that a

copper containing enzyme system (e.g. a peroxidase) could be operating in the apple tissues, as addition of copper chelating agents inhibited ethylene production.

Following the report⁴⁸ that an extract obtained from pea seedlings could produce ethylene in an enzyme or light mediated reaction, Yang *et al* showed in 1966⁴⁹, that the active precursor of ethylene in the photochemical reaction was methionine. In tracer experiments using ¹⁴C labelled methionine they demonstrated the origin of the ethylene carbons to be C-3 and C-4 of methionine, in a reaction mediated by flavinmononucleotide and light. They suggested that the overall conversion could be represented by:



The following year the same authors reported a cell free chemical system⁵⁰, which could convert methionine, methional, and 4-methylthio-2-oxobutanoate, KMBA, (20) into ethylene, in the presence of flavinmononucleotide (or riboflavin) and light. Both methional and KMBA were converted into ethylene more efficiently than methionine, and by ¹⁴C labelling Yang showed the fate of the carbon atoms of methionine to be as follows:

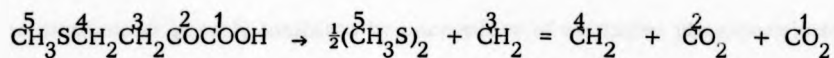


Two enzyme systems which could convert methionine analogues to ethylene were reported in 1967^{51,52}, one isolated from pea seedlings, and the other from cauliflower florets. The system from pea seedlings⁵² could convert methional but not methionine itself, to ethylene. The cauliflower

derived system⁵¹ could convert methionine or methional to ethylene, if both particulate and non-particulate fractions were present, but only methional was converted if the particulate fraction was removed. Both these systems resembled peroxidases, and Yang developed a cell free system involving horseradish peroxidase, which could convert methional into ethylene⁵³.

Also in 1967, the fate of the carbon atoms of ¹⁴C-labelled methionine infused into ethylene producing vegetative tissue, bananas, and apple tissue was reported⁵⁴, as follows: C-1 was released as CO₂, C-2 was retained in the tissue and further metabolised, C-3 and C-4 were released as ethylene, and the sulphur atom and C-5 were retained in the tissue. The fate of C-2 of methionine was established in a later study as formic acid⁵⁴.

In 1969⁵⁵, Yang et al showed that the peroxidase system⁵³ could convert either methional, or KMBA, into ethylene, and showed by ¹⁴C labelling the fate of the carbon atoms of KMBA to be as follows:



20

A similar result was reported for methional, except C-1 (corresponding to C-2 of KMBA) was converted into formic acid. The isolation of two more enzymic systems, one from apples, and one from tomatoes, were reported^{56,57} each capable of converting methional or KMBA into ethylene. Bauer and Yang reported⁵⁸ that apple tissue could not convert methional into ethylene, and although KMBA was converted to ethylene, it was a much less effective precursor than methionine. Also in 1969, KMBA was reported to stimulate the production

of ethylene by cauliflower florets⁵⁹, but this result was later shown to be in error⁶⁰.

The production of ethylene by the peroxidase systems described above differs from the in vivo pathway leading to ethylene in a number of respects³⁶. The peroxidase systems utilise methionine, methional, and KMBA, whereas in intact tissues, methionine is the sole effective precursor³⁶. Compounds known to inhibit the in vitro peroxidase systems (i.e. phenols) have no effect on tissues producing ethylene⁶¹. Furthermore, catalase (a known inhibitor of peroxidase) has no effect when administered to tissues producing ethylene⁶⁰, and no correlation could be found between catalase levels of tissues and their ability to produce ethylene⁶². Based on this evidence, Yang concluded in 1974 "that the available results fail to support the proposal that KMBA, methional, and peroxidase are involved in ethylene biosynthesis in vivo"³⁶.

Recently, a new pathway for the conversion of methionine to ethylene in plant tissues has been proposed. The observation that the conversion of methionine to ethylene is greatly inhibited by uncouplers of oxidative phosphorylation led Burg⁶³ and later Murr and Yang⁶⁴ to propose that SAM was an intermediate in ethylene production.

While the research reported here was in progress, three important papers on the biosynthesis of ethylene from methionine appeared. In 1977 Adams and Yang⁶⁵ observed radioactivity in 5'-methyl-thioadenosine (and its hydrolysis products) in ethylene producing tissues infused with [³⁵S] methionine, thus supporting the proposal that SAM is an intermediate in ethylene biosynthesis. Early this year (1979)⁶⁶ Adams and Yang presented evidence that 1-aminocyclopropane-1-carboxylic acid (21) ACC, is an intermediate in the production of

ethylene from SAM by apple tissues. They infused ^{14}C -labelled methionine into apple tissue, and isolated labelled ACC, and then after inferring its structure, infused labelled ACC and obtained labelled ethylene. The pathway they proposed to account for these observations is shown in Fig. 1-B-6. Boller et al have very recently⁶⁷ reported the development of a sensitive assay for ACC, and the characteristics of an enzyme isolated from tomato tissues which converts SAM to ACC. They used this assay to detect ACC in extracts of tomato fruits, and to assess the activity of the soluble enzyme which converts SAM to ACC in these tissues.

These latest results are discussed more fully in conjunction with our own results in the text (Chapter 7).

e) The Biosynthesis of ethylene from methionine by microorganisms

The first reported fungal production of a gas which affected the maturation of plants was in 1934³⁵, but it was not conclusively proven that microorganisms naturally produced ethylene until 1951, when Young et al demonstrated ethylene production by pure cultures of P. digitatum⁴¹. During the following years many fungi were shown to produce ethylene when grown in pure culture⁴³, but essentially all the work on the biosynthesis of ethylene production by fungi was carried out with P. digitatum, due to its very high rate of ethylene production, and until 1974 only one bacteria had been shown to produce ethylene, Pseudomonas solanacearum⁶⁸. Much of this early work was performed before the role of methionine as the precursor of ethylene in plants was realised.

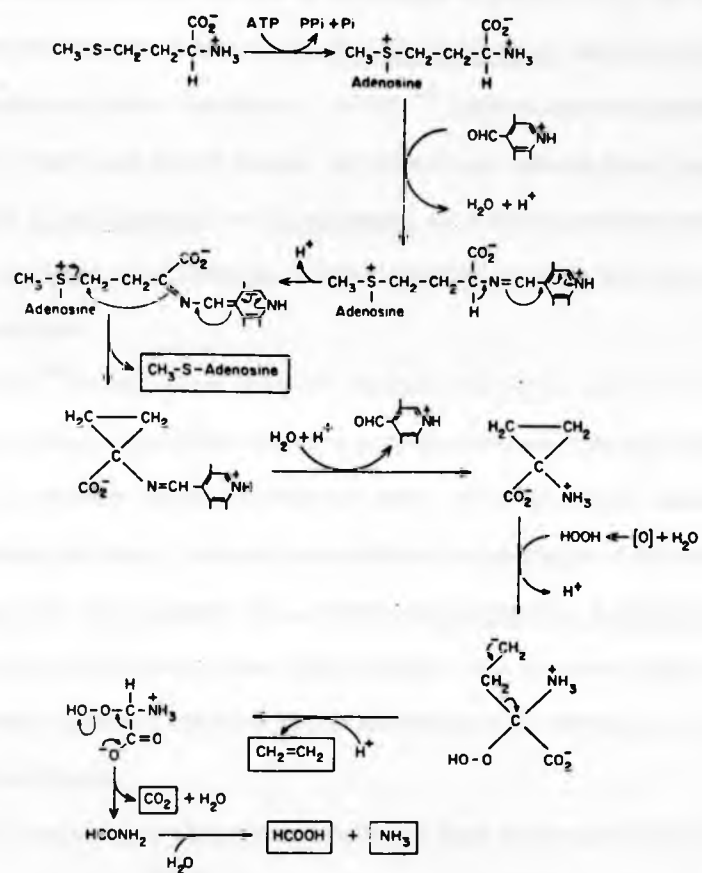


FIG. 1-B-6

The pathway proposed by Adams and Yang to account for the intermediacy of ACC in the formation of ethylene from methionine by apple tissues (Taken from Ref. 66)

The first deliberate addition of methionine to growth media was in 1970⁶⁹, but the organisms in question, Erwinia carotovora, failed to produce ethylene under any growth conditions. In 1972⁴⁴ Lynch deliberately added methionine to soils and growth media, and from them isolated three fungi, M. hiemalis, C. cartiovaaria, and T. cutaneum, all of which produced ethylene only in the presence of methionine. Of four bacteria tested in this study, none produced ethylene.

In 1976⁴³ Primrose and Dilworth obtained five bacterial isolates from water logged soils, and all were shown to produce ethylene from methionine. Following an extensive search of soils and water, 65 isolates were obtained which produced ethylene. Amongst these ethylene producing bacteria were Escherichia coli, Pseudomonas spp., Aeromonas hydrophila, Arthrobacter, and 37 enteric bacteria (our research has extended this list even further). All of these isolates produce ethylene only in the presence of methionine, or certain methionine analogues.

The stimulation of ethylene production by light has been reported for M. hiemalis and E. coli^{70, 71}, and so has the light dependent release of ethylene from cell free filtrates of cultures grown in the presence of methionine⁷⁰. Primrose suggested an intermediate was accumulating in the culture media, which was then photochemically degraded to ethylene; support for this suggestion came from the observations that addition of riboflavin to cell free filtrates increased ethylene release from them over 25 fold⁴², and some of the best ethylene producing cultures secrete flavins⁷². Ethylene is also released from these filtrates in the absence of light, and so a second non-photochemical pathway must exist which degrades the intermediate to ethylene. Here a peroxidase is implicated

as addition of catalase inhibits ethylene production⁴², and an inverse correlation exists between the catalase activity of a culture and its ability to produce ethylene.

On the basis of the above observations, Primrose⁴² suggested that the intermediate was either KMBA or methional, as both had been shown to be converted to ethylene by riboflavin/light, and peroxidase model systems (see earlier). Comparison of the pH dependence of the photochemical reaction producing ethylene from the intermediate, to that of KMBA and methional under the same conditions, led Primrose to favour KMBA as the intermediate⁷².

3. The Biosynthesis of other metabolites from methionine

Methionine is believed to be the biosynthetic precursor of 2-azetidine carboxylic acid (22) and possibly the precursor of thiamine (23) in vivo. The acid (22) is found in lily of the valley plants⁷³, red algae⁷⁴, sugarbeet, and tobacco⁷⁵. It may be present in trace amounts in many more plants⁷⁵. Tracer studies using radiolabelled methionines, and other materials, have provided numerous results as to the labelling pattern observed in the acid (22), and these findings have been reviewed⁷⁵. Currently it is believed that the acid (22) arises from methionine via a diamine (22a) as shown in Fig. 1-B-7. The diamine (22a) is proposed to arise from either SAM(5) or homoserine (22b).

Thiamine (23) arises biosynthetically from the coupling of hydroxyethylthiazole (24) and hydroxymethylpyrimidine (25) (Fig. 1-B-8). The biosynthetic route which produces thiazole (24) is still unknown. Numerous radiolabelling experiments have provided conflicting information as to the incorporation of methionine, and other precursors, into thiazole (24), and the

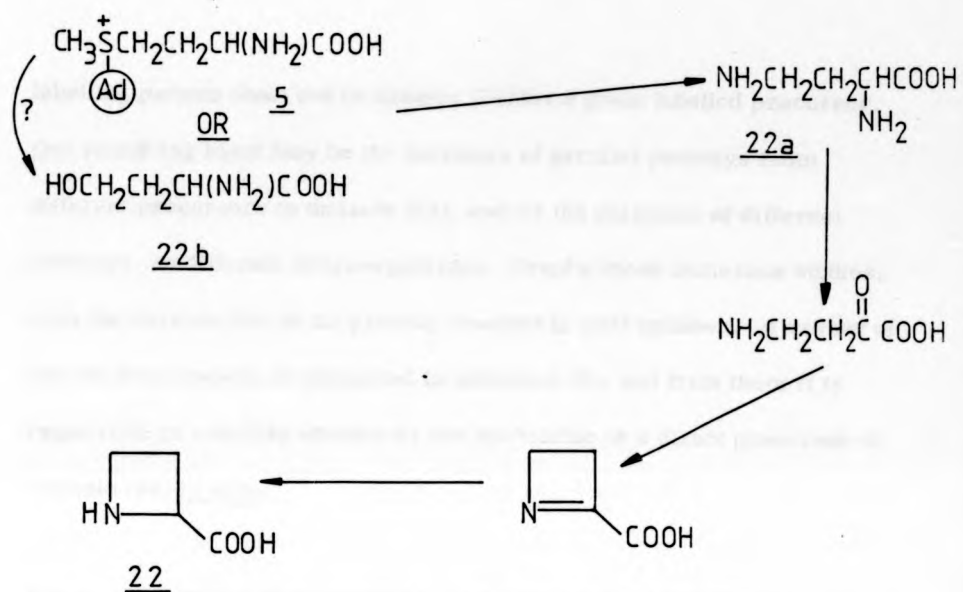


FIG 1-B-7

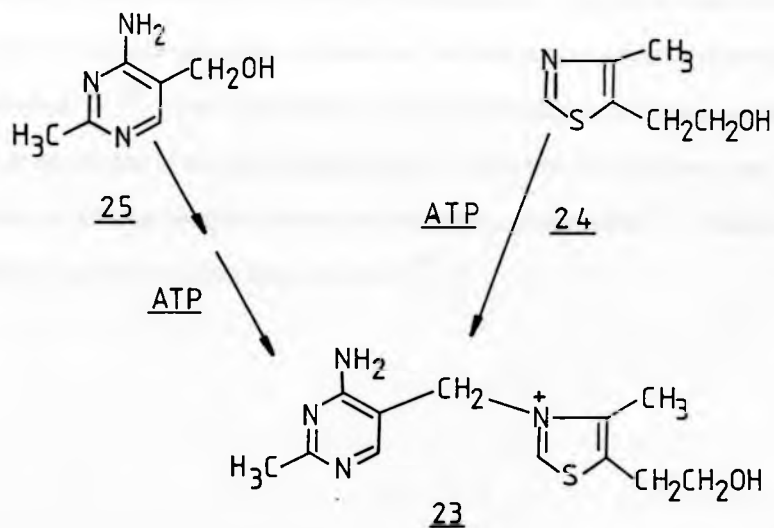


FIG 1-B-8

FIG. 1-B-7

THE PROPOSED PATHWAY FOR THE BIOSYNTHESIS OF 2-AZETIDENE CARBOXYLIC ACID (22) FROM SAM (5)

FIG. 1-B-8

THE BIOSYNTHETIC ORIGIN OF THIAMINE (23)

labelling pattern observed in thiazole (24) for a given labelled precursor. One stumbling block may be the existence of parallel pathways from different precursors to thiazole (24), and/or the existence of different pathways in different microorganisms. Despite these numerous studies, even the bare outline of the pathway involved is still unknown. A review of the available results is presented in reference 76, and from them it is impossible to conclude whether or not methionine is a direct precursor of thiazole (24) in vivo.

1-B-4 OTHER BIOCHEMICAL FUNCTIONS OF METHIONINE

Methionine has one important biochemical function, as an intact molecule, in the formation of N-formylmethionine. This compound is an initiator of protein synthesis in bacteria, in the form of sRNA-N-formylmethionine^{77,78}. The formylation of methionine appears to increase the rate of formation of the first peptide bond, whilst the sRNA directs the position of the methionine residue in the growing polypeptide⁷⁹. Recent progress in this area has been reviewed⁸⁰.

1-C THE USE OF ISOTOPES AS LABELS

1-C-1 INTRODUCTION

1. General

Most naturally occurring elements exist in a number of isotopic forms. The percentage of an isotopic form in natural materials is termed that isotope's natural abundance. For example, all carbon compounds of natural origin contain 98.9% of their carbon atoms in the isotopic form ^{12}C , and 1.1% in the isotopic form ^{13}C . Thus the natural abundance of the heavier isotope of carbon, ^{13}C , is 1.1%. The natural abundance of the isotopes of carbon, hydrogen, oxygen and nitrogen are:

^{12}C , 98.9%; ^{13}C , 1.1%; ^1H , 99.98%; ^2H , 0.005%; ^{14}N , 99.63%; ^{15}N , 0.366%; ^{16}O , 99.76%; ^{17}O , 0.037%; ^{18}O , 0.20%.

Isotopes may be either stable or radioactive. Radioactive isotopes (e.g. ^{14}C) decay by the continuous and spontaneous emission of characteristic types of radiation. Stable isotopes (e.g. ^{12}C , ^{13}C) do not decay or emit radiation. No radioisotopic natural abundances are given above, because although the radioisotopes do occur naturally, their natural abundances are so low as to be almost insignificant (e.g. ^3H , ca $5 \times 10^{-6}\%$).

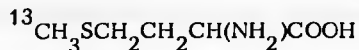
By enriching a compound with a given isotope, ie raising the percentage of the isotope above natural abundance, materials may be labelled without altering their chemical structure. The fate of the labelled molecule, or molecular fragment, may then be followed through a chemical or biochemical reaction. For example, by growing plants or bacteria in an atmosphere enriched in radioactive $^{14}\text{CO}_2$, and then analysing the organism's components for radioactivity, it is possible to demonstrate that CO_2 is fixed into complex

molecules by photosynthesis.

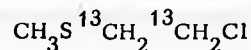
Compounds enriched in radioisotopes are normally prepared by chemical synthesis from a simple radioactive species, which is obtained in a nuclear reaction. For example ^{14}C may be produced by neutron bombardment of nitrogen.

The stable isotopes of C, H, O, and N, all have significant natural abundances, and thus they may be separated from simple natural materials by physical or chemical methods. This provides a simple compound, highly enriched in the isotope in question, which may be used as a starting material in the chemical synthesis of labelled compounds. For example, by cryogenic distillation of natural carbon monoxide (98.9% ^{12}CO , 1.1% ^{13}CO), carbon monoxide highly enriched in ^{13}C can be obtained (e.g. 98% ^{13}CO , 2% ^{12}CO).

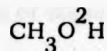
Labelled compounds are named by inserting the position of the labelling isotopic nuclide, and its symbol, in square brackets into the name of the compound. The complete rules for naming isotopically labelled compounds are presented in ref. 81. These rules are followed in this thesis, and as an aid to clarity, many of the labelled compound's structures are also presented. Examples of this are provided by [methyl- ^{13}C] methionine (a), 1-chloro-2-(methylthio)-[1,2- $^{13}\text{C}_2$] ethane (b), and methan[^2H]ol (c).



a



b



c

For compounds labelled with stable isotopes, isotopic content is quoted as atom %. This is defined as the ratio of the number of atoms of the isotope in question, to the total number of atoms, and unless otherwise stated, refers only to the labelled position(s). Thus in sodium [2- ^{13}C] acetate 90 atom %, 90% of the CH_3 carbon atoms are present in the form of ^{13}C , whereas the ^{13}C content of the carboxyl group is 1.1% (natural abundance). In double labelled materials, the isotopic content refers to both positions, and unless otherwise stated, reflects a statistical distribution of label. Thus sodium[1,2- $^{13}\text{C}_2$] acetate 90 atom% consists of 81% $^{13}\text{CH}_3$ $^{13}\text{CO}_2$ Na, 9% $^{13}\text{CH}_3$ $^{12}\text{COONa}$, 9% $^{12}\text{CH}_3$ $^{13}\text{COONa}$, and 1% $^{12}\text{CH}_3$ $^{12}\text{COONa}$.

2. A Short History of Isotope Labelling

The potential of isotopes as biological tracers was realised by Hevesy before the availability of isotopically enriched compounds. In 1923⁸², he published an account of the uptake, distribution, and displacement of lead in plants, which he studied by administering small quantities of the naturally occurring, radioisotope, thorium B, (^{212}Pb).

Following the discovery of stable isotopes of the biologically important elements carbon (^{13}C), hydrogen (^2H), nitrogen (^{15}N) and oxygen (^{17}O and ^{18}O) in the period 1927 - 1932⁸³, procedures were developed for the enrichment of simple compounds with these isotopes⁸³. These enriched compounds were quickly realised to be of value in tracer studies of metabolic processes. A major problem in the early use of these isotopes as tracers was the tedious and difficult techniques which were required to detect and assay them. The isotopes ^2H and ^{18}O , for example, could only be assayed by conversion of the

labelled material to water, followed by comparative refractive index, or density measurements. By the late 1930's the isotopes ^{13}C , ^{18}O , and ^{15}N could be assayed by conversion of labelled compounds to either CO_2 or N_2 , and mass spectroscopy, the instruments were not commercially available however, and were difficult to build and maintain.

Despite the problems involved in their analysis, many important metabolic studies were performed with these isotopes⁸³. The time a water molecule resides in the body was estimated using $^2\text{H}_2\text{O}$ ⁸⁴, and the de novo biosynthesis of fatty acids was also demonstrated⁸⁵. Stearic acid labelled with ^2H was used to verify the existence of fatty acid de-saturation mechanisms in animals⁸⁶. Extensive de novo biosynthesis of amino acids and proteins involving amino-group transfer was observed after the administration of ^{15}N labelled amino acids⁸⁷. The fixation of atmospheric CO_2 was studied using $^{13}\text{CO}_2$ ⁸⁸, and the reactions of the Krebs' cycle were also investigated in this way⁸⁹. Using ^{18}O labelled water, the origin of the oxygen produced in photosynthesis was shown to be H_2O , and not CO_2 ⁹⁰.

In 1939 a second isotope of carbon became available, the radioactive isotope ^{11}C . As sensitive techniques already existed for the detection and quantification of radiation, this isotope was soon used in biological tracer experiments, despite its inconveniently short half-life ($t_{1/2} = 20$ min).

Experiments carried out at Harvard for example demonstrated the incorporation of radioactive ^{11}C , from $\text{NaH}^{11}\text{CO}_3$, into newly formed rat liver glycogen^{91,92}. The results of many metabolic studies using these isotopes were presented at a symposium held in 1947⁹³, and this early work has been reviewed⁸³.

The results of these studies pointed to a continuous synthesis and degradation of bodily constituents, fats, proteins, amino acids, etc., in contrast to previous ideas of an essentially static biochemical system. Schoenheimer realised that this reflected metabolic pathways which were constantly in action, even in supposedly steady state individuals, and formulated the dynamic-state concept of cell and bodily constituents⁹⁴.

The discovery of a second isotope of hydrogen, ^3H , was reported in 1934⁹⁵, and in 1939⁹⁶ it was shown to be radioactive, emitting low energy β particles ($t_{1/2} = 12.3$ years)⁹⁷. In 1941⁹⁸ a third isotope of carbon, ^{14}C , was discovered, and it was demonstrated to be an energetic β particle emitter, with a long half life ($t_{1/2} = 5600$ years). The first samples of ^{14}C labelled compounds became generally available in 1945. As sensitive techniques already existed for the detection and quantification of radioactivity, when ^{14}C became widely available it quickly supplanted ^{13}C as an isotopic label in tracer studies. This was due both to the tedious techniques required to detect and assay ^{13}C , and to the relatively small amounts of this isotope that were available at the time. Similarly, ^3H became more popular as an isotopic label than ^2H , especially after the introduction of scintillation counting techniques, which overcame many of the early problems associated with the detection of the weak ^3H β emissions. However, tracer studies were still carried out with stable isotopes during this period (ca 1940 - 1965) especially with ^{15}N , ^{17}O , and ^{18}O , because no suitable radioactive isotopes of these elements exist. The vast majority of tracer studies in this period used the radioactive isotopes ^{14}C and ^3H .

Many of these tracer studies investigated the metabolic pathways on which Schoenheimer's dynamic-state concept had focussed attention. Using these isotopes (^{14}C and ^3H) the foundations of our current understanding of metabolism were laid⁹⁹.

In the last ten years there has been a renaissance in the use of stable isotopes as labels. This is due both to technical developments in the field of isotope separation¹⁰⁰ (enabling large quantities of highly enriched material to be produced at reasonable cost) and to the development of new sensitive spectroscopic techniques applicable to the detection and assay of stable isotopes^{100, 101}. Compounds highly enriched in the isotopes ^{13}C , ^{15}N , ^{17}O , and ^{18}O are now produced by industrial scale isotopic separations, and a wide range of labelled compounds is commercially available. These isotopes can be detected by high resolution mass spectroscopy. For those isotopes whose nuclei have non-zero nuclear spin, [^{13}C ($\frac{1}{2}$), ^2H (1), ^3H ($\frac{1}{2}$), ^{15}N ($\frac{1}{2}$), and ^{17}O ($\frac{5}{2}$)] Fourier transform n. m. r. techniques may be used.

The above developments mean that in some cases stable isotope labelling is now preferably to radioisotopic labelling. In the study of biosynthetic pathways for example¹⁰², where ^{13}C labelled precursors are used, the position of isotopic incorporation into a metabolite can often be directly determined by ^{13}C n. m. r. This makes unnecessary the complex and tedious degradations required to pin-point the position of ^{14}C labelling in similar studies. The stable isotopes are also useful as labels in situations where the use of radioisotopes is undesirable (e.g. in experiments on certain classes of human subjects)¹⁰⁰.

A large amount of important work concerning the recent applications of stable isotopes in the life sciences has been published in the proceedings of international symposia¹⁰³⁻¹⁰⁸, and review articles^{100-102, 109, 110}. A summary of these applications is presented in section 1-D.

1-C-2 SEPARATION OF THE STABLE ISOTOPES OF CARBON,
NITROGEN AND OXYGEN

1. Introduction

The current increase in the use of stable isotopes as tracers centres on the isotopes of hydrogen, carbon, oxygen, and nitrogen^{100, 101, 107, 111}.

Substitution of an atom by an isotopic atom of the same element is accompanied by small changes in the chemical and physical properties of the element concerned¹¹². These effects are due to the different masses of the isotopes, and the effect is largest where the ratio of isotopic masses is largest. The techniques employed commercially in the separation of simple molecules enriched in stable isotopes exploit these small differences. The main processes used commercially for the separation of stable isotopes are distillation, chemical exchange, and thermal diffusion^{110, 113}.

Separations involving distillation rely on the small differences in vapour pressures of the isotopic species concerned. For example, the equilibrium constant for the process $^{12}\text{CO}(\text{liq}) + ^{13}\text{CO}(\text{gas}) \rightleftharpoons ^{12}\text{CO}(\text{gas}) + ^{13}\text{CO}(\text{liq})$ is 1.008, indicating that preferential partition of the heavier isotope of carbon into the liquid phase will occur. Chemical exchange separations exploit the small differences that exist at equilibrium between two isotopic species in a reversible reaction involving two chemical compounds. For the

reaction $^{15}\text{NH}_3(\text{gas}) + ^{14}\text{NH}_4^{\oplus}(\text{aq}) \rightleftharpoons ^{14}\text{NH}_3(\text{gas}) + ^{15}\text{NH}_4^{\oplus}(\text{aq})$ for example the equilibrium constant is 1.034, and thus the heavier isotope of nitrogen will accumulate in the aqueous phase. The establishment of a thermal gradient in a gas mixture gives rise to diffusion, and the separation of the components of the mixture. Convection currents generated in the gas mixture may be used to make this separation cumulative. Isotopic species may be separated in this way (e.g. $^{13}\text{CH}_4$ and $^{12}\text{CH}_4$), the lighter isotope normally migrating up the temperature gradient. Using the above techniques the less abundant isotopes of C, H, N, and O may be separated from natural materials, to provide isotopically enriched compounds.

2. Separation of the isotopes of carbon

Almost all the ^{13}C produced at the present time arises from the cryogenic distillation of carbon monoxide¹¹⁴. The separation factor for this process is 1.008 (i.e. the equm. constant given above) and thus many separations need to be performed in order to obtain high enrichment of ^{13}C . The system employed by Prochem in the U.K.^{100, 105} involves the distillation of carbon monoxide in columns 20m long, randomly packed with steel helices. Two identical columns with stepped diameters (6.5 cm - 2.5 cm) distil natural carbon monoxide, producing at their bases carbon monoxide containing ca 12 atom % ^{13}C . The enriched liquid from these columns is fed on a batch basis to a third column of constant diameter (2.5 cm), which raises the isotopic content of the liquid carbon monoxide to 90 - 93 atom % ^{13}C . This can be raised to 98 atom % ^{13}C by a further distillation¹⁰⁷. An initial equilibrium time of 10 - 12 weeks was required

on start-up, and the plant has been running continuously and automatically for over 6 years. The output is of the order of a few Kg. of 90 - 93 atom % ^{13}C per year. A similar operation at the Los Alamos Scientific Laboratories in the U.S.A. produces enriched CO by distillation, at up to 99 atom % ^{13}C .

3. Separation of the isotopes of nitrogen and oxygen

Isotopes of nitrogen are separated at Prochem by a chemical exchange process, giving H^{15}NO_3 at up to 99.5 atom %, and by cryogenic distillation of NO at Los Alamos. The latter process is also used to effect separation of the isotopes of oxygen, simultaneously. Oxygen isotopes are also separated by the distillation of water (H_2O or $^2\text{H}_2\text{O}$) at the Weizmann Institute (H_2^{17}O , 20 atom %; H_2^{18}O , 98 atom %; $^{17}\text{O}_2$, 96 atom %) ¹¹⁵ and at the Karlsruhe Nuclear research centre (H_2^{18}O , 99.9 atom %; and H_2^{17}O , 30 atom %) ¹⁰⁷.

4. Other Separations

The separation of carbon isotopes by other methods (thermal diffusion of CH_4 ; chemical exchange in the system $2\text{R}_2\text{NH} + \text{CO}_2 \rightleftharpoons (\text{R}_2\text{NCO}_2)^- + (\text{H}_2\text{NR}_2)^+$; selective laser induced photolysis of CF_3I ; and photolysis of dibenzyl ketone in soap solutions) have been reported ¹¹⁴, but only the cryogenic distillation of CO is of any commercial importance at present. This is also true for the isotopes of nitrogen (laser induced photo-dissociation of NH_3 ; g.l.c. at 77°K) and oxygen (laser induced photo-dissociation of CH_2O) ¹¹⁴.

1-C-3 SYNTHESIS OF COMPOUNDS LABELLED WITH STABLE ISOTOPES

1. Industrial Syntheses

It will be apparent from the previous section that the forms in which stable isotopes are obtained (e.g. CO for ^{13}C and HNO_3 for ^{15}N) are not usually suitable as synthetic starting materials in the research laboratory. The manufacturers who separate stable isotopes, and a number of other suppliers, offer a range of isotopically labelled compounds. Many of these are simple synthetic intermediates (e.g. KCN, CH_3OH , CH_3I) and some are more complex specialised products (e.g. ascorbic acid, tyrosine). The cost of "raw isotope" (e.g. ^{13}CO) has reached a steady level due to the efficient methods used for isotope separation¹⁰⁰. Coupled with the fact that all small scale industrial chemical syntheses are very labour intensive, this means that a large proportion of the cost of any labelled compound arises from the labour (i.e. time) required to synthesise it¹¹⁴. For complex molecules, whose production involves a number of synthetic steps, the cost of isotope is almost negligible compared to the cost of the labour involved in its synthesis. This being the case, the cost of many complex labelled materials is governed by the commercial demand for them, as it is only fractionally more expensive to produce 100g of such a compound, than 1g¹¹⁴.

The synthesis of a number of key intermediates from ^{13}CO and H^{15}NO_3 , as performed industrially are depicted in Fig's. 1-C-1 and 1-C-2. Most of these syntheses are scaled down versions of tonne scale industrial processes, and proceed in high yields. Many of them use specialist apparatus not available in the research laboratory. For example [^{13}C] methanol is

FIG 1-C-1

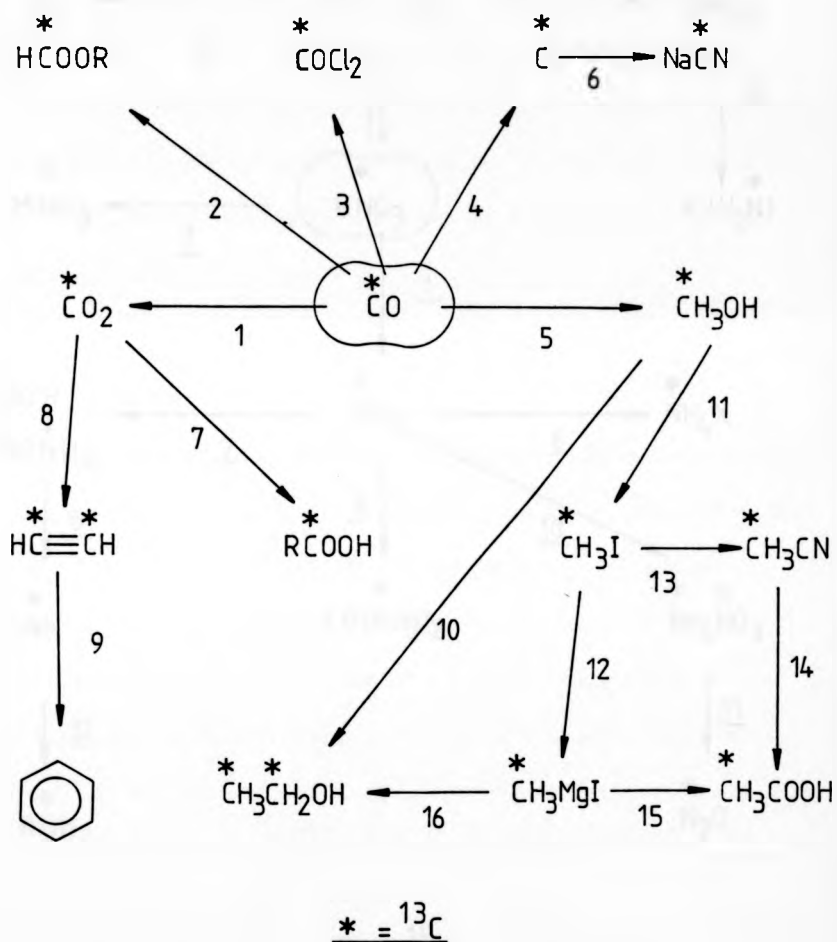


FIG. 1-C-1

THE INDUSTRIAL SYNTHESIS OF INTERMEDIATES FROM ^{13}C .

REAGENTS : 1) CuO ; 2) ROH ; 3) Cl_2 ; 4) Fe ; 5) H_2 , Cu-Zn-Cr ;
 6) NH_3/Na ; 7) RMgX ; 8a) Li , 8b) H_2O ; 9) $\text{V}_2\text{O}_5/\text{Al}_2\text{O}_3$;
 10) $^*\text{CO}$, RCl_3/HI ; 11) HI ; 12) Mg ; 13) KCN ; 14) NaOH ; 15) CO_2 ;
 16) $^*\text{CO}_2$

FIG 1-C-2

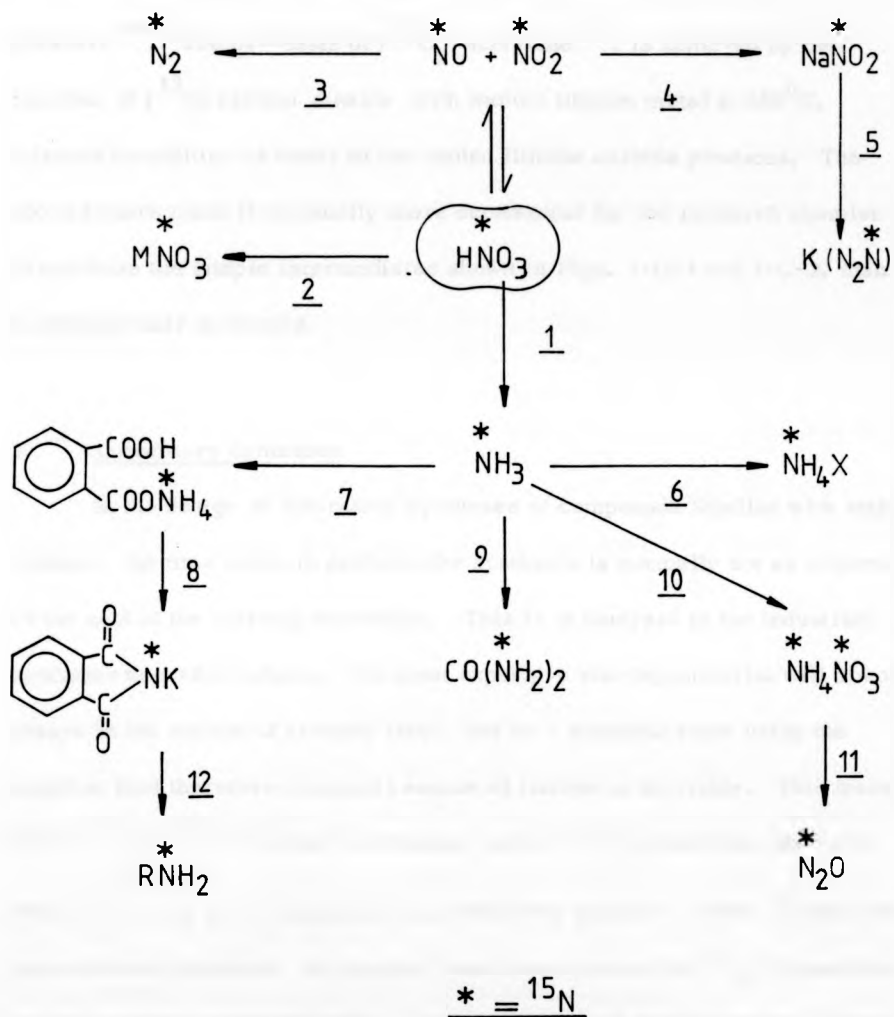


FIG. 1-C-2

THE INDUSTRIAL SYNTHESIS OF INTERMEDIATES FROM H^{15}NO_3 .

REAGENTS : 1) DEVARDAS ALLOY ; 2) MOH ; 3) Fe ;

4) NaOH ; 5) N_2H_4 ; 6) HX ; 7) PHTHALIC ACID ; 8a) HEAT,

8b) KOH/EtOH ; 9) Ph_2CO_3 ; 10) H^{15}NO_3 ; 11) HEAT ; 12a) RX,

12b) HCl

prepared from [^{13}C] carbon dioxide by catalytic reduction under pressure¹¹⁶. The synthesis of [$^{13}\text{C}_2$] acetylene¹¹⁷, is achieved by the reaction of [^{13}C] carbon dioxide with molten lithium metal at 600°C , followed by addition of water to the cooled lithium carbide produced. The above factors mean it is usually more economical for the research chemist to purchase the simple intermediates shown in Figs. 1-C-1 and 1-C-2, than to attempt their synthesis.

2. Laboratory Syntheses

In the design of laboratory syntheses of compounds labelled with stable isotopes, the time taken to perform the synthesis is normally not as important as the cost of the starting materials. This is in contrast to the industrial syntheses described above. The most expensive starting material will almost always be the source of isotopic label, and so a synthetic route using the simplest (and therefore cheapest) source of isotope is desirable. This means that for ^{13}C or ^{15}N labelled compounds, one of the intermediates shown in Figs. 1-C-1, or 1-C-2 should be used wherever possible. When ^2H labelled materials are prepared, the original label source should be $^2\text{H}_2\text{O}$ if possible, for the reasons described above. The reactions used should proceed in high yields, and not require difficult purifications of intermediates. For economic reasons the incorporation of the label into the molecule at as late a stage as possible is desirable. If the synthesis is to be performed on a relatively large scale, the recovery of unused isotopic label from reactions which do not proceed quantitatively may be appropriate. Another factor which may

influence the synthetic procedure adopted is the degree of isotopic enrichment required. This is normally as high as possible (e.g. 90 - 99 atom % for ^{13}C) in cases where large dilutions of label may occur during an experiment (e.g. in biosynthetic or metabolic tracer studies). For studies of chemical reaction mechanisms, where normally no dilution occurs, levels of 5 - 10 % enrichment may be adequate. Once the synthesis is complete, the verification of the labelled position(s) in the compound is important.

An ideal synthesis would incorporate all of the desirable features outlined above, and a good researcher would balance one against another to achieve the desired material in good yield at reasonable cost. Methods applicable to the synthesis of labelled compounds have been reviewed^{118, 119, 120, 121}.

3. Preparation of labelled compounds by biosynthesis

For the production of labelled natural products, biosynthetic pathways may be exploited in some cases^{100, 101}. Photosynthetic production of ^{13}C labelled sugars is an example of biological production of uniformly labelled compounds¹¹⁴. Uniformly labelled glucose, sucrose, fructose, and starch, may be obtained from tobacco leaves after incubation in $^{13}\text{CO}_2$ ¹²². Uniformly labelled amino acids have been isolated in good yield from algae grown in the presence of sodium [^{13}C] bicarbonate¹²³. Small amounts of specifically labelled antibiotics and other natural products have been obtained during studies of their metabolism, using labelled precursors¹⁰⁰. Other compounds may be obtained specifically labelled by using cell free enzyme systems as chemical reagents, in the presence of labelled compounds. For

example 2-oxoglutaric acid has been enzymatically reduced, in the presence of $^{15}\text{NH}_4^+$, to yield L-[^{15}N] glutamic acid¹²⁴.

1-C-4 DETECTION OF ISOTOPIC LABELS

1. Radioactive Isotopes¹²⁵

The three most commonly used radioactive tracers in biochemical experiments are ^{14}C , ^{32}P , and ^3H , all of which decay by the emission of β particles¹²⁵. These particles are ejected with an average velocity less than 0.1 that of light, and their energies form characteristic unsymmetrical gaussian distributions for each isotope. Both ^{14}C (medium energy particles) and ^3H (very low energy particles) are soft β emitters, whereas ^{32}P (energetic particles) is an emitter of hard radiation.

The technique most widely used for the detection and quantification of these emissions is scintillation counting¹²⁵. Modern instruments can achieve counting efficiencies of over 90% for ^{14}C and over 60% for ^3H . Coupled with the very low natural abundance (background) of these isotopes, this means that the incorporation of a small fraction of a percent of a radio labelled precursor into a metabolite can be reliably assessed.

The main drawback in the use of radiolabels is the determination of the position of labelling in a molecule. The detection of radioactivity is non-specific, and so the labelled position must be determined by degradation. These degradations may be very tedious and complex where a large molecule is concerned, and in some cases the desired degradative reactions may not exist. In addition each step of the degradative sequence consumes precious material. When assaying a material for radioactivity care must be taken to ensure it is

not contaminated with small quantities of highly radioactive impurities. The purification of these materials is often achieved by exhaustive recrystallisation to constant activity.

Recently the detection of ^3H labels by ^3H n.m.r. has become possible^{126, 127}. This method has the advantage of directly determining the label's position, as well as its presence.

Another drawback in the use of radioisotopic tracers is their unsuitability in studies involving infants, children, or women of childbearing age¹⁰⁰.

2. Stable Isotopes

The detection and assay methods used for stable isotopes depend on the different physical properties exhibited by the isotopes of an element. The techniques mainly used are, mass, n.m.r., optical emission, and infra red spectroscopy.

(a) Mass spectroscopy^{100, 109}

In addition to the well known high resolution (analytical) mass spectrometer, a second instrument is used for the analysis of stable isotopes, the isotope mass spectrometer^{100, 103, 107, 114}. In contrast to the analytical mass spectrometer, which provides a mass:energy spectrum by variation of either the magnetic field, or the accelerating voltage, in the isotope mass spectrometer, these conditions are held constant throughout an analysis. The ratios of a given pair of isotopes are measured alternately in a gas sample of unknown isotopic composition, and a reference sample. In a typical experiment, the ^{13}C content of a reference sample of CO_2 is compared with that of a sample of CO_2 of unknown isotopic content. Results are expressed

in the $\delta\%$ notation, where for the above case

$$\delta^{13}\text{C} = \frac{^{13}\text{C}/^{12}\text{C}_{\text{sample}} - ^{13}\text{C}/^{12}\text{C}_{\text{standard}}}{^{13}\text{C}/^{12}\text{C}_{\text{standard}}} \times 1000$$

Accuracy of ± 0.1 to $0.5 \delta\%$ can be attained for analysis of ^2H : ^1H , ^{13}C : ^{12}C , ^{15}N : ^{14}N , ^{18}O : ^{16}O , and ^{34}S : ^{32}S . These mass spectrometers are used for many clinical experiments and the only drawback of the technique is the need to convert the sample to be analysed to a gas (H_2 , CO_2 , or N_2) before analysis^{100, 103, 107}.

Owing to the substantial progress that has been made in the interpretation of mass spectral fragmentation patterns, and the correlation of mass spectra with molecular structure, the incorporation and position of a stable isotopic label may be measured directly by mass spectroscopy, in some cases. For example, a favourable fragmentation occurred in the analysis of the TMS esters of ^{13}C labelled amino acids obtained from algae grown in the presence of $^{13}\text{CO}_2$ ¹²⁸. It was possible to demonstrate a slight preference for ^{13}C incorporation into the side chains of the amino acids concerned, relative to the ^{13}C content of their carboxyl groups. Conversely, the use of stable isotopic labelled compounds has been of value in the elucidation of mass spectral fragmentation patterns, especially in complex molecules¹⁰¹.

Due to the relatively high natural abundance of the stable isotopes, the detection of singly labelled species is not as sensitive as the detection of singly labelled radioisotopic species. Significant gains in sensitivity may be achieved by the use of double labelled materials. For example the natural abundance of $^{13}\text{C}^{18}\text{O}_2$ is only $4.4 \times 10^{-6}\%$, and in principle CO_2 transport

studies could be conducted in which dilution of $^{13}\text{C}^{18}\text{O}_2$ occurred to the extent of 1 part in 10^8 . Multiple labelling is often used to produce shifts of more than one mass unit in the mass spectrum of a labelled compound, facilitating exact isotopic analysis¹¹⁴. The availability of combined g. l. c./mass spectrometry systems has provided a very powerful tool for the analysis of complex mixtures at the microgram level.

Using compounds labelled with stable isotopes, mass spectroscopy may become a quantitative detection technique. The quantitative analysis of drugs in physiological fluids provides an example of this. When a sample has been obtained (e.g. plasma, spinal fluid, etc.) an exactly known quantity of a labelled counterpart of the drug is added to the physiological sample, which is then worked up (e.g. g. l. c.) to provide a pure sample of the drug. As the labelled drug is essentially chemically identical to the unlabelled drug, no separation occurs, and by comparison of the relative sizes of the mass spectral peaks for the two compounds, quantification can be achieved. For these studies, multiple deuterium labelling is often used to provide shifts of more than 1 mass unit. Detection and quantification of materials in the pico-gram range has been achieved in this way¹²⁹.

(b) n. m. r. spectroscopy

The majority of work reported to date has involved the use of ^1H and ^{13}C n. m. r. spectroscopy. Recent instrumental advances have led to the recording of ^2H , ^{14}N , ^{15}N , and ^{17}O spectra. The main advantage of n. m. r. as a detection method lies in the wealth of structural information present in the n. m. r. spectrum, which allows the position of the labelling nuclide to be directly determined in many cases.

The presence of both ^2H and ^{13}C isotopic labels may be detected by ^1H n.m.r. spectroscopy¹¹⁴. Under the conditions used for the observation of ^1H n.m.r. signals, the ^2H nucleus does not give rise to n.m.r. signals, and thus may be used as a "spinless" label. Many tracer studies have been performed in this way^{120, 111}. Protons directly attached to ^{13}C nuclei (spin $\frac{1}{2}$) show spin-spin couplings, and so each ^1H resonance is accompanied by two "satellite" bands. These are not normally observed in unlabelled materials, owing to the low natural abundance of ^{13}C . Enrichment with ^{13}C leads to quantitative enhancement of these bands, and thus they may be used to both detect and quantify ^{13}C labelling. The method has obvious disadvantages for complex molecules which exhibit many ^1H n.m.r. bands. However the ^{13}C enrichment of simple materials is often assayed by this technique¹¹⁴, and it has been used to detect ^{13}C isotopic labels in biosynthetic studies¹³⁰.

The direct observation of ^{13}C resonances is clearly preferable to the satellite method, but due to the low natural abundance of ^{13}C , and its low n.m.r. sensitivity, ^{13}C resonances from natural materials are ca 6000 fold weaker than ^1H resonances¹³¹. The advent of Fourier transform n.m.r. techniques in the 1960's overcame this sensitivity problem. The principles of ^{13}C n.m.r. and Fourier transform techniques in general are well documented^{130, 131, 132, 133}, and familiarity with them is assumed.

Owing to the very large range of chemical shifts observed for ^{13}C n.m.r. signals, even in complex molecules each carbon atom normally gives rise to a distinct resonance. The low natural abundance of ^{13}C (1.1%) means that these resonances normally appear as singlets (as the probability of two adjacent ^{13}C nuclei is $0.01 \times 0.01 = 0.012\%$). Any enrichment of ^{13}C at a

given position will lead to enhanced lines in the ^{13}C n. m. r. spectrum of the compound, and thus the position of ^{13}C labelling may be directly determined by ^{13}C n. m. r. spectroscopy. The natural abundance of ^{13}C means that incorporations below ca 0.5% (of a 90 atom% substrate) cannot be reliably assessed, and this is the main drawback in the use of ^{13}C n. m. r. as a detection method in studies of biosynthetic pathways, using ^{13}C labelled materials. Sensitivity gains may be obtained by growing the organism in question in a medium depleted in ^{13}C before addition of a ^{13}C labelled substrate, and in some cases by the use of ^{13}C double labelled materials (see section 1-D). In studies of chemical reaction mechanisms, normally no dilution of label occurs, and ^{13}C labelling coupled with ^{13}C n. m. r. is often the method of choice for chemical tracer experiments ¹³¹.

The natural abundance spectra of many other nuclei, including ^2H , ^{14}N , ^{15}N and ^{17}O , may now be recorded, with modern F. T. instruments ^{126, 134, 135, 136}. Labelling studies using n. m. r. detection for these nuclei have been reported (e. g. ref 134). It is probably that as the n. m. r. spectrometers necessary for the observation of these nuclei become more generally available, many more tracer studies using the above isotopes and n. m. r. detection techniques will be performed.

(c) Optical emission spectroscopy

Assay of the isotopes of nitrogen by optical emission spectroscopy depends on the wavelength separation of the three species $^{14}\text{N}_2$, $^{14}\text{N}^{15}\text{N}$, and $^{15}\text{N}_2$ ¹¹⁴. The compound to be assayed is converted to nitrogen gas, and this is excited by a radiofrequency discharge, in a flow discharge tube. The technique is simple and sensitive, and is commonly used for the assay of ^{15}N

in agricultural studies¹³⁷. It has also been used in studies of protein turnover rates in man¹⁰⁶. Reproducibility can approach $\pm 1\%$ for measurements of 0.25 to 0.35% ¹⁵N enrichment, requiring a sample of $< 100 \mu\text{g}$.

(d) Infra red spectroscopy

Isotopic substitution causes characteristic changes in the i. r. spectrum of the compound concerned. Infra red data on many labelled compounds is available¹³⁸. The detection and assay of isotopes is normally only possible for simple materials (e.g. gases), using i. r. spectroscopy¹¹⁴. For example, the absorption peaks of ¹²CO at 2193 cm^{-1} and ¹³CO at 2144 cm^{-1} are sufficiently well separated to allow the ¹³C content of CO to be determined¹⁰¹. At enrichments of 1 - 20%, the mean error reported was ca 2% and the standard deviation, 6% in these measurements¹³⁹. The technique may be employed to distinguish between isomeric labelled compounds in some cases (e.g. E-[1,2-²H₂], Z-[1,2-²H₂] and [1,1-²H₂] ethylene)¹³⁸.

1-D APPLICATIONS OF STABLE ISOTOPES¹⁰⁰⁻¹¹⁰

1. Clinical applications

There are a large number of studies of human metabolism, using compounds labelled with stable isotopes^{100, 103, 109}. Studies of infants, children, and women of childbearing age are now possible, whereas the risks to these groups involved in the use of radioisotopes have previously precluded tracer studies involving these groups¹¹⁴.

Water labelled with ^2H has been extensively in studies of intercompartmental water transport, and in the estimation of total body water¹⁰⁰. The latter provides the clinician with indirect information on body composition, and energy balance. The efficiency of haemodialysis has been assayed by measurement of $^2\text{H}_2\text{O}$ equilibration between renal fluid, and body water¹⁰⁰. Studies on the rates of serum cholesterol biosynthesis have been carried out, by maintaining patients at a higher than normal $^2\text{H}_2\text{O}$ level¹⁰⁰.

The early diagnosis of metabolic disorders has been aided recently by the development of ^{13}C breath tests^{100, 107}. After the administration of a ^{13}C labelled compound to a patient, the exhaled CO_2 is monitored for ^{13}C content at intervals. These measurements can give an estimate of the rate of metabolism of a given compound. The metabolism of glucose in diabetic and obese patients has been compared to that of normal subjects in this way¹⁰⁰. The investigation of a number of liver and gastrointestinal disorders has been possible, using specifically labelled substrates. This type of test is particularly useful for the mass screening of patients because it is non-invasive. The study of lung efficiency has benefited from the use of ^{13}CO , and preliminary results of studies on protein and fat metabolism using ^{13}C labelled compounds have been reported^{100, 140}.

The conversion of urea into ammonia in the gastrointestinal tract provides nitrogen for protein synthesis. Many studies of urea uptake have been reported, using ^{15}N labelled urea. Substrates labelled with ^{15}N have been used in examinations of hereditary enzyme defects. Studies of uric acid metabolism (defects in which cause gout) have also benefited from the use of ^{15}N labelled compounds. Glycine labelled with ^{15}N has been used in studies of haemoglobin synthesis and metabolism, and in examinations of protein turnover rates in man. The above examples are taken mainly from Refs. 100 and 107.

2. Pharmaceutical Applications

The use of stable isotope labelled drugs in conjunction with mass spectroscopy allows the detection and quantification of drugs and drug metabolites in physiological fluids. Compounds labelled with stable isotopes make ideal internal standards for quantitative g.l.c./m.s. studies of physiological fluids (see above). Detection and quantification in the picogram range can be achieved in this way^{114, 136}.

The bio-availability of a drug when administered orally, compared to when administered intravenously, is normally studied by giving identical doses on different occasions. The uptake of the drug is then assessed for both methods of administration. In these experiments it is necessary to assume that drug kinetics remain constant during the time interval between the two doses (typically 7 - 14 days). This assumption is not always valid (e.g. dietary fluctuations affect the metabolism of some drugs) and may be avoided by simultaneous administration of a drug orally, and its labelled counterpart intravenously. The efficiency of each method of administration may be assessed

by parallel assays of the drug, and its labelled counterpart (or their metabolites) in the same physiological sample^{114,141}.

3. Environmental Applications¹¹⁴

The nitrogen cycle has been extensively studied using ¹⁵N labelled fertilizers, and other compounds^{114,107}. The determination of the environmental fate of pesticides hinges on the analysis of very small quantities of pesticide residues. Quantitative g.l.c./m.s. using stable isotopically labelled internal standards may be used to advantage in this area. Picogram levels of pesticide residues may be detected in samples of soil, water, crops, and animal tissue, provided the necessary labelled counterpart of the compound in question is available as an internal standard¹¹⁴. In a study of continental air movements^{105,106}, ¹³C²H₄ methane was released from a given location, and its presence detected 1500 - 2500 Km away at concentrations of 2×10^{-17} parts by volume, using mass spectrometry. This very high sensitivity was possible owing to the almost negligible natural abundance of mass 21 methane in the atmosphere¹¹⁴.

4. Agricultural Applications

ca 50% of the work published in the life sciences on ¹⁵N labelling involves agricultural applications¹¹⁴. The assimilation of nitrogen by plants has been widely studied using ¹⁵N labelled materials^{105,137}. Many studies of the dissimilation of nitrogen fertilizers in the biosphere have also been performed using ¹⁵N labelled compounds, mainly ammonium salts, nitrates, and urea¹¹⁴.

5. Biochemical Applications

(a) Introduction

Stable isotopes have been used as biochemical tracers since their availability in the 1930's (section 1-A). Recently the availability of ^{13}C n.m.r. spectrometers has made the use of ^{13}C labelled compounds very attractive in the study of biosynthetic pathways^{102, 142}.

(b) ^{13}C labelling and the study of metabolic pathways

After isolation and structural elucidation of a metabolite, its biogenesis can be tested using labelled precursor(s). In such studies, two major problems are (1) incorporation of sufficient labelled precursor into a metabolite to render the results of isotopic analysis meaningful, (2) demonstration of the exact position occupied by the isotopic label in the metabolite.

The incorporations achieved using microorganisms, or cell free systems from animal tissues, are much higher (ca 1 - 10%) than those observed when plants or whole animals are used (often $<10^{-2}\%$). This has meant that most biosynthetic studies are carried out on the former systems¹⁴². It is important to be aware that administration of a large quantity of a potential intermediate may distort the metabolic pool, and hence the biosynthetic pathways in operation for a given organism. This is normally only a problem where ^{13}C labelled precursors are used, and distortion of the metabolic pool has only been conclusively demonstrated in a very few cases¹⁴³.

The popularity of radioisotopic labels, (e.g. ^{14}C) in biosynthetic studies stems mainly from the very low incorporations which can be reliably assayed. However to determine the exact position that has been labelled in

a metabolite, tedious degradations are usually necessary. For complex metabolites, the degradative reactions needed may not exist, and it is often impossible to determine the exact position of the label. The use of ^{13}C labelled precursors, in conjunction with ^{13}C n.m.r., can overcome this problem in most cases^{102,130}.

After administration of a ^{13}C labelled precursor, the isolated metabolite is examined by ^{13}C n.m.r. spectroscopy. Provided an assigned spectrum of the metabolite is available, the selective enhancement of signals due to labelled positions exactly locates the position of label incorporation. Owing to the relatively high natural abundance of ^{13}C , incorporations of at least 0.5% of a 90 atom % ^{13}C precursor are normally required to give meaningful results. This problem may be somewhat reduced by growing the organisms under study in ^{13}C depleted media before addition of the ^{13}C labelled precursor¹³⁰. This technique may raise the acceptable dilution from ca 1:200 to ca 1:1000 in favourable cases.

It has been demonstrated that double labelling techniques can provide unique information about biosynthetic pathways¹⁴⁴, and also overcome somewhat the sensitivity problems associated with ^{13}C labelling studies¹⁴⁵. Because the natural abundance of ^{13}C is 1.1%, the probability of finding two adjacent ^{13}C nuclei in natural material is 0.011^2 or 1.2×10^{-4} . This is the reason natural abundance ^{13}C n.m.r. spectra do not exhibit any $^{13}\text{C} - ^{13}\text{C}$ spin-spin couplings. If two adjacent centres in (e.g. C-3 and C-4), a molecule are enriched to 90 atom % ^{13}C , then 81% of the molecules present will contain a $^{13}\text{C} - ^{13}\text{C}$ fragment, and so show $^{13}\text{C} - ^{13}\text{C}$ couplings in the ^{13}C n.m.r. spectrum. In addition, the material will also contain 8% ^{13}C -3

^{12}C -4 species, and 8% ^{13}C -4 ^{12}C -3 species (see section 1-C-1), and these molecules will each give rise to singlet signals ca 8 fold the intensity of natural abundance signals. The resulting ^{13}C n.m.r. spectrum will exhibit an AB, or AX doublet centred on a singlet, for each labelled carbon atom. This is exemplified in the spectrum of $\text{CH}_3\text{S}^{13}\text{CH}_2^{13}\text{CH}_2\text{Cl}$ 90 atom %, shown in Fig. 4-B-6. Incorporation of a double labelled compound of this type can provide information on which units are derived intact from a given precursor¹⁰², because incorporation of intact ^{13}C - ^{13}C units will lead to spin-spin couplings in the ^{13}C n.m.r. spectrum of the metabolite. In Fig. 1-D-1a, the intact $^{13}\text{CH}_2$ - $^{13}\text{CH}_2$ unit is incorporated into the product, and the ^{13}C n.m.r. spectra shows an AX system superimposed on a natural abundance singlet, for each CH_2 group. In case (b) the $^{13}\text{CH}_2$ - $^{13}\text{CH}_2$ grouping is split before incorporation, and enhancement of the signal due to each labelled CH_2 group in the product is observed, but no spin-spin coupling. This information can be used for instance to deduce the folding pattern of polyketide chains in the biosynthesis of acetate derived natural products. Information of this type is almost always lost in the degradative sequence associated with ^{14}C labelling.

Where intact two carbon units are incorporated from a precursor, greater sensitivity in the detection of ^{13}C incorporation by ^{13}C n.m.r. can be achieved, using double labelled materials¹⁰². This is possible because the signals due to the labelled positions are not coincident with the natural abundance signals, due to unlabelled molecules. For example, incorporation of 0.2% of a ^{13}C double labelled precursor would give rise to satellite signals, each ca 10% of the natural abundance peaks, which could be easily detected. This is in contrast to the ca $\frac{1}{6}$ enhancement of a ^{13}C line which would occur

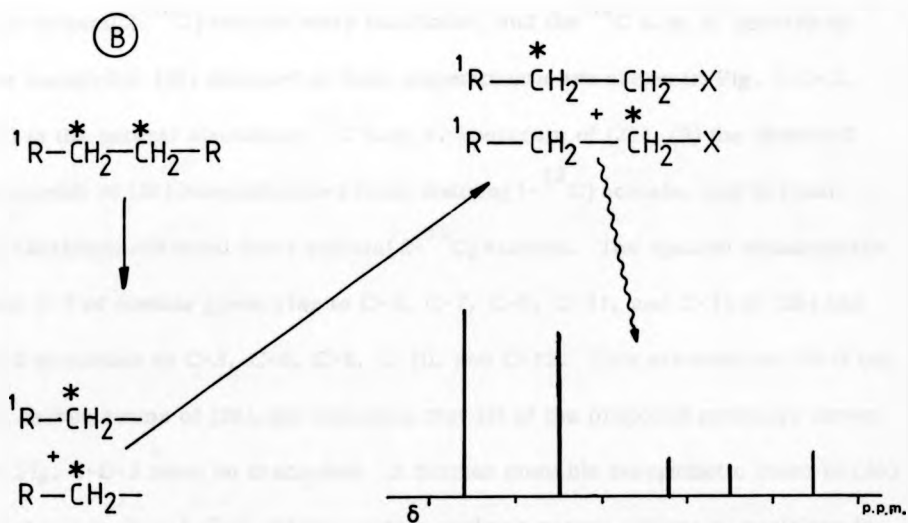
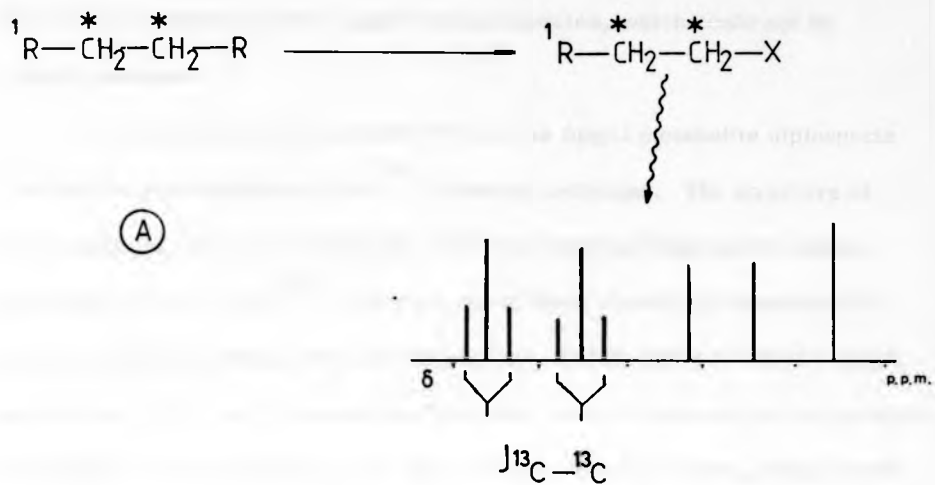


FIG 1-D-1

INCORPORATION OF A ${}^{13}\text{C}$ DOUBLE LABELLED PRECURSOR INTO A METABOLITE

A) INTACT INCORPORATION LEADS TO ${}^{13}\text{C}-^{13}\text{C}$ SPLITTINGS

B) FISSION THEN INCORPORATION LEADS TO ENHANCEMENT, BUT NO SPLITTINGS

with 0.2% incorporation of a single labelled species, which could not be reliably observed.

A recent study of the biosynthesis of the fungal metabolite diplosporin (26) serves as an example of the ^{13}C labelling technique. The structure of the metabolite (26) was established, and three possible biosynthetic routes postulated (Fig. 1-D-2)¹⁴⁶. The first two of these involve the condensation of two polyketide chains, and alkyl migrations, and the third involves a single polyketide chain, and introduction of the ethyl chain by successive methylations. The expected labelling pattern for each of these routes is shown, when acetate is used as a precursor. Feeding experiments using sodium[1- ^{13}C] acetate¹⁷ and sodium[2- ^{13}C] acetate were concluded, and the ^{13}C n.m.r. spectra of the metabolite (26) obtained in these experiments are shown in Fig. 1-D-3. (A) is the natural abundance ^{13}C n.m.r. spectrum of (26), (B) the observed spectrum of (26) biosynthesised from sodium[1- ^{13}C] acetate, and (C) that of (26) biosynthesised from sodium[2- ^{13}C] acetate. The spectra demonstrate that C-1 of acetate gives rise to C-4, C-7, C-9, C-11, and C-13 of (26) and C-2 of acetate to C-3, C-6, C-8, C-10, and C-12. This accounts for 10 of the 12 carbon atoms of (26), and indicates that all of the proposed pathways shown in Fig. 1-D-2 must be discarded. A further possible biosynthetic route to (26) is shown in Fig. 1-D-4, where acetate carbons occupy alternate positions in the diplosporin skeleton, and two C-1 units are incorporated from methionine. Evidence for the folding of the polyketide chain as depicted in Fig. 1-D-4 was obtained by examination of the ^{13}C n.m.r. spectrum of the (26) obtained when sodium[1,2- $^{13}\text{C}_2$] acetate was used as precursor. The ^{13}C n.m.r. of the (26) obtained in this way (Fig. 1-D-3D) shows couplings for all signals except C-2

FIG 1-D-2

26

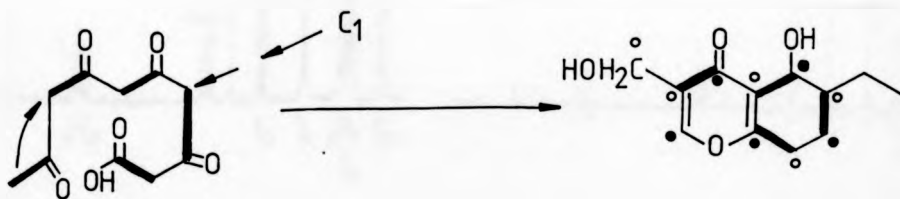
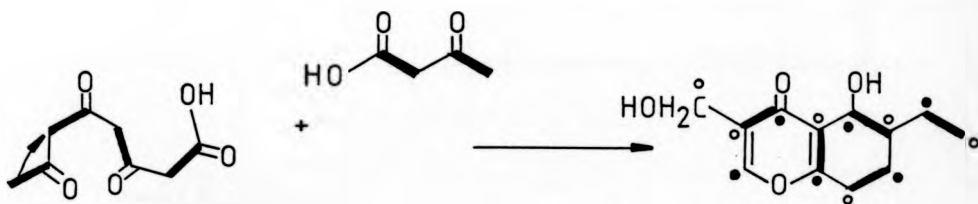
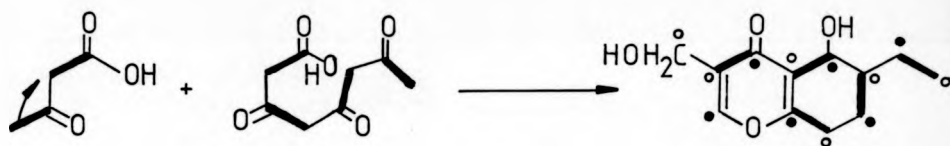
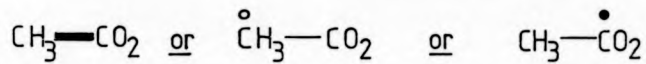
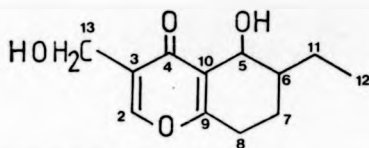


FIG. 1-D-2

THE STRUCTURE OF DIPLOSPORIN (26). SHOWN ARE THREE POSSIBLE BIOSYNTHETIC ROUTES FROM ACETATE TO DIPLOSPORIN, AND THE EXPECTED LABEL DISTRIBUTION WHEN $[1\text{-}^{13}\text{C}]$, $[2\text{-}^{13}\text{C}]$ AND $[1,2\text{-}^{13}\text{C}_2]$ ACETATE ARE INCORPORATED INTO (26)

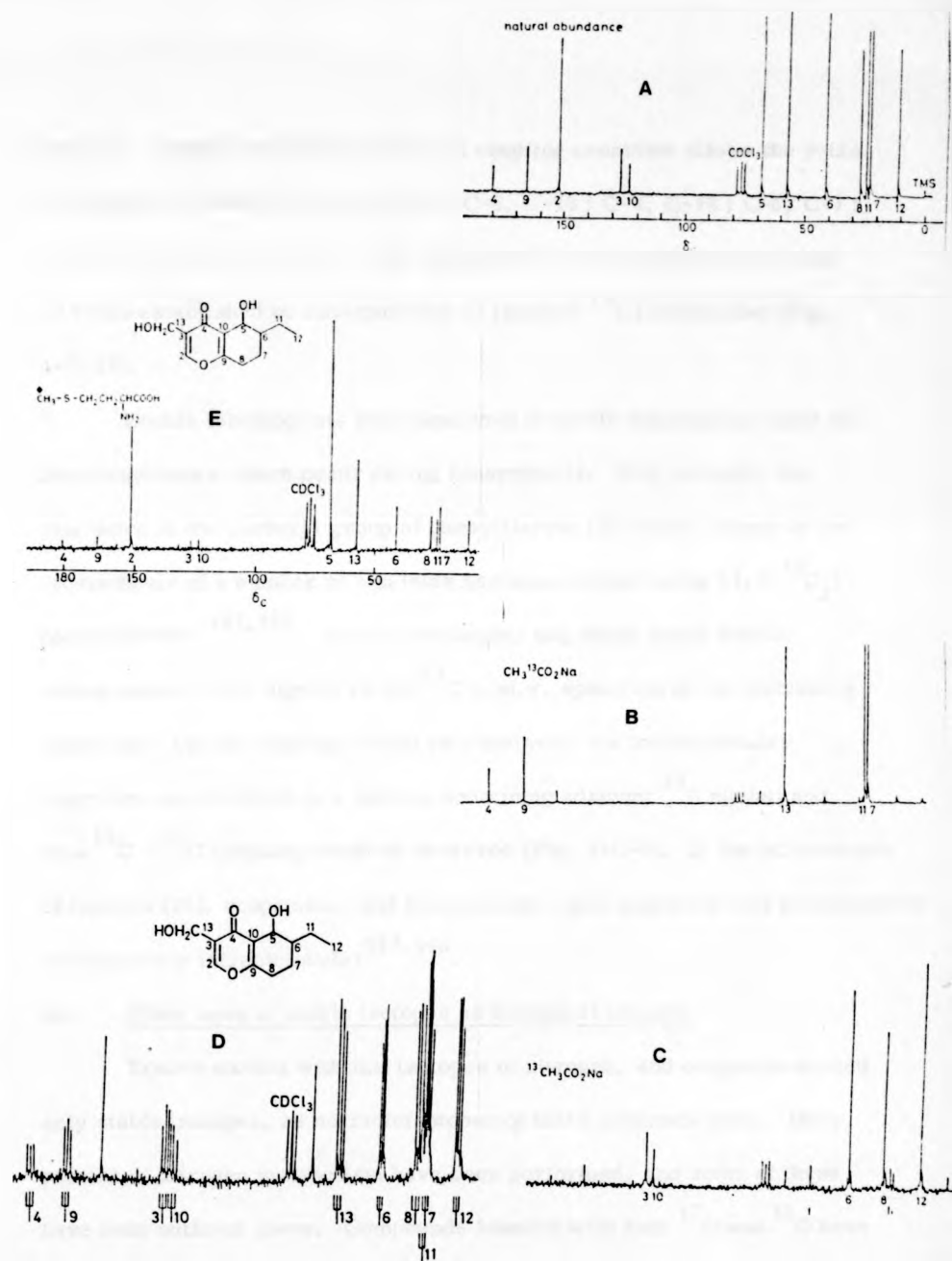


FIG. 1-D-3

^{13}C n. m. r. spectra of diposporin (26) obtained after incorporation of (B) [1- ^{13}C] acetate, (C) [2- ^{13}C] acetate, (D) [1,2- $^{13}\text{C}_2$] acetate, (E) [methyl- ^{13}C] methionine and (A) at natural abundance (Taken from Ref. 143).

and C-5. Comparison of the observed coupling constants allows the pairs of coupled carbons to be identified as C-3, C-13 ; C-4, C-10 ; C-6, C-7 ; C-8, C-9 ; and C-11, C-12. The origin of the carbon atoms at C-2 and C-5 was established by incorporation of [methyl- ^{13}C] methionine (Fig. 1-D-3E).

Double labelling has also been used to obtain information about the rearrangements which occur during biosynthesis. For example, the migration of the carboxyl group of phenylalanine (27) which occurs in the biosynthesis of a number of alkaloids has been studied using [1,3- $^{13}\text{C}_2$] phenylalanine ^{147, 148}. An intermolecular migration would lead to enhancement of two signals in the ^{13}C n. m. r. spectrum of the metabolite concerned, but no couplings would be observed. An intramolecular migration would result in a species containing adjacent ^{13}C nuclei; and thus $^{13}\text{C} - ^{13}\text{C}$ coupling would be observed (Fig. 1-D-5). In the biosynthesis of tenellin (28), scopamine, and hyoscyamine, this migration was demonstrated to be entirely intramolecular ^{147, 148}.

(c) Other uses of stable isotopes as biological tracers

Tracer studies with the isotopes of nitrogen, and oxygen have used only stable isotopes, as no radioisotopes of these elements exist. Many studies of nitrogen metabolism have been performed, and some of these have been outlined above. Compounds labelled with both ^{17}O and ^{18}O have been used as tracers for oxygen, for example, in establishing the origin of oxygen atoms in metabolic products as H_2O , or O_2 ¹⁰⁰.

Materials doubly labelled with ^{15}N and ^{13}C have been used in studies of drug metabolism in humans and animals ¹⁰⁷.

FIG 1-D-4

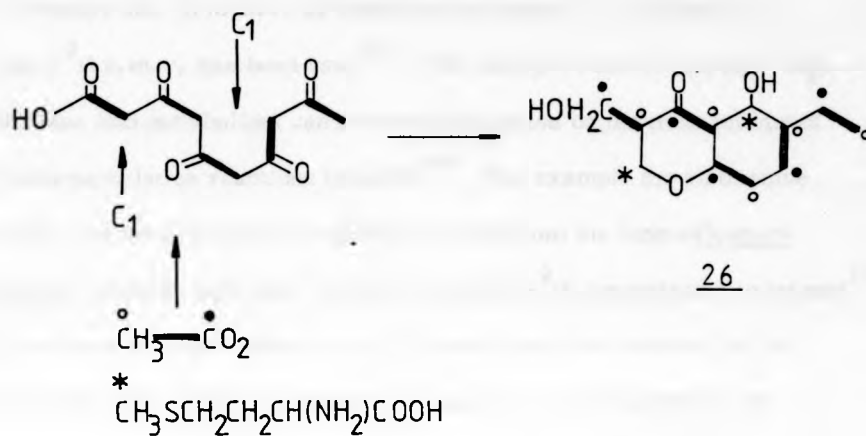


FIG 1-D-5

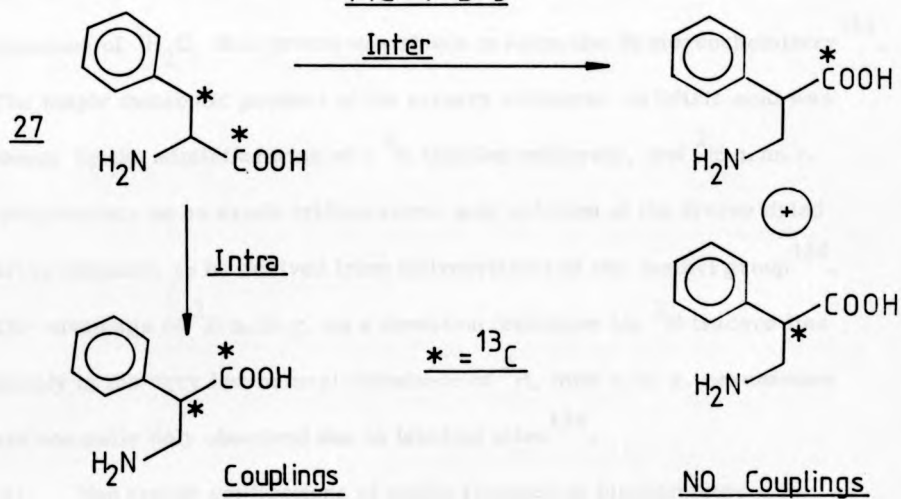


FIG. 1-D-4 THE BIOSYNTHETIC ORIGIN OF DIPLOSPORIN (26) AS DEMONSTRATED BY ^{13}C LABELLING STUDIES

FIG. 1-D-5 THE CONSEQUENCES OF INTER AND INTRA MOLECULAR MIGRATION ON THE LABEL DISTRIBUTION OBSERVED FOR A METABOLITE DERIVED FROM [1,3- $^{13}\text{C}_2$] PHENYLALANINE

Studies using ^2H as a tracer for hydrogen have used mass spectroscopy and ^1H n.m.r. as detection techniques¹¹¹, and more recently ^2H n.m.r. has been used¹³⁴. The incorporation of [methyl- $^2\text{H}_3$] methionine into metabolites can provide information on the mechanisms of the transmethylation reactions involved¹⁴⁹. For example the methionine derived C-24 methyl group of ergosterol isolated from the fungus Oospora irresiens contains only two ^2H atoms if [methyl- $^2\text{H}_3$] methionine is infused¹⁵⁰. This indicates the involvement of a C-24 methylene intermediate in this transmethylation. In the cyclisation of squalene to tetrahymanol in Tetrahymana puriformis, a proton is introduced, and by ^2H n.m.r. spectroscopy on the material obtained from a culture incubated in the presence of $^2\text{H}_2\text{O}$, this proton was shown to adopt the 3β stereochemistry¹⁵¹. The major metabolic product of the urinary antibiotic nalidixic acid was shown by the administration of a ^2H labelled substrate, and ^2H n.m.r. spectroscopy on an crude trifluoroacetic acid solution of the freeze dried urine obtained, to be derived from hydroxylation of the methyl group¹³⁴. The advantage of ^2H n.m.r. as a detection technique for ^2H tracers lies mainly in the very low natural abundance of ^2H , thus n.m.r. resonances are normally only observed due to labelled sites¹³⁴.

(d) Non tracer applications of stable isotopes in biochemistry

In biochemical systems, ^{13}C , ^1H , ^2H , and ^{17}O n.m.r., as well as n.m.r. observation of other nuclei, has been used to study the three dimensional structure, and molecular motion of macromolecules¹⁵². In many of these studies specific enrichment with the isotope under observation can be of value. For example, the degree of molecular motion of the fatty

acyl chains of plasma membranes has been studied using ^2H n. m. r. and ^2H labelled materials¹⁵³. The degree of quadrupole splitting observed could be directly correlated with the degree of molecular motion at a given site in the side chain. Using a series of lipids selectively deuterated at different positions along their alkyl chains, an overall picture of the motion of these chains was built up^{154, 155, 156}.

^{13}C n. m. r. has been used more widely than ^2H n. m. r. for molecular motion studies, for example in studies of nucleic acids, carbohydrates, and peptides¹⁵⁷. ^{17}O has also been employed as a structural probe¹³⁶, but to date any application to biological systems is very limited. Recently the metabolism of ^{13}C labelled substrates has been directly observed in whole live microorganism cells, using ^{13}C n. m. r.¹⁵⁸

6. Chemical Applications

Molecular structure, and the mechanisms of chemical reactions, may be elucidated using stable isotopes.

Structural studies involving mass spectroscopy can benefit greatly from the use of isotopic labelling, for the determination of molecular fragmentation patterns^{101, 159}. Isotopic substitution may be used as an assignment aid in n. m. r. studies. For example, the substitution of ^1H by ^2H causes predictable chemical shift effects in the ^{13}C n. m. r. spectrum of the molecule in question^{132, 133, 160}. Use of ^2H labelled compounds, and ^2H n. m. r. may also be used as an assignment aid in ^1H n. m. r. studies¹³⁴. In studies of molecular dynamics using n. m. r. spectroscopy, isotopic enrichment may allow the selective observation of specific resonances, as in the biological examples above¹³³.

In a tracer context, isotopes have been used to elucidate the mechanisms of many chemical reactions. Most of these studies were performed with radioisotopes¹¹⁸, until the late 1960's. Using ¹⁴C and ³H labelling, the mechanisms of many reactions, including the pinacol rearrangement, and the Claisen rearrangement, were investigated¹¹⁸. More recently these studies have used stable isotopes, coupled with n. m. r. detection techniques. For example, the rearrangements of alkyl cobaloximes have been studied using ¹³C labelling and ¹³C n. m. r.^{161, 162}. In a recent study¹⁶², the rearrangement of [1-¹³C] cyclopropylmethyl-(pyridine)cobaloxime to [4-¹³C] but-3-enyl(pyridine)cobaloxime was followed by ¹³C n. m. r. spectroscopy. The conversion of C-1 labelled material into C-4 labelled material, and not C-2 labelled material, demonstrated that the mechanism operating involved a cobalt migration, and not a carbon migration. Detailed mechanistic studies have also been performed using ²H labelling. For example, the pyrolysis of anti-1,5-bishomocycloheptatriene was shown to proceed by a two-fold 1,5 homodienyl rearrangement, using ²H labelling, rather than other postulated mechanisms¹⁶³.

The use of specifically labelled molecules may also provide mechanistic information via the kinetic isotope effect¹¹². ²H labelling is of particular use in this context, and has been widely employed¹¹².

1-E OUTLINE OF THE PROJECT

1. General

A major problem facing the biochemist wishing to investigate metabolic pathways using isotopic labelling techniques, is the synthesis of the desired specifically labelled precursor. The aim of this project was to develop quick, efficient, syntheses of certain specifically labelled methionines, and to use these molecules in examinations of the metabolism of methionine's alkyl chain. A number of potentially useful labelled methionines were identified, and their synthesis has been investigated. When these syntheses had been achieved, metabolic studies on the biochemical utilisation of methionine's alkyl chain were undertaken, using these labelled materials. This thesis describes the synthesis of a number of these methionines, and reports preliminary results of metabolic studies using them.

2. Potentially useful labelled methionines, whose syntheses have been investigated

(a) [3-¹³C], [4-¹³C] and [3,4-¹³C₂] methionine, (29), (30) and (31)

Radiolabelling studies of the biosynthesis of spermidine(6) and spermine(7) have established the incorporation of intact putrescine (8), and of C-2 of methionine ¹² ¹⁶⁴, ¹⁶⁵. No incorporation of radioactivity from [1-¹⁴C] methionine was observed in these studies. The incorporation of (29) and/or (30) into polyamines (6) and (7), followed by ¹³C n.m.r. analysis would establish the precursor role of the entire methionine alkyl chain (C-2 to C-4). Incorporation of (31) into polyamines (6) and (7) could provide evidence as to whether or not the methionine C-3, C-4 fragment

is incorporated intact. Given intact incorporation of the methionine alkyl chain, the degree of incorporation which could be reliably assessed would be much lower for (31) than for (29) or (30), due to reasons outlined in Section 1-D. The labelled polyamines (6) and (7) obtained in these experiments would be of value in studies of the metabolism of (6) and (7). For example, ^{13}C n.m.r. studies of the binding of these polyamines to nucleic acids and membranes could benefit from selective observation of resonances. Specifically labelled (6) and (7) (especially (6) and (7) derived from methionine (31)) would also be of value in studies of the precursor roles of the polyamines in the biosynthesis of alkaloids, as discussed above.

The ethylene produced by plants has been demonstrated to arise from C-3 and C-4 of methionine, by radiolabelling experiments^{36,54}. The origin of the ethylene produced by bacteria is assumed to be the same, although this has not yet been proven. Isolation of the ethylene produced by the above systems from methionine (31), as a derivative (e.g. 2,4-Dinitrobenzenesulphenyl chloride adduct) followed by ^{13}C n.m.r. analysis could establish whether or not the intact C-3, C-4 fragment of methionine becomes ethylene.

(b) [3,4- $^2\text{H}_2$] methionines ; stereospecificlabelling

The precursor role of methionine in ethylene biosynthesis has been established, but no details of the reactions involved are yet known. Stereospecifically labelled [3,4- $^2\text{H}_2$] methionines (e.g. (3R,4R)-[3,4- $^2\text{H}_2$] methionine (32) and (3R,4S)-[3,4- $^2\text{H}_2$] methionine (33)) could be used to determine the stereochemistry of the elimination involved in the production of ethylene from methionine. This is outlined in Fig. 1-E-1. Labelled methionine of the type described above would also be useful in the determination

FIG 1-E-1

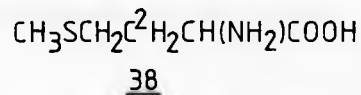
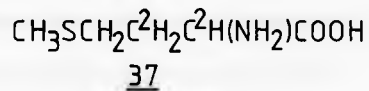
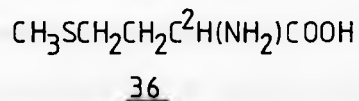
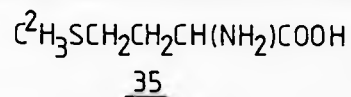
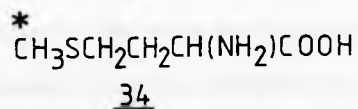
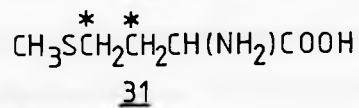
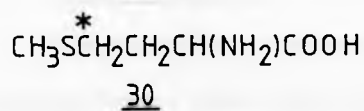
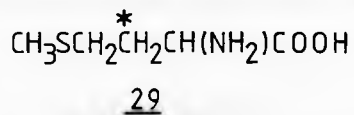
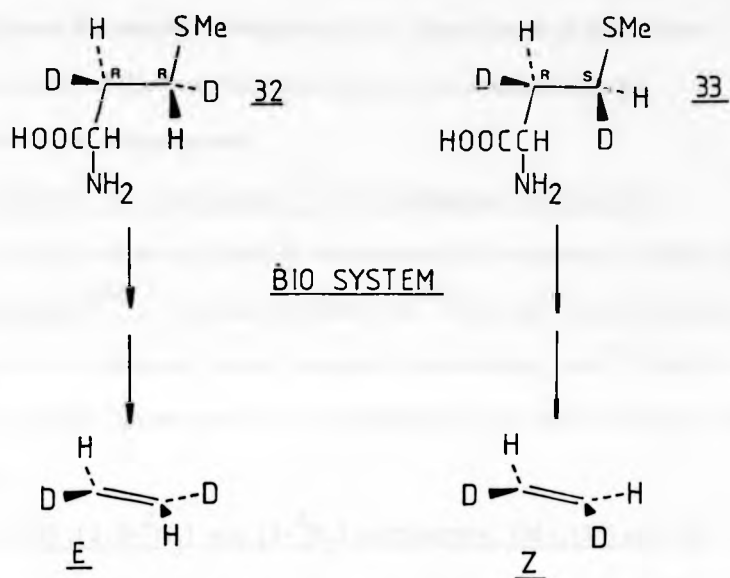


FIG. 1-E-1

THE GEOMETRY OF THE [1,2-²H₂] ETHYLENES WHICH WOULD RESULT FROM THE PRODUCTION OF ETHYLENE FROM STEREOSPECIFICALLY LABELLED METHIONINES, IF A TRANS ELIMINATION OCCURRED

of the mechanism of formation of spermidene (6) from putrescine (8) and decarboxylated S-adenosyl methionine (5a). Elucidation of the stereochemical course of the reaction could allow alternate mechanistic possibilities to be distinguished.

(c) [methyl- ^{13}C] and [methyl- $^2\text{H}_3$] methionines, (34) and (35)

The value of methyl labelled methionines in biosynthetic studies is well established^{6,99}. Material labelled with ^{13}C can be used to determine the origin of C-1 units and methyl groups in metabolites, and ^2H labelled material to obtain information on the mechanisms involved in methyl transfer reactions.

(d) [2- ^2H], [2,3- $^2\text{H}_3$] and [3- $^2\text{H}_2$] methionines, (36), (37) and (38)

A method has been described for labelling certain amino acids at their 2 and/or 3 positions with ^2H ¹⁶⁶. We have extended this method to the preparation of the above labelled methionines, for use in mechanistic studies.

3. Metabolic Studies

The intermediates involved in the production of ethylene from methionine are not known. Early work was concerned with the identification of a possible intermediate in this conversion which accumulates in ethylene producing cultures. When stereospecifically labelled methionines became available, they were used to examine the stereochemistry of the reactions involved in ethylene biosynthesis.

[3,4- $^{13}\text{C}_2$] methionine has been used in studies of the biosynthesis of spermidene (6) from methionine and putrescine (8), in E. coli. Studies on the stereochemical course of the transfer of the amino propyl group of

methionine to diamine (8) to give polyamine (6), using incorporation of stereospecifically labelled [3,4-²H₂] methionines are in progress.

CHAPTER 2MATERIALS AND METHODS2-A MATERIALS1. Solvents

All solvents used were of laboratory grade unless otherwise indicated. Anhydrous or absolute solvents were purified and dried according to standard procedures¹⁶⁷. Chloroform was obtained ethanol free by chromatography on basic alumina. Anhydrous, absolute, and deuterated solvents were stored in tightly stoppered bottles with parafilm seals, and were used only in a dry box.

2. Chemicals

All chemicals were of the highest purity commercially available, and in most cases were purified before use, by standard procedures¹⁶⁷. Labelled compounds were obtained from BOC Prochem Limited, Deer Park Road, London SW19, and were used as received. $^2\text{H}_2\text{O}$ was stored and used in a dry box, as was [$^2\text{H}_4$] methanol.

2-B INSTRUMENTAL

1. NMR spectra

^1H n.m.r. spectra were recorded by the author, using the following instruments:

- a) Perkin Elmer (model R-12) 60 MHz ^1H n.m.r. spectrometer
- or b) Bruker (model WH90) 90 MHz ^1H n.m.r. spectrometer
- or c) Perkin Elmer (model R-34) 220 MHz ^1H n.m.r. spectrometer

Peaks are designated by their chemical shift (δ) in parts per million, followed in brackets by their relative integral value (e.g. 1H, 2H) in hydrogens, their multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, and m = multiplet), and the spin-spin coupling constant (J) in Hertz, where appropriate. Spectra were recorded at 60 MHz, in CDCl_3 , using TMS as a reference (zero δ) unless otherwise stated.

^{13}C n.m.r. spectra were recorded by the author on a Bruker (model WH90) 22.63 MHz ^{13}C n.m.r. spectrometer, equipped with a variable temperature accessory. Peaks are designated as above, and refer to samples in CDCl_3 and referenced against TMS unless otherwise stated. All spectra were run with broad band ^1H decoupling, and consist of singlets only, unless otherwise indicated.

All n.m.r. spectra were assigned by reference to tables of chemical shift data (e.g. Refs. 131, 133), comparison to the n.m.r. spectra of model compounds, or comparison to the n.m.r. spectra of authentic materials, unless otherwise indicated.

2. Infra red spectra

Infra red (i. r.) spectra were recorded on a Perkin Elmer (model 457) grating infra red spectrophotometer. Spectra were calibrated using the 1603.4 peak of polystyrene and were run using NaCl plates. Samples were either mulls (nujol), thin films, solutions, or gases (10 cm gas cell). Peaks are designated by their wave number (cm^{-1}) as strong (s), medium (m), weak (w) or very weak (vw) and as broad (Br) or sharp (sh). Peaks showing pqr structure are designated by the letters pqr following the band type.

3. Optical Rotations

Optical rotations were measured with a Bendix NPL automatic polarimeter (model 143D) using a 1.00 cm x 0.708 cm^2 cell. The instrument was calibrated against a standard sucrose solution before each measurement. Values are expressed as specific rotations $([\alpha])^{168}$.

4. Gas liquid chromatography (g.l.c.)

G.l.c. analyses were carried out using a Perkin Elmer (model F-11) flame ionisation gas chromatograph, using N_2 as carrier gas. Samples were compared to authentic materials, and co-injections run wherever possible.

5. Ultra Violet (u. v.) spectra

Ultra violet spectra were recorded with a Pye-Unicam (model SP800) ultra violet spectrophotometer.

6. Mass spectra

Mass spectra were recorded on a Micromass MS9. Peaks are quoted as m/e , followed by their percentage of the base peak. The base peak is indicated as 100%. The molecular ion is designated M^+ .

2-C METHODS

- a) Solutions in organic solvents were dried using MgSO_4 , which had been stored at 110°C for at least 48 h. Evaporation under reduced pressure refers to the removal of bulk solvent at 20°C and 15 mm Hg, using a Büchi rotary evaporator, unless otherwise stated.
- b) Glassware was dried by storing at 110°C overnight, and cooling under dry nitrogen. Glassware used in exchange, or other very moisture sensitive reactions, was dried as above, and then flamed and allowed to cool either in a dry box, or under a blanket of dry nitrogen.
- c) Exchange reactions were carried out under strictly anhydrous conditions, all manipulations being performed in a dry box or under a blanket of dry nitrogen.
- d) Solutions of sodium methoxide in methanol were prepared in a dry box from anhydrous methanol (or deuteromethanol) and freshly cut clean sodium. Sodium was cleaned by dipping in a small sample of the solvent methanol (anhydrous or deuterated, as appropriate) and was then dissolved in the solvent methanol. Aliquots were titrated against aqueous standard hydrochloric acid solutions (prepared from BDH Limited CVS ampoules) using phenolphthalein as indicator.
- e) Flash chromatography was carried out according to the method of Ref. 169, using Merck Kiesel gel 60 (230 - 400 mesh) cat. no. 9385). The progress of columns was followed by microslide t.l.c.
- f) Thin layer chromatography (t.l.c.) was carried out on plates pre-coated with silica gel (Merck, Kiesel gel, 60 F₂₅₄, cat. no. 5554) or on glass plates coated with Merck, Kiesel gel G (type 60, cat. no. 7731) in

0.25 mm layers. Spots were applied as solutions in the eluting solvent, or in the case of 24 DNP's as solutions in EtOAc. Systems used were:

- (1) Merck 5554 plate, 880 ammonia/absolute ethanol 23:77 as eluant, followed by drying at 100°C, spraying with a 0.5% solution of ninhydrin in butanol, and heating to develop spots.
- (2) Microslide plate, methanol eluant, followed by drying, spraying with 35% H₂SO₄, and charring to develop spots.
- (3) For 24 DNP's, Merck 5554 plates, eluant as given in the text, and visible and u. v. light used for visualisation.

Solvents were made up freshly, and are quoted as volume/volume ratios, standards were included on plates wherever possible. Pre-coated plates were stored in a dessicator over self indicating silica gel, and home-made plates dried at 110°C overnight, followed by storage as above. Plates were used within 8 weeks (Merck) or 2 weeks (home made), or re-activated at 110°C, as above.

g) Free SH was detected and quantified by using Ellman's test¹⁷⁰. Aliquots (0.5 cm³) of liquid ammonia reactions were tested by evaporating in a stream of nitrogen, dissolving the residue in 1 cm³ of TRIS buffer (pH 8) and adding a solution of Ellman's reagent (100 μl of a 4 mg/cm³ solution). A yellow colouration indicated the presence of free SH, and was sensitive to < 0.5% free SH.

h) Gases were handled on an all-glass vacuum line system. Standard techniques were employed. Gases were dried by distillation through a trap cooled in a dry ice/acetone freezing mixture.

CHAPTER 3

SYNTHESES OF METHYL LABELLED METHIONINES

3-A INTRODUCTION

The use of methyl labelled methionines in studies of methionine metabolism is well established^{6,99}. An improved synthesis of (S)-[methyl-²H₃] methionine (35) was published in 1970 by Dolphin and Endo¹⁷¹. In our hands however this procedure suffered from a number of serious drawbacks. A modified procedure which overcomes these problems has been devised.

(1R, 3S)-dehydromethionine (39) is readily available by oxidation of (S)-methionine (e.g. with I₂ in MeOH)¹⁷², and its conversion to methionine by thiols has been observed¹⁷³. Dehydromethionine undergoes base catalysed methyl exchange, and is ring opened to methionine sulphoxide, in ²H₂O containing NaO²H. In [²H₄] methanol, containing C²H₃ONa, exchange of the methyl protons without conversion to methionine sulphoxide can be observed (Chapter 6). Therefore (S)-[methyl-²H₃] methionine (35) could be prepared from (1R, 3S)-dehydromethionine (39), by proton exchange in CH₃O²H containing CH₃ONa, followed by treatment with H₂S (Fig. 3-A-1). The isotopic content of this labelled methionine was assayed by mass spectroscopy on its N-trifluoroacetyl butyl ester (Fig. 3-A-2).

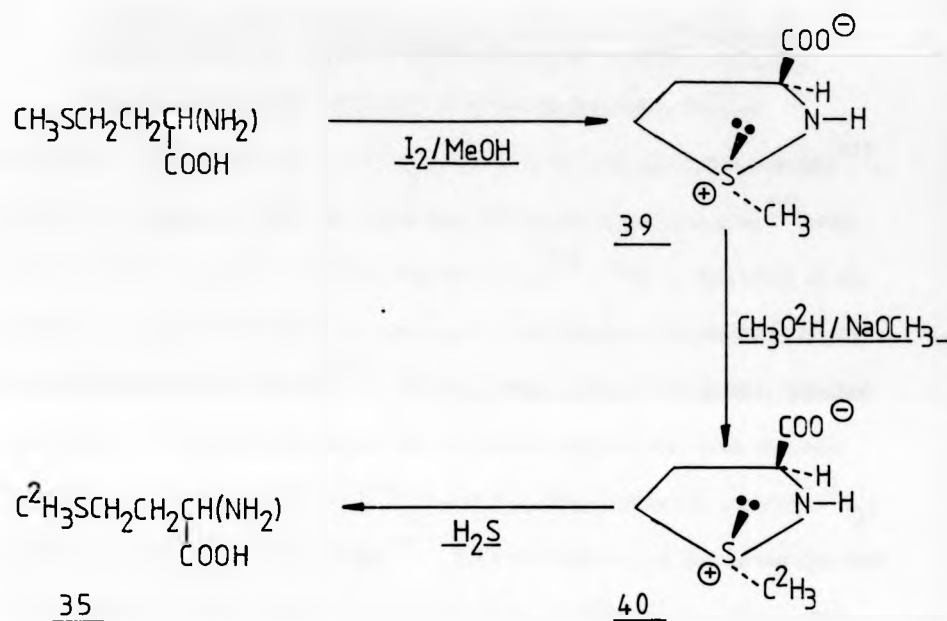


FIG 3-A-1

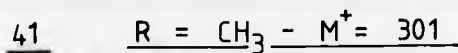
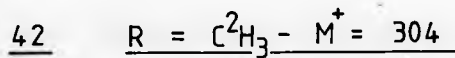
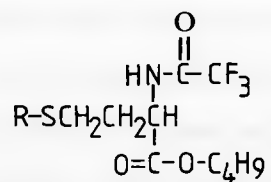


FIG 3-A-2

FIG. 3-A-1 PREPARATION OF (S)-[METHYL- ^{13}C] METHIONINE VIA (1R,3S)DEHYDROMETHIONINE

FIG. 3-A-2 DERIVATIVES OF METHIONINE USED FOR MASS SPECTRAL ANALYSIS

3-B PREPARATION OF METHYL LABELLED METHIONINES BY
ALKYLATION OF THE SODIUM SALT OF HOMOCYSTEINE

Classical syntheses of methyl labelled methionines involve alkylation of the sodium salt of homocysteine with labelled methyl iodide¹⁷⁴. The salt is prepared either by reduction of S-benzyl homocysteine¹⁷⁵ with sodium/liquid ammonia, or from homocysteine¹⁷⁶. The preparation of the sodium salt of homocysteine by reduction of methionine with sodium/liquid ammonia, has been reported¹⁷⁷. An improved synthesis of methyl labelled methionines, in which the sodium salt of homocysteine was obtained from methionine, and alkylated with [²H₃] methyl iodide to give (S)-[methyl-²H₃] methionine, was reported in 1970¹⁷¹. This procedure has the advantage that methyl labelled methionines are obtained from methionine in a single step, which is of value in the synthesis of double labelled materials (e.g. $\text{CH}_3\text{SCH}_2\text{CH}_2\text{}^{13}\text{CH}(\text{NH}_2)\text{COOH} \rightarrow \text{}^{13}\text{CH}_3\text{SCH}_2\text{CH}_2\text{}^{13}\text{CH}(\text{NH}_2)\text{COOH}$). In this synthesis¹⁷¹, (S)-methionine (0.49 mol) was reduced with sodium (1.5 mol) in liquid ammonia, to the sodium salt of homocysteine. Solid ammonium chloride (0.5 mol) was added (to prevent racemisation¹⁷⁷), followed by [²H₃] methyl iodide (0.52 mol). Evaporation of the solvent and workup (neutralisation of an aqueous solution of the residue with HCl, followed by recrystallisation from aqueous ethanol) gave (S)-[methyl-²H₃] methionine in 87% overall yield. The only analytical data reported for the product was its isotopic content (identical to that of the methyl iodide used), and its optical rotation, $[\alpha]_{\text{D}}^{23} = 21.5^\circ$ in 1M HCl cf authentic material 21.6° in 1M HCl.

In our hands the published procedure¹⁷¹ gave low yields (50 - 60%), and the methionine obtained showed a slow running impurity on t.l.c. This was

identified as homocysteine (2) by comparison with authentic material, and quantified by Ellman's test¹⁷⁰ as 2 - 3%. Increasing the ethanol content of the recrystallisation solvent, in an attempt to obtain better yields, resulted in co-crystallisation of inorganic salts (NaCl, NH₄Cl).

To overcome these problems, the following procedure was adopted. Use of lithium in place of sodium for the reduction, gives lithium chloride on workup, which is more soluble in ethanol than sodium chloride, and therefore remains in solution when the crude methionine is recrystallised from aqueous ethanol. Hope and Humphries reported¹⁷⁷ that reduction of methionine with sodium/liquid ammonia, followed by oxidation of the homocysteine obtained to homocystine, led to a racemised product unless a weak acid was present. By addition of NH₄Cl after the reduction and before the oxidation, this racemisation could be avoided. In order to avoid racemisation, Dolphin and Endo¹⁷¹ added NH₄Cl in their preparation of methyl labelled methionine as described above. By running a series of trial reactions, the addition of ammonium chloride was found to be unnecessary, as no racemisation was observed, and so this addition was dispensed with. After alkylation of the homocysteine, and evaporation of the liquid ammonia solvent, the residue was evaporated from water several times to remove all traces of ammonia (formed by $\text{LiNH}_2 + \text{H}_2\text{O} \rightarrow \text{NH}_3 + \text{LiOH}$). Neutralisation of an aqueous solution of the residue, and recrystallisation from ethanol water gave methionine in good yield (79 - 83%). However, this material still showed a spot due to homocysteine on t.l.c., which was quantified by Ellman's test as 1 - 3%. It has been pointed out¹⁷⁴ that pure products are not obtained from this type of reaction, unless an excess of methyl iodide is used. In

order to ensure complete alkylation of the sodium salt of homocysteine, the progress of the reaction was therefore monitored by Ellman's tests on aliquots¹⁷⁰ of the reaction mixture*.

Small portions of labelled methyl iodide were added as necessary, until an Ellman's test showed a negative result. Work up of the reaction gave (S)-[methyl-²H₃] methionine in 80 - 83% yield, pure by t.l.c. A further 8 - 10% was available by ion exchange chromatography of the mother liquor from the final recrystallisation. The optical rotation of the methionine obtained was not significantly different from that of the (S)-methionine used as starting material.

Using this procedure, (S)-[methyl-²H₃] methionine 99 atom % and (S)-[methyl-¹³C]methionine 91 atom % were prepared. From our results it is considered probable that the material prepared in Ref. 171 contained homocysteine as a contaminant, which was not detected by optical rotation measurements. The procedure reported here provides material free from homocysteine, and involves a simpler work up than that of Ref. 171.

* See materials and methods, Chapter 2

3-C PREPARATION OF (S)-[METHYL-²H₃] METHIONINE VIA
PROTON EXCHANGE IN (1R,3S)-DEHYDROMETHIONINE

Exchange of the methyl protons of (1R,3S)dehydromethionine (39) occurs with $t_{\frac{1}{2}}$ ca 3 min at 37°C in [²H₄] methanol containing 2 mole % [²H₃] CH₃ONa (chapter 6). Because the reduction of dehydromethionine to methionine by thiols had been observed¹⁷³, the reaction between hetrocycle (39) and H₂S was investigated. Bubbling H₂S through a methanolic solution of hetrocycle (39) resulted in almost quantitative precipitation of (S)-methionine. The optical rotation of this material was not significantly different from that of the (S)-methionine used in the preparation of (39).

In the preparation of (S)-[methyl-²H₃] methionine (35) via proton exchange in (39), the use of methan[²H] ol provides a considerably economy over [²H₄] methanol. Methan[²H] ol is easily prepared from ²H₂O and dimethyl carbonate¹⁷⁸. After exchange, the (1R,3S)-[methyl-²H₃] dehydro-methionine (40) was converted into amino acid (35) by bubbling H₂S through the methanolic solution of (40). The recovered (35) showed a small (ca 2%) slow running impurity on t.l.c., which was identified as methionine sulphoxide, by comparison with authentic material. This impurity was removed by recrystallising the methionine from methanol/water.

The preparation of labelled compounds by exchange with labelled solvent is an equilibrium process¹⁷⁹. The percentage of label present in the substrate at equilibrium is the maximum incorporation possible in a single exchange. By using a large excess of solvent, or multiple exchanges with fresh solvent, the label content of the substrate may approach that of the solvent. For a substrate exchanging labile protons for ²H solvent atoms, the ²H content of

the substrate at equilibrium is given ¹⁷⁹ by:

$$\gamma = 1 - \left(\frac{x}{x+y} \right) \quad \text{eqn. (1)}$$

where a) γ = the molar ratio $\left(\frac{x^{2\text{H}}}{x^{\text{H}}} \right)$, for a substrate containing x exchangeable protons, $x^{2\text{H}}$ of which have been exchanged at equilibrium, leaving x^{H} unexchanged (i. e. $x = x^{\text{H}} + x^{2\text{H}}$)

and b) y = the number of ^2H atoms in the solvent available for exchange.

For n exchanges eqn. (1) becomes :

$$\gamma_n = 1 - \left(\frac{x}{x+y} \right)^n \quad \text{eqn. (2)}$$

For example in the exchange of dehydromethionine (0.1 g, 6.8×10^{-4} mol) where $x = 4$ ($\text{SCH}_3 + \text{NH}$) in $\text{CH}_3\text{O}^2\text{H}$ (1.2 cm^3 , 2.66×10^{-2} mol, 40 mol excess) where $y = 1$, eqn. (2) becomes:

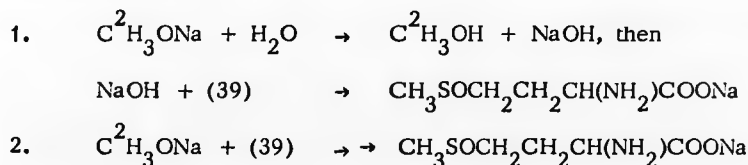
$$\gamma_n = 1 - \left(\frac{4}{40+4} \right)^n \quad \text{eqn. (3)}$$

For $n = 1$, $\gamma_1 = 0.91$. Thus one exchange in $\text{CH}_3\text{O}^2\text{H}$, 100 atom %, gives [methyl- $^2\text{H}_3$] dehydromethionine, 91 atom %. For 2 exchanges (i. e. $n = 2$) $\gamma_2 = 0.99$ and so the dehydromethionine product will have an isotopic content of 99 atom %.

The ^2H content of the methan[^2H] ol obtained by the method of Ref. 178, was $97\% \pm 1\%$ as determined by 220 MHz ^1H n. m. r. analysis. Because a

solvent excess of 43 fold was used in the preparation of methyl labelled (39), the maximum label incorporation possible in one exchange was 88% ($\gamma_1 = 0.91$; $0.91 \times 97\% = 88\%$) and in two exchanges was 96% ($\gamma_2 = 0.992$, $0.992 \times 97\% = 96\%$).

Experiments described in chapter 6 show that exchange of the methyl protons in dehydromethionine in $^2\text{H}_2\text{O}$ containing NaO^2H , is accompanied by rapid formation of methionine sulphoxide. In [$^2\text{H}_4$] methanol containing 2 mol % $\text{C}^2\text{H}_3\text{ONa}$, the methyl protons of (39) exchange with $t_{1/2} = \underline{\text{ca}}$ 3 min, but the observed rate of reaction is non linear. This indicates consumption of the basic catalyst is occurring. Two reactions which would consume the catalyst are :



The exchange reactions were therefore conducted under strictly anhydrous conditions, and using only a small amount (2 mol %) of catalyst.

Using 2 mol % CH_3ONa in $\text{CH}_3\text{O}^2\text{H}$, 97 atom %, (S)-[methyl- $^2\text{H}_3$] methionine, 93 atom % was obtained from (1R,3S)dehydromethionine, via two exchanges in 43 fold excess of solvent, and treatment with H_2S . This represents 97% of the maximum incorporation available using this solvent at this excess. The reaction of the catalyst with dehydromethionine presumably accounts for both the failure of the exchange reactions to reach equilibrium and the presence of a small quantity of methionine sulphoxide in the product. In preliminary experiments, where the incorporation of ^2H into (35) was ca

50 - 80%, ^1H n. m. r. was used to analyse isotopic content. For higher incorporations of ^2H it was better to use mass spectroscopy on a volatile derivative of the labelled methionine. The N-trifluoroacetyl butyl ester of methionine (41)¹⁸⁰ which could be easily purified by fast column chromatography¹⁶⁹ was found to be suitable. Analysis of the mass spectrum of N-trifluoroacetyl-(S)-[methyl- $^2\text{H}_3$] methionine butyl ester (42) compared with that of (41) indicated an overall ^2H content of 93% for the labelled methionine (35) prepared as described above. The major source of ^1H in this material was (S)-[methyl- $^2\text{H}_2$] methionine, and the products overall isotopic composition was 81% [$^2\text{H}_3$], 16% [$^2\text{H}_2$], 1% [$^2\text{H}_1$] and 1% [$^2\text{H}_0$].

Preparation of methyl labelled (S)-methionines via exchange in (1R,3S) dehydromethionine is both rapid and economical. The procedure is especially suited to small scale preparations of doubly-methyl labelled materials, e.g. $^{13}\text{C}_3\text{H}_3\text{SCH}_2\text{CH}_2\text{CH}(\text{NH}_2)\text{COOH} \rightarrow ^{13}\text{C}^2\text{H}_3\text{SCH}_2\text{CH}_2\text{CH}(\text{NH}_2)\text{COOH}$. It avoids the use of expensive double labelled methyl iodides, and is much more amenable to synthesis on a 50 or 100 mg scale than sodium/liquid ammonia reactions. Material doubly labelled with two different isotopes of hydrogen could be prepared by partial exchange of, for example [methyl- $^2\text{H}_3$] dehydromethionine in tritiated water, which would give [methyl- $^2\text{H}/^3\text{H}$] methionine on treatment with H_2S .

3-D EXPERIMENTAL

1. Preparation of (S)-[methyl-¹³C] methionine, 91 atom %, by alkylation of the sodium salt of homocysteine (cf ref. 171)

Using dry apparatus, liquid ammonia (ca 400 cm³) was distilled from a small quantity of sodium (ca 0.5 g) onto (S)-methionine (9.08 g, 0.0609 mol) over 1 h under dry N₂. During 30 min lithium (1.28 g, 0.185 mol) was added in three portions to the refluxing solution, which was maintained under an atmosphere of dry nitrogen. The resulting blue solution was stirred, and after 1.5 h the colour of the solution had been discharged. [¹³C] methyl iodide (9.5 g, 0.0665 mol), 91 atom %, was added, and the reaction boiled under reflux for 2 h, when an Ellman's test on a 0.5 cm³ aliquot of the solution showed the presence of free SH. A further portion of labelled methyl iodide (0.5 g, 0.0035 mol) was added, and the solution was boiled under reflux for another hour. An Ellman's test at this time still showed the presence of free SH, and a further portion of labelled methyl iodide (0.4 g, 0.0028 mol) was added. After refluxing the solution for a further hour, no free SH could be detected by Ellman's test. The solvent was removed by surrounding the reaction with a bath at 25°C, and passing a stream of nitrogen through the solution (2 h). The yellow residue was dissolved in water (200 cm³) and evaporated to dryness under reduced pressure (50°C, 20 mm Hg). This procedure was repeated twice. The solid residue was dissolved in water (400 cm³) and the pH of the solution reduced to 5.7 by the addition of hydrochloric acid (5 M). Evaporation to ca 30 cm³ gave white crystals, which redissolved on boiling, and after the addition of boiling ethanol (600 cm³) the solution was stored at -20°C for 12 h. The crystalline solid which formed was collected

at the pump, and washed with ice cold ethanol/water (10 cm^3), ethanol ($2 \times 10 \text{ cm}^3$), and with ether ($2 \times 10 \text{ cm}^3$). Air drying gave (S)-[methyl- ^{13}C] methionine (7.55 g, 83%), 91 atom %, as a white crystalline solid, m. p. $273 - 277^\circ\text{C}$, δ (90 MHz, D_2O , TSS) 2.1 (0.9 x 3 H, d, $^{13}\text{C}-^1\text{H} = 138.6 \text{ Hz}$, $^{13}\text{CH}_3\text{S}$; and 0.1 x 3 H, s, $^{12}\text{CH}_3\text{S}$), 2.28 (2 H, m), 2.7 (2 H, m), and 4.22 (1 H, t) p.p.m., pure by t.l.c. (system 1, Rf 0.45), no detectable free SH (Ellman's test), $[\alpha]_{\text{D}}^{22} = 20.8^\circ$ (C = 1.15, 1 M HCl) cf authentic (S)-methionine used as starting material $[\alpha]_{\text{D}}^{22} = 21.5^\circ$ (C = 1.1, 1 M HCl), containing no Li ions (flame test).

Ion exchange chromatography

The mother liquor from the above recrystallisation was evaporated to dryness under reduced pressure, and the residue dissolved in hydrochloric acid (20 cm^3 , 2.5 M). This solution was chromatographed on a column of Dowex 50 x 8 ion exchange resin (500 g) using hydrochloric acid (2.5 M) as eluate. The progress of the column was monitored by optical rotation measurements on the neat eluant. Early fractions were coloured (I_2) and later fractions contained (S)-[methyl- ^{13}C] methionine, constituting a symmetrical band. The fractions containing product were combined and evaporated to dryness under reduced pressure (60°C , 20 mm Hg) and the solid residue was redissolved in water (50 cm^3). The pH of this solution was adjusted to 5.7 by the addition of saturated lithium hydroxide solution. Evaporation and recrystallisation as described above gave pure (S)-[methyl- ^{13}C] methionine, 91 atom %, (0.73 g, 8%), as a white crystalline solid.

2. Preparation of (1R,3S)dehydromethionine¹⁷²

In a dry box, sodium methoxide solution (70 cm³ of a 1.15 M solution, 0.0805 mol) was added to a suspension of (S)-methionine (6.01 g, 0.04 mol) in absolute methanol (370 cm³). After swirling to dissolve the methionine, iodine (10.19 g, 0.0403 mol) was added, and the dark red solution was sealed and stirred for 12 h. The almost colourless solution which resulted, was evaporated under reduced pressure to ca 20 cm³, and chromatographed on a column of silica gel (240 g, 80 - 200 mesh) using methanol as eluant. The progress of the column was followed by t.l.c. on the neat eluant (system 2). Iodine was eluted first, followed by methionine and dehydromethionine. The fractions containing (1R,3S)dehydromethionine (39) were combined and evaporated under reduced pressure to give (39) (4.97 g, 84%) as a white powder, the material darkens and decomposes with gas evolution (in agreement with the literature¹⁵⁴), at 206 - 208°C, pure by t.l.c. (system 2, Rf 0.15), δ (D₂O, TSS) 2.72 (2 H, m), 2.82 (3 H, s), 3.72 (2 H, m) and 4.3 (1 H, 2 x d) p.p.m., 220 MHz ¹H n.m.r. δ (D₂O, TSS) 2.47 (1 H, m), 2.83 (3 H, s), 2.90 (1 H, m), 3.63 (1 H, m), 3.85 (1 H, m) and 4.4 (1 H, 2 x d) p.p.m., $[\alpha]_D^{25} = 79.6^\circ$ (C = 1.23, H₂O). The compound was stored in vacuo and recrystallised twice from methanol/ether, immediately before use. Samples were discarded after ca 20 days, because t.l.c. showed same decomposition to methionine sulphoxide after ca 40 days.

3. Preparation of methan[²H]ol¹⁷⁸

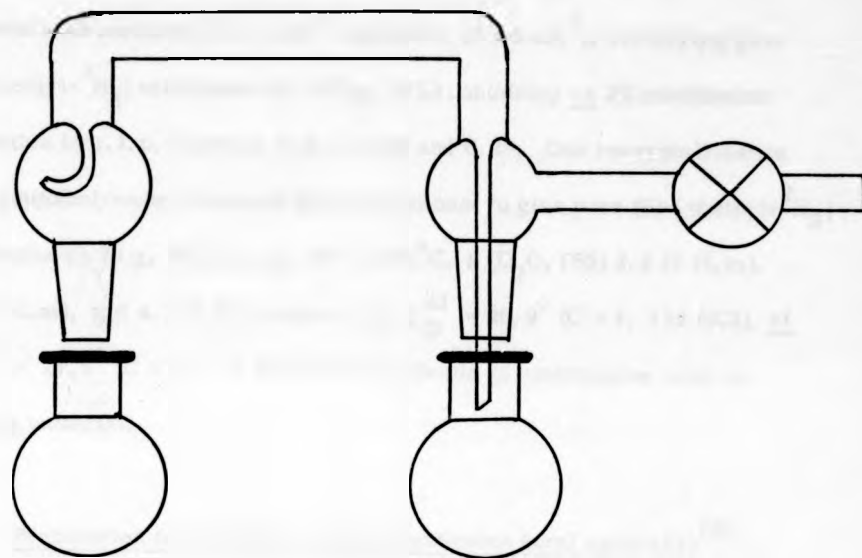
²H₂O (25 g, 25 cm³, 1.25 mol), 99.8 atom %, was added to a mixture of dimethylcarbonate (100 g, 94 cm³, 0.11 mol) and dimethylsulphate (4 g,

3 cm³, 0.03 mol), and the resulting solution was boiled under reflux, with the exclusion of atmospheric moisture, for 96 h. The product was fractionally distilled, and the fraction boiling between 66 and 72°C was collected. The methan[²H]ol thus obtained (ca 50 cm³) was fractionally distilled twice from clean sodium (0.03 g), under anhydrous conditions, the colourless fraction boiling between 65.5 and 67°C being collected :
 220 MHz ¹H n.m.r. (neat, TMS) δ 3.35 (3 H, s) and 4.8 (0.026 x 1 H, s) p.p.m. The ²H content was determined by the addition of known percentages of CH₃OH and rerunning the n.m.r. spectrum. ²H content 97% ± 1%.

4. Preparation of (S)-[methyl-²H₃] methionine via proton exchange of (1R,3S)dehydromethionine

The following reaction was carried out under scrupulously dry conditions, all manipulations being performed in a dry box, or in sealed, apparatus. (1R,3S)dehydromethionine (0.5 g, 3.4 m mol) was dissolved in dry methan[²H]ol (6 cm³), 97 atom %, and sodium methoxide in methan[²H]ol (57 μl of a 1.2 M solution, 0.068 m mol)* was added. The reaction flask was incorporated into the apparatus shown in Fig. 3-D-1, and the sealed apparatus stored for 3 h at 37°C. Solvent was distilled off by evacuating the system (10 mm Hg) and cooling the receiver in liquid nitrogen. The apparatus was transferred to a dry box, and opened. Methan[²H]ol (6 cm³), 97 atom %, and sodium methoxide in methan[²H]ol (57 μl of a 1.2 M solution, 0.068 mol) were added, and the reaction was sealed and stored for a further 3 h at 37°C. The reaction flask was opened, and immediately H₂S was rapidly bubbled through the solution, for 1.5 min. The resulting suspension was diluted with methanol

* See chapter 2 for preparation of sodium methoxide in deuterio methanol



REACTION
FLASK

RECEIVER

FIG 3-D-1

FIG. 3-D-1

Apparatus used to perform the exchange of (1R, 3S)-dehydromethionine in methan[²H]ol with the total exclusion of atmospheric moisture.

(4 cm³) and the solid product was collected at the pump. After washing the solid with methanol (3 x 6 cm³) and ether (2 x 6 cm³), air drying gave (S)-[methyl-²H₃] methionine (0.497 g, 97%) containing ca 2% methionine sulphoxide by t.l.c. (system 1) Rf's 0.45 and 0.29. One recrystallisation from methanol/water removed this contaminant to give pure (S)-[methyl-²H₃] - methionine (0.39 g, 76%), m.p. 274 - 276°C, δ (D₂O, TSS) 2.2 (2 H, m), 2.6 (2 H, m), and 4.2 (1 H, t) p.p.m., $[\alpha]_D^{23} = 20.9^\circ$ (C = 1, 1 M HCl), cf $[\alpha]_D^{22} = 21.5^\circ$ (C = 1.1, 1 M HCl) for authentic (S)-methionine used as starting material.

5. Preparation of N-trifluoroacetyl methionine butyl ester (41)¹⁸⁰

A solution of dry hydrogen chloride in absolute butanol (HCl/BuOH) was prepared by the dropwise addition of dry acetyl chloride (17.6 cm³, 19.5 g, 0.25 mol) to ice cold, stirred absolute butanol (200 cm³), under an atmosphere of dry nitrogen. HCl/BuOH (40 cm³ of a 1.25 M solution, 0.05 mol) was added to (S)-methionine (0.6 g, 0.004 mol), and the resulting suspension was heated on a steam bath for 2 h, with the exclusion of atmospheric moisture. The solvent was removed from the cooled solution by evaporation under reduced pressure (50°C, 20 mm Hg, the last traces being removed at 35°C, 0.1 mm Hg), to provide a pale yellow oil. This oil was taken up in dichloromethane (40 cm³) and trifluoroacetic anhydride (4 cm³, 5.8 g, 0.028 mol) was added. The yellow reaction mixture was sealed, and stored at 25°C for 90 min, during which time it became colourless. The solvent, and excess trifluoroacetic anhydride, were removed under reduced pressure (30°C, 20 min then 30°C 1 mm) to give a pale yellow oil (ca 1 g). T.l.c. (silica gel, CH₂Cl₂,

H_2SO_4 spray and char) showed a major spot (ca 90%) at Rf 0.38, and a minor impurity at the origin (ca 10%). The derivative (41) (0.4 g) was purified by flash column chromatography, according to Ref. 169, on a 20 mm column of Merck Kieselgel 60 (200 - 400 mesh), eluting with CH_2Cl_2 . The progress of the column was monitored by t.l.c. (see above). Evaporation of the relevant fractions gave pure (41), (0.35 g) as a pale yellow oil, pure by t.l.c. (see above, Rf 0.38), δ 0.95 (3 H, m), 1.5 (4 H, m), 2.1 (3 H, s), 2.2 (2 H, m), 4.2 (2 H, t), 4.8 (1 H, t) and 7.4 (1 H, Br. s) p.p.m., m/e 227 (100%), 301 (45%, M^+ for (41)), 302 (7%) and 303 (2%).

6. Preparation of N-trifluoroacetyl-(S)-[methyl- $^2\text{H}_3$] methionine butyl ester (42)

Derivative (42) was prepared from (S)-[methyl- $^2\text{H}_3$] methionine (0.03 g, 0.19 m mol), as described for derivative (41) above. Flash column chromatography on a 5 mm dia. column of Merck Kieselgel 60 (200 - 400 mesh), as above, gave (42) as a pale yellow oil (0.36 g, 60%), m/e 227 (100%), 301 (0.6%), 302 (0.6%), 303 (9.6%), and 304 (47%, M^+ for (42)), indicating a ^2H content of 93%, and a composition of 81% [$^2\text{H}_3$], 16% [$^2\text{H}_2$], 1% [$^2\text{H}_1$] and 1% [$^2\text{H}_0$] methionine.

CHAPTER 4

SYNTHESES OF METHIONINES LABELLED AT C-3, AND/OR C-4WITH ^2H OR ^{13}C 4-A INTRODUCTION

The potential value of methionines labelled at C-3, and/or C-4 in studies of the metabolic fate of methionine's alkyl chain was outlined in chapter 1. The syntheses of several of these methionines are described.

A synthesis of stereospecifically labelled rac-[3,4- $^2\text{H}_2$] methionine i.e. $\text{CH}_3\text{SCH}^2\text{HCH}^2\text{HCH}(\text{NH}_2)\text{COOH}$ of known relative configuration at C-3 and C-4 has been developed, and is described. The stereochemical identity, and homogeneity of the material synthesised has been demonstrated by conversion to a 5 membered heterocycle, followed by 220 M Hz ^1H n. m. r. analysis, and by studies on the mechanisms of the reactions involved in the synthesis. The route used for the above methionine has been adapted for the synthesis of rac-[3,4- $^{13}\text{C}_2$] methionine. A synthesis of this material from $^{13}\text{CO}_2$, via [$^{13}\text{C}_2$] acetylene is described. A literature method¹⁶⁶ has been extended to prepare rac-[2- ^2H], [2,3- $^2\text{H}_3$] and [3- $^2\text{H}_2$] methionine from rac-methionine and $^2\text{H}_2\text{O}$.

4-B THE SYNTHETIC ROUTES

4-B-1 rac-[3,4-¹³C] METHIONINE

1. Rationale

The synthesis of a material designated as "[3-¹⁴C] methionine" was reported in 1974¹⁸¹, using the route depicted in Fig. 4-B-1. One step of this synthesis is the conversion of 2-(methylthio)-[1-¹⁴C] ethanol into "1-chloro-2-(methylthio)-[1-¹⁴C] ethane", by trioctylphosphine/ CCl_4 reagent. An additional product from this reaction could be 1-chloro-2-(methylthio)-[2-¹⁴C] ethane, produced due to the intermediacy of a symmetrical species with homotropic C-1 and C-2 (e.g. the 1-methylthiiranium ion, Fig. 5-A-1). In order to test this possibility, 2-(methylthio)-[1-¹³C] ethanol (43) was synthesised as described below, and its conversion into 1-chloro-2-(methylthio)ethane (44) studied by ¹³C n.m.r. spectroscopy. The results of these studies are presented in chapter 5, and show that for $\text{R}_3\text{P}/\text{CCl}_4$ (R=octyl, phenyl, or isopropyl) the chloride (44) produced is a ca 1:1 mixture of C-1 and C-2 labelled material. A similar label distribution was observed when other reagents were used to convert alcohol (43) to chloride (44), indicating that chloride (44) is not a suitable intermediate for the production of C-3 or C-4 labelled methionines. However, a mixture of C-3 and C-4 labelled material i.e. rac-[3,4-¹³C] - methionine is useful in biosynthetic studies, and its synthesis via the chloride (44) is convenient.

2. Synthesis of 2-(methylthio)-[1-¹³C] ethanol (Fig. 4-B-2)

Using a literature procedure for ¹⁴C labelled material¹⁸², [1-¹³C] - acetic acid, 90 atom % was converted into ethyl[1-¹³C] bromoacetate 90 atom %.

FIG 4-B-1

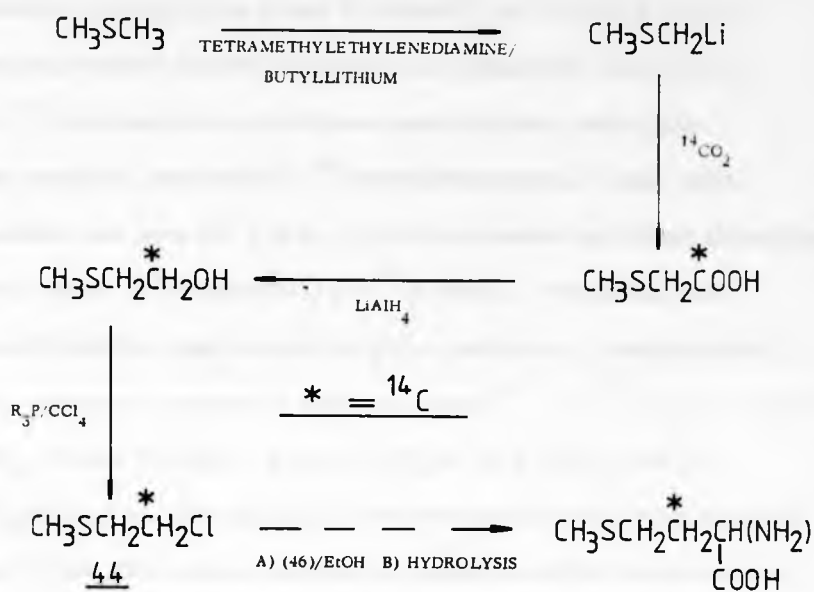


FIG 4-B-2

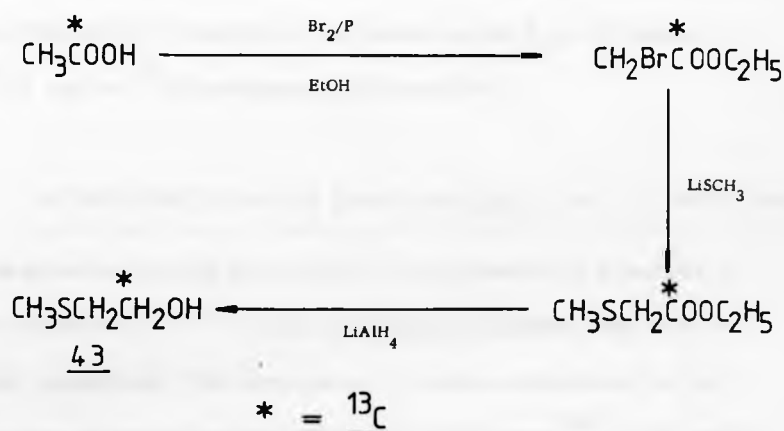


FIG. 4-B-1 THE ROUTE USED BY PICHAT AND BEAUCOURT TO PREPARE "[3- ^{14}C] METHIONINE

FIG. 4-B-2 THE ROUTE USED TO PREPARE 2-(METHYLTHIO)-[1- ^{13}C] ETHANOL

This material was diluted to either 11.4 atom %, or 20 atom %, by the addition of unlabelled ethylbromoacetate (see chapter 5). Reaction of ethyl[1-¹³C] bromoacetate with lithium methylthiolate, under phase transfer catalysis, gave ethyl[1-¹³C] methylthioacetate, in good yield. This material was pure by ¹H n.m.r., and was reduced by lithium aluminium hydride in ether, to 2-(methylthio)-[1-¹³C] ethanol. The product was fractionally distilled, and was pure by g.l.c. and n.m.r., compared with authentic material, prepared by a different route¹⁸³. Its ¹³C n.m.r. spectrum in CDCl₃, showed 3 singlets, at δ 14.2 (CH₃S), 35.6 (SCH₂), and 59.4 (CH₂CH₂OH) p.p.m., the signal from the enrichment carbon being enhanced ca 10 or 20 fold above natural abundance, depending on the isotopic content. Treatment of this alcohol with any of the reagents described in chapter 5 leads to a ca 1:1 mixture of C-1 and C-2 labelled chloride (44). Condensation of this mixture of chlorides with sodium salt of diethyl acetamidomalonate (46) in ethanol, followed by hydrolysis of the product gives a ca 1:1 mixture of rac-[3-¹³C] and [4-¹³C] methionines, (29) and (30).

4-B-2 STEREOSPECIFICALLY LABELLED RAC-[3,4-²H₂] METHIONINES

The chloride (44) has been used as an intermediate in a number of methionine syntheses^{184, 185}. Modern syntheses avoid this compound due to its vesicant properties. The preparation of β-chloro thio ethers by the addition of alkyl sulphenyl halides to olefins is well known¹⁸⁶. This addition proceeds in a trans manner, and the observed stereochemistry has been explained by the proposal of an intermediate thiliranium ion in the reaction^{187, 188} (Figs. 4-B-3 and 4-B-4). It has been observed however that these

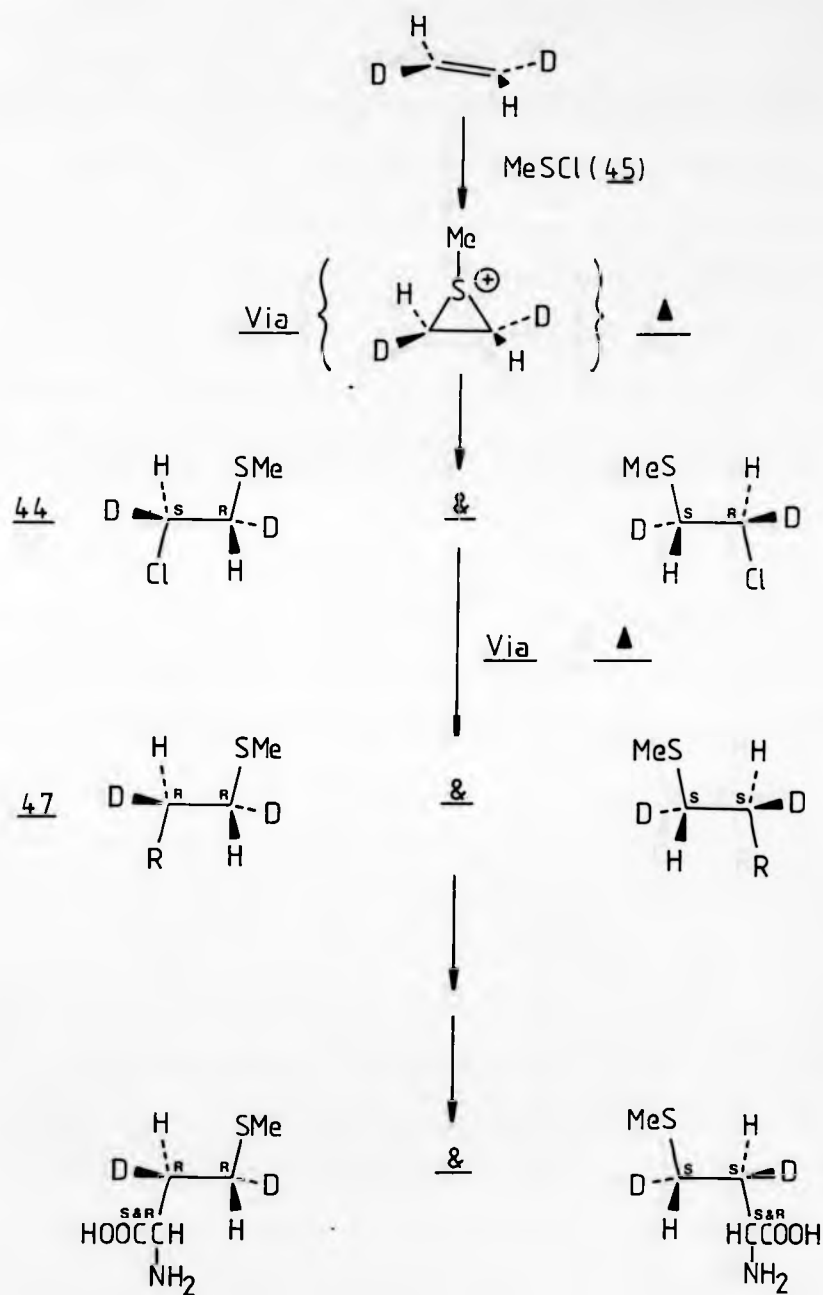


FIG 4-B-3

THE SYNTHESIS OF RAC-(3R,4R)METHIONINE

R = ETHYL 2-ACETAMIDO-4-ETHOXYCARBONYL

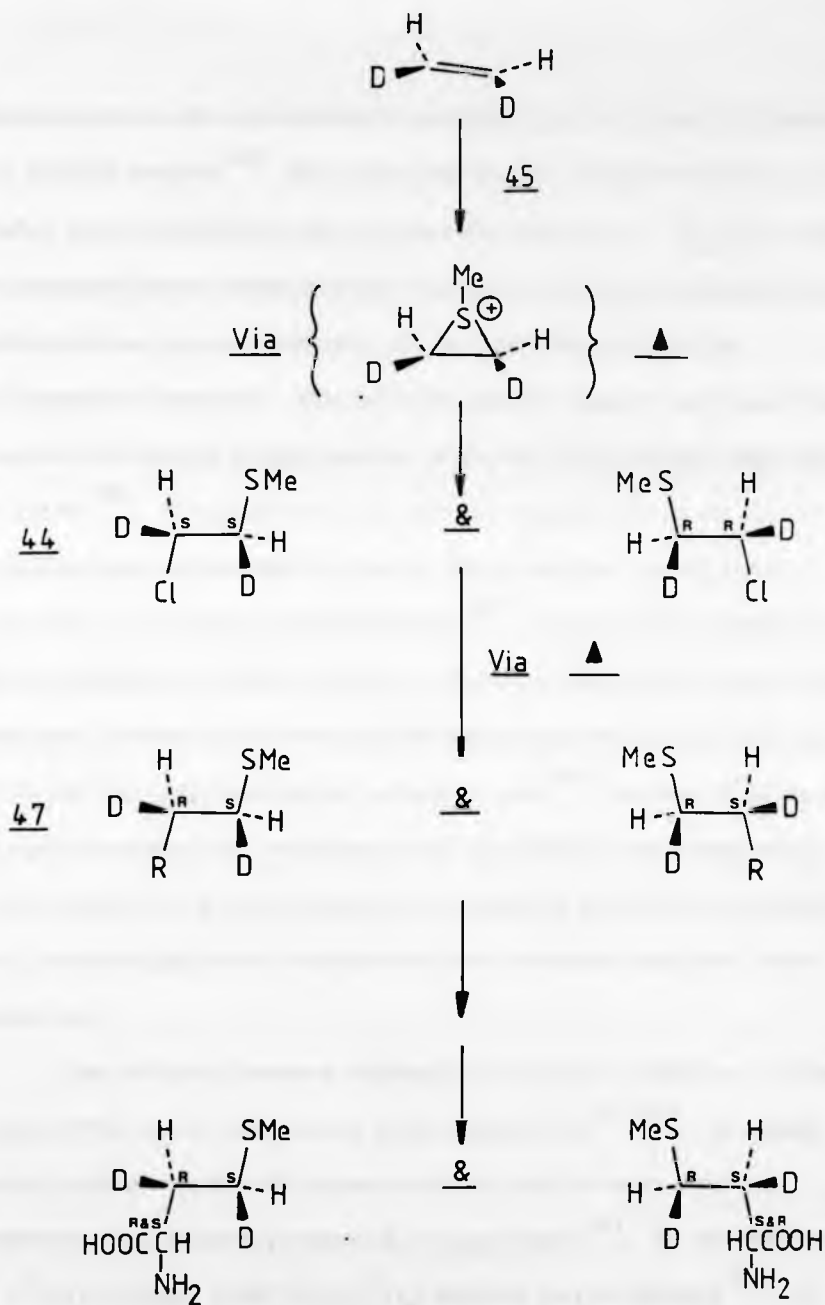


FIG 4-B-4

THE SYNTHESIS OF RAC-(3R,4S)METHIONINE

R = ETHYL 2-ACETAMIDO-4-ETHOXYCARBONYL

reactions do not show the expected characteristics, if catinoid intermediates are actively involved¹⁸⁹. The typical addition of a sulphenyl halide to an olefin, at low temperature and in a non-polar solvent (e.g. CH_2Cl_2), is not accompanied by the rearrangements or stereoconversions normally associated with carbonium ion intermediates, and no competition by external nucleophiles is observed. This led to the postulate that the thiiranium ions involved are strongly bridged species, with very little positive charge localised on carbon¹⁹⁰. This postulate is however not supported by recent results which indicate that nucleophilic attack on thiiranium ions occurs almost exclusively at carbon, rather than sulphur¹⁸⁹. It is currently considered that the addition of sulphenyl halides to olefins, at low temperatures and in non-polar solvents proceeds through bridged species of an unspecified nature, which are less polar than normal thiiranium ions¹⁸⁹. In polar solvents, and at higher temperatures, reactions typical of carbonium ion intermediates can be observed e.g. rearrangements, competition by external nucleophiles, etc., which suggests the involvement of true thiiranium ions under these conditions.

The addition of methane sulphenyl chloride (45) to ethylene, in a non polar solvent at low temperature, gives chloride (44)^{187, 191}. By analogy to the addition of halide (45) to unsymmetrical olefins under identical conditions, this reaction proceeds in a trans manner¹⁹¹. By the reduction of [²H₂] acetylene, E and Z-[1,2-²H₂] ethylene may be obtained¹⁹². It was considered that stereospecifically labelled rac-[3,4-²H₂] methionines could be obtained from E and Z-[1,2-²H₂] ethylene via chloride (44) (produced by reaction of halide (45) with the labelled ethylenes).

Trial experiments showed that bubbling ethylene through a solution of halide (45) in CH_2Cl_2 at -20°C gave chloride (43), by ^1H n.m.r. analysis. The same reaction performed at high temperatures (e.g. 0°C and 20°C) gave mixtures of products, which included chloride (44). E and Z-[1,2- $^2\text{H}_2$] ethylenes were obtained from [$^2\text{H}_2$] acetylene by modification of literature procedures¹⁹², and their purity was checked by i.r. analysis¹³⁸.

The reaction between E or Z-[1,2- $^2\text{H}_2$] ethylene and halide (45) in CH_2Cl_2 at low temperature will produce a racemic mixture of chlorides (44). The stereochemical course of these additions are depicted in Figs. 4-B-3 and 4-B-4. The reaction is shown to proceed via the 1-methylthiiranium ion for convenience, but it should be recognised that other less polar intermediates (having homotropic C-1 and C-2) may be involved under these conditions¹⁸⁹. In order to reduce the possibility of racemisation at C-1 by $\text{S}_{\text{N}}2$ attack of Cl^\ominus on chloride (44) (i.e. $\text{CH}_3\text{SCH}_2\text{CH}_2\text{CH}_2\text{Cl} + \text{CH}_3\text{SCH}_2\text{CH}_2\text{CH}_2\text{Cl}^\ominus \rightarrow \text{CH}_3\text{SCH}_2\text{CH}_2\text{CH}_2\text{Cl} + \text{Cl}^\ominus + \text{CH}_3\text{SCHCH}_2$) the material was carried through to the next stage of the reaction at once.

Condensation of chloride (44) with salt (46) followed by hydrolysis of the product with aqueous acid, was considered the most convenient route from chloride (44) to rac-[3,4- $^2\text{H}_2$] methionine. Other routes are available (e.g. Ref. 193, 194) but were not considered suitable. The reaction between chloride (44) and salt (46) would be expected to proceed via a thiiranium ion, under the conditions used (NaOEt/EtOH). Any reaction of salt (46) with chloride (44) via an $\text{S}_{\text{N}}2$ pathway would lead to products of inverted configuration at C-3. It was therefore necessary to ensure that an $\text{S}_{\text{N}}2$ pathway was not followed. Under good $\text{S}_{\text{N}}2$ conditions, KI/acetone) chloride (44)

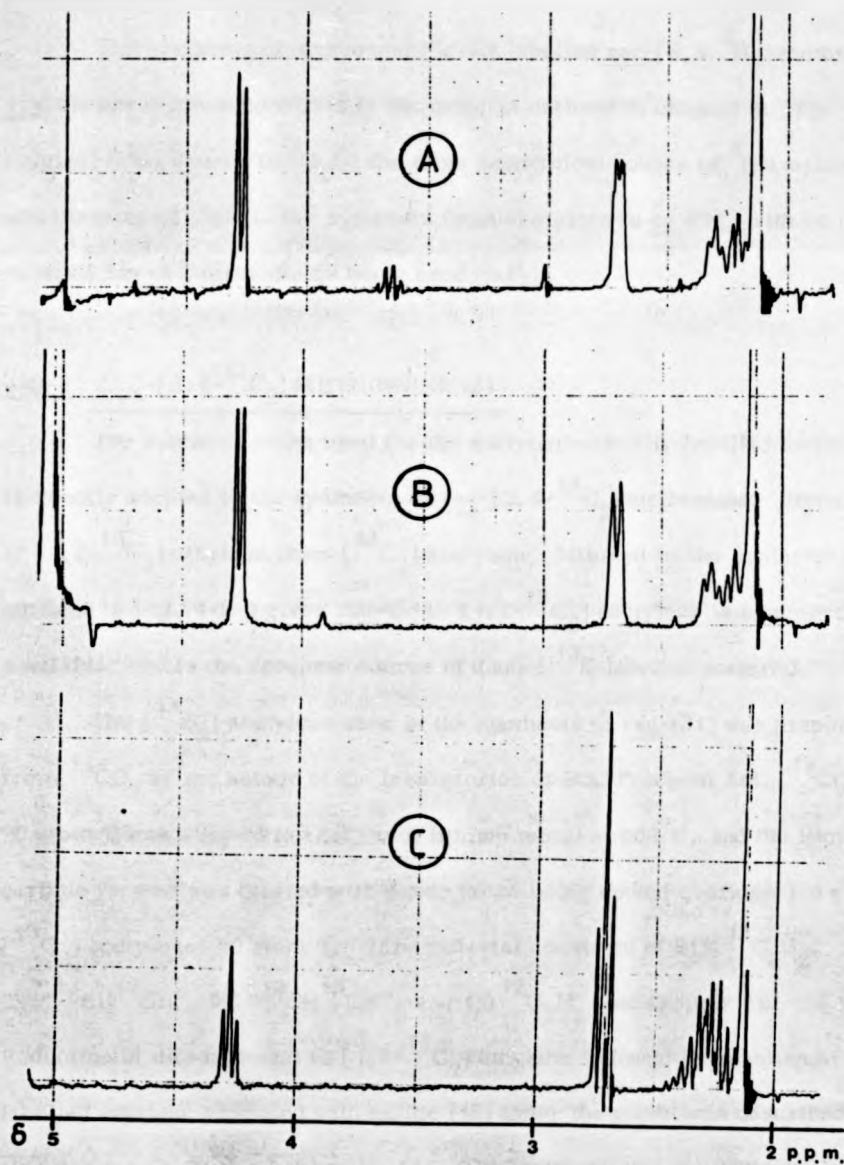
reacts ca 1.5 times as fast as butyl chloride (under the same conditions)¹⁹⁵. Butyl chloride can only react via an S_N2 pathway, whereas chloride (44) can react either in this way, or via a thiiranium ion intermediate. A competitive reaction was conducted, in which chloride (44) and butyl chloride were in competition for a limited amount of salt (46), under the conditions used to prepare the labelled adducts. The product from this reaction was analysed by ^{13}C n.m.r. spectroscopy and consisted of the adduct derived from chloride (44) and unreacted salt (46), with no evidence of product derived from butyl chloride. This demonstrates that the rate of reaction of chloride (44) is much faster than that of butyl chloride, under these conditions, and indicates that chloride (44) reacts via neighbouring group participation of sulphur (i.e. via a thiiranium ion intermediate).

The stereochemistry of the ethyl 2-acetamido-2-ethoxycarbonyl-4-(methylthio)butanoates (47) obtained from the reactions involving the chlorides derived from E and Z-[1,2- 2H_2] ethylene, is shown in Figs. 4-B-3 and 4-B-4. Hydrolysis of derivative (47) gives rac-methionine directly. It can be seen that the rac-methionine derived from E-[1,2- 2H_2] ethylene will be a mixture of (2R,3R,4R); (2S,3R,4R); (2R,3S,4S); and (2S,3S,4S)-[3,4- 2H_2] - methionines. This material will be abbreviated as rac-(3R,4R)methionine. The material derived from Z-[1,2- 2H_2] ethylene will be a mixture of (2R,3R,4S); (2S,3R,4S); (2R,3S,4R); and (2S,3R,4S)-[3,4- 2H_2] methionines, and this will be abbreviated as rac-(3R,4S)methionine.

It was considered desirable to prove the relative configurations of the labelled methionines, rac-(3R,4R) methionine, and rac-(3R,4S)methionine, by physical methods if possible. The protons at C-3 and C-4 of methionine

are diastereotopic. However due to the large number of conformations which the open chain amino acid can assume, they are not distinguishable by ^1H n. m. r. By conversion of methionine to a cyclic compound, the number of possible conformations would be greatly reduced, and it could be possible to distinguish individual proton resonances, and proton spin-spin couplings. Dehydromethionine (39) is easily prepared from methionine, is a 5 membered hetrocycle, and has diastereotopic protons at C-4 and C-5 (derived from C-3 and C-4 of methionine). The complete analysis of the ^1H n. m. r. spectrum of hetrocycle (39) would enable the spectra of the dehydromethionines derived from the stereospecifically labelled methionines to be predicted. The methionines could be distinguished as the dehydromethionines derived from rac-(3R,4R)methionine would be expected to show only cis couplings, and those derived from rac-(3R,4S)methionine only trans couplings (Fig. 6-B-3). The analysis of the 220 MHz ^1H n. m. r. spectrum of dehydromethionine proved more difficult than expected, owing to the equal values of 1 cis and 1 trans coupling constant, in the C-4, C-5 fragment, which meant conventional analysis techniques could not be used (see chapter 6). The non-equal values of the other two coupling constants in the ethane fragment (C-4, C-5), allows distinction between the two labelled dehydromethionines. By decoupling experiments it was possible to demonstrate the stereochemical homogeneity of the two labelled hetrocycles (39) derived from the two stereospecifically labelled methionines. The results of these ^1H n. m. r. experiments are presented, and their relevance to the ^1H n. m. r. spectrum of dehydromethionine discussed, in chapter 6. The 220 MHz ^1H n. m. r. spectra of rac-(3R,4S)methionine and rac-(3R,4R)methionine are presented in Fig. 4-B-5a and b. The 220 MHz

FIG. 4-B-5



220 MHz ^1H n. m. r. of (A) rac-(3R, 4S)methionine, (B) rac-(3R, 4R)-methionine and (C) rac-methionine

^1H n. m. r. spectra of the dehydromethionines obtained from these materials are shown in Figs. 6-B-4 and 6-B-5, and the results of the decoupling experiments in Fig. 6-B-6.

The synthesis of stereospecifically labelled rac-[3,4- $^2\text{H}_2$] methionine via the above route conforms to the criteria outlined in chapter 1. The original label source is $^2\text{H}_2\text{O}$, the most economical source of ^2H available, and the overall yield of the synthesis from acetylene is ca 40%, with no purification of intermediates being necessary.

4-B-3 RAC-[3,4- $^{13}\text{C}_2$] METHIONINE (31)

The synthetic route used for the stereospecifically labelled methionines is readily adapted to the synthesis of rac-[3,4- $^{13}\text{C}_2$] methionine. Preparation of [1,2- $^{13}\text{C}_2$] ethylene from [$^{13}\text{C}_2$] acetylene, followed by the synthetic route outlined in Fig. 4-B-3 gives rac-(31). [1,2- $^{13}\text{C}_2$] acetylene is commercially available, and is the cheapest source of double ^{13}C labelled material.

The [$^{13}\text{C}_2$] acetylene used in the synthesis of rac-(31) was prepared from $^{13}\text{CO}_2$ by the author at the laboratories of BOC Prochem Ltd. $^{13}\text{CO}_2$ 90 atom % was allowed to react with lithium metal at 600°C , and the lithium carbide formed was treated with water (after being cooled overnight) to give [$^{13}\text{C}_2$] acetylene, 90 atom %. This material consisted of 81% $^{13}\text{C}_2\text{H}_2$, 9% " $^{13}\text{CH}^{12}\text{CH}$ ", 9% " $^{12}\text{CH}^{13}\text{CH}$ ", and 1% $^{12}\text{C}_2\text{H}_2$ (see chapter 1). Reduction of this material to [1,2- $^{13}\text{C}_2$] ethylene followed by reaction of the labelled ethylene produced with halide (45) under the conditions described above, gave [1,2- $^{13}\text{C}_2$] chloride (44). This material has the same distribution of ^{13}C as the acetylene used in its preparation, and the ^{13}C n. m. r.

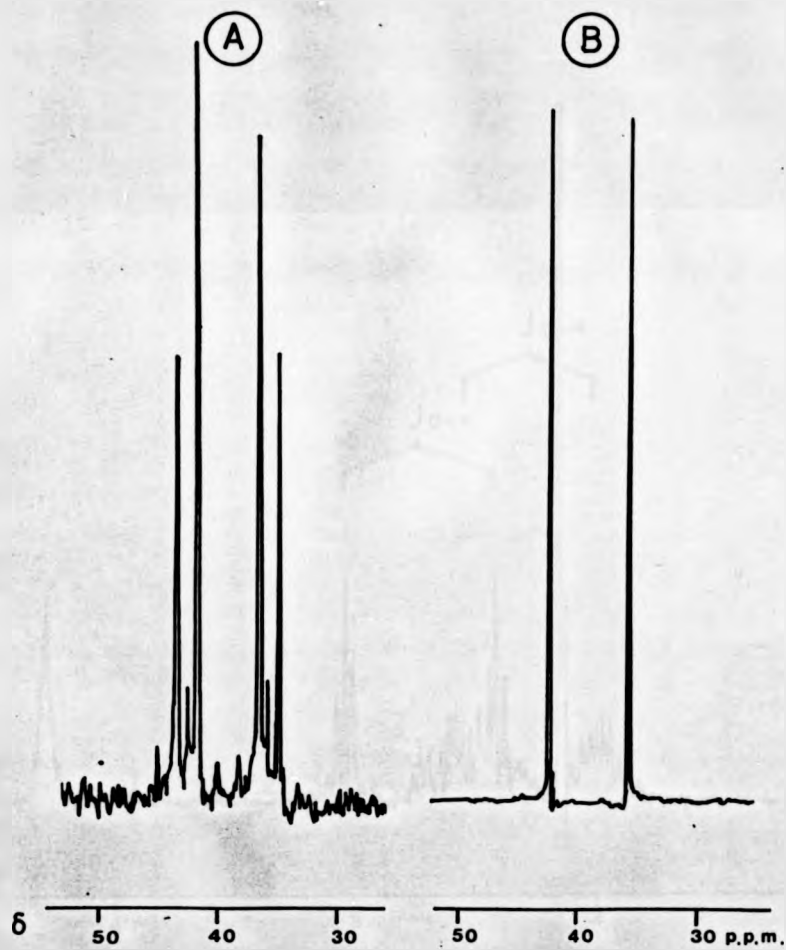
spectrum of the methylene carbons of this material is shown in Fig. 4-B-6. An intense doublet astride a singlet is observed for each carbon resonance. The doublets arise from the 81% ^{13}C - ^{13}C species present, and the singlets from the ^{13}C single labelled species (9% for each methylene position). Condensation of this chloride (44) with salt (46), and hydrolysis of the product with aqueous acid, gave rac-(31). The 220 MHz ^1H n.m.r. spectrum of this material (Fig. 4-B-7) shows ^{13}C satellites for each methylene signal, amounting to ca 90% of the total intensity of each signal (cf the 220 MHz ^1H n.m.r. spectrum of unlabelled rac-methionine, Fig. 4-B-5c). Owing to the very small chemical shift difference between the two methylene carbon resonances in methionine, ca 6 Hz, no splitting was observed in the ^{13}C n.m.r. spectrum of rac-(31).

The synthesis of rac-(31) described above fulfils the criteria of chapter 1. The cheapest source of double labelled material is used and the overall yield of the synthesis is ca 40%, with no purification of intermediates being necessary.

4-B-4 RAC-[2- ^2H], [2,3- $^2\text{H}_3$] AND [3- $^2\text{H}_2$] METHIONINE

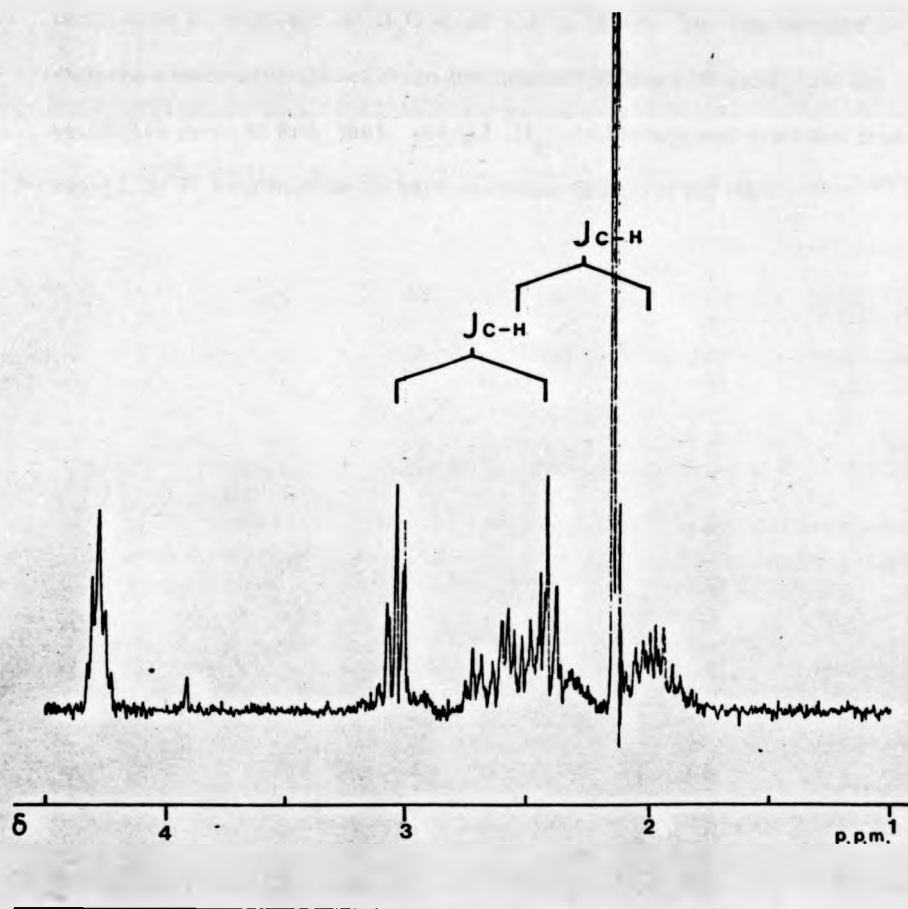
In 1974 a method for the selective deuteration of amino acids at either the α or β positions was published¹⁶⁶. This procedure made use of the observation that the relative rate of exchange of α and β protons in amino acid/aluminium (III)/pyridoxal complexes was pH dependent. It was observed that at pH ca 5.1, the relative rates of α and β exchange are almost equal, whereas at pH ca 10.2 α exchange is much faster than β exchange. This observation was applied to the preparation of α , $\alpha\beta$, and

FIG. 4-B-6



The methylene region of the ^{13}C n.m.r. spectra of A) $\text{CH}_3\text{S}^{13}\text{CH}_2^{13}\text{CH}_2\text{Cl}$ and B) $\text{CH}_3\text{SCH}_2\text{CH}_2\text{Cl}$. The vertical scales are ca 1:50, A:B

FIG. 4-B-7



220 MHz ^1H n. m. r. spectrum of *rac*-[3,4- $^{13}\text{C}_2$] methionine, showing ^{13}C - ^1H , cf Fig. 4-B-5, C

by back exchange in H_2O , β ^2H labelled alanine, amino butyric acid, and valine. Trial reactions showed that selective deuteration of methionine under similar conditions could be observed, and followed by ^1H n. m. r. spectroscopy. Reaction times were optimised to produce rac-[2- ^2H]-methionine by exchange in $^2\text{H}_2\text{O}$ at pH 10.2 ($p^2\text{H}$ 10.6¹⁶⁶) and rac-[2,3- $^2\text{H}_3$]-methionine by exchange in $^2\text{H}_2\text{O}$ at pH 5.2 ($p^2\text{H}$ 5.6¹⁶⁶). The labelled materials were crystallised from the reaction mixture in good yield (no yields are given in Ref. 166). rac-[3- $^2\text{H}_2$]-methionine was produced from rac-[2,3- $^2\text{H}_2$]-methionine by back exchange in H_2O at pH 10.2.

4-C EXPERIMENTAL

4-C-1 SYNTHESIS OF 2-(METHYLTHIO)-[1-¹³C] ETHANOL

1. ethyl[1-¹³C] bromoacetate, 90 atom %¹⁸²

Bromine (7.5 cm³, 23.4 g, 0.29 mol) was added dropwise with stirring, to an ice cold suspension of purified red phosphorus (0.355 g, 0.014 mol) in [1-¹³C] acetic acid (2 g, 0.033 mol), 90 atom %. The mixture was boiled under reflux for 5 h, with the exclusion of atmospheric moisture, and then cooled to 0°C. Anhydrous ethanol (6 cm³, 4.68 g, 0.1 mol) was added, and the mixture was warmed to 20°C over 20 min. After dilution with water (20 cm³) the solution was extracted with ether (3 x 40 cm³). The combined organic extracts (containing ethyl [1-¹³C]bromoacetate, Br₂, and HBr) were quickly washed with small portions of saturated aqueous sodium carbonate, (total 150 cm³) until the washings were just alkaline, 5% aqueous sodium thiosulphate, until the organic phase was just colourless, and finally with 50% aqueous potassium bromide. Drying and evaporation under reduced pressure gave ethyl[1-¹³C] - bromoacetate, (5.1 g, 93%), 90 atom %, as a colourless liquid: δ 1.3 (3 H, t), 3.8 (2 H, d, _J¹³C-¹H = 6 Hz) and 4.18 (2 H, 2 x q, _J¹³C-¹H = 4 Hz) p.p.m. This material was diluted to either 11.4 atom % or 20 atom % by addition of unlabelled ethyl bromoacetate.

2. Ethyl[1-¹³C] methylthioacetate, 20 atom %

To an ice cold solution of lithium methylthiolate (10.8 g, 0.2 mol)* in water (100 cm³) was added a solution of ethyl[1-¹³C] bromoacetate (22.6 g, 0.134 mol) 20 atom %, and benzyl tri-n-butyl ammonium bromide (1.28 g,

* prepared from lithium hydroxide (8.4 g, 0.2 mol) and methane thiol (9.6 g, 0.2 mol)

0.0041 mol; 3 mol %) in dichloromethane (125 cm³). The mixture was vigorously stirred at room temperature for 2 h, when analysis of the organic layer by ¹H n.m.r. showed no residual BrCH₂- at δ 3.8 p.p.m. The layers were separated, and the aqueous phase extracted with dichloromethane (2 x 25 cm³). The combined organic phases were dried, and evaporated at reduced pressure, the bulk of the solvent (ca 80%) being removed at 25 mm Hg and 25°C. The remaining solvent was distilled off at room pressure to avoid loss of volatile product. The residue (15.81 g) contained phase transfer catalyst (1.28 g) (which was removed by dissolving the product in ether and filtering off the insoluble catalyst), and ethyl [1-¹³C] methylthioacetate (14.53 g, 81%) 20 atom %: δ 1.3 (3 H, t), 2.2 (3 H, s), 3.2 (2 H, s) and 4.2 (2 H, q) p.p.m. ¹³C satellites arising from signals at δ 3.2 and 2.2 p.p.m. (each ca 10% intensity of main peak), ¹³C - ¹H = 8 and 54 Hz respectively are present.

3. 2-(methylthio)-[1-¹³C] ethanol, 20 atom %

Lithium aluminium hydride (3.3 g, 0.086 mol) was added to anhydrous ether (100 cm³) and the suspension was boiled under reflux for 1.5 h, under an atmosphere of dry nitrogen. A solution of ethyl[1-¹³C] methylthioacetate (14.5 g, 0.108 mol) 20 atom % in anhydrous ether (50 cm³) was added dropwise over 20 min, and the reaction was boiled under reflux for a further 20 h. The excess of lithium aluminium hydride was destroyed by the careful addition of water (3.3 cm³) followed by 15% aqueous sodium hydroxide (3.3 cm³) and finally a further portion of water (10 cm³). The precipitated solids were filtered off under suction, and washed well with ether (4 x 20 cm³).

The combined organic filtrates were dried and evaporated under reduced pressure, to provide a pale yellow oil. Fractional distillation gave 2-(methylthio)-[1- ^{13}C] ethanol, 20 atom %, as a colourless liquid (7.47 g, 74%), b.p. 70 - 71 $^{\circ}\text{C}$ at 20 mm Hg, pure by g.l.c., (20% D.E.G.S., 150 $^{\circ}\text{C}$, N_2 at 20 cm^3/min) R_t identified to that of authentic material produced by another route¹⁸³: δ 2.12 (3 H, s), 2.7 (2 H, t), 3.12 (1 H, s) and 3.74 (2 H, t) p.p.m. ^{13}C satellites arising from the signal at δ 3.74 p.p.m. (each ca 10% of the main peak) $^{13}\text{C}-^1\text{H} = 144.5$ Hz are present. ^{13}C n.m.r. (C_6H_6 , TMS) δ 14.2 ($-\text{SCH}_3$), 35.6 ($\text{S}-\text{CH}_2-$) and 59.4 ($\text{CH}_2-\text{CH}_2\text{OH}$) p.p.m. The signal at δ 59.4 p.p.m. corresponding to the enriched carbon is ca 20 fold enhanced over natural abundance.

2-(methylthio)ethanol¹⁸³

Methane thiol (25 g, 22 cm^3 , 0.52 mol) was distilled into a solution of ethylene oxide (22 g, 19 cm^3 , 0.50 mol) in dry methanol (200 cm^3) maintained at -15 $^{\circ}\text{C}$, over 45 min. Sodium methoxide (1 cm^3 of a 1 M solution, in dry methanol) was added, and the reaction was stirred for 3 h. Evaporation under reduced pressure and fractional distillation of the residue gave 2-(methylthio)ethanol as a colourless liquid (40.15 g, 86% lit. 75%) b.p. 76-68 $^{\circ}\text{C}$ at 20 mm Hg, pure by g.l.c. (20% D.E.G.S., 150 $^{\circ}\text{C}$, N_2 at 20 cm^3/min); δ 2.1 (3 H, s), 2.65 (2 H, t), 3.63 (2 H, t), and 4.5 (1 H, s) p.p.m.; ^{13}C n.m.r. (C_6H_6 , TMS) δ 14.2 (CH_3S), 35.6 (CH_2S), and 59.6 (CH_2OH) p.p.m.

1-chloro-2-(methylthio)ethane*^{183a}

Purified thionyl chloride (20.4 g, 12.7 cm^3 , 0.17 mol) in ethanol-free chloroform (10 cm^3), was added over 1.5 h to a solution of 2-(methylthio)-

*the compound is a potent vesicant and should be handled with care.

ethanol (15 g, 0.16 mol) in ethanol-free chloroform (14 cm³), which was boiling under reflux. The reaction was boiled under reflux for 4 h and subsequently cooled. Solvent was distilled off at room pressure, and the residue was fractionally distilled to give 1-chloro-2-(methylthio)ethane, as a colourless liquid (15.77 g, 88%), b.p. 56°C at 30 mm Hg; δ 2.16 (3 H, s), 2.79 (2 H, t) and 3.67 (2 H, t) p.p.m.; ¹³C n.m.r. (C₆H₆, TMS) δ 14.9 (CH₃S), 35.5 (SCH₂), and 42.1 (CH₂Cl) p.p.m.

4-C-2 SYNTHESIS OF RAC-(3R, 4R)METHIONINE AND RAC-(3R, 4S)METHIONINE

1. Preparation of methanesulphenyl chloride ¹⁹³

Sulphuryl chloride (14.3 g, 0.1 mol) was added dropwise over 15 min to stirred dimethyl disulphide (9.4 g, 0.1 mol) at -15°C. The reaction was allowed to warm to room temperature over 2 h, and the product was then fractionally distilled. The fraction boiling between 30 and 32°C at 110 mm Hg was collected, the receiver being cooled at -78°C, to give methanesulphenyl chloride as a deep orange liquid (12 g, 70%), δ 2.9 (s) CH₃SCl, n.b. absence of peak at δ 2.40 (s) due to (CH₃S)₂. The compound has a very strong smell and was stored in a well sealed container at -20°C. The material was used within 2 days of preparation, residues being destroyed with sodium hypochlorite solution.

2. E-[1, 2-²H₂] ethylene ¹⁹²

Using all dry apparatus, ²H₂O (10 cm³, 10 g, 0.5 mol), 99.8 atom %, was added dropwise to calcium carbide (10 g, 0.15 mol), and the [²H₂] acetylene which was generated was collected by the displacement of water

from a 2200 cm³ conical flask (for a detailed description of this procedure see Ref. 192). The addition of ²H₂O was stopped when 1800 cm³ of acetylene had been collected (ca 1 cm³ ²H₂O remaining). The remaining water was removed from the flask using an aspirator, and replaced by chromium II solution (250 cm³)*. The flask was sealed and shaken at room temperature in an orbital shaker, the progress of the reaction being monitored by g.l.c. at intervals (chromosorb 101, N₂ at 20 p.s.i., 25°C). After 40h no acetylene could be detected by g.l.c. The ethylene was transferred to the vacuum line, and dried to give Z-[1,2-²H₂] ethylene (1700 cm³), free from E-[1,2-²H₂] ethylene by i.r. analysis¹³⁸: i.r. (gas cell, 50 mm Hg) 3060 (m.Br), 2300 (m, shoulder), 1300 (m, sh, pqr), 730 (m, sh), and 680 (m, sh) cm⁻¹, n.b. absence of peaks at 1342 and 842 cm⁻¹ characteristic of E-[1,2-²H₂] ethylene.

3. Z-[1,2-²H₂] ethylene¹⁹²

Using all dry apparatus, ²H₂O (10 cm³, 0.5 mol) 99.8 atom %, was added dropwise to calcium carbide (10 g, 0.15 mol), and the [²H₂] - acetylene which was generated was collected by the displacement of water

* potassium chromium IV sulphate dodecahydrate (100 g, 0.2 mol) was dissolved in hydrochloric acid (250 cm³, 3M) and 2% zinc amalgam (50 cm³) added. The mixture was shaken under an atmosphere of CO₂ until the colour changed from dark green, to translucent blue. The amalgam was run off and the chromium II solution used at once.

from a 2200 cm³ conical flask, containing a zinc/copper couple*.

The addition of ²H₂O was stopped when 1500 cm³ of [²H₂] acetylene had been collected. The remaining water was removed with an aspirator and replaced by dilute hydrochloric acid (38.5 cm³ of a 2 M solution). The flask was vented to atmospheric pressure after ca 2 min, and shaken in an orbital shaker for 2 days, when g.l.c. (chromosorb 101, N₂ at 20 p. s. i., 25°C) showed no residual acetylene. The ethylene was transferred to the vacuum line, and dried, to give E-[1,2-²H₂] ethylene (1100 cm³), free from Z-[1,2-²H₂] ethylene by i. r. analysis¹³⁸: i. r. (gas cell, 50 mm Hg), 3060 (m, Br), 2250 (m, Br), 2300 (m, shoulder), 1342 (m, sh, pqr), and 842 (s, sh, pqr) cm⁻¹, n.b. absence of peaks at 1300, 990, and 727 cm⁻¹, characteristic of Z-[1,2-²H₂] ethylene.

4. Rac-(3R,4S)methionine (Fig. 4-B-4)

a) Reaction between Z-[1,2-²H₂] ethylene and methanesulphenyl chloride

A solution of methanesulphenyl chloride (12 g, 0.145 mol) in CH₂Cl₂ (100 cm³) was placed in a 500 cm³ flask, cooled to -25°C, and evacuated to ca 2 cm Hg on the vacuum line. Dry Z-[1,2-²H₂] ethylene was admitted, and the solution swirled. The admission of ethylene was continued until no

* prepared by the addition of finely powdered zinc (53.5 g, 0.82 mol) to copper-sulphate (13 g, 0.05 mol) in water (200 cm³). The solid couple was filtered off and used at once.

further absorption occurred, and the solution was colourless (ca 40 min), whilst the temperature of the solution was kept below -25°C (total ethylene absorbed 4025 cm^3 , 0.179 mol). The solvent CHCl_2 was distilled off at 20°C and 50 mm Hg, during 25 min to give 1-chloro-2-(methylthio)-[1,2- $^2\text{H}_2$] ethane (44), (which was used immediately for the next stage of the reaction) δ 2.12 (3 H, s), 2.8 (1 H, broad, $d, J = 9\text{ Hz}$), and 3.63 (1 H, broad, $d, J = 9\text{ Hz}$) p.p.m. (cf $\text{CH}_3\text{SCH}_2\text{CH}_2\text{Cl}$, 4-C-1, 5) containing ca 9% CH_2Cl_2 by ^1H n.m.r. analysis.

b) Reaction between [1,2- $^2\text{H}_2$] chloride (44) and salt (46)

Using all dry apparatus, sodium (2.5 g, 0.108 mol) was dissolved in anhydrous ethanol (75 cm^3) by boiling the mixture under reflux with the exclusion of atmospheric moisture. Diethylacetamido malonate (22.5 g, 0.166 mol) was added and the mixture boiled under reflux until a clear solution resulted. The product from the above reaction (consisting of ca 0.145 mol chloride (44) + CH_2Cl_2) was added and the reaction was boiled under reflux, with the exclusion of atmospheric moisture for 5 h. After cooling to 0°C , the precipitated sodium chloride was filtered off under suction, and washed with cold ethanol ($3 \times 10\text{ cm}^3$). The combined filtrates were evaporated to dryness under reduced pressure to provide an orange oil. This oil was extracted into CH_2Cl_2 ($3 \times 100\text{ cm}^3$), the solution filtered through a pad of celite, under suction, and was evaporated under reduced pressure, (20°C , 20 mm Hg then 20°C , 1 mm Hg) to give ethyl 2-acetamido-2-ethoxycarbonyl-4-(methylthio)-[1,2- $^2\text{H}_2$] butanoate (31.7 g, 93%) (47), as an orange crystalline mass, δ 1.25 (6 H, t), 2.04 (3 H, s), 2.08 (3 H, s), 2.50 (2 H, broad, q) 4.3 (4 H, q) and 6.9 (1 H, broad, s) p.p.m. This

material was hydrolysed without further purification.

c) Hydrolysis of the above [1,2-²H₂] (47) to rac-(3R,4S)methionine

The above product (31.7 g, 0.108 mol) was dissolved in hydrochloric acid (122 cm³ of a 2 M solution) and the mixture boiled under reflux for 6 h, with magnetic stirring. A further quantity of acid was added (122 cm³ of a 2 M solution) and the reaction boiled under reflux for a further 3 h. The orange solution which resulted was evaporated to dryness under reduced pressure (90°C, 15 mm Hg), to give an orange oil. This oil was taken up in water (60 cm³) and the pH of the solution was adjusted to 7 by the addition of saturated aqueous LiOH. The solution was again evaporated to dryness under reduced pressure, and the solid residue was dissolved in boiling water (80 cm³) and boiling absolute ethanol added (1000 cm³). The solution was stored at -20°C for 36 h, and the solid precipitate was then filtered off under suction and washed with cold ethanol (3 x 20 cm³) and ether (3 x 20 cm³). Air drying gave rac-(3R,4S)methionine (9.12 g, 0.0612 mol) as a pink crystalline solid, treatment with charcoal, and recrystallisation from aqueous ethanol gave rac-(3R,4S)methionine as brilliant white crystals, m.p. 275-277°C, 220 MHz ¹H n.m.r., δ (D₂O/DCl, TSS) 2.12 (3 H, s), 2.25 (1 H, broad, 2 x t), 2.7 (1 H, broad, d, *J* = 4.8 Hz) and 4.28 (1 H, d), pure by t.l.c. (system, 1, R_f 0.45). The overall yield of this synthesis, based on ethylene was 34%.

5. Rac-(3R,4R)methionine (Fig. 4-B-3)

The reaction between E-[1,2-²H₂] ethylene and methanesulphenyl chloride was performed in the same manner as 4a above, producing [1,2-²H₂] chloride (44) from E-[1,2-²H₂] ethylene (1100 cm³) and methane-

sulphenyl chloride (3.5 g, 0.042 mol). Evaporation of the CH_2Cl_2 solvent gave [1,2- $^2\text{H}_2$] chloride (44) (which was used immediately in the next stage of the reaction), δ 2.15 (3 H, s), 2.8 (1 H, broad, m) and 3.6 (1 H, broad, m) p.p.m. Reaction of this chloride (44) with salt (46), prepared from sodium (0.84 g, 0.036 mol), anhydrous ethanol (30 cm^3) and diethyl acetamido malonate (7.40 g, 0.034 mol), under the conditions described above, 4b, gave ethyl 2-acetamido-2-ethoxy-carbonyl-4-(methylthio)-[1,2- $^2\text{H}_2$] butanoate (47)(ca 8 g) as an orange oil, δ 1.25 (6 H, t), 2.04 (3 H, s), 2.08 (3 H, s), 2.50 (2 H, broad, q), 4.3 (4 H, q) and 6.9 (1 H, broad, s) p.p.m. This material was hydrolysed as described above, 4c, to give rac-(3R,4R)methionine, (2.21 g, 0.015 mol) as white crystals, m.p. 275-278, δ ($\text{D}_2\text{O}/\text{DCl}$, TSS) 2.12 (3 H, s), 2.25 (1 H, broad, 2 x t), 2.7 (1 H, broad, d, $J = 7.3$ Hz), and 4.28 (1 H, d) p.p.m., pure by t.l.c. (system 1, Rf 0.45). The overall yield of this synthesis based on ethylene was 30%.

4-C-3 SYNTHESIS OF RAC-[3,4- $^{13}\text{C}_2$] METHIONINE

1. Preparation of [$^{13}\text{C}_2$] acetylene 90 atom % (carried out at BOC Prochem Ltd)

Lithium metal (21 g, 3 mol) was placed in a stainless steel reaction vessel, which was evacuated to 0.01 mm Hg. The reactor was heated to ca 600°C using a propane burner, and the vacuum seal checked. $^{13}\text{CO}_2$ (8000 cm^3 , 0.35 mol) 90 atom %, was admitted at a rate sufficient to maintain a vigorous reaction (total time ca 3 h). After cooling overnight, water (3000 cm^3) was added slowly and the [$^{13}\text{C}_2$] acetylene which was generated was passed through a trap at -73°C, and then collected in a series of traps cooled at -196°C, on the vacuum line. When no further gas evolution was

evident, the gaseous contents of the reactor were pumped through a trap at -73°C into a trap at -196°C on the vacuum line. The $[\text{}^{13}\text{C}_2]$ acetylene 90 atom %, was purified by pumping through traps at -73°C , -95°C , and into a trap at -196°C . Yield 3600 cm^3 , 88%.

2. Preparation of $[\text{}^{13}\text{C}_2]$ ethylene, 90 atom %

The $[\text{}^{13}\text{C}_2]$ acetylene prepared as described above was reduced by the method of 4-C-2, 2 to $[\text{}^{13}\text{C}_2]$ ethylene.

3. Preparation of $[1,2\text{-}^{13}\text{C}_2]$ chloride (44)

Reaction of $[\text{}^{13}\text{C}_2]$ ethylene (2000 cm^3 , 0.089 mol) with methane-sulphenyl chloride (6 g, 0.073 mol) by the method described above (4-C-2, 4a) and evaporation of solvent, gave $[1,2\text{-}^{13}\text{C}_2]$ chloride (44) (ca 7.6 g) as a colourless liquid, δ 2.15 (0.8 x 3 H, d, $J^{13}\text{C}\text{-}^1\text{H} = 4\text{ Hz}$), 2.8 (2 H, 2 x M, $J^{13}\text{C}\text{-}^1\text{H} = 47\text{ Hz}$), and 3.6 (2 H, 2 x M, $J^{13}\text{C}\text{-}^1\text{H} = 48\text{ Hz}$) p.p.m. ^{13}C n.m.r. (C_6H_6 , TMS) δ 35.7 (d, $J^{13}\text{C}\text{-}^{13}\text{C} = 37\text{ Hz}$) and 42.1 (d, $J^{13}\text{C}\text{-}^{13}\text{C} = 37\text{ Hz}$) p.p.m., each doublet being astride a singlet of ca 10% total signal intensity, arising from single labelled species, see discussion, and cf Fig. 4-B-6.

4. Reaction between $[1,2\text{-}^{13}\text{C}_2]$ (44) and salt (46)

The reaction between the above chloride (44) and salt (46), (prepared from sodium (1.38 g, 0.06 mol), ethanol (30 cm^3), and diethyl acetamidomalonate (12.15 g, 0.055 mol)), was performed as described above (4-C-2, 4b). Workup gave $[1,2\text{-}^{13}\text{C}_2]$ adduct (47) (15.5 g, 0.053 mol) as a brown oil,

δ 1.25 (6 H, t), 2.08 (6 H, 3 x s), 2.4 (4 H, m) 4.3 (4 H, q), and 6.9 (1 H, s, broad) p.p.m. This material was hydrolysed without further purification.

5. Preparation of $\text{rac-[3,4-}^{13}\text{C}_2\text{] methionine}$

The above $[3,4-^{13}\text{C}_2]$ adduct (47) was hydrolysed as described above (4-C-2, 4c) and work up gave $\text{rac-[3,4-}^{13}\text{C}_2\text{] methionine}$ (4.76 g, 0.032 mol) as faintly yellow crystals. Treatment with charcoal, and recrystallisation from aqueous ethanol gave $\text{rac-[3,4-}^{13}\text{C}_2\text{] methionine}$ as white crystals, m.p. 274-276°C, 220 MHz ^1H n.m.r., δ ($\text{D}_2\text{O}/\text{DCl}$, TSS), 2.15 (3 H, d), 2.28 (0.9 x 2 H, 2 x m, $^1\text{J}^{13}\text{C-}^1\text{H} = 135$ Hz, and 0.1 x 2 H, t), and 4.29 (1 H, m) p.p.m., pure by t.l.c. (system 1, Rf 0.45), (Fig. 4-B-7 cf Fig. 4-B-5c).

The overall yield of this synthesis based on $[^{13}\text{C}_2]$ ethylene was 39%.

4-C-4 SYNTHESIS OF $\text{RAC-[2-}^2\text{H]}$, $[2,3-^2\text{H}_3]$ AND $[3-^2\text{H}_2]$ METHIONINE¹⁶⁶

1. $[2,3-^2\text{H}_3]$ methionine

Rac-methionine was lyophilised from $^2\text{H}_2\text{O}$. In a dry box, $^2\text{H}_2\text{O}$ (14 cm^3) 99.8 atom % was added to lyophilised rac-methionine (1.45 g, 9.7 mmol), followed by aluminium sulphate solution in $^2\text{H}_2\text{O}$ (1 cm^3 of a 0.25 M soln., 2.5 mole %, prepared according to ref. 166), and pyridoxal hydrochloride (0.19 g, 0.1 mmol). Under an atmosphere of N_2 , the pH of the solution was adjusted to 5.2 ($\text{p}^2\text{H } 5.6$)¹⁶⁶ by the addition of NaO^2H (5M). The mixture was refluxed under an atmosphere of nitrogen for 24 h, the progress of the exchange reaction being followed by ^1H n.m.r. analysis, and then boiling ethanol (150 cm^3) was added. After standing the solution

at -20°C for 12 h, the crystalline precipitate was collected at the pump, and was washed with cold ethanol/water ($2 \times 10 \text{ cm}^3$), and ether ($2 \times 10 \text{ cm}^3$). Air drying gave rac-[2,3- $^2\text{H}_3$] methionine (1.27 g, 87%) as a white crystalline solid, m.p. $275\text{-}277^{\circ}\text{C}$, δ (D_2O , TSS) 2.15 (3 H, s) and 2.65 (2 H, broad, s), p.p.m., n.b. absence of peaks at δ 2.75 and 3.95 p.p.m., ^2H content by 220 MHz ^1H n.m.r. analysis $\geq 98\%$, pure by t.l.c. (system 1, Rf 0.45).

2. Rac-[3- $^2\text{H}_2$] methionine

To rac-[2,3- $^2\text{H}_3$] methionine (1.0 g, 6.58 mmol) in H_2O (30 cm^3) was added aluminium sulphate solution (0.66 cm^3 of a 0.25 M solution, 2.5 mole %), and pyridoxal hydrochloride (0.136 g, 0.68 mmol). The pH of the solution was adjusted to 10.2 by the addition of sodium hydroxide solution (5 M). The rate of α exchange was monitored by ^1H n.m.r. analysis, and after standing for 18 h at 37°C , the reaction mixture was evaporated under reduced pressure to ca 10 cm^3 . Hydrochloric acid (5 M) was added until the pH fell to 5.3, and the precipitated solid was redissolved by boiling. Boiling ethanol (50 cm^3) was added, and the solution stored for 12 h at -20°C . The crystals which formed were collected at the pump, and were washed with ethanol ($2 \times 10 \text{ cm}^3$) and ether ($2 \times 10 \text{ cm}^3$). Air drying gave rac[3- $^2\text{H}_2$] methionine (0.96 g, 97%) as a white crystalline solid, m.p. $275\text{-}277^{\circ}\text{C}$, 90 MHz ^1H n.m.r., δ ($\text{D}_2\text{O}/\text{DCl}$, TSS), 2.15 (3 H, s), 2.65 (2 H, broad, s) and 4.05 (1 H, broad, s) p.p.m. pure by t.l.c. (system 1, Rf 0.46). Exact comparison of 90 MHz ^1H n.m.r. integrals gave a ^2H content of $\geq 94\%$ at C-3 and $\leq 8\%$ at C-2.

CHAPTER 5

THE CONVERSION OF 2-(METHYLTHIO)ETHANOL INTO 1-CHLORO-
2-(METHYLTHIO)ETHANE; A ^{13}C LABELLING STUDY

5-A INTRODUCTION

A synthesis of "rac-[3- ^{14}C] methionine" from $^{14}\text{CO}_2$ was published in 1974¹⁸¹, one step of which was the conversion of 2-(methylthio)-[1- ^{14}C]-ethanol into "1-chloro-2-(methylthio)-[1- ^{14}C] ethane" by trioctyl phosphine/ CCl_4 , (cf Fig. 4-B-1). An additional product from this reaction could be 1-chloro-2-(methylthio)-[2- ^{14}C] ethane, produced via the 1-methylthiiranium ion as shown in Fig. 5-A-1. To test this possibility, 2-(methylthio)-[1- ^{13}C] ethanol (43) was synthesised (see chapter 4), and its conversion into 1-chloro-2-(methylthio)ethane (44) by reagents of the type $\text{R}_3\text{P}/\text{CCl}_4$ (R=phenyl, isopropyl, and octyl) examined, by ^{13}C n.m.r. spectroscopy. This investigation showed that for each case that the product was a 1:1 mixture of 1-chloro-2-(methylthio)-[1- ^{13}C] ethane and 1-chloro-2-(methylthio)-[2- ^{13}C] ethane. Because it would be synthetically valuable to convert an alcohol such as 2-(methylthio)ethanol to the corresponding chloride without neighbouring group participation, other reagents were examined which perform the general conversion $\text{ROH} \rightarrow \text{RCl}$. The reagents chosen operate under very mild conditions, therefore minimising the possible thermal interconversion of 1-chloro-2-(methylthio)-[1- ^{13}C] ethane with its [2- ^{13}C] isomer, via the 1-methylthiiranium ion (Fig. 5-A-2). The results of an examination of 3 reagents are reported.

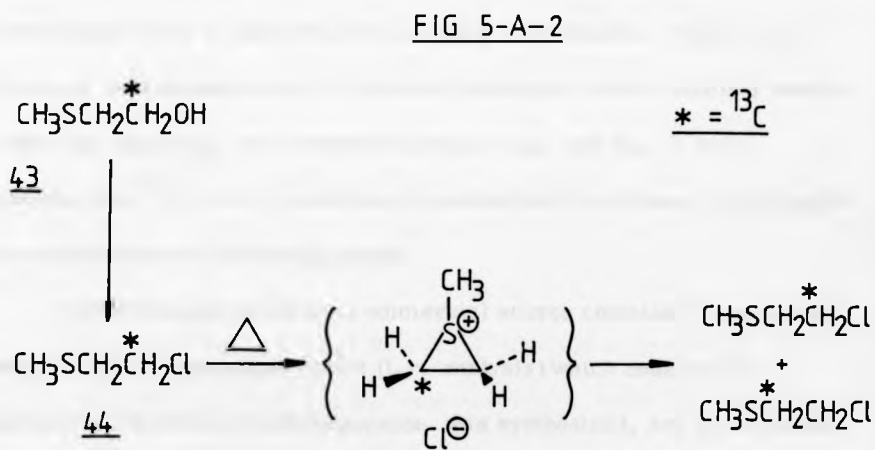
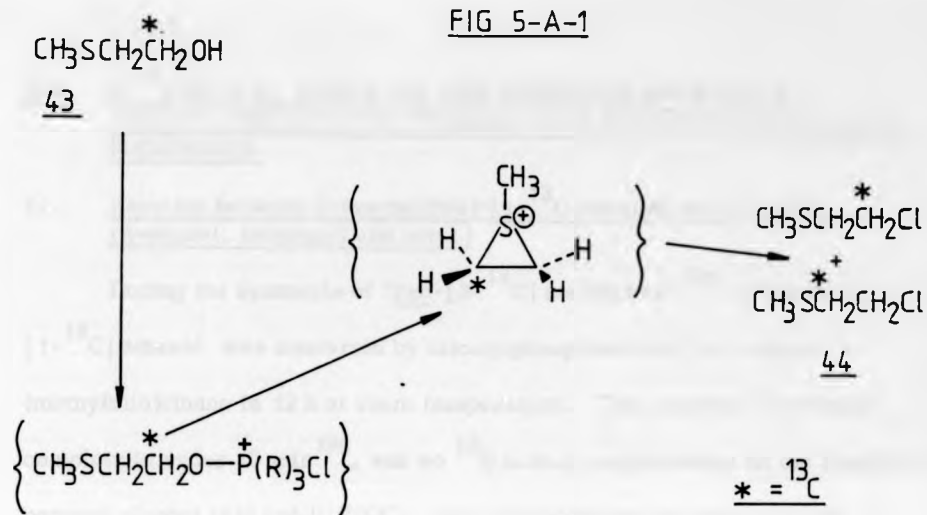


FIG. 5-A-1 THE RESULT OF THE INTERMEDIACY OF THE 1-METHYLTHIRANIUM ION IN THE CONVERSION $\text{ROH} \rightarrow \text{RCI}$ VIA $\text{R}_3\text{P}/\text{CCl}_4$

FIG. 5-A-2 POSSIBLE THERMAL INTERCONVERSION OF C-1 LABELLED (44) TO A MIXTURE OF C-1 AND C-2 LABELLED MATERIAL

5-B A ^{13}C N.M.R. STUDY OF THE REACTION BETWEEN 2-(METHYLTHIO)-[1- ^{13}C] ETHANOL AND CARBONTETRACHLORIDE/PHOSPHINES

1. Reaction between 2-(methylthio)-[1- ^{13}C] ethanol and $\text{R}_3\text{P}/\text{CCl}_4$ (R=phenyl, isopropyl and octyl)

During the synthesis of "rac-[3- ^{14}C] methionine"¹⁸¹, 2-(methylthio)-[1- ^{14}C] ethanol was converted by trioctylphosphine/ CCl_4 to 1-chloro-2-(methylthio)ethane in 12 h at room temperature. This reaction is probably complete in under 15 min¹⁹⁶, and so ^{13}C n.m.r. experiments on the reaction between alcohol (43) and $\text{R}_3\text{P}/\text{CCl}_4$ were performed at low temperatures, in order to follow the process. The observation that the product chloride (44) is a mixture of C-1 and C-2 labelled material could be due either to the intermediacy of the 1-methylthiiranium ion in its formation, (Fig. 5-A-1) or thermal rearrangement of C-1 labelled product to its C-2 labelled isomer on standing (again via the 1-methylthiiranium ion, see Fig. 5-A-2). Recording the ^{13}C n.m.r. spectrum of product as it is formed, might enable these possibilities to be distinguished.

Trioctylphosphine from a commercial source contained a significant quantity of trioctylphosphine oxide (i.r. analysis) which could not be separated. Therefore trioctylphosphine was synthesised, and by exclusion of oxygen at all times, a pure product was obtained (showing no P=O in its i.r. spectrum). For comparison, the reactions of alcohol (43) with triisopropyl phosphine and triphenyl phosphine/carbon tetrachloride reagents were also examined. Both triisopropyl phosphine and triphenyl phosphine were obtained commercially. Triphenyl phosphine, being relatively air-stable, was pure enough to use directly, whereas triisopropyl phosphine was distilled under nitrogen just before use (providing a material

showing no P=O in its i. r. spectrum). In the discussion which follows, peaks from the phosphine component of the reaction systems are ignored.

The reaction between alcohol (43) (prepared as in chapter 4) and triisopropyl or trioctyl phosphine/ CCl_4 was much faster than the reaction between alcohol (43) and triphenyl phosphine/ CCl_4 . For triphenyl phosphine the reaction was followed by recording ^{13}C n. m. r. spectra at intervals, over a long period at room temperature. The initial spectrum consisted of a single large peak at $\delta 59.4$ p. p. m., due to the labelled alcohol. Subsequent spectra show the disappearance of this peak, and the appearance of two additional peaks at $\delta 42.2$ and 35.4 p. p. m. corresponding to the two CH_2 groups of $\text{CH}_3\text{SCH}_2\text{CH}_2\text{Cl}$. The equal intensity of these two peaks (corresponding to the enriched methylene carbons in $\text{CH}_3\text{SCH}_2^{13}\text{CH}_2\text{Cl}$ and $\text{CH}_3\text{S}^{13}\text{CH}_2\text{CH}_2\text{Cl}$ respectively) throughout the reaction is consistent with the intermediacy of the 1-methylthiiranium ion in the formation of chloride (44), rather than formation of C-1 labelled material, followed by redistribution of label.

Owing to the sensitivity of the trialkyl phosphines to atmospheric oxygen, all manipulations with them were carried out under an atmosphere of dry nitrogen. The reaction between triisopropyl phosphine/ CCl_4 and alcohol (43) is violently exothermic at -5°C and produces a 50/50 mixture of C-1 and C-2 labelled chloride (44) (by ^{13}C n. m. r. analysis). After mixing the reactants at -78°C , and allowing the heterogeneous system obtained to become homogeneous by slowly warming to -10°C , a ^{13}C n. m. r. spectrum was recorded which showed a large peak at $\delta 59.4$ p. p. m. due to the labelled alcohol, and a smaller doublet at $\delta 68$ p. p. m. ($J = 8.5$ Hz). Warming the

FIG. 5-B-1

^{13}C N.M.R. SPECTRA RECORDED DURING THE REACTION OF
2-(METHYLTHIO)-[1- ^{13}C] ETHANOL WITH $\text{R}_3\text{P}/\text{CCl}_4$ (R = ISOPROPYL)

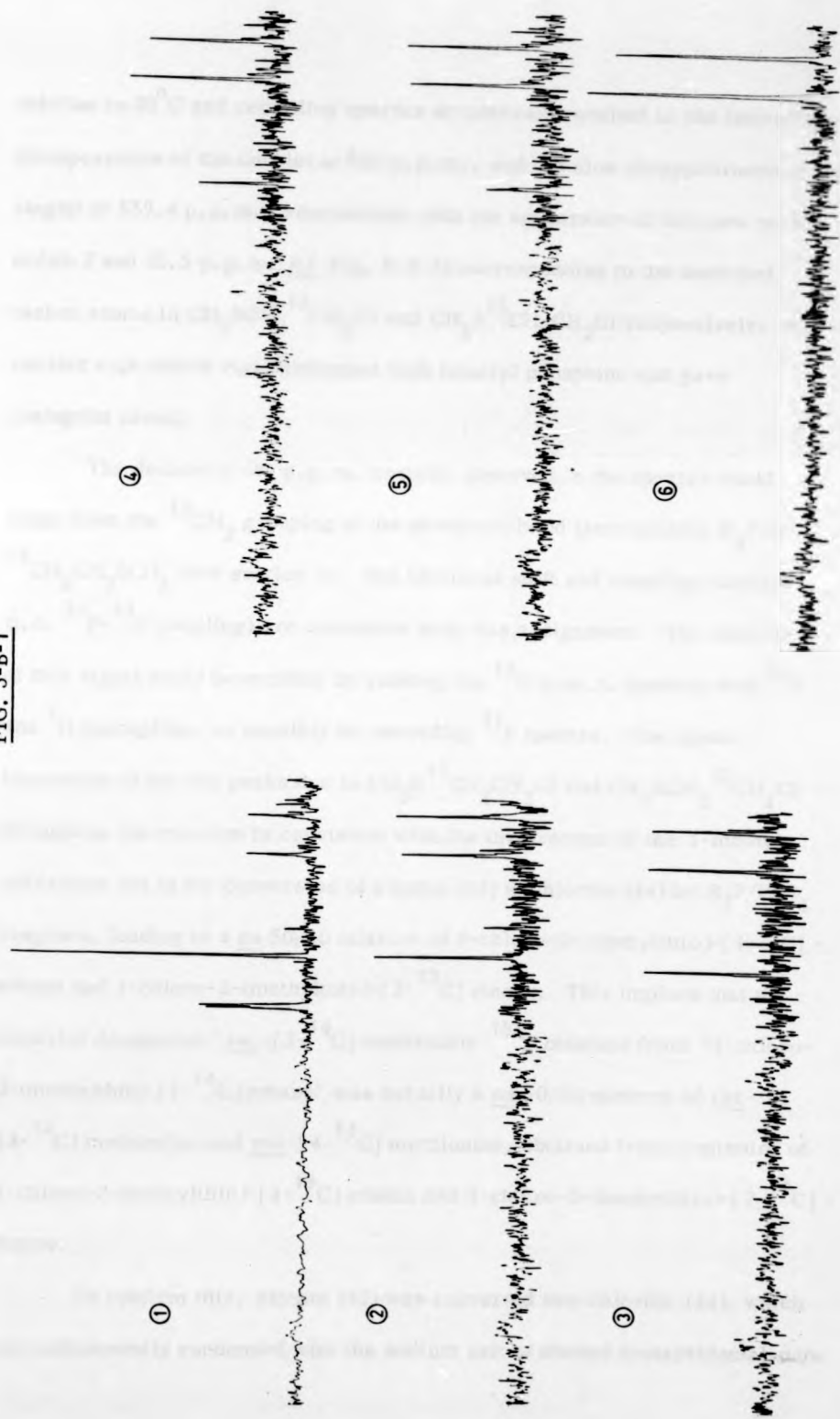
Spectra were each 2000 scans taking ca 2 min 15 sec to accumulate.

Times are time that accumulation began.

1. Immediately on becoming homogeneous at -10°C , Time = zero
2. After 15 min at 30°C
3. After 25 min at 30°C
4. After 45 min at 30°C
5. After 75 min at 30°C
6. After 17 h at 30°C

The total width of each plot is ca 100 p. p. m.

FIG. 5-B-1



solution to 30°C and recording spectra at intervals resulted in the immediate disappearance of the doublet at δ68 p.p.m., and the slow disappearance of the singlet at δ59.4 p.p.m., concomitant with the appearance of two new peaks at δ42.2 and 35.5 p.p.m. (cf Fig. 5-B-1) corresponding to the enriched carbon atoms in $\text{CH}_3\text{SCH}_2^{13}\text{CH}_2\text{Cl}$ and $\text{CH}_3\text{S}^{13}\text{CH}_2\text{CH}_2\text{Cl}$ respectively. A similar experiment was performed with trioctyl phosphine and gave analogous result.

The doublet at δ68 p.p.m. initially observed in the spectra could arise from the $^{13}\text{CH}_2$ grouping of the phosphorylated intermediate $\text{R}_3\text{P-O-}^{13}\text{CH}_2\text{CH}_2\text{SCH}_3$ (see section 3). The chemical shift and coupling constant (i.e. $^{31}\text{P-}^{13}\text{C}$ coupling) are consistent with this assignment. The identity of this signal could be verified by running the ^{13}C n.m.r. spectra with ^{31}P and ^1H decoupling, or possibly by recording ^{31}P spectra. The equal intensities of the two peaks due to $\text{CH}_3\text{S}^{13}\text{CH}_2\text{CH}_2\text{Cl}$ and $\text{CH}_3\text{SCH}_2^{13}\text{CH}_2\text{Cl}$ throughout the reaction is consistent with the involvement of the 1-methylthiiranium ion in the conversion of alcohol (43) to chloride (44) by $\text{R}_3\text{P/CCl}_4$ reagents, leading to a ca 50/50 mixture of 1-chloro-2-(methylthio)-[1- ^{13}C]-ethane and 1-chloro-2-(methylthio)-[2- ^{13}C]-ethane. This implies that the material designated "rac-[3- ^{14}C] methionine"¹⁸¹, obtained from "1-chloro-2-(methylthio)-[1- ^{14}C]-ethane" was actually a ca 50/50 mixture of rac-[3- ^{14}C] methionine and rac-[4- ^{14}C] methionine, obtained from a mixture of 1-chloro-2-(methylthio)-[1- ^{14}C]-ethane and 1-chloro-2-(methylthio)-[2- ^{14}C]-ethane.

To confirm this, alcohol (43) was converted into chloride (44), which was subsequently condensed with the sodium salt of diethyl acetamidomalonate

(46), both reactions being conducted under conditions identical to those of Ref. 181. The ^{13}C n.m.r. spectrum of the ethyl 2-acetamido-2-ethoxy-carbonyl-4-(methylthio)butanoate (47) thus obtained showed it to be a ca 50/50 mixture of C-3 and C-4 labelled material. Thus, rac-methionine obtained by the hydrolysis of this material would be a ca 50/50 mixture of [$3\text{-}^{13}\text{C}$] and [$4\text{-}^{13}\text{C}$] methionines.

The material designated as "rac-[$3\text{-}^{14}\text{C}$] methionine" has since been re-examined by mass spectroscopy, and confirmed to be a 50/50 mixture of [$3\text{-}^{14}\text{C}$] and [$3\text{-}^{14}\text{C}$] methionines¹⁹⁷.

2. Thiiranium ions as reaction intermediates

In general, compounds of the type $\text{R-S-CH}_2\text{-CH}_2\text{-X}$ where X is a leaving group, can undergo substitution reactions with a nucleophile Y (neutral or negatively charged), either by an $\text{S}_{\text{N}}2$ process (direct displacement of X by Y) or by an $\text{S}_{\text{N}}1$ process (prior ionisation of X assisted by sulphur, followed by capture of the resulting thiiranium ion by Y, Fig. 5-B-2).

Thiiranium ions are established intermediates in many solvolytic and pyrolytic reactions of β -haloalkyl thioethers^{198, 199}. Thus, nucleophilic substitutions of β -haloalkyl thioethers (e.g. $\text{S}(\text{CH}_2\text{CH}_2\text{Cl})_2 \xrightarrow{\text{H}_2\text{O}} \text{S}(\text{CH}_2\text{CH}_2\text{OH})_2$) occur much faster than substitutions with the corresponding halides in which S is replaced by CH_2 , owing to neighbouring group participation by sulphur¹⁹⁸; pyrolysis reactions of isomeric materials (e.g. $\text{CH}_3\text{CHClCH}_2\text{SAr}$ and $\text{CH}_2\text{ClCH}(\text{CH}_3)\text{SAr}$) yield identical product mixtures via a common intermediate thiiranium ion¹⁹⁹. The addition of sulphenyl halides to olefins is also believed to proceed via thiiranium intermediates¹⁹⁹, but recent evidence suggests that less polar symmetrical intermediates may be involved¹⁸⁹.

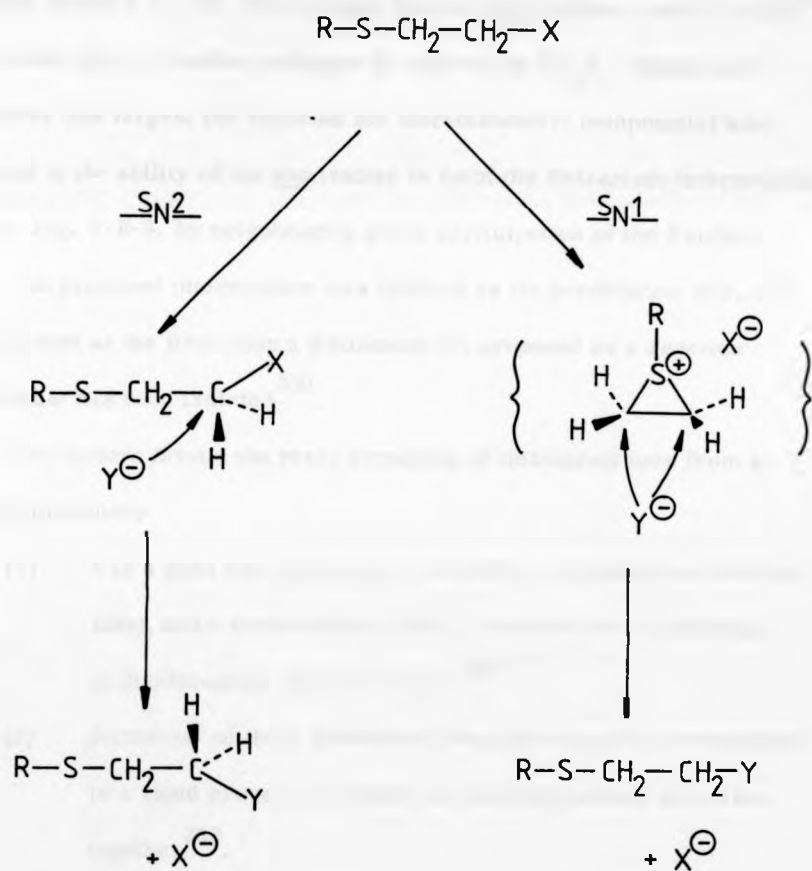


FIG 5-B-2

THE TWO POSSIBLE REACTION PATHWAYS OPEN TO β -HALOALKYL THIOETHERS, UNDERGOING
NUCLEOPHILIC SUBSTITUTION

A recent study²⁰⁰ of the solvolysis of the endo-sulphonate, and exo-*p*-nitrobenzoate, esters of 2-thiabicyclo[2.2.1]-heptan-6-ols, (Fig. 5-B-3) showed that, (after correcting for solvent and leaving group) the exo ester reacts 3.7×10^{14} times faster than its endo isomer, and 3.1×10^{10} times faster than its carbon analogue (S replaced by CH_2). These rate differences (the largest yet reported for stereoisomeric compounds) were attributed to the ability of the exo isomer to form the thiiranium intermediate shown in Fig. 5-B-3, by neighbouring group participation of the β sulphur atom. The proposed intermediate was isolated as its perchlorate salt, this being claimed as the first time a thiiranium ion proposed as a reaction intermediate has been isolated²⁰⁰.

Two factors favour the ready formation of thiiranium ions from β -haloalkyl thioethers

- (1) S is a good nucleophile (e.g. in dialkyl sulphides and thiolate ions), more reactive than O (e.g. in ethers and alkoxides), in displacement reactions ($\text{S}_{\text{N}}2$)²⁰¹.
- (2) formation of three membered rings from acyclic precursors is a rapid process, because the reacting centres are close together²⁰².

With a sufficiently reactive nucleophile, under favourable $\text{S}_{\text{N}}2$ conditions (aprotic solvent), direct displacement of halide ion from β -haloalkyl thioethers can be observed (e.g. reaction of $\text{CH}_3\text{SCH}_2\text{CH}_2\text{Cl}$ with I^\ominus in acetone¹⁹⁵).

Solvolysis Rates

$$\underline{A/B}, \quad \underline{3.7 \times 10^{14} / 1}$$

$$\underline{A/C}, \quad \underline{3.1 \times 10^{10} / 1}$$

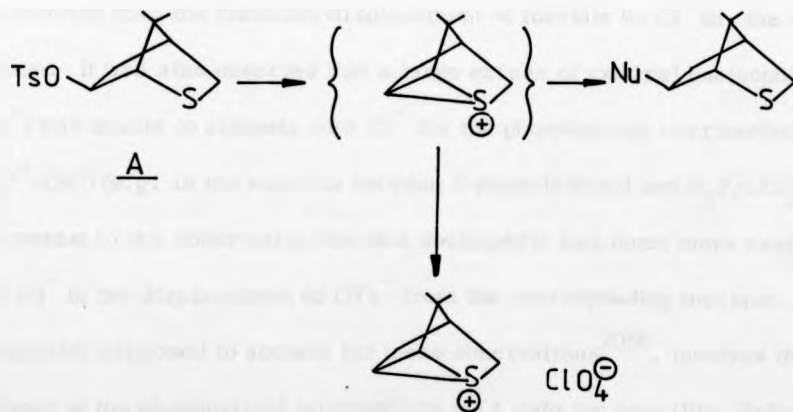
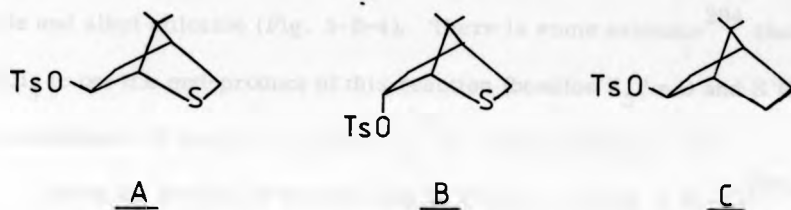


FIG 5-B-3

RELATIVE RATES OF SOLVOLYSIS FOR ISOMERIC TOSYLATE ESTERS, AND THE ISOLATION OF THE THIIRANIUM INTERMEDIATE INVOLVED IN THE SOLVOLYSIS OF A

3. The mechanism of the reaction between alcohols and reagents of the type R_3P/CCl_4

The conversion of alcohols into alkyl chlorides by R_3P/CCl_4 was reported in 1966²⁰¹, (R = phenyl) and shown to proceed with inversion in 1968¹⁹⁶ (octan-2-ol, R = octyl). The reaction is generally accepted^{202, 203} to proceed via a phosphorylated intermediate which collapses to phosphine oxide and alkyl chloride (Fig. 5-B-4). There is some evidence²⁰⁴ that $CHCl_3$ is not the end-product of this reaction (besides $R_3P = O$ and $R'Cl$), but compounds of the type $[R_3PCHCl_2]^+Cl^-$ and $[R_3PCH_2Cl]^+Cl^-$.

Detailed studies of the reaction $R_3P/CCl_4 + R'OH \rightarrow R'-Cl$ ^{205a, 205b, 205c} showed, as well as a remarkable tendency for inversion, even in systems prone to rearrangement, that the reaction exhibited some characteristics distinctly different from normal S_N2 reactions. For example, the reaction of neo-pentyl alcohol with R_3P/CCl_4 results in a greater degree of inversion than the classical displacement of tosylate by Cl^- in the same system. It was also observed that a large excess of external nucleophile (CN^-) was unable to compete with Cl^- for the phosphonium intermediate (R_3P^+-OR') (e.g. in the reaction between 2-phenylethanol and R_3P/CCl_4), in contrast to the observation that this nucleophile was much more reactive than Cl^- in the displacement of OTs^- from the corresponding tosylate. One mechanism proposed to account for these observations^{205b}, involves the collapse of the phosphorylated intermediate via a tight ion pair (Fig. 5-B-5). In this so-called 4-centre mechanism P-Cl bond cleavage precedes O-R' bond cleavage, giving a tight ion pair, which collapses in an S_N2 manner.

Aneja et al²⁰⁶ observed that treatment of 1,3-distearoylglycerol with Ph_3P/CCl_4 gave 2-chlorodeoxy-1,3-distearoylglycerol, in contrast to

FIG 5-B-4

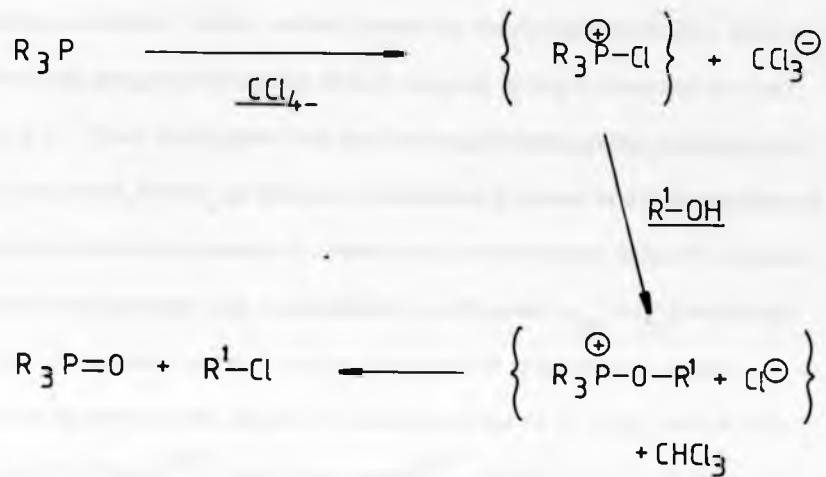


FIG 5-B-5

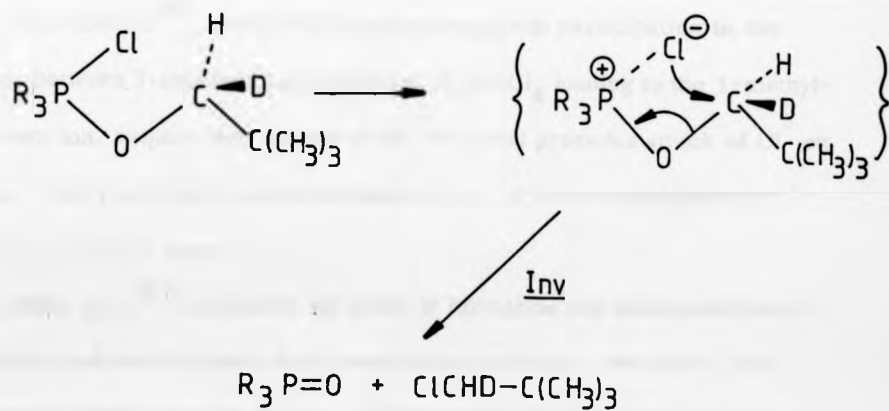


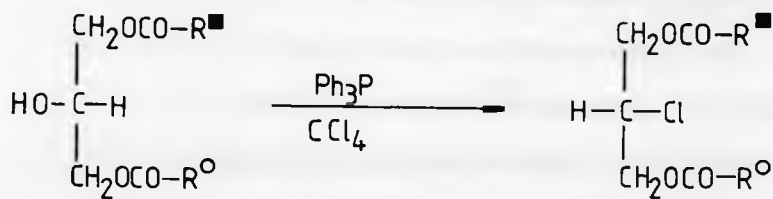
FIG. 5-B-4 THE OVERALL MECHANISM OF THE CONVERSION OF ROH INTO RCl BY R_3P/CCl_4 REAGENTS

FIG. 5-B-5 THE 4 CENTRE MECHANISM PROPOSED BY SCHNIDER ET AL (205b)

the reaction between the tosyl ester of 1-palmitoyl-3-stearoylglycerol and lithium chloride, which yielded primarily rearranged products, (due to neighbouring group participation of acyl oxygen, giving a dioxolenium ion) Fig. 5-B-6. They interpreted this lack of neighbouring group participation in the case of $\text{Ph}_3\text{P}/\text{CCl}_4$ to indicate a concerted process in the breakdown of the phosphorylated intermediate, rather than prior fission of the P-Cl bond. This concerted process was rationalised as a thermal $\sigma_{2s} + \sigma_{2a}$ pericyclic reaction. It has been pointed out that this lack of neighbouring group participation reflects the degree of cleavage of the O-R' bond, rather than that of the P-Cl bond²⁰². In a later report²⁰⁷, Aneja *et al* obtained some rearranged products from the reaction between $\text{Ph}_3\text{P}/\text{CCl}_4$ and cholesterol. These results were interpreted as reflecting rearrangement of the phosphorylated intermediate before its concerted fragmentation into products, but others believe these results suggest decomposition of the phosphorylated intermediate via a carbonium ion²⁰⁸.

Our results²⁰⁹, indicating neighbouring group participation in the reaction between 2-(methylthio)ethanol and $\text{R}_3\text{P}/\text{CCl}_4$ leading to the 1-methylthiuranium ion, require that fission of the O-C bond precedes attack of Cl^- on carbon. This rules out a concerted mechanism, of the type suggested by Aneja *et al*, in this case.

Jones *et al*²⁰² compared the rates of formation and decomposition of phosphorylated intermediates from reactions of primary, secondary, and neo-pentyl alcohols with $\text{Ph}_3\text{P}/\text{CCl}_4$. They observed the order of reactivity primary > secondary > neo-pentyl for both the rates of formation, and decomposition of these intermediates. In the neo-pentyl case the intermediate



————— / BUT \ —————

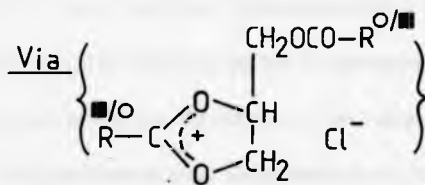
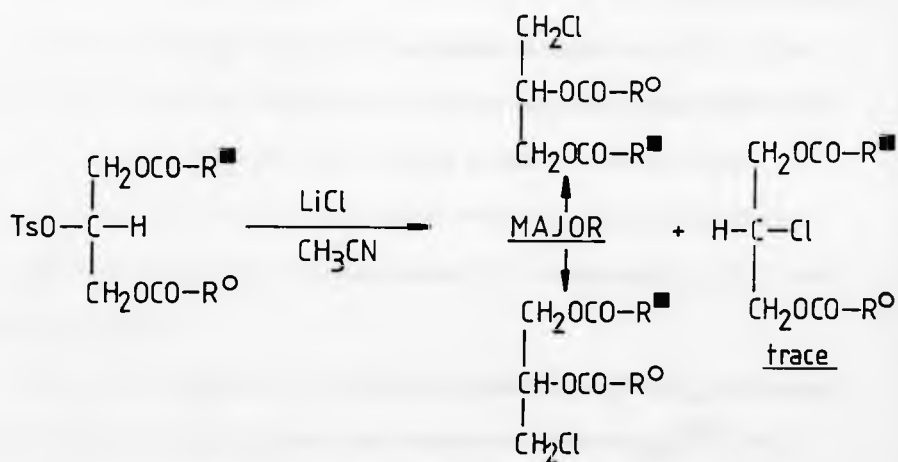


FIG 5-B-6

THE RESULTS OF ANEJA ET AL'S EXPERIMENTS, DEMONSTRATING NO NEIGHBOURING GROUP PARTICIPATION WHEN $\text{R}_3\text{P}/\text{CCl}_4$ REAGENT IS USED

was isolated, and its structure elucidated as $(\text{CH}_3)_3\text{CCH}_2\text{OP}(\text{C}_6\text{H}_5)_3\text{Cl}$. The $\text{CH}_2\text{-O-P}$ grouping (which had previously only been assumed) was identified by ^1H n. m. r. experiments, aided by ^{31}P decoupling. For primary alcohols the rate of decomposition of the phosphorylated intermediate was found to be greater than its rate of formation. It was concluded that the mechanism of the conversion of alcohols into chlorides by $\text{R}_3\text{P}/\text{CCl}_4$ in best accord with available results, is an $\text{S}_{\text{N}}2$ type intramolecular displacement from a tight ion pair, as previously suggested^{205b}.

In contrast, Cristol *et al*²⁰⁸ have shown that in the reaction of certain allylic alcohols with $\text{Ph}_3\text{P}/\text{CCl}_4$, rearrangement products can occur. The reactions of a number of benzobicyclooctadienyl and benzabicyclononatrienyl alcohols with $\text{Ph}_3\text{P}/\text{CCl}_4$ were shown to give products resulting from competing processes: direct displacement reactions, Wagner-Meerwein rearrangements, and allylic rearrangements (i. e. competing $\text{S}_{\text{N}}1$, $\text{S}_{\text{N}}2$, and other mechanisms).

The reaction between 2-(methylthio)ethanol and $\text{R}_3\text{P}/\text{CCl}_4$ represents one extreme of the reaction spectrum proposed by Cristol *et al*²⁰⁸. In this case the presence of a β sulphur atom provides a favourable neighbouring group participation giving the 1-methylthiiranium ion. From our results, and those of others therefore, it is concluded that the decomposition of the phosphorylated intermediate in the reaction between alcohols and $\text{R}_3\text{P}/\text{CCl}_4$ can occur in a number of ways. The mechanism of this process ranges from almost pure $\text{S}_{\text{N}}2$, through a situation where competing $\text{S}_{\text{N}}1$, $\text{S}_{\text{N}}2$, and other mechanisms can exist, to an almost pure $\text{S}_{\text{N}}1$ type mechanism, depending on the structure of the substrate alcohol.

5-C STUDIES OF OTHER REAGENTS WHICH EFFECT THE GENERAL
CONVERSION R-OH→R-Cl, USING 2-(METHYLTHIO)-[1-¹³C]
ETHANOL AND ¹³C N.M.R. SPECTROSCOPY

1. Alkyl chlorides from the decomposition of imidate hydrohalides

By the synthesis attributed to Pinner²¹⁰, an alcohol (ROH) and a nitrile (R'CN) may be condensed in the presence of a hydrogen halide (HX), to give an imidate hydrohalide (R'C(=NH₂X)OR). These imidate hydrohalides can be isolated as crystalline solids²¹¹ and on pyrolysis yield the corresponding alkyl halides (RCl) and amides (R'CONH₂), Fig. 5-C-1. If trichloroacetonitrile and an alcohol are condensed in the presence of anhydrous hydrogen chloride, the imidate hydrohalide produced decomposes rapidly at room temperature²¹² to trichloroacetamide, and the corresponding alkyl chloride. This reaction has been used²¹³ to produce 6-halo-6-deoxysugars from the corresponding protected sugars in good yield, under mild conditions.

The mechanism of decomposition of solid imidate hydrohalides on pyrolysis is considered to be S_N2²¹¹, and so is that of the thermal decomposition of imidate hydrohalides in solution²¹⁴. The former reaction has been shown to proceed with inversion in the case of the imidate hydrohalide derived from optically active butan-2-ol²¹⁵. In studies on the decomposition of the imidate hydrohalides obtained from trichloroacetamide and alcohols²¹², a mechanism involving formation of a tight ion pair, followed by collapse to products in an S_N2 manner has been proposed (Fig. 5-C-2).

2-(methylthio)-[1-¹³C] ethanol, trichloroacetamide, and anhydrous hydrogen chloride were mixed in chloroform at -20°C, and a ¹³C n. m. r. spectrum recorded at once. This showed a single large peak at δ 59.9 p. p. m.,

FIG 5-C-1

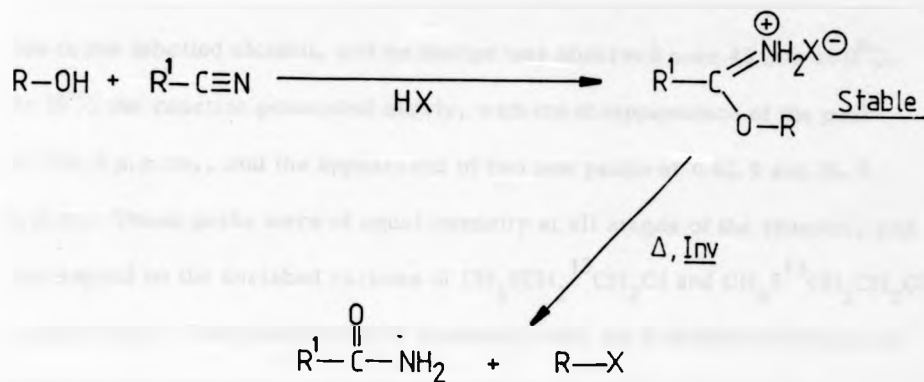


FIG 5-C-2

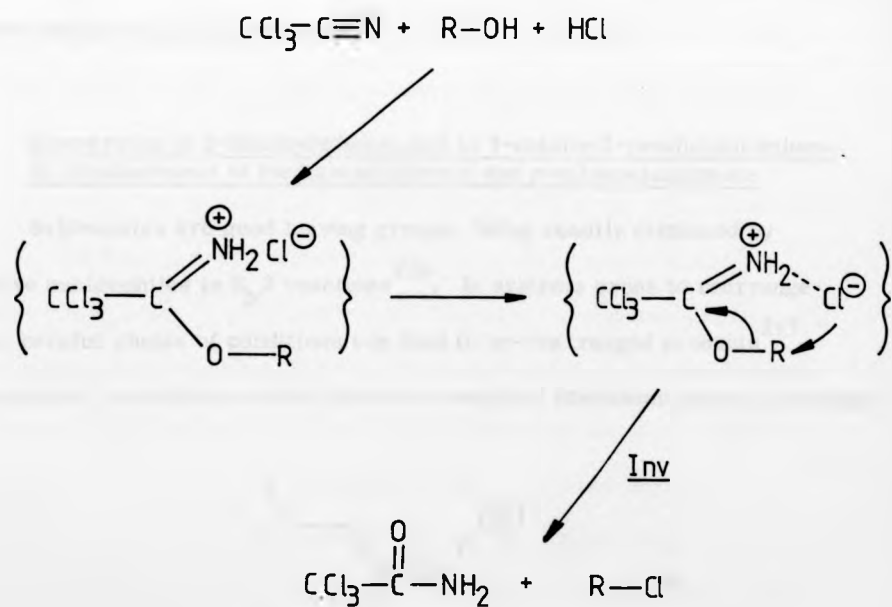


FIG. 5-C-1

PREPARATION AND DECOMPOSITION OF IMIDATE HYDROHALIDES

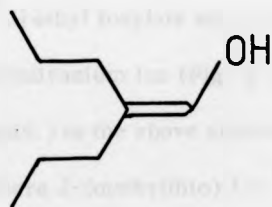
FIG. 5-C-2

THE TIGHT ION PAIR MECHANISM PROPOSED TO ACCOUNT FOR THE OBSERVED STEREOCHEMISTRY IN THE DECOMPOSITION OF CCl_3CN DERIVED IMIDATE HYDROHALIDES

due to the labelled alcohol, and no change was observed over 45 min at 0°C. At 25°C the reaction proceeded slowly, with the disappearance of the peak at δ 59.9 p.p.m., and the appearance of two new peaks at δ 42.9 and 36.5 p.p.m. These peaks were of equal intensity at all stages of the reaction, and correspond to the enriched carbons of $\text{CH}_3\text{SCH}_2^{13}\text{CH}_2\text{Cl}$ and $\text{CH}_3\text{S}^{13}\text{CH}_2\text{CH}_2\text{Cl}$ respectively. This observation is consistent with the 1-methylthiiranium ion being an intermediate in the formation of chloride (44), formed by the decomposition of 2-(methylthio)ethyl trichloromethylimidate. It is to be expected therefore that this reaction, previously held to be an exclusive $\text{S}_{\text{N}}2$ process, will exhibit a spectrum of mechanisms, as does the conversion of ROH to RCl by $\text{R}_3\text{P}/\text{CCl}_4$ reagents²⁰⁸.

2. Conversion of 2-(methylthio)ethanol to 1-chloro-2-(methylthio)ethane by displacement of methanesulphonate and p-toluenesulphonate

Sulphonates are good leaving groups, being readily displaced by reactive nucleophiles in $\text{S}_{\text{N}}2$ reactions²¹⁶. In systems prone to rearrangement, careful choice of conditions can lead to un-rearranged products²¹⁷. For example, the allylic alcohol (48) was converted (methanesulphonyl chloride/



48

collidene/lithium chloride/DMF, 0°C, 1.5 h)²¹⁷ into the corresponding allylic chloride, without rearrangement. A contaminating non-allylic alcohol

which was present in the starting material, was converted into its mesyl ester, but failed to undergo nucleophilic substitution with Cl^- under these conditions. Conversion of alcohol (43) via its sulphonate ester into chloride (44) was examined, as a possible route to 1-chloro-2-(methylthio)-[1- ^{13}C]-ethane.

Sulphonate esters may be prepared from sulphonyl chlorides, and the corresponding primary alcohols, at low temperatures²¹⁸. The reaction between alcohol (43) and p-toluenesulphonyl chloride in pyridine at -20°C produces 2-(methylthio)-[1- ^{13}C] ethyl tosylate in under 3 h. ^{13}C n. m. r. spectra recorded during the reaction show the disappearance of the peak due to enriched alcohol at $\delta 60.1$ p. p. m., and the appearance of a single new peak at $\delta 68.7$ p. p. m., corresponding to the enriched carbon atom of 2-(methylthio)-[1- ^{13}C] ethyl tosylate. Over 72 h at -20°C two additional peaks appear in the spectrum (accounting for ca 10% of the overall intensity) at $\delta 43.2$ and 36.3 p. p. m. These correspond to the enriched methylene carbons of $\text{CH}_3\text{SCH}_2^{13}\text{CH}_2\text{Cl}$ and $\text{CH}_3\text{S}^{13}\text{CH}_2\text{CH}_2\text{Cl}$ respectively, and are of ca equal intensity. As no peak due to 2-(methylthio)-[2- ^{13}C] ethyl tosylate was observed, this result is consistent with the formation of chloride (44) from 2-(methylthio)-[1- ^{13}C] ethyl tosylate and pyridinium hydrochloride occurring via the 1-methylthiiranium ion (Fig. 5-C-3(a)). Addition of lithium chloride (2 mol. equiv.) to the above solution, and warming to 20°C , resulted in conversion of more 2-(methylthio)-[1- ^{13}C] ethyl tosylate into C-1 and C-2 labelled chloride (44), and some decomposition to other unidentified products. ^{13}C n. m. r. spectra recorded during the reaction show the peak at $\delta 68.7$ p. p. m. decreasing in intensity, and the two peaks

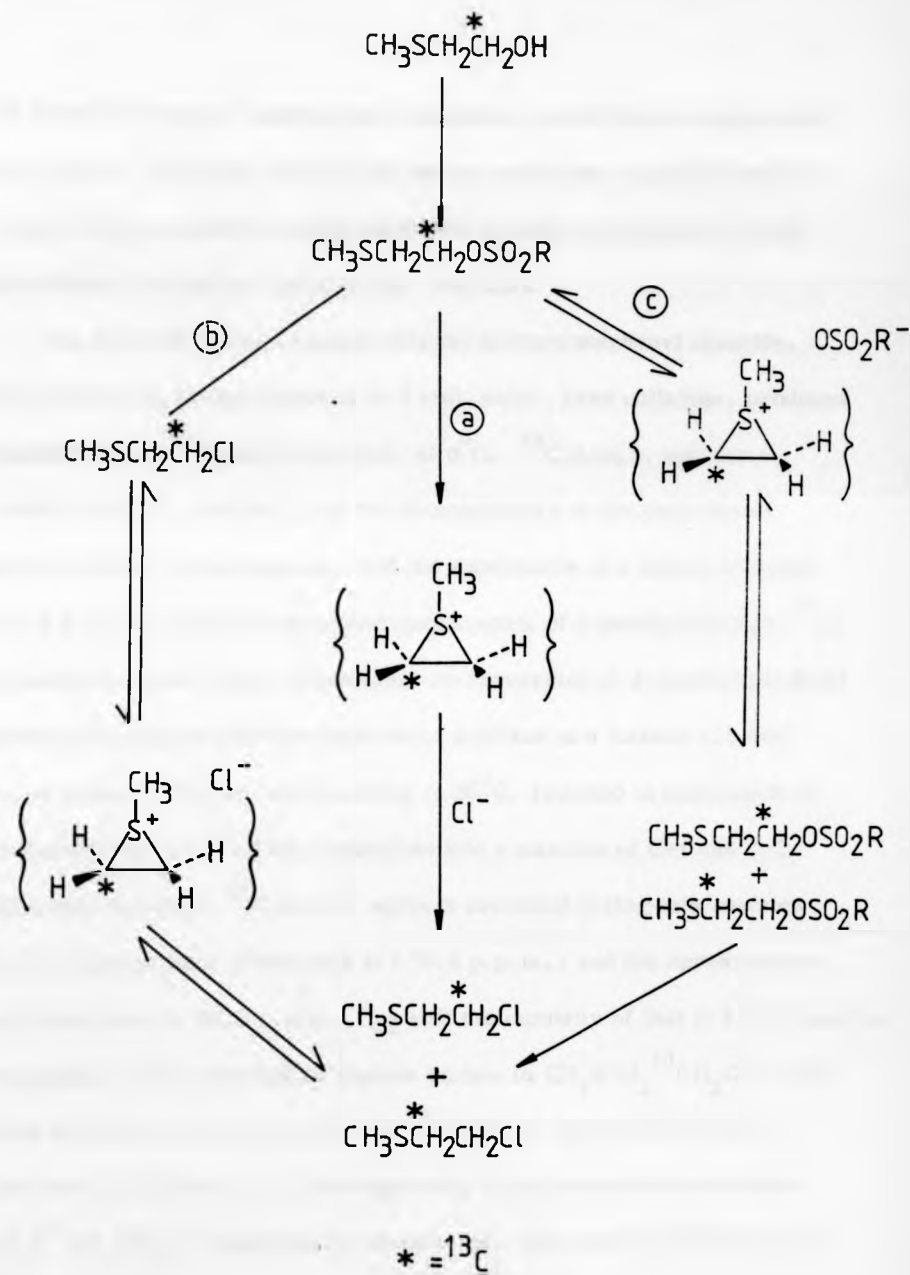


FIG 5-C-3

THREE POSSIBLE MECHANISMS WHICH WOULD GIVE RISE TO A MIXTURE OF C-1 AND C-2 LABELLED (44) IN THE PREPARATION OF (44) FROM (43) BY Cl^- DISPLACEMENT OF THE CORRESPONDING SULPHONATE ESTER

at δ 43.2 and 36.3 p.p.m. increasing in intensity, as well as the appearance of other peaks. The equal intensity of the two peaks due to chloride (44) at all stages of this reaction is again consistent with the intermediacy of the 1-methylthiiranium ion as shown in Fig. 5-C-3(a).

The reaction between alcohol (43) and methanesulphonyl chloride, in DMF containing lithium chloride (1.1 mol. equiv.) and collidine, produced 2-(methylthio)-[1- ^{13}C] ethyl mesylate, at 0°C . ^{13}C n.m.r. spectra recorded during the reaction show the disappearance of the peak due to labelled alcohol at δ 61.5 p.p.m., and the appearance of a single new peak at δ 70.8 p.p.m., due to the enriched carbon atom of 2-(methylthio)-[1- ^{13}C] ethyl mesylate. Under these conditions, no conversion of 2-(methylthio)ethyl mesylate into chloride (44) was observed. Addition of a further 1.1 mol. equiv. of lithium chloride, and warming to 20°C , resulted in conversion of the 2-(methylthio)-[1- ^{13}C] ethyl mesylate into a mixture of C-1 and C-2 labelled chloride (44). ^{13}C n.m.r. spectra recorded during this reaction show the disappearance of the peak at δ 70.8 p.p.m., and the appearance of a single new peak at δ 43.5 p.p.m., (ca half the intensity of that at δ 70.8 p.p.m.), corresponding to the enriched methylene carbon in $\text{CH}_3\text{SCH}_2^{13}\text{CH}_2\text{Cl}$. After addition of benzene to the solution, the ^{13}C n.m.r. spectrum revealed a second peak, at δ 35.9 p.p.m. corresponding to the enriched carbon atom of $\text{CH}_3\text{S}^{13}\text{CH}_2\text{CH}_2\text{Cl}$, which had previously been obscured by solvent peaks. Repeating the reaction in the presence of 3 mol. equiv. of lithium chloride at 10°C gave an analogous result, i.e. formation of single labelled mesylate occurring at 10°C , and formation of chloride (44) only occurring on warming to 20°C . The product of this reaction was again a mixture of C-1 and C-2.

labelled chloride (44), as shown by ^{13}C n. m. r. spectra obtained after the addition of benzene.

These results indicate, that under conditions suitable for the displacement of OMs^- or OTs^- from the sulphonate esters of alcohol (43) by Cl^- , a mixture of C-1 and C-2 chloride (44) results. For the tosylate, this mixture of products arises from the intermediacy of the 1-methylthiiranium ion in the conversion of tosylate to chloride. For the mesylate, the observed products could arise either via the above mechanism, or by thermal equilibration of 1-chloro-2-(methylthio)-[1- ^{13}C] ethane with its C-2 labelled isomer. Thus, displacement of sulphonates from the sulphonate esters of alcohols (43) is not a suitable route for the preparation of 1-chloro-2-(methylthio)-[1- ^{13}C] ethane.

It is concluded that none of the reagents investigated is suitable for the conversion of alcohol (43) into chloride (44), without neighbouring group participation by sulphur.

5-D EXPERIMENTAL

1. Synthesis of 2-(methylthio)-[1-¹³C] ethanol (43)

2-(methylthio)-[1-¹³C] ethanol was prepared as described in chapter 4. For the studies reported in section 5-B, material containing 11.4 atom % ¹³C was used, and for those in section 5-C, material containing 20 atom % ¹³C. This dilution was achieved by addition of unlabelled ethyl bromoacetate to the ethyl-[1-¹³C] bromoacetate, 90 atom %, obtained from [1-¹³C] acetic acid, 90 atom %.

Authentic samples of 2-(methylthio) ethanol and 1-chloro-2-(methylthio) ethane were prepared as described in chapter 4.

2. Trioctylphosphine 218a*

1-bromooctane (20 g, 18 cm³, 0.104 mol) in anhydrous ether (25 cm³) was added to magnesium turnings (2.58 g, 0.106 mol) and iodine (ca 5 mg), under an atmosphere of dry nitrogen, at a rate sufficient to maintain vigorous reaction. After boiling the solution under reflux for 15 min, and cooling to -5°C, phosphorous trichloride (4.72 g, 3 cm³, 0.034 mol) in anhydrous ether (10 cm³) was added to the stirred solution over 30 min. Stirring was continued for a further 20 min and the reaction subsequently allowed to warm up to room temperature. After boiling under reflux for 96 h, and cooling to room temperature, water (15 cm³) was added, followed by hydrochloric acid (24 cm³)

* Both trioctyl and triisopropyl phosphine are unstable with respect to atmospheric oxidation. All manipulations of these materials were carried out under a blanket of dry nitrogen, or in a dry box.

of a 2M solution) and aqueous ammonia (29 cm³ of a 2M solution). The organic phase was separated, filtered through glass wool, and evaporated under reduced pressure to give a yellow oil (13.6 g, 90%). Kugelrohr distillation (210°C, 0.1 mm Hg) gave trioctylphosphine as a colourless liquid, pure by i.r.:i.r. (film) 2900 (s), 1650 (m), 1460 (s), 1370 (m), and 710 (m) cm⁻¹. N.B. absence of P=O stretch at 1138 cm⁻¹. Exposure of an i.r. film to the atmosphere gave rise to a broad band centred at 1138 cm⁻¹ due to P=O.

3. Triisopropyl phosphine and triphenyl phosphine

Commercial triphenyl phosphine was used without purification, commercial triisopropyl phosphine was distilled under dry nitrogen just before use, to provide a material showing no P=O in its i.r. spectrum.

4. Ethyl 2-acetamido-2-ethoxycarbonyl-4-(methylthio)butanoate (cf Ref. 185)

Diethylacetamidomalonate (14.27 g, 66 mmol) was added to a solution of sodium (1.62 g, 70 mmol) in anhydrous ethanol (31 cm³) and the mixture was boiled under reflux, with exclusion of atmospheric moisture, until a homogeneous solution resulted. 1-chloro-2-(methylthio)ethanol (9 g, ca 9 cm³, 81 mmol) was added, and the reaction was boiled under reflux for a further 5 h. After cooling the solution to 0°C, the precipitated sodium chloride was filtered off under suction, washed with ethanol (3 x 10 cm³), and the combined filtrates were evaporated under reduced pressure. Pumping overnight (25°C, 1 mm Hg) gave a yellow oil (15.88 g) containing ca 5%

CH_2Cl_2 by ^1H n.m.r. analysis. This oil crystallised on scratching, and after low temperature recrystallisation from ether/petroleum ether, gave ethyl 2-acetamido-2-ethoxycarbonyl-4-(methylthio)butanoate (47) (13.33 g, 70%) as white crystals: m.p. $45-46^\circ\text{C}$ (lit $50-52^\circ$), pure by t.l.c. (silica gel, ethyl acetate, H_2SO_4 spray and char, Rf 0.38), δ 1.25 (6 H, t), 2.0 (3 H, s), 2.02 (3 H, s), 2.5 (4 H, m) and 4.3 (4 H, q) p.p.m., ^{13}C n.m.r. δ 13.9 ($\underline{\text{C}}\text{H}_3\text{CH}_2$), 15.4 ($\underline{\text{C}}\text{H}_3\text{S}$), 22.9 ($\underline{\text{C}}\text{H}_3\text{CO}$), 28.4 ($\text{SCH}_2-\underline{\text{C}}\text{H}_2$), 31.9 (SCH_2), 56.6 ($\text{CH}_2-\underline{\text{C}}-\text{NH}$), 62.2 ($\underline{\text{C}}\text{H}_2-\text{O}$), 167.4 ($\underline{\text{C}}\text{OO}$), and 169.5 ($\underline{\text{C}}\text{ONH}$) p.p.m.

5. ^{13}C n.m.r. study of the reaction between $\text{R}_3\text{P}/\text{CCl}_4$, and 2-(methylthio)-[1- ^{13}C] ethanol, where R = phenyl, isopropyl and octyl

a) A solution of redistilled triisopropyl phosphine (0.83 g, 5.2 mmol) in [$^2\text{H}_6$] benzene (0.5 cm^3) in a 10 mm dia. n.m.r. tube was degassed by argon bubbling, and frozen by cooling to -78°C . A degassed solution of 2-(methylthio)-[1- ^{13}C] ethanol (0.2 g, 2.2 mmol), 11.4 atom %, in CCl_4 (0.8 g, 5.2 mmol) was added, and the mixture maintained at -78°C . The above operations were carried out in a dry box. The capped n.m.r. tube was transferred to the pre-cooled (-10°C) probe of the ^{13}C n.m.r. spectrometer. When the contents of the tube became a homogeneous solution (ca 3 min), a spectrum was recorded at once, (200 scans, ca 2 min 15 sec). The reaction was then warmed to 30°C and spectra recorded at intervals (each 200 scans, taking 2 min 15 sec to accumulate) and the results are depicted in Fig. 5-B-2, and discussed in the text. When no further reaction was evident, the sample was allowed to stand at room temperature for 17 h, and a t_∞ spectrum was recorded.

b) The above experiment was repeated, using trioctylphosphine in place

of triisopropyl phosphine. After mixing at -78°C the reactants were warmed to -13°C and an initial ^{13}C n.m.r. spectrum recorded. The reaction was then warmed to 17°C and spectra recorded at intervals. The results are discussed in the text, and were analogous to those of the triisopropyl phosphine reaction.

c) To a solution of 2-(methylthio)-[1- ^{13}C] ethanol, 11.4 atom %, (0.2 g, 2.2 mmol) in [$^2\text{H}_6$] benzene (0.5 cm^3) was added a solution of triphenyl phosphine (1.37 g, 5.2 mmol) in CCl_4 (2.4 g, 15.8 mmol). The reaction was incubated at room temperature in a 10 mm dia. n.m.r. tube and ^{13}C spectra recorded at intervals, over 7 days. The results are discussed in the text.

6. Conversion of 2-(methylthio)-[1- ^{13}C] ethanol into ethyl 2-acetamido-2-ethoxy carbonyl-4-(methylthio)butanoate (by the method of Ref. 181)

To stirred 2-(methylthio)-[1- ^{13}C] ethanol (0.146 g, 1.58 mmol) 11.4 atom %, at 0°C a solution of trioctyl phosphine (1.18 g, 3.19 mmol) in CCl_4 (0.488 g, 0.3 cm^3 , 3.19 mmol) was added, over 2 min, under an atmosphere of dry N_2 . The reaction was sealed, and stirred for 5 min at 0°C , and then overnight at room temperature. Dimethylformamide (3 cm^3) was added, and the mixture distilled at 20 mm Hg to give a colourless oil, b.p. $58-60^{\circ}\text{C}$, 20 mm Hg (ca 3 cm^3). This solution of 1-chloro-2-(methylthio)ethane (ca 1.58 mmol) in dimethylformamide (ca 3 cm^3) was added to a solution of the sodium salt of diethylacetamido malonate (0.762 g, 3.19 mmol)* in anhydrous ethanol (3.8 cm^3), and the reaction stirred for 20 h at 55°C , with the exclusion

* prepared from diethylacetamido malonate (0.692 g, 3.19 mmol) as described previously

of atmospheric moisture. The solution was filtered, the solid residue washed with ethanol ($2 \times 5 \text{ cm}^3$), and the combined filtrates evaporated under reduced pressure to provide an orange oil. This was dissolved in dichloromethane, filtered through cellite, and evaporated under reduced pressure; dimethylformamide was distilled off at 80°C , 1 mm Hg and the residue was purified by column chromatography on 40 g silica gel (80 - 200 mesh), using dichloromethane/methanol, 97:3 as eluant. Evaporation of fractions gave a product which was ca 50/50 diethylacetamido malonate (A) and ethyl 2-acetamido-2-ethoxycarbonyl-4-(methylthio)butanoate (B) by ^{13}C n. m. r. ^{13}C n. m. r., δ 14.0 ($\underline{\text{C}}\text{H}_3\text{CH}_2$ (A) and (B)), 15.4 ($\underline{\text{C}}\text{H}_3\text{S}$, (B)), 22.7 ($\underline{\text{C}}\text{H}_3\text{CO}$, (A) and (B)), 28.4 ($\underline{\text{S}}\text{CH}_2\text{CH}_2$, (B)), 31.9 ($\text{SCH}_2\underline{\text{C}}\text{H}_2$, (B)), 56.6 ($\text{CH}_2-\underline{\text{C}}-\text{N}$, (A) and (B)), 62.2 ($\underline{\text{C}}\text{H}_2-\text{O}$, (A) and (B)), 167.4 ($\underline{\text{C}}\text{OO}$, (A) and (B)) and 170 ($\underline{\text{C}}\text{ONH}$, (A) and (B)) p. p. m. Each of the peaks corresponding to the methylene carbons of the $\text{CH}_3\text{SCH}_2\text{CH}_2-\text{C}$ grouping were enhanced ca 5 fold over natural abundance (cf the ^{13}C n. m. r. spectrum a 50/50 mixture of unlabelled (B) with (A)).

7. ^{13}C n. m. r. study of the reaction between 2-(methylthio)-[1- ^{13}C]-ethanol and trichloroacetonitrile/HCl

Trichloroacetonitrile (0.17 g, $117 \mu\text{l}$, 1.18 mmol) was added to a solution of 2-(methylthio)-[1- ^{13}C] ethanol (0.1 g, 1.07 mmol), 20 atom %, in dry deuteriochloroform (2 cm^3) at -20°C (in a 10 mm dia. n. m. r. tube), and the resulting solution saturated with dry HCl at -20°C . These operations were carried out under an atmosphere of dry nitrogen. The capped n. m. r. tube was then transferred to the pre-cooled (-10°C) probe of the ^{13}C n. m. r.

spectrometer. The solution was allowed to warm up to 25°C, and ^{13}C n.m.r. spectra were recorded at intervals. The results are discussed in the text.

8. The conversion of the mesyl and tosyl esters of 2-(methylthio)-[1- ^{13}C] ethanol into 1-chloro-2-(methylthio)ethane; a ^{13}C n.m.r. study

1. p-toluenesulphonyl ester

To a solution of 2-(methylthio)-[1- ^{13}C] ethanol (0.1 g, 1.07 mmol), 20 atom %, in [$^2\text{H}_5$] pyridine (0.5 cm³), contained in a 10 mm dia. n.m.r. tube and cooled to -20°C, was added a solution of purified p-toluenesulphonyl chloride (0.22 g, 1.18 mmol) in dry pyridine (0.5 cm³). This was performed under an atmosphere of dry nitrogen. The capped n.m.r. tube was transferred to the pre-cooled (-20°C) probe of the ^{13}C n.m.r. spectrometer, and a spectrum recorded at once. Spectra were recorded at intervals, over 72 h, and the results are discussed in the text. Lithium chloride (0.09 g, 2.11 mmol) was added, and the reaction warmed to 20°C to obtain a homogeneous solution. ^{13}C n.m.r. spectra were recorded at intervals and the results are discussed in the text.

2. Methane sulphonyl ester (cf Ref. 217)

To a solution of 2-(methylthio)-[1- ^{13}C] ethanol (0.1 g, 1.07 mmol), 20 atom %, lithium chloride (0.05 g, 1.1 mmol) and collidene (0.143 g, 1.18 mmol) in dry dimethylformamide (1.5 cm³) contained in a 10 mm n.m.r. tube and cooled to -30°C, was added methanesulphonylchloride (0.134 g, 91 μl , 1.18 mmol). These operations were carried out under an atmosphere

of dry nitrogen. The capped n. m. r. tube was transferred to the pre-cooled (-20°C) probe of the ^{13}C n. m. r. spectrometer, and a spectrum recorded at once. The reaction was then warmed to 0°C and ^{13}C n. m. r. spectra recorded at intervals. The results are discussed in the text. When no further reaction was evident, lithium chloride (0.05 g, 1.1 mmol) was added, and the solution warmed to 20°C . ^{13}C n. m. r. spectra were recorded at intervals, and the results are discussed in the text.

The above reaction was repeated, in the presence of 3 mol. equiv. of lithium chloride (rather than 1 mol equiv) and an analogous result was obtained, which is discussed in the text.

CHAPTER 6

SOME CHEMISTRY OF DEHYDROMETHIONINE

6 - A INTRODUCTION

Dehydromethionine (S-methylisothiazolidene-3-carboxylate) was first prepared by Lavine²¹⁹, and he correctly assigned its structure (without regard to relative stereochemistry) in 1945²²⁰, Fig. 6-A-1a. A crystal structure of the racemic compound was published in 1976¹⁷², and this revealed the carboxyl group to be on the opposite side of the ring plane to the sulphur methyl group. The structure of dehydromethionine derived from (S)methionine is therefore as shown in Fig. 6-A-1b, having the (1R, 3S) configuration (39).

The compound is easily prepared by oxidation of methionine, e.g. by iodine in methanol, and although this oxidation normally produces sulphoxides from thioethers¹⁷³, dehydromethionine arises from methionine owing to a very favourable intramolecular attack of the amino group on an iodo-sulphonium intermediate^{173, 221}. The decomposition of dehydromethionine to methionine sulphoxide in aqueous alkali has been reported²²², and so has its reduction to methionine by thiols in buffer solutions¹⁷³. Recently²²³ dehydromethionine has been identified as an intermediate in the rose bengal sensitised photooxygenation of methionine to methionine sulphoxide. Despite the possible bacteriostatic properties of dehydromethionine²²⁴, and the fact that it seems to be the only unicyclic isothiazolidine isolated to date, the literature on it is limited to the references cited above.

TABLE 6-B-2

CHEMICAL SHIFTS (δ)	COUPLING CONSTANTS (Hz)
(1) H-3 = 4.39	(1,2) = 7.4
(2) H _R -4 = 2.8	(1,3) = 5.9
(3) H _S -4 = 2.4	(1,4) = 0
(4) H _S -5 = 3.8	(1,5) = 0
(5) H _R -5 = 3.6	(2,3) = -13.9
	(2,4) = 8.2
	(2,5) = 6.9
	(3,4) = 8.2
	(3,5) = 5.0
	(4,5) = -13.1

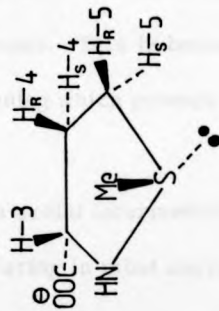
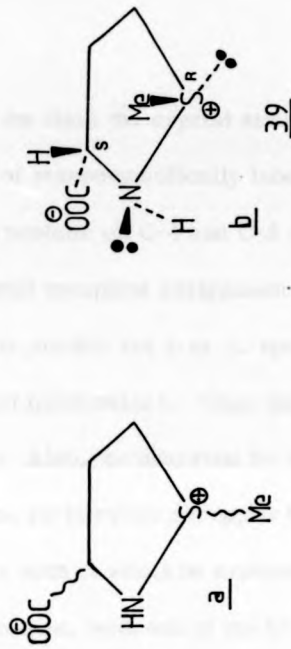


FIG 6-B-2

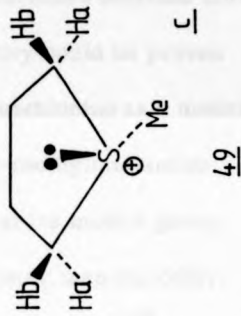
FIG 6-A-1



39

FIG. 6-A-1

- A) THE STRUCTURE OF DEHYDROMETHIONINE
 B) THE STEREOCHEMISTRY OF DEHYDROMETHIONINE DERIVED FROM (S)-METHIONINE
 C) THE STRUCTURE OF 1 METHYL-THIOLANUM IODIDE



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At the time the crystal structure appeared, we were engaged in the syntheses of stereospecifically labelled $[3,4-^2\text{H}_2]$ methionines (see chapter 4), and as the protons on C-4 and C-5 of dehydromethionine are diastereotopic, we reasoned that complete assignment of the ^1H n. m. r. of dehydromethionine would enable us to predict the n. m. r. spectra of the dehydromethionines obtained from our labelled methionines. Thus their stereochemical identity could be proven physically. Also, as observed by Glass ¹⁷², dehydromethionine is a model, save for the carboxylate group, for the ylid obtained from 1-methylthiolanium iodide. As such it would be expected to undergo exchange at its methyl group, and C-5 protons, with one of the C-5 protons exchanging faster than the other. This is in analogy to the effects first observed by Barbarella in 1971 ²²⁵, when he reported a rate difference of 400:1 for the diastereotopic ring protons α to the sulphonium centre in 1-methylthiolanium iodide (49), the protons cis to the S-methyl group exchanging faster than those trans ²²⁶ (Fig.6-A-1c). However more recent work has reduced this rate difference to between 12:1 and 28:1 ²²⁷, and work on the stereochemistry of exchange α to a sulphoxide centre ²²⁸, has cast doubt on the assignment of the faster exchanging pair of protons. This is because solvent effects were shown to be very important in determining which protons α to a sulphoxide centre exchange the faster.

Nevertheless, dehydromethionine could be a useful intermediate for the production of specifically labelled methionines, (bearing in mind conversion of dehydromethionine back to methionine by thiols as previously mentioned).

Another aspect of the chemistry of dehydromethionine which interested us was its conversion to methionine sulphoxide by aqueous alkali ²²², and in buffer solutions ¹⁷³. We thought that this conversion might be stereospecific,

leading to a single diastereoisomer, by mechanisms discussed later in the text. For these reasons we investigated the chemistry of dehydromethionine as discussed below.

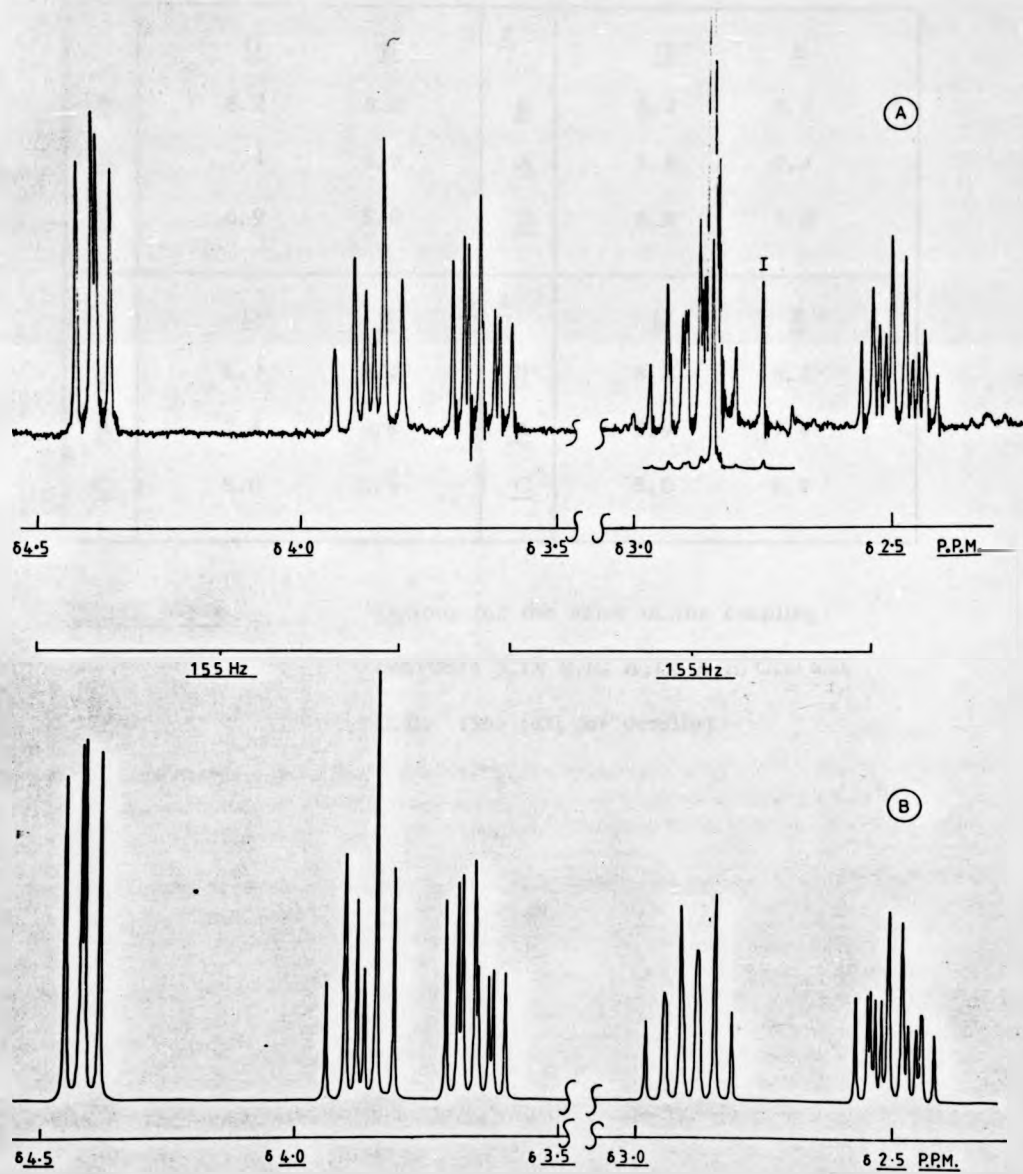
6 - B THE ^1H N.M.R. SPECTRUM OF (1R,3S) DEHYDROMETHIONINE

The 220 MHz ^1H n.m.r. spectrum (Fig. 6 - B - 1A) of (1R,3S) dehydromethionine (Fig. 6 - B - 2) in $^2\text{H}_2\text{O}$ shows 5 multiplets, at δ 4.39, 3.8, 3.6, 2.8 and 2.4 p.p.m., each corresponding to one proton, and a singlet at δ 2.8 p.p.m. corresponding to the methyl protons. The signal at δ 4.39 p.p.m. (A) can be assigned to the proton α to the carboxylate group²²⁹, because of its multiplicity and chemical shift, and the remaining 4 signals may be assigned to 2 geminal pairs. The signals at δ 3.8 (B) and 3.6 (C) p.p.m. are assigned as the pair attached to C-5²²⁵, and those at δ 2.8 (D) and 2.4 (E) as the pair attached to C-4²³⁰. The assignment of individual protons within these geminal pairs is discussed later.

First order analysis of the signals at δ 4.39, 3.8 and 3.6 p.p.m. leads to the following structures:

δ 4.39 (A) = 2 x d; δ 3.8 (B) = 2 x t; and δ 3.6 (C) = 2 x q; and approximate values for the coupling constants involved in the signals can be obtained directly from the spectrum. This gives values of 5.8 Hz and 7.4 Hz for the 2 couplings involved in signal (A); 8.2 Hz, 8.2 Hz, and 13.1 Hz for the 3 couplings involved in signal (B); and 5.0 Hz, 6.9 Hz and 13.0 Hz for the 3 couplings involved in signal (C). The presence of a coupling of ca 13 Hz in both signals (B) and (C) lends weight to their assignment as a geminal pair. A value for the geminal coupling involved in signal (E) can be obtained by consideration of the overall width of the signal, leading to a value of ca 13.8 Hz. This gives values for all the coupling constants expected in this system (assuming no 4 bond couplings), and the chemical shift of each of the signals has already been obtained directly

FIG. 6-B-1



220 MHz ^1H n.m.r. spectrum of (1R,3S)-dehydromethionine, (A) observed spectrum (B) calculated spectrum using the parameters of Table 6-B-2.

TABLE 6-B-1

	<u>D</u>	<u>E</u>	<u>1</u>	<u>2</u>	<u>D</u>	<u>E</u>
<u>B</u>	8.2	8.2		<u>B</u>	8.2	8.2
<u>A</u>	7.4	5.9		<u>A</u>	5.9	7.4
<u>C</u>	6.9	5.0		<u>C</u>	6.9	5.0
	<u>D</u>	<u>E</u>	<u>3</u>	<u>4</u>	<u>D</u>	<u>E</u>
<u>B</u>	8.2	8.2		<u>B</u>	8.2	8.2
<u>A</u>	7.4	5.9		<u>A</u>	5.9	7.4
<u>C</u>	5.0	6.9		<u>C</u>	5.0	6.9

TABLE 6-B-1

Options for the value of the coupling constants B,D; B,E; A,D; A,E; C,D and C,E. (See text for details)

from the spectrum. Considering the previous assignment of the signals, and the structure of (1R, 3S) dehydromethionine, 4 possible combinations of coupling constants and chemical shifts exist (Table 6 - B - 1). Using these coupling constants and chemical shifts, the subspectrum consisting of signals (A), (B), and (C) was computer simulated, and the chemical shifts and coupling constants adjusted to give the best fit for signals (A), (B), and (C). A simulation was then run for each option in Table 6 - B - 1, and the closest fit to the observed spectrum resulted from option 1 (Fig. 6 - B - 1B).

In analysis of 5 ring systems it is usual at this point^{229, 230}, to obtain a configurational assignment by applying the observation that in 5 membered rings approaching planarity, $J_{\text{trans}} < J_{\text{cis}}$ generally holds. (For a discussion of the validity of this approach in 5 membered heterocycles see references 231 and 232. This approach will not work here, however, because for signal (B) both J_{trans} and J_{cis} are equal to 8.2 Hz. Presumably the equivalence of J_{trans} and J_{cis} in this case stems from a combination of the distortion from pseudo-trigonal projection symmetry which exists in 5 membered ring systems²³¹, and the effect of S^+ and tetrahedral NH¹⁷² on the conformational equilibria which exist in solution^{232, 233}. Thus, with dehydromethionine, it is not possible to assign the members of the geminal pairs without further information, and we were unable to prove the stereochemistry of our labelled methionines by reference to the n. m. r. spectra of the dehydromethionines derived from them. Rather we decided to prove their configurations in another way, and use the dehydromethionines derived from them to complete the assignment of the n. m. r. spectrum of (1R, 3S) dehydromethionine.

The routes used to synthesise the stereochemically labelled methionines are depicted in Figs. 4 - B - 3 , and 4 - B - 4 (for a full discussion of these routes see chapter 4). [$^2\text{H}_2$] acetylene was reduced stereospecifically to E or Z [$^2\text{H}_2$] ethylene, and the stereochemical purity of these ethylenes checked by i. r. spectroscopy. The ethylenes were then converted into 1-chloro-2-(methylthio)ethanes via reaction with methanesulphenyl chloride. As this reaction is a trans addition involving a symmetrical cyclic intermediate^{190, 191}, the product obtained is a 50/50 mixture of enantiomers in each case, i. e. 50/50 (R,R)/(S,S) from Z [$^2\text{H}_2$] ethylene and 50/50 (R,S)/(S,R) from E [$^2\text{H}_2$] ethylene.

These chlorides were then condensed with the sodium salt of diethylacetamidomalonate, to give the labelled ethyl 2-acetamido-2-ethoxycarbonyl-4-(methylthio)butanoates. We expected this reaction to proceed by an $\text{S}_{\text{N}}1$ pathway, via an epi-sulphonium ion. Any reaction by an $\text{S}_{\text{N}}2$ pathway would give product inverted at the substitution centre, and so it was necessary to ensure that an $\text{S}_{\text{N}}2$ mechanism was not operating. Reference to the literature¹⁹⁵ shows that 1-chloro-2-(methylthio)ethane reacts under $\text{S}_{\text{N}}2$ conditions (KI/ acetone) 1.5 times faster than 1-chlorobutane. We set up a competitive experiment, involving the reaction of 1-chloro-2-(methylthio)ethane (14mmol) and 1-chlorobutane (14 mmol) with the sodium salt of diethylacetamidomalonate (24 mmol), the sole product of which was derived from 1-chloro-2-(methylthio)ethane i. e. no product derived from 1-chlorobutane was observed. Full details of this experiment are given in chapter 4. This result indicates that under these conditions an $\text{S}_{\text{N}}1$ pathway operates for 1-chloro-2-(methylthio)ethane (i. e. an epi-sulphonium ion is involved). So for each pair of ethyl 2-acetamido-

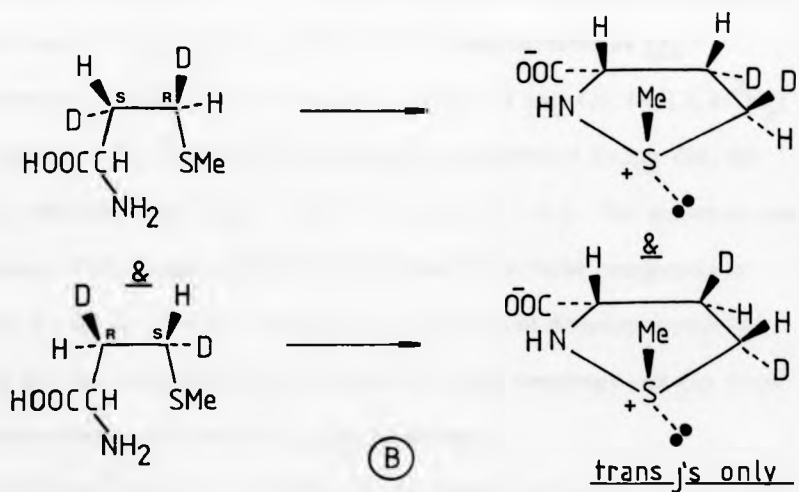
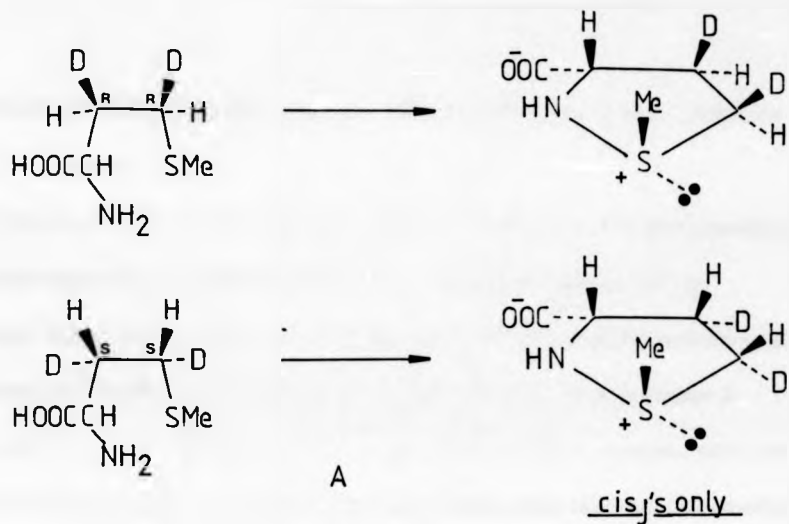


FIG 6-B-3

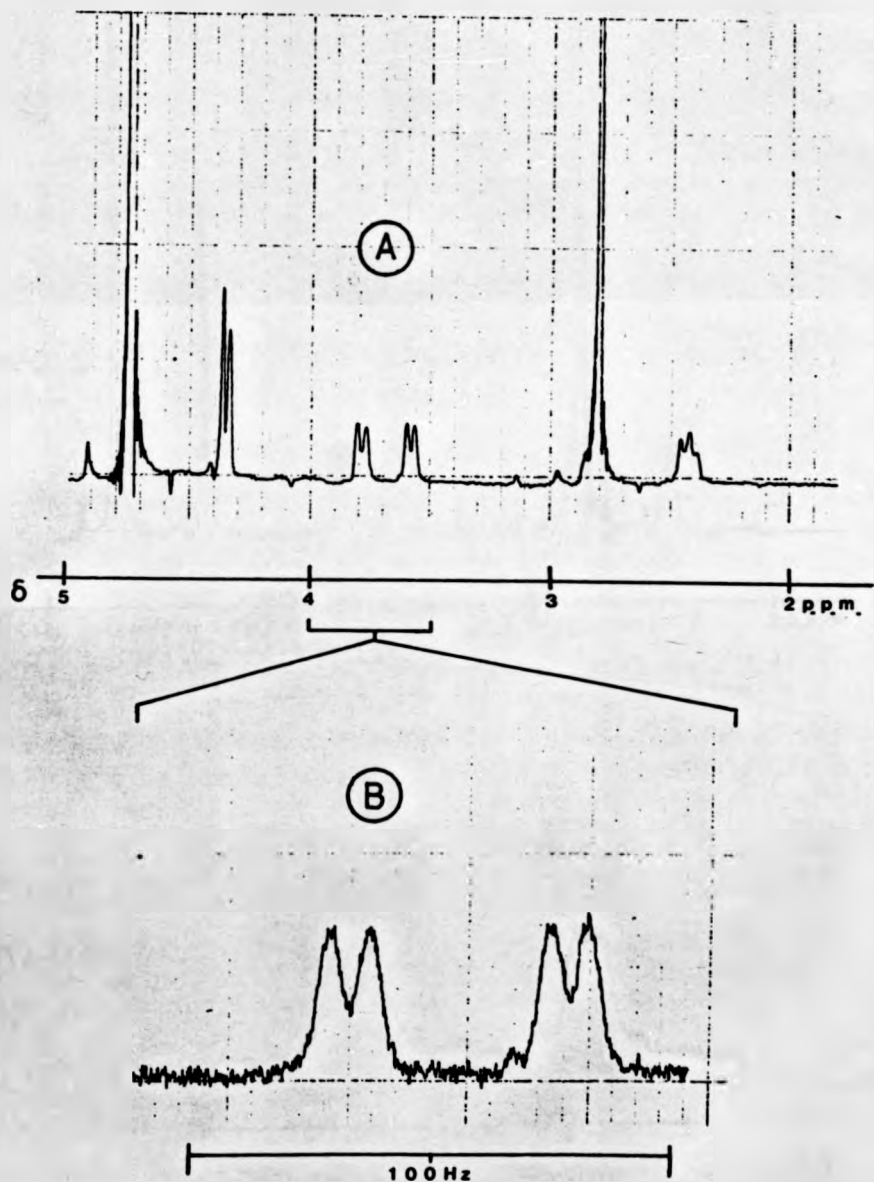
THE STEREOCHEMISTRY OF THE DEHYDMETHIONINES DERIVED FROM (A) RAC(3R,4R) AND (B) RAC(3R,4S)METHIONINE. ONLY THE MATERIALS DERIVED FROM (S)METHIONINE ARE SHOWN

2-ethoxycarbonyl-4-(methylthio)butanoates, the stereochemistry is as shown in Figs. 4 - B - 3 , and 4 - B - 4 .

Hydrolysis of ethyl 2-acetamido-2-ethoxycarbonyl-4-(methylthio)butanoate in aqueous hydrochloric acid leads directly to racemic methionine (as no stereochemical induction is expected in cases where stereoisomerism arises from the substitution of ^2H for H, in the case of the labelled ethyl 2-acetamido-2-ethoxycarbonyl-4-(methylthio)butanoates, the product will be a racemic mixture of (R) and (S) methionines). The product isolated from each labelled intermediate will be a mixture of 4 stereoisomers: i. e. a mixture of (2R, 3R, 4R); (2S, 3R, 4R); (2R, 3S, 4S); and (2S, 3S, 4S) [$3, 4\text{-}^2\text{H}_2$] methionine will result from the synthesis which starts from $\text{E}[^2\text{H}_2]$ ethylene, and this will be abbreviated as rac - (3R, 4R)methionine. From $\text{Z}[^2\text{H}_2]$ ethylene a mixture of rac-(3S, 4R) [$3, 4\text{-}^2\text{H}_2$] methionine and rac-(3R, 4S) [$3, 4\text{-}^2\text{H}_2$] methionine (abbreviated as rac-(3R, 4S) methionine) is obtained (see Figs. 4 - B - 3 , and 4 - B - 4). The structure and stereochemistry of the dehydromethionines derived from these compounds is shown in Fig. 6 - B - 3. It can be seen that the mixture of dehydromethionines derived from rac-(3R, 4R)methionine will show only cis couplings and that from rac-(3R, 4S)methionine will show only trans couplings.

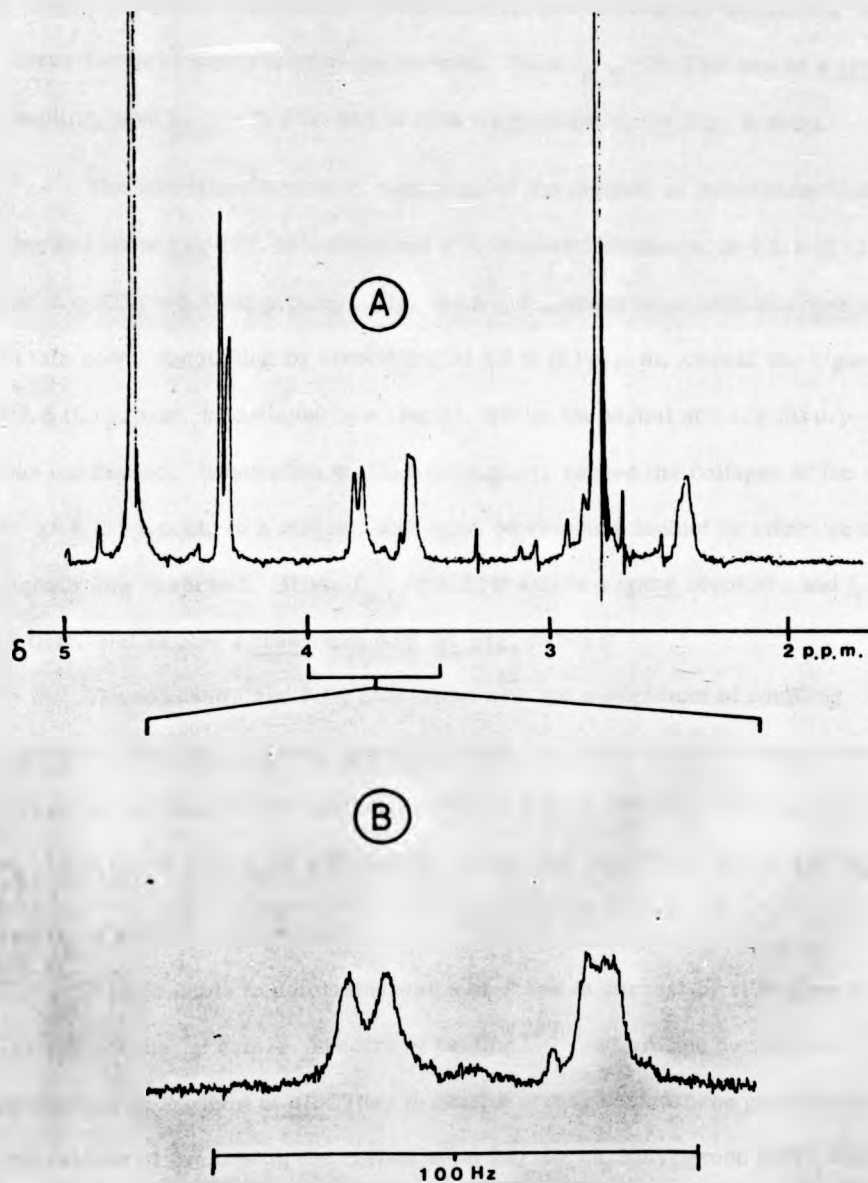
The 220 MHz ^1H n. m. r. spectrum of the mixture of the dehydromethionines obtained from rac-(3R, 4R)methionine shows doublets at δ 3.8 (B) ($J = 8.2$ Hz), and 3.6 (C) ($J = 6.9$ Hz) p. p. m., Fig. 6 - B - 4 , which must both be cis couplings. Decoupling by irradiating at δ 2.4 (E) p. p. m. collapsed the signal at δ 3.8 (B) p. p. m. to a singlet, whereas the signal at δ 3.6 (C) p. p. m. was unaffected. Irradiation at δ 2.8 (D) p. p. m. collapsed the signal at δ 3.6 (C)

FIG. 6-B-4



The 220 MHz ^1H n. m. r. spectrum of the dehydromethionine derived from rac (3R,4R)methionine (A) and expanded as shown, (B)

FIG. 6-B-5



The 220 MHz ^1H n. m. r. spectra of the dehydromethionines derived from rac-(3R, 4S)methionine (A) and expanded as shown, (B).

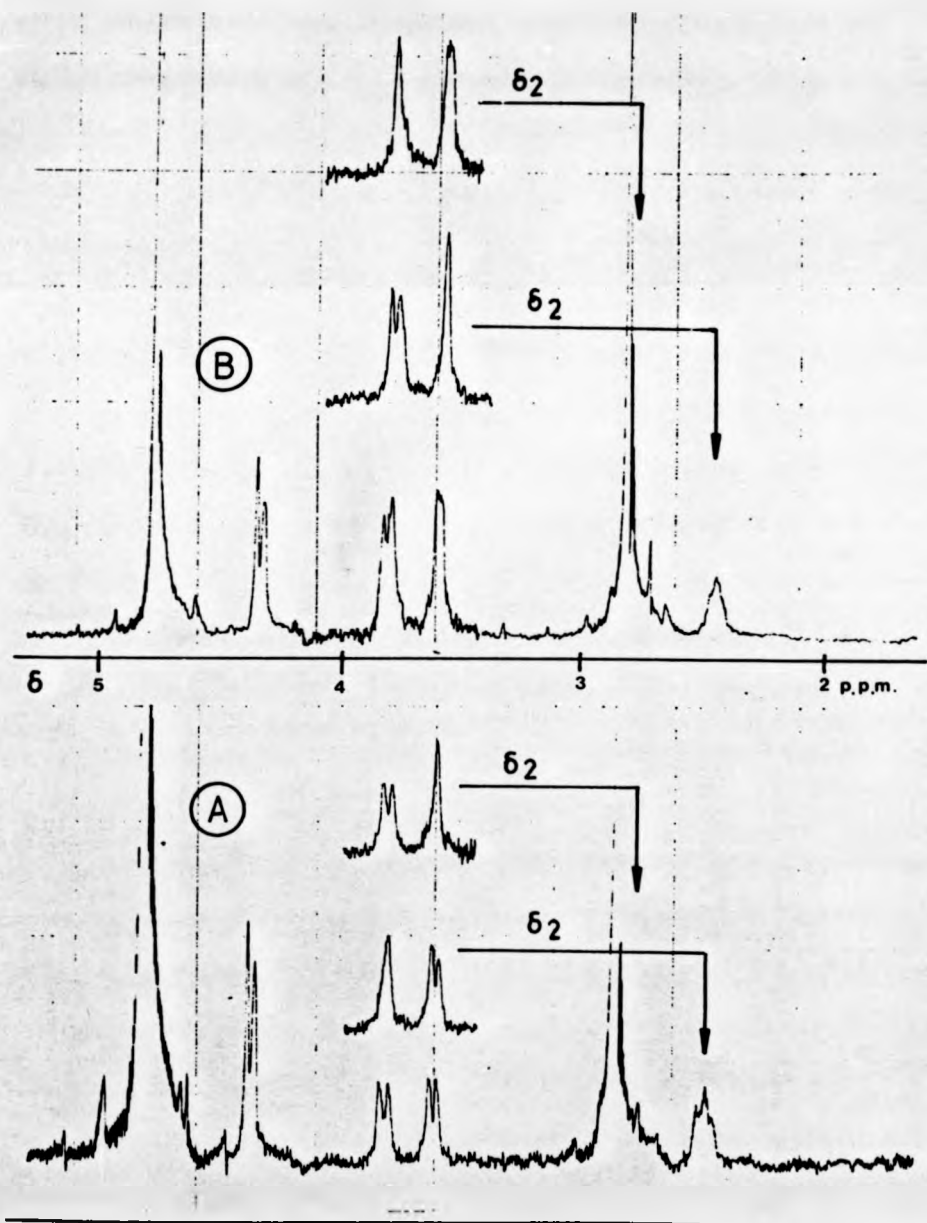
p.p.m., whilst the signal at $\delta 3.8$ (B) p.p.m. was unchanged, confirming the stereochemical homogeneity of the sample. Thus $J_{B,E} = 8.2$ Hz and is a cis coupling, and $J_{C,D} = 6.9$ Hz and is also a cis coupling (cf Fig. 6-B-6).

The 220 MHz ^1H n.m.r. spectrum of the mixture of dehydromethionines obtained from rac-(3R,4S)methionine also showed 2 doublets, at $\delta 3.8$ (B) ($J = 8.2$ Hz), and 3.6 (C) ($J = 5.0$ Hz p.p.m.), Fig. 6 - B - 5, which must both be trans couplings. In this case, decoupling by irradiating at $\delta 2.4$ (E) p.p.m. caused the signal at $\delta 3.6$ (C) p.p.m. to collapse to a singlet, whilst the signal at $\delta 3.8$ (B) p.p.m. was unaffected. Irradiation at $\delta 2.8$ (D) p.p.m. caused the collapse of the signal at $\delta 3.8$ (B) p.p.m. to a singlet, and again no residual doublet or effect on other signals was observed. Thus, $J_{B,D} = 8.2$ Hz and is a trans coupling, and $J_{C,E} = 5.0$ Hz, and is also a trans coupling (cf Fig. 6-B-6).

These results are fully consistent with the assignment of coupling constants and chemical shifts given in option 1 of Table 6 - B - 1, and allow only 2 possible configurational assignments (c.f. Fig. 6 - B - 2). That is, either $H_S^{-5} = B, H_R^{-5} = C, H_S^{-4} = E,$ and $H_R^{-4} = D,$ OR $H_S^{-5} = C, H_R^{-5} = B, H_S^{-4} = D,$ and $H_R^{-4} = E.$

It is possible to determine which of these is correct by reference to the analyses of the ^1H n.m.r. spectra of proline²²⁹ and proline derivatives^{233, 234}, performed by Pogliani et al. They present a strong case for the geminal non-equal equivalence of protons on the carbon atom β to the carbonyl group being due to an anisotropic field shift effect of the carboxyl group on protons lying in its plane. They conclude that the proton in a cis orientation to the carboxyl group appears at higher field to that in a trans orientation. Thus in dehydromethionine the proton

FIG. 6-B-6



The effect of decoupling at the frequencies indicated, on the ¹H n. m. r. spectra of the dehydromethionines derived from A) rac(3R, 4R) and B) rac (3R, 4S)methionine

H_S-4 must give rise to the signal at δ 2.4 p.p.m., i.e. $H_S-4 = E$. This gives a unique solution to the configurational assignment of dehydromethionine, and the full assignment of its n.m.r. spectrum is given in Table 6 - B - 2.

6 - C BASE CATALYSED EXCHANGE REACTIONS OF DEHYDROMETHIONINE

Following the results of Ref. ²²⁵, obtained in 5 M NaO²H solution, we initially examined the exchange of dehydromethionine in ²H₂O containing NaO²H. Addition of an excess (25%) of NaO²H to a solution of dehydromethionine in ²H₂O, gave a solution which after standing for 2 min at 37⁰C, contained no dehydromethionine by ¹H n. m. r. analysis. By t. l. c. and comparison to reference spectra, we determined the product to be methionine sulphoxide having ca 70% deuterium in its methyl group. No further exchange of the methionine sulphoxide was observed over 24 h at 37⁰C. Further experiments involving addition of 0.1, 0.25, 0.50, and 1 mol. equiv. of NaO²H to dehydromethionine solutions demonstrated that two reactions were occurring, the exchange of the methyl protons in dehydromethionine, and the stoichiometric, irreversible conversion of dehydromethionine to methionine sulphoxide, which did not undergo further exchange. The partial deuterium incorporation observed in the methyl group of the methionine sulphoxide indicates that the rates of the two reactions are comparable. In all cases the reaction was complete in under 2 min.

Because the reaction leading to methionine sulphoxide requires eventual deprotonation of the attacking O²H⁻, we reasoned that exchange without decomposition might be effected by an alkoxide ion in the corresponding alcohol. To this end we examined the exchange of dehydromethionine in [²H₄] methanol, containing catalytic NaOC²H₃. Addition of 0.018 mol. equiv. of NaOC²H₃ to a solution of dehydromethionine in [²H₄] methanol led to exchange of the methyl protons with t_{1/2} of \leq 3 min at 37⁰C. The rate of the reaction was monitored by running ¹H n. m. r. spectra at intervals, and comparing the size of the integral

for the CH_3 protons. A plot of $\log [H]$ (where $H = \% \text{ of protons present in the partially deuterated species}$) versus time is shown in Fig. 6 - C - 1. The non-linearity of the plot is attributed to consumption of base by irreversible reaction of base with dehydromethionine (possibly due to trace amounts of water) to give methionine sulphoxide, however, an estimate of $t_{\frac{1}{2}}$ for the reaction is obtainable from this treatment. The values of $[H]$ are corrected to allow for the fact that at equilibrium the dehydromethionine would contain 91% ^2H in its methyl group (see chapter 3 - C).

At higher concentrations of base (0.05, 0.10, and 0.50 mol. equiv.) exchange of the methyl protons was too fast to be followed by this method. At these higher concentrations of base, no exchange of protons at C-5 could be detected over 96 h, (by 90 MHz ^1H n. m. r.), rather the solutions of dehydromethionine decomposed at varying rates to methionine sulphoxide and methionine, identified by ^1H n. m. r. and t. l. c., until all of the base present had been consumed. Thus, due to the instability of dehydromethionine in basic media, we were unable to observe any exchange at C-5 under these conditions.

All of the above reactions in [$^2\text{H}_4$] methanol were very sensitive to trace amounts of water ($\text{NaOC}^2\text{H}_3 + \text{H}_2\text{O} \rightarrow \text{NaOH} + \text{C}^2\text{H}_3\text{OH}$, and then NaOH reacts irreversibly with dehydromethionine), i. e. in the exchange which is represented in Fig. 6 - C - 1, the amount of water necessary to consume effectively all the base would be 6×10^{-6} mol or 0.1 mg. The exchange of dehydromethionine in methan[^2H]ol at low base concentrations is however useful for preparing methyl labelled methionines, and this is developed into a synthetic procedure in chapter 3.

The stereochemical implications of the stoichiometric conversion of dehydromethionine to methionine sulphoxide are exploited later in this chapter (6 - D).

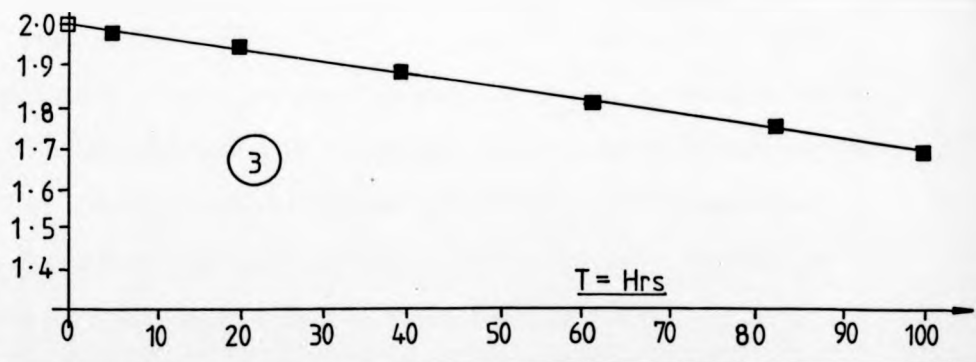
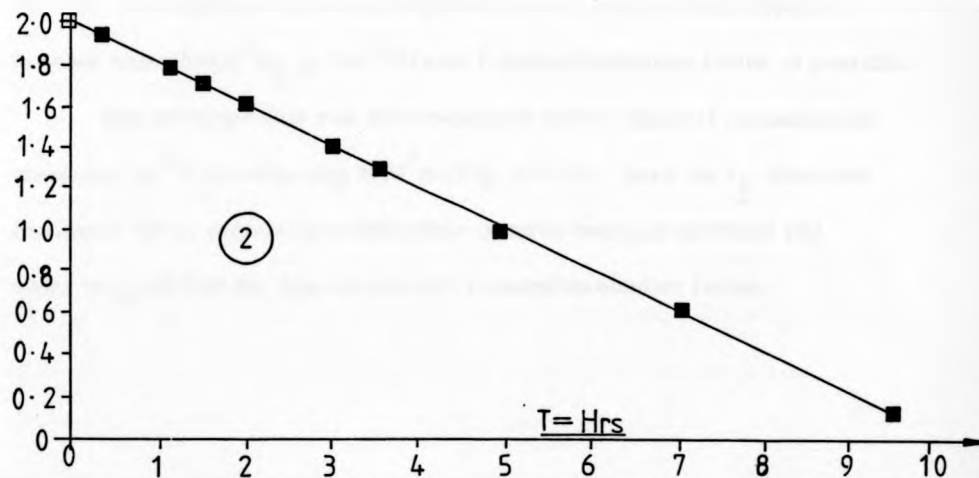
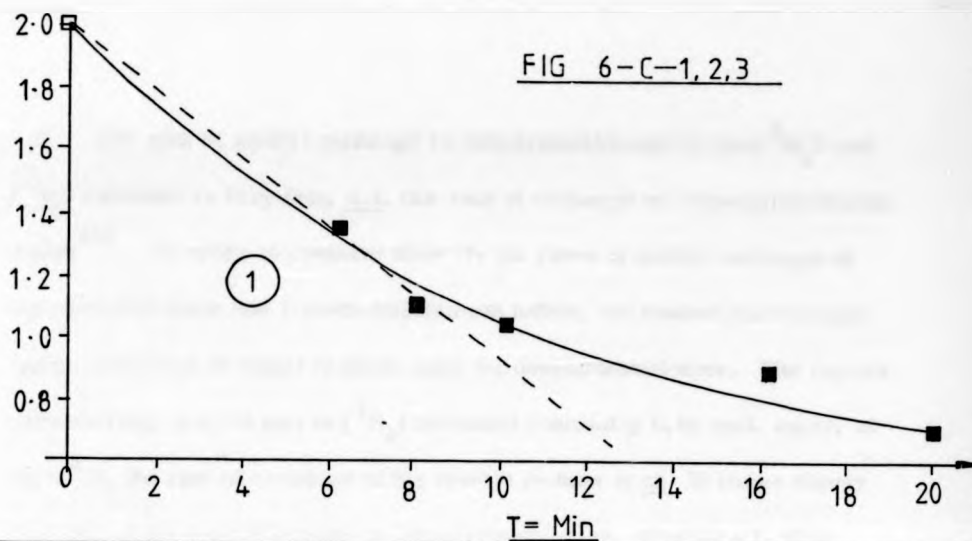


FIG. 6-C-1, 2 AND 3

PLOTS OF RATES OF EXCHANGES OF (39) AND (49). FOR
CONDITIONS SEE TEXT AND EXPERIMENTAL

The rate of methyl exchange in dehydromethionine in both $^2\text{H}_2\text{O}$ and $[^2\text{H}_4]$ methanol is very fast, c.f. the rate of exchange of 1-methylthiolanium iodide²²⁵. In order to compare directly the rates of methyl exchange of dehydromethionine and 1-methylthiolanium iodide, we studied its exchange under conditions identical to those used for dehydromethionine. The results indicate (Fig. 6-C-2) that in $[^2\text{H}_4]$ methanol containing 0.02 mol. equiv. of NaOC^2H_3 the rate of exchange of the methyl protons is ca 30 times slower than that of the methyl protons in dehydromethionine, showing a $t_{\frac{1}{2}}$ of ca 90 min. As expected, linearity is observed, as no irreversible reaction between base (NaOC^2H_3 or NaO^2H) and 1-methylthiolanium iodide is possible.

The exchange rate was also examined under identical concentration conditions in $^2\text{H}_2\text{O}$ containing NaO^2H (Fig. 6-C-3). Here the $t_{\frac{1}{2}}$ observed is almost 100 h, indicating a difference in rates between methanol and water of ca 60 fold for this reaction of 1-methylthiolanium iodide.

6-D THE PREPARATION OF OPTICALLY ACTIVE METHIONINE
SULPHOXIDE FROM DEHYDROMETHIONINE

Lavine reported²³⁵ in 1947 the oxidation of methionine to methionine sulphoxide by a number of reagents, one of which was alcoholic iodine. In this case a sulphoxide was produced which he assayed as 92.8% L-methionine (d) sulphoxide ((S)-methionine-(S)-sulphoxide²³⁶), and he identified dehydromethionine as an intermediate in this very slow reaction (165 h). In the light of the crystal structure of dehydromethionine¹⁷², we considered that treatment of (1R, 3S)dehydromethionine (derived from (S)-methionine) with base should lead to (S)-methionine-(S)-sulphoxide^{222, 236}, via the mechanism shown on Fig. 6-D-1. Attack of OH^- along the axis of the S-N bond (least repulsion would be experienced by attack along this line), could lead to a trigonal bipyramidal (t. b. p.) intermediate of the type shown (A). This is in analogy to the intermediates proposed by Cram²³⁷, and Mislow²³⁸. The existence of intermediates of this nature is now supported by a large body of evidence. For a review of this topic, and nucleophilic substitution at tricoordinate sulphur in general, see Ref. 199. The above t. b. p. (Fig. 6-D-1) is the most stable arrangement of the substituents present (maximum number of electronegative substituents apical²³⁹), and would therefore not be expected to Berry pseudo-rotate before decaying by fission of the apical S-N bond. This bond fission would then lead to (S)-methionine-(S)-sulphoxide.

Trial experiments had previously shown (6-C) the stoichiometric conversion of dehydromethionine to methionine sulphoxide, by NaO^2H in $^2\text{H}_2\text{O}$, the reaction taking ca 2 min with 1 mol. equiv. of NaO^2H . In order to obtain methionine sulphoxide in a pure state, dehydromethionine was opened by aqueous LiOH, and after neutralisation, the addition of a large excess of

FIG 6-D-1

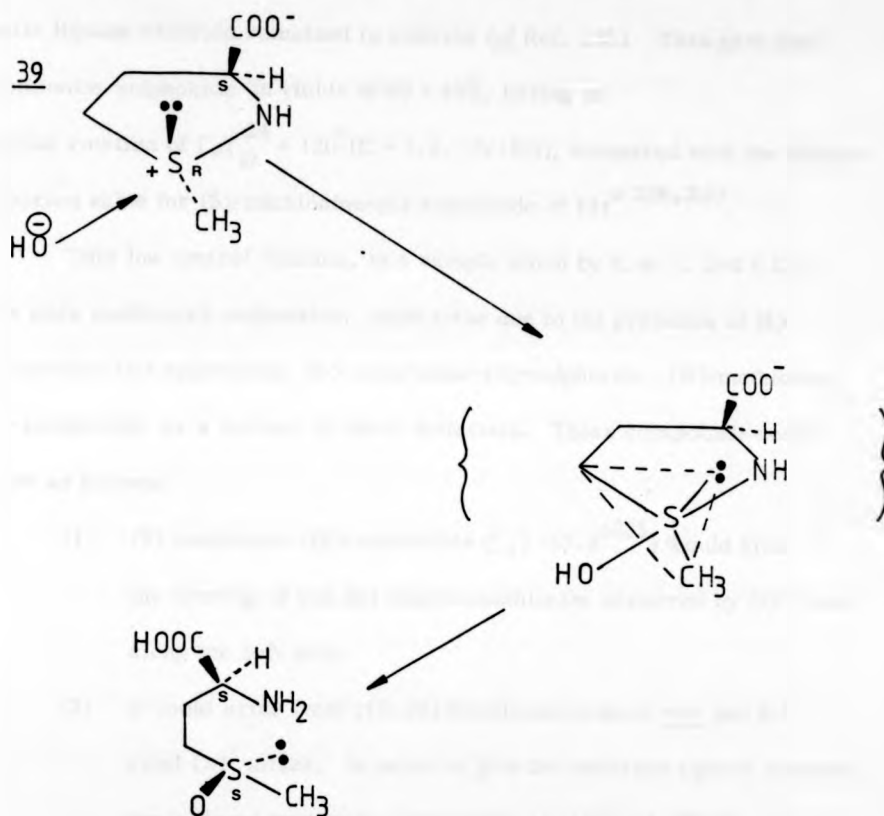


FIG. 6-D-1

A POSSIBLE MECHANISM FOR THE PRODUCTION OF
(S)-METHIONINE-(S)-SULPHOXIDE FROM (1R,3S)
DEHYDROMETHIONINE DUE TO ATTACK BY BASE.
THE TRIGONAL BIPYRAMIDAL INTERMEDIATE SHOWN
IS THE MOST STABLE AVAILABLE WITH THE ABOVE
SUBSTITUENTS (MAXIMUM NUMBER OF ELECTRO-
NEGATIVE SUBSTITUENTS APICAL)

of acetone to the reaction led to the precipitation of methionine sulphoxide, while lithium chloride remained in solution (cf Ref. 235). This gave pure methionine sulphoxide in yields of 80 - 85%, having an optical rotation of $[\alpha]_D^{24} = 120^\circ$ (C = 1.8, 1N HCl), compared with the highest reported value for (S)-methionine-(S)-sulphoxide of 131° 236,240.

This low optical rotation, in a sample which by n. m. r. and t. l. c. was pure methionine sulphoxide, could arise due to the presence of (S)-methionine-(R)-sulphoxide, (R)-methionine-(R)-sulphoxide, (R)-methionine-(S)-sulphoxide, or a mixture of these materials. These compounds could arise as follows:

- (1) (S)-methionine-(R)-sulphoxide ($[\alpha] -57.6^{o235}$) would arise if the opening of (1S,3S) dehydromethionine occurred by OH^- attack along the S-N axis.
- (2) It could arise from (1R,3S) dehydromethionine via non S-N axial OH^- attack. In order to give the observed optical rotation, the isolated materials composition would be 94.2% (S)-methionine-(S)-sulphoxide and 5.8% (S)-methionine-(R)-sulphoxide.
- (3) The presence of (R)-methionine in commercial (S)-methionine would be expected to give (1S,3R) dehydromethionine on oxidation. Opening of this material by the mechanism shown in Fig. 6-D-1, would give (R)-methionine-(R)-sulphoxide ($[\alpha] = -131^\circ$). To give the observed rotation, the composition of the methionine sulphoxide isolated above would be 95.8% (S)-methionine-(S)-sulphoxide, and 4.2% (R)-methionine-(R)-sulphoxide.
- (4) (R)-methionine-(S)-sulphoxide ($[\alpha] = +57.6^{o235}$) would arise

from (1S,3R)dehydromethionine as outlined above for (S)-methionine-(R)-sulphoxide.

Of these possible explanations for the low optical rotation observed in the isolated methionine sulphoxide, the most plausible are (1), (2) and (3). (1) would require the presence of ca 6% of the dehydromethionine formed from (S)-methionine to have the opposite configuration at sulphur to that reported by Glass and Duchek¹⁷². They may have removed this other stereoisomer by repeated recrystallisations, in order to obtain crystals for X-ray analysis. However, no evidence of diastereomeric (39) could be found in any samples prepared (220 MHz ¹H n.m.r. analysis). (2) would require non S-N axial attack of OH⁻ on (1R,3S)dehydromethionine to occur to the extent of ca 6%, which is not unreasonable. (3) would require the presence of ca 4% (R)-methionine in the commercial (S)-methionine used to make (1R,3S)dehydromethionine. This is considered unlikely, but is being investigated using an (R)-amino acid oxidase (cf Ref. 241).

The preparation of optically active methionine sulphoxide via the above method ((S)-methionine-(S)-sulphoxide from (S)-methionine via (1R,3S)-dehydromethionine; and (R)-methionine-(R)-sulphoxide from (R)-methionine via (1S,3R)-dehydromethionine) is not as stereochemically efficient as the resolution of the picrates^{235, 236}, or as the oxidation of methionine by auric chloride²⁴⁰. However, it has the advantages of being rapid, experimentally simple, and of using readily available, cheap starting materials. It is also readily adapted to the synthesis of [methyl-²H₃] labelled methionine sulphoxide (cf the preparation of (S)-[methyl-²H₃] methionine via proton exchange in dehydromethionine, chapter 3 and Ref. 244).

6-E EXPERIMENTAL

1. Preparation of (1R,3S)dehydromethionine (39)

This material was prepared as described in chapter 3.

2. Preparation of [4,5-²H₂]dehydromethionines

[4,5-²H₂]dehydromethionines (cf Fig. 6-B-3) were prepared from rac-(3R,4R)methionine, and rac-(3R,4S)methionine by the method described in chapter 3. On a 100 mg scale it was convenient to purify the dehydromethionine by "flash column chromatography" using a 10 mm dia. column, according to the method of Ref. 169.

3. ¹H n.m.r. analysis of (1R,3S)dehydromethionine, and [4,5-²H₂]dehydromethionines

220 MHz ¹H n.m.r. spectra were recorded on samples of dehydromethionine (0.05 g) in ²H₂O, (0.5 cm³), 99.8%, and referenced against TSS. The spectrometer was carefully tuned to achieve the best possible resolution by locking, and tuning, on the resonance of 2% tert-butanol added to the samples. The broad nature of the signals at δ 3.8 and 3.6 p.p.m. in Figs. 6-B-4, 6-B-5, and 6-B-6, arises due to the unresolved ²H-¹H couplings present in the signals, and due to the broadening effect of adjacent ²H nuclei. Spectra were computer simulated using a Nicolet NIC-80/S-7117-D (NMRCA L) computer programme²⁴². Details of the theory and operation of the programme are presented in Refs. 242 and 243. After simulation, a line width of 0.7 Hz was added to the calculated spectrum to enable plotting via the normal spectrometer plotting table.

As the analysis of the ^1H n. m. r. spectrum of (1R,3S)dehydromethionine was performed, but the [4,5- $^2\text{H}_2$] dehydromethionines were racemic (i. e. 50/50 (1R,3S), (1S,3R) derived from (S) and (R) i. e. rac-[3,4- $^2\text{H}_2$] methionine), the ^1H n. m. r. spectra of dehydromethionine derived from rac-methionine was also examined. The spectrum was identical to that of dehydromethionine derived from (S) methionine. No significant differences existed in the n. m. r. spectra of crude samples, compared with samples which had been purified by "flash column chromatography" and samples which had been so purified, and then recrystallised.

4. Preparation of 1-methylthiolanium iodide (49)

Methyl iodide (24.2 g, 10.6 cm³, 0.17 mol) was added dropwise to stirred tetrahydrothiophene (10 g, 10 cm³, 0.11 mol) over 10 min at 0°C. After standing for 12 h at room temperature, the solid product was broken up, and washed by decantation with ethanol (3 x 20 cm³) and then ground with ethanol (100 cm³). The resulting suspension was filtered at the pump and washed with ethanol (5 x 20 cm³). Drying in vacuo gave 1-methylthiolanium iodide as a white solid, with a faint odour of tetrahydrothiophene. Two recrystallisations from ethanol/ether gave (49) as pure white odourless needles (19.5 g, 75%), volatalises without melting at 190-192°C, δ ($^2\text{H}_2\text{O}$, TSS) 2.38 (2 H, m), 2.87 (3 H, s), and 3.55 (2 H, m).

5. Preparation of (S)-methionine-(S)-sulphoxide from (S)-methionine via (1R,3S)-dehydromethionine

(1R,3S) dehydromethionine (39) was prepared as described in chapter 3.

To a solution of (39) (0.1 g, 0.67 mmol) in water (1.75 cm³) was added saturated lithium hydroxide solution (0.25 cm³ of a 3.3 M solution, 0.825 mmol). After allowing the reaction to stand at room temperature for 15 min, the pH of the solution was adjusted to 7 by the addition of hydrochloric acid (2 M). Acetone (ca 20 volumes) was slowly added, and the white solid which formed allowed to settle over 20 min. The white crystalline precipitate was collected at the pump, washed with acetone (2 x 5 cm³) and dried in vacuo to give (S)-methionine-(S)-sulphoxide (0.9 g, 81%) as white crystals : darkens and sinters at 240-247°C (in agreement with the literature²³⁶), pure by t. l. c. (systems 1, Rf 0.29 and 2, Rf 0.21), δ (²H₂O, TSS) 2.3 (2 H, m), 2.7 (3 H, s), 3.0 (2 H, m) and 3.9 (1 H, t) p. p. m., $[\alpha]_D^{24} = 120^\circ \pm 1^\circ$ (C = 1.8, 1M HCl), containing no Li ions (flame test). The isomeric composition of this material is discussed in the text.

Exchange Reactions

Reactions in [²H₄] methanol were carried out under scrupulously anhydrous conditions, all manipulations being performed in a dry box, using very dry apparatus. Standard solutions of NaOC²H₃ in [²H₄] methanol were prepared as described in chapter 2.

1. Exchange of (1R, 3S)dehydromethionine (39), and 1-methylthiolanium (49) iodide in [²H₄] methanol

In a dry box, sodium methoxide solution in [²H₄] methanol (10 μl of a 0.625 M solution, 0.0063 mol; 1.8 mol %) was added to a solution of (1R, 3S) (39) (0.05 g, 0.34 mmol) in [²H₄] methanol [0.6 cm³], and the solution was stored in a tightly capped 5 mm dia. n. m. r. tube at 37°C. 60 MHz ¹H n. m. r.

spectra were recorded at intervals, and integrated in order to compare the ^2H content of the methyl signal to that of the un-exchanged C-4 protons. The results were corrected to allow for the equilibrium ^2H content of (39) being 91% (see chapter 3), and were then plotted as $\log [\text{H}\%]$ present, versus time. The results are depicted in Fig. 6-C-1 and discussed in the text.

The rate of methyl exchange in (49) was studied under identical conditions. Thus, sodium methoxide in [$^2\text{H}_4$] methanol (12.5 μl of a 0.505 M solution, 0.0063 mmol; 1.8 mol%) was added to a solution of (49) (0.078 g, 0.34 mmol) in [$^2\text{H}_4$] methanol (0.6 cm^3). The tightly capped 5 mm dia. n. m. r. tube containing the solution was stored at 37°C, and 60 MHz ^1H n. m. r. spectra were recorded at intervals. The ^2H content of the methyl group was assayed by the comparison of integrals as above. Correction of these values to allow for the ^2H content at equilibrium gave the values of H% present, the log of which are plotted versus time in Fig. 6-C-2, and the result is discussed in the text.

2. Exchange of (49) in $^2\text{H}_2\text{O}$

To a solution of (0.078 g, 0.34 mmol) in $^2\text{H}_2\text{O}$ (0.6 cm^3) was added NaO^2H (9 μl of a 0.7 M solution, 0.063 mmol, 1.8 mol%) in $^2\text{H}_2\text{O}$, and the solution was stored as above. 60 MHz ^1H n. m. r. spectra were recorded, and integrated as above. Correction of the H% present thus obtained, to allow for the presence of 98% ^2H at equilibrium, gave the values of H% present, the logs of which are plotted versus time in Fig. 6-C-3. The result is discussed in the text.

3. Attempts to exchange protons at C-5 of (39)

Solutions were made up as for the exchange of (39) in [$^2\text{H}_4$] methanol described above, containing 0.05, 0.1 and 0.5 mol. equiv. of NaOC^2H_3 . The samples were incubated at 37°C , and 90 MHz ^1H n.m.r. spectra were recorded, and integrated, at intervals. These spectra do not show any evidence of exchange at C-5 of (39), rather from the n.m.r. spectra, and t.l.c. run at the same time as spectra were recorded (system 2) the decomposition of (39) to methionine (1) and methionine sulphoxide (MS) is evident.

4. Exchange of (39) in $^2\text{H}_2\text{O}$; reaction between (39) and NaO^2H

Solutions of (39) (0.05 g, 0.34 mmol) in $^2\text{H}_2\text{O}$ (0.6 cm^3) were made up in the dry box, and NaO^2H added (addition of 9 μl ; 45 μl ; 112 μl ; 224 μl ; and 450 μl of a 0.7 M solution, i.e. 2 mol %; 10 mol %; 25 mol %; 50 mol %; and 100 mol %). In each case, reaction was complete before an n.m.r. spectrum could be recorded (ca 2 min). Each sample showed the presence of an amount of methionine sulphoxide (MS) corresponding to the amount of base added, which was partially exchanged in the methyl group, and also partially exchanged residual (39). In each case no further exchange or conversion of (39) to (MS) was observed on storage at 37°C for 12 h, indicating stoichiometric consumption of NaO^2H . The presence of (50) was confirmed by t.l.c. (system 2). The addition of successive 10 mol % aliquots of NaO^2H to the solution containing 10 mol % NaO^2H , demonstrated stepwise stoichiometric conversion of partially exchanged (39) into partially exchanged (MS) until at 1 mol. equiv. NaO^2H , only partially exchanged (MS) was present. This was confirmed by t.l.c. (system 2).

CHAPTER 7

STUDIES ON THE BIOSYNTHESIS OF ETHYLENE AND SPERMIDENE FROM METHIONINE : APPLICATIONS OF STABLE ISOTOPE LABELLED METHIONINES

7-A STUDIES ON ETHYLENE BIOSYNTHESIS

7-A-1 INTRODUCTION

The mechanisms involved in the biosynthesis of ethylene from methionine by plants are not clear at present (cf chapter 1). Recently the study of ethylene production by microorganisms has been undertaken³⁷. As Dr S. B. Primrose of the Biological Sciences Department of the University of Warwick was engaged in studies on ethylene biosynthesis by bacteria, a collaboration was arranged.

Using material obtained from cultures grown by Dr Primrose, a possible intermediate in the production of ethylene by bacteria has been isolated, and identified as 4-methylthio-2-oxobutanoate (KMBA)²⁴⁵. In order to examine the mechanism of the elimination involved in ethylene biosynthesis, stereospecifically labelled rac-[3,4-²H₂] methionines were synthesised, as described in chapter 4. The results of preliminary studies of the conversion of stereospecifically labelled methionine to ethylene, by cultures of coryneform bacterium D7F are presented. These results are discussed in the light of current ideas on the mechanisms involved in the biosynthesis of ethylene by bacteria and plants.

7 - A - 2 KMBA AS AN INTERMEDIATE IN THE BIOSYNTHESIS OF
ETHYLENE FROM METHIONINE BY BACTERIA

Primrose observed in 1977⁷² that addition of 2,4-dinitrophenylhydrazine (24DNP) to culture fluids of ethylene producing bacteria gave an orange precipitate, but after illumination in the presence of a flavin, the culture fluids yielded no precipitate when treated with 24DNP. This suggests that the intermediate which accumulates in ethylene-producing cultures is an aldehyde or ketone, and preliminary studies led Primrose to favour KMBA as the intermediate.

Our work has demonstrated that this compound which accumulates in culture media is KMBA, but as with all studies of this type, it is possible that KMBA is not an actual biochemical intermediate in the production of ethylene from methionine, but is an artifact arising chemically from the actual intermediate, either naturally or due to the conditions used in the compound's isolation. If this were the case, then all assays of KMBA could in fact be assaying either the active intermediate itself, or KMBA (formed as an artifact, or as an intermediate), or both. This possible origin of the KMBA which accumulates in bacterial cultures growing in the presence of methionine is discussed later in the text, in relation to possible ethylene-producing mechanisms, and should be borne in mind during the discussion which follows.

We have identified and characterised the 2,4-dinitrophenyl hydrazone (DNP) of this compound, as follows. Authentic samples of KMBA DNP were obtained by chemical synthesis²⁴⁶, and by the reaction of commercial KMBA (20) with 24 DNP in acidic solution. Authentic samples of the DNP's of methional,

TABLE 7-A-1

T. L. C. CHARACTERISTICS OF SELECTED DNP'S

Compound's DNP/ Solvent	A	B	C	D
KMBA (a)	0.39 and 0.58	0.14	0.074 and 0.23	Origin
KMBA (b)	0.38 and 0.59	0.14	0.075 and 0.23	Origin
Pure Isomer KMBA (c)	0.57	0.14	0.23	Origin
D7F ppt	0.40 and 0.58	0.14	0.074 and 0.23	Origin
B12E ppt	0.40 and 0.58	0.14	0.074 and 0.23	Origin
Methional	0.94	0.83	0.46	0.25
α -oxobutyric acid	0.52	0.15	0.29	Origin
Acetone	0.92	0.83	0.49	0.36
24 DNP	0.78	0.66	0.37	-

TABLE 7-A-2

¹H n.m.r. data for the 2,4-dinitrophenylhydrazones (DNP's) of 4-methylthio-2-oxobutanoate derived from natural sources and authentic materials

Spectra were of solutions in [²H₆]dimethyl sulphoxide run at 90 MHz. The chemical shifts of aromatic protons are especially sensitive to the conditions.

	Chemical shifts from tetramethylsilane (p. p. m.)				
Dinitrophenylhydrazone	CH ₃ S	CH ₂ CH ₂	H-6'	H-5'	H-3' NH
*Synthetic KMBA DNP	2.12(s), 2.17(s)	2.82(s)	8.01(d), 8.08(d)	8.48(q), 8.50(q)	8.86(d) 11.09(s), 11.50(s)
† Single isomer of KMBA DNP	2.12(s)	2.81(s)	7.95(d)	8.37(q)	8.81(d) 11.53(s)
‡ KMBA DNP from Coryneform D7F	2.12(s), 2.17(s)	2.83(s)	8.03(d), 8.09(d)	8.39(q), 8.48(q)	8.86(d) 11.10(s), 11.49(s)

*Synthesized by the method of Cahill and Rudolph

† Synthesized by a modification of the method of Cahill and Rudolph

‡ Material was obtained by addition of 2,4-DNP reagent to culture fluids of Coryneform D7F

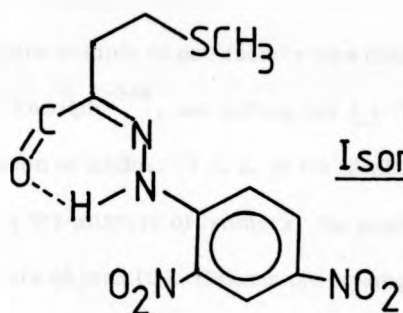
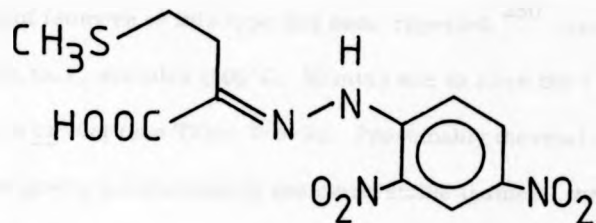
α -oxobutyric acid, and acetone were obtained from the commercial compounds in the same manner. T.l.c. systems were developed which achieved good separations on silica gel plates between the DNP's of acetone/methional (50)/(19) α -oxobutyric acid (57), KMBA, and unreacted 24DNP, using the following solvent systems: dichloromethane/methanol, 7:3 (A); dichloromethane/methanol, 9:1 (B); and propan-2-ol (C). In each of these systems the Rf's of acetone and methional DNP's were very similar, but adequate separation between these two DNP's could be achieved by using pentane/diethylether, 2:1 (D) as solvent system. The collected Rf values for these DNP's in the above solvent systems are given in Table 7-A-1.

KMBA DNP prepared from commercial KMBA (a) showed 2 spots in solvent systems (A) and (C), as did KMBA DNP obtained by chemical synthesis (b). The literature shows^{247, 248, 249, 250}, that the DNP's of α -keto acids can exist in 2 isomeric forms (Fig. 7-A-1), as E and Z isomers around C=N. One of these isomers is more stable than the other, because of an intramolecular hydrogen bond between C=O and N-H. The separation of such isomers by chromatography has been reported. The ratio of the 2 spots observed on t.l.c. for KMBA DNP was ca 1:1 and we considered it probable these were the two isomers of KMBA DNP shown in Fig. 7-A-1, (E) and (Z).

The 90 MHz ¹H n.m.r. spectra of each of these samples of authentic KMBA DNP (see Table 7-A-2) showed 2 methyl singlets (integral ratio ca 1:1) δ 2.12 and 2.17 p.p.m., assigned as the methylthio groups of the two isomers (c.f. work on isomeric DNP's and their n.m.r. spectra, Refs 251 and 252). Other features indicative of the presence of two isomers were 2 NH signals, δ 11.1 and 11.5 p.p.m., overlapping quartets around δ 8.45 p.p.m. (H - 5')

FIG 7-A-1

Isomer-Z



Isomer-E

FIG. 7-A-1

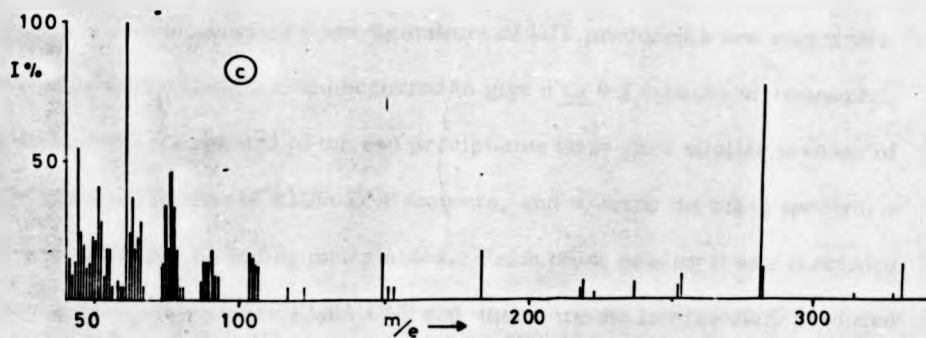
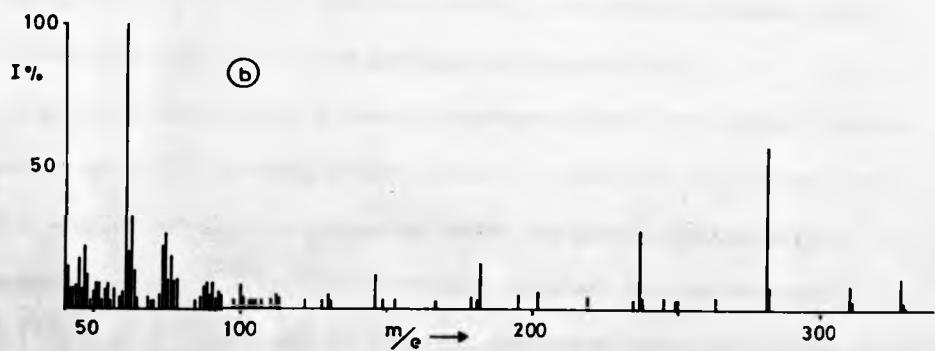
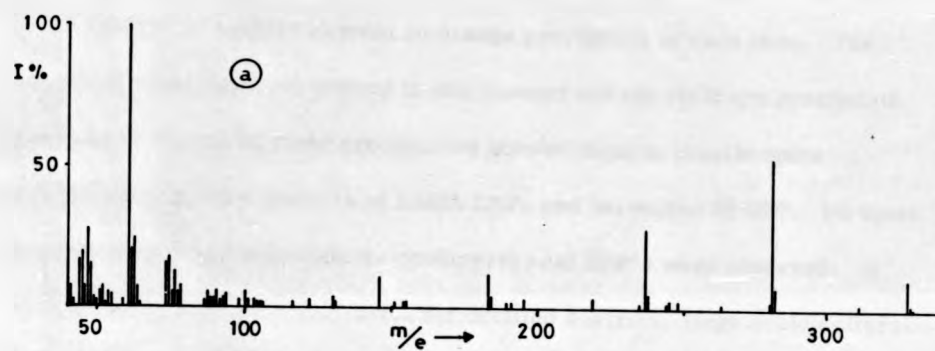
The two geometric isomers of KMBA DNP. The E-isomer is stabilised relative to the Z isomer by an intramolecular hydrogen bond between N-H and C=O as shown.

and overlapping doublets at around δ 8.02 and 8.08 p.p.m. (H - 6'). The thermal interconversion of isomers of this type has been reported,²⁵⁰ and the effect of heating the n.m.r. samples (100°C, 30 min) was to alter the ratio of the two sets of peaks to ca 9:1 (see Table 7-A-2). Presumably thermal equilibration occurs here giving predominantly the more stable isomer (the Z isomer, in Fig. 7-A-1).

A pure sample of one isomer was obtained by repeating the procedure of Cahill and Rudolph²⁴⁶, and adding hot (ca 70°C) 24 DNP solution to a hot (ca 70°C) solution of KMBA. T.l.c. of the KMBA DNP obtained in this way showed it to be a ca 9:1 mixture of isomers, the predominant isomer having the higher Rf in solvents (A) and (C). Column chromatography on silica gel with solvent (A) provided a pure sample of this isomer.

The 90 MHz ¹H n.m.r. spectrum of this pure isomer (see Table 7-A-2) showed a singlet at δ 2.12 p.p.m. (but no signal at δ 2.17 p.p.m.), a single NH resonance at δ 11.15 p.p.m., and no duplication of signals at δ 8.45, 8.02 and 8.08 p.p.m. The i.r. spectrum of this isomer showed an intense band at 1624 cm⁻¹, assigned as a hydrogen bonded C=O stretch²⁴⁷ but no significant absorption at higher wave number, which would have arisen from free C=O. This isomer can therefore be assigned as the Z isomer (c.f. fig. 7-A-1), having an intramolecular hydrogen bond, and the result of the thermal equilibration of the n.m.r. sample given previously is therefore an alteration from a ca 1:1 ratio of Z/E isomers to a ratio of ca 9:1, Z/E isomers. The mass spectra (Fig. 7-A-2) of the mixtures of isomers (a) and (b) and of the pure single isomer were very similar, showing peaks at m/e 328 (M⁺), 281, and 237 amongst others. Metastable peaks were observed in each spectrum at m/e 240.7 and 200.

FIG. 7-A-2



Mass spectra of (A) KMBA DNP (b), (B) DNP p.p.t. from $D7F_2$
(C) DNP p.p.t. from B12E grown in the presence of [methyl- 2H_3]-
methionine

After growing cultures of Aeromonas hydrophila B12E and B19E , and the Coryneform bacteria D7F and D19E, in the presence of methionine, 24DNP reagent was added to yield an orange precipitate in each case. The cultures of other bacteria treated in this manner did not yield any precipitate (see later). T.l.c. of these precipitates showed them to contain spots corresponding to both isomers of KMBA DNP, and unreacted 24 DNP. No spots corresponding to methional or α -oxobutyric acid DNP's were observed. In order to obtain sufficient derivative for detailed analysis, large scale cultures of the best "intermediate" producers (D7F and B12E) were grown as described later. These cultures converted almost 50% of the added methionine to the unknown intermediate, assayed gravimetrically as its DNP.

T.l.c. examination of these precipitates (Table 7-A-1) showed them to contain spots corresponding to both isomers of KMBA DNP, (ca 1:1) and a very faint spot corresponding to unreacted 24DNP. No spots corresponding to methional or α -oxobutyric acid DNP's were observed, even at very high loadings. The 90 MHz ^1H n.m.r. spectra of the two precipitates were very similar to the spectra of the authentic KMBA DNP's (Table 7-A-2). Heating the n.m.r. sample of the precipitate obtained from the culture of D7F produced a new spectrum, showing that equilibration had occurred to give a ca 9:1 mixture of isomers. The solution i. r. spectra of the two precipitates were very similar to those of the authentic mixture of KMBA DNP isomers, and so were the mass spectra, showing all peaks including metastables. From these results it was concluded that the precipitates were KMBA DNP and therefore the intermediate produced by these cultures was KMBA. In order to demonstrate that this KMBA was derived directly from methionine added to the cultures, a culture of B12E was

grown in the presence of [methyl-²H₃] methionine. The mass spectrum of the KMBA DNP isolated from this culture showed m/e 331 (M⁺) (Fig. 7-A-2), thus demonstrating the precursor role of methionine.

The amount of KMBA produced by other cultures was too small to be detected directly by its conversion to KMBA DNP. However, by incubating cultures in the presence of [³⁵S]methionine, radio-labelled KMBA was produced, which was isolated (after addition of "cold" KMBA as a carrier) as its DNP. T.l.c. followed by autoradiography, showed the presence of radiolabelled KMBA DNP by the fogging observed of a photographic plate placed over the chromatogram, in areas corresponding to the KMBA DNP spots on the chromatogram. In this way KMBA was shown to be a metabolic product of Pseudomonas aeruginosa PAT, E. coli E603, E. coli SPAO, Saccharomyces cerevisiae DD1, and Penicillium digitatum, grown in the presence of methionine.

To ensure no methional DNP had been formed, but not detected, the culture fluids obtained were extracted with dichloromethane, after the addition of 24 DNP reagent and recovery of the precipitate. Evaporation of this extract gave a solid which was mainly unreacted 24 DNP. T.l.c. showed no methional DNP for any culture examined, except the large scale culture of B12E. In this case a very faint spot corresponding to methional DNP was observed, the yield relative to the yield of KMBA DNP being below 1%.

Following these results, Dr Primrose developed a sensitive photochemical assay for KMBA, involving the conversion of KMBA to ethylene in the presence of a flavin and light²⁴⁵. The assay conditions were arranged such that no photochemical conversion of methionine to ethylene occurred, and although methional was also active in the assay (to a much lesser extent than KMBA), because no significant

methional DNP had been observed from any of the cultures grown in the presence of methionine, it was assumed that in all cases KMBA was being assayed. Using this assay, KMBA was detected in the culture fluids of Escherichia, Pseudomonas, Bacillus, Acinetobacter, Aeromonas, Rhizobium, and Corynebacterium species

When the assay was applied to cultures grown in the presence of varying concentrations of glucose, it showed that glucose concentration had little effect of the production of KMBA by a given culture, but a marked effect on the production of ethylene. This result may explain the failure of earlier workers to observe ethylene formation by their cultures. Using the assay, he was able to show that the production of both KMBA and ethylene are enzymatic reactions (in the dark), rather than chemical reactions dependent on low molecular weight co-enzymes²⁴⁵. This deduction follows from the observation that the formation of both KMBA and ethylene is induced by addition of methionine, and addition of chloramphenicol inhibited this induction (presumably due to its inhibition of protein synthesis). In the enzymic degradation of KMBA a peroxidase is implicated, because addition of catalase seriously inhibits ethylene production. This enzymic production of ethylene is considered to be the only pathway operating in vivo, as the production of ethylene by cultures grown in the presence of methionine is the same, in the presence or absence of light⁷².

The cultures shown to produce KMBA by the KMBA assay also produce ethylene, but in much smaller quantities than KMBA. This, coupled with the fact that KMBA accumulates in cultures of D7F, B12E, etc., to such an extent, indicates that the synthesis of KMBA is proceeding faster than its degradation. These observations support the previously advanced idea⁷² that the failure

of some bacteria to produce ethylene is due to their failure to degrade the active intermediate, rather than their failure to synthesise it.

This accumulation of KMBA in cultures, despite at least two degradative pathways to ethylene, is not surprising. Only in the case of exceptional flavin over-producers⁷⁰ is sufficient flavin likely to be present to allow the photochemical conversion of KMBA to ethylene, and so this degradative pathway is probably not open. In the enzyme-mediated reaction a peroxidase has been implicated, and peroxidases are not normally considered to be constitutive in bacteria. Any peroxidase present (normally, or induced by the presence of methionine) would have its activity moderated by the catalase normally present in bacterial cells. The known effect of glucose concentration on catalase activity may account for the observed effect of glucose on a culture's ability to degrade KMBA to ethylene. Possible reaction pathways leading to the production of KMBA from methionine during ethylene biosynthesis are discussed in section 7-A-4.

7-A-3 PRELIMINARY RESULTS OF AN EXAMINATION OF THE STEREO-CHEMISTRY INVOLVED IN THE BIOSYNTHESIS OF ETHYLENE FROM METHIONINE BY BACTERIA

The syntheses of stereospecifically labelled $\text{rac-}[3,4\text{-}^2\text{H}_2]$ methionines have been described in chapter 4, and here preliminary results of incubating cultures of organism D7F in the presence of these methionines are reported.

As Fig. 1-E-1 shows, if a trans elimination occurs (see discussion later) in the production of ethylene from methionine, then this elimination would produce E-[1,2-²H₂] ethylene from $\text{rac-}(3R,4R)$ methionine, and Z-[1,2-²H₂] ethylene from $\text{rac-}(3R,4S)$ methionine. By using the organism which produced most ethylene from a given quantity of methionine (D7F) and combining the atmospheres

from a large number of cultures, we were able to obtain sufficient ethylene for i. r. analysis. Cultures were incubated in the presence of rac-(3R,4R)methionine and the atmospheres from these cultures combined. Isolation of the ethylene present, and i. r. analysis, showed the presence of [1-²H] ethylene (ca 50%) and a mixture of E and Z, [1,2-²H₂] ethylenes (ca 25% of each). The cultures used were grown for 48 h in the dark, and immediately after the isolation of their atmospheres, the culture flasks were vented to atmospheric pressure, riboflavin was added, and they were then illuminated for 3 h under conditions identical to those used for the photochemical assay of KMBA. Again the ethylene was isolated from the combined atmospheres of these flasks, and its i. r. spectrum showed [1-²H] ethylene and a mixture of E and Z, [1,2-²H₂] ethylenes. In this case the percentage represented by [1-²H] ethylene was higher than in the previous case. The experiment was repeated and the results were not significantly different.

At first these results were surprising, because the ²H content and stereochemical purity of the labelled methionines used was reasonably certain (see chapters 4 and 6). The most probable cause of loss of ²H seemed to be exchange with solvent water, during the biochemical production of ethylene from methionine. This exchange could occur at a number of places in this biosynthetic pathway. These include, exchange of an enzyme-bound intermediate in the formation of KMBA (or other intermediate, catalysed by the presence of metal ions or enzyme co-factors perhaps) in the medium before its conversion to ethylene, or exchange in an enzyme bound intermediate involved in the production of ethylene from KMBA (or other intermediate). As the exchange

of protons α to a carbonyl function is well known ¹²⁰, and the synthesis of KMBA proceeds faster than its degradation to ethylene, the chemical exchange of KMBA in the medium, before its conversion to ethylene, was a possibility. Note also that proton exchange during enzyme-mediated transamination reactions (i.e. $\text{RCH}_2\text{CH}(\text{NH}_2)\text{COOH} \xrightarrow{^2\text{H}_2\text{O}} \text{RC}^2\text{H}_2\text{COCO}(\text{OH})$) has been observed (e.g. see Ref. 252a).

Incubation of the sodium salt of KMBA in $^2\text{H}_2\text{O}$ at 37°C resulted in exchange (followed by ^1H n. m. r.) of the protons on C-3 with a $t_{\frac{1}{2}}$ of ca 12 h at pH 6.2 and ca 8 h at pH 4.3 (n.b. the pH of the culture media used to produce ethylene from rac-(3R,4R)methionine varied from 5.9 to 6.4). In the presence of magnesium ions these $t_{\frac{1}{2}}$ values were reduced to ca 1 h at pH 6.2 and ca 1.5 h at pH 4.3. As the cultures had been incubated at 30°C for 48 h, and the synthesis of KMBA proceeds faster than its degradation, this means that if KMBA is an intermediate in the production of ethylene from methionine, chemical exchange of $[3,4\text{-}^2\text{H}_2]$ KMBA would have occurred in the medium before its conversion to ethylene.

$[3\text{-}^2\text{H}_2]$ KMBA (52) was prepared by incubation of KMBA in $^2\text{H}_2\text{O}$ at 37°C , and when subjected to the riboflavin/light assay, this $[3\text{-}^2\text{H}_2]$ KMBA yielded $[1\text{-}^2\text{H}_2]$ ethylene, by i. r. analysis, and no $[1\text{-}^2\text{H}]$ ethylene was observed. This indicates that no exchange occurs during the photochemical conversion of KMBA to ethylene, and so the ethylene obtained by the photochemical assay from culture fluids of D7F cultures incubated in the presence of rac-(3R,4R)methionine reflects the ^2H content of the KMBA present in the media when the assay was performed. Thus the KMBA present in the medium at the time the assay was performed had undergone chemical exchange, as

more [$1-^2\text{H}$] ethylene was present in the ethylene produced by the riboflavin assay, than in the ethylene produced naturally by the culture.

The demonstration that exchange of KMBA could occur during its time in the medium, before conversion to ethylene, did not however prove that this was the cause of the loss of ^2H observed in the ethylene produced by the cultures. It was still necessary to examine the possibility that exchange occurs in the biosynthetic route between methionine and KMBA. To this end, the shortest time possible was used to produce KMBA from cultures of D7F grown in the presence of rac-[$3-^2\text{H}_2$] methionine (38) (in order to minimise exchange of KMBA in the medium). Isolation of this KMBA as its DNP and assay of its ^2H content by ^1H n. m. r. seemed the best approach, as exchange of KMBA DNP does not occur after its formation.

Trial experiments showed that isolation of KMBA DNP from KMBA (52) using normal 24 DNP reagent led to a loss of $\geq 20\%$ ^2H . As this ^2H loss probably occurred via exchange of KMBA under the strongly acidic conditions used to form KMBA DNP, an alternative 24 DNP reagent was developed, which would produce KMBA DNP under less acidic conditions. This reagent allowed isolation of KMBA DNP showing $\geq 90\%$ of the ^2H content of the KMBA (52) used.

Trial experiments had shown, that the production of KMBA by cultures of D7F grown in the presence of rac-methionine for 3-4 h, was seriously inhibited by concentrations of greater than $200 \mu\text{g}/\text{cm}^3$ rac-methionine. By incubating cultures of D7F for 3.5 h in the dark, in the presence of $200 \mu\text{g}/\text{cm}^3$ rac-methionine, sufficient KMBA was produced to allow its isolation as KMBA DNP, using the modified 24 DNP reagent, and a modified recovery procedure.

Addition of the modified 24 DNP reagent to culture fluids did not result

in precipitation of KMBA DNP, but by extraction of the solution with dichloromethane, and evaporation of the dried extract, a solid containing KMBA DNP and unreacted 24 DNP was obtained. Column chromatography proved unsuitable for the removal of this contaminating 24 DNP, as such a large quantity was present. However repeated extraction of the solid with sodium carbonate solution gave a solution containing pure KMBA DNP, which could be precipitated by acidification. The solubilities of the 2 isomers of KMBA DNP in sodium carbonate solution would be expected to differ²⁵⁰, by a factor of ca 5, but due to the small quantities present, and the relatively large quantity of sodium carbonate used both isomers were normally obtained in a ca 1:1 ratio. Trials showed that by this means pure KMBA DNP could be isolated in ca 50% yield from solutions containing KMBA at concentrations of less than 0.086 mg/cm³.

Incubation of a culture of D7F in the presence of 200 $\mu\text{g}/\text{cm}^3$ of methionine (38), for 3.5 h in the dark, followed by the isolation of KMBA DNP by the above method gave sufficient pure KMBA DNP for ¹H n. m. r. analysis. The 220 MHz ¹H n. m. r. spectrum of this KMBA DNP showed it to contain only ca 20% ²H at the carbon atom α to C=N. Incubation of KMBA (52) in sterile growth medium, in the dark, for the same period, followed by isolation of KMBA DNP in an identical manner gave a material whose 220 MHz ¹H n. m. r. showed ca 90% ²H at the carbon atom α to C=N.

This result indicates that under these conditions (3.5 h culture) exchange in the medium during the time required to produce KMBA is not significant, and therefore the exchange observed in the conversion of (38) to KMBA must occur primarily during the formation of KMBA. This

exchange could be due to either catalysis by low molecular weight co-enzymes produced by the organism (e.g. pyridoxal phosphate, see later) or exchange of an enzyme bound intermediate which has labile protons at C-3 (e.g. methionine \rightarrow KMBA via a transaminase).

These results demonstrate that a major part of the observed ^2H loss in the production of ethylene from rac-(3R,4R)-[3,4- $^2\text{H}_2$] methionine occurs during the early steps of the biochemical pathway involved, if KMBA is an intermediate in this pathway, and that stereochemically labelled methionines are not suitable for studying the elimination involved in the production of ethylene from methionine in bacteria, using whole cell cultures.

7-A-4 POSSIBLE BIOSYNTHETIC ROUTES TO ETHYLENE

The results described above, and in Ref. 245, indicate that in ethylene producing cultures, a major metabolic fate of added methionine is KMBA, or a compound which is converted to KMBA during the isolation procedure. Indeed up to 50% of the methionine metabolised by these cultures is converted into KMBA²⁴⁵. This, coupled with the conversion of KMBA into ethylene by enzymic^{72,245}, photochemical⁷², and model systems^{50,53} indicates that KMBA is probably the major metabolic precursor of ethylene in bacteria.

Adams and Yang have presented evidence⁶⁶ that in apple tissues, 2-azetidine carboxylic acid (ACC) is an intermediate in the production of ethylene from methionine. The scheme which they propose to account for their results, and those of others^{63,64,66}, is shown in Fig. 1-B-6. Methionine is converted into SAM, which reacts with pyridoxal phosphate to give the intermediate shown (53). This intermediate then fragments to ACC (21) and 5' methylthio adenosine (CH₃-S-Ad). ACC is then shown reacting with enzyme generated H₂O₂ to give ethylene, and other products. Although the preliminary steps of this pathway are plausible, the mechanism shown which yields ethylene from ACC is not. This is because it involves two very unfavourable processes, (1) the attack of a nucleophile (HOO[⊖]) on an unactivated, saturated carbon atom, and (2) the generation of a free -CH₂[⊖], with no stabilising influences present.

Hex^eolytic, olefin forming reactions can be either concerted, or proceed in a step wise manner²⁵³. In concerted processes, rigid stereochemical and stereoelectronic requirements exist, as to the conformation of

FIG 7-A-3

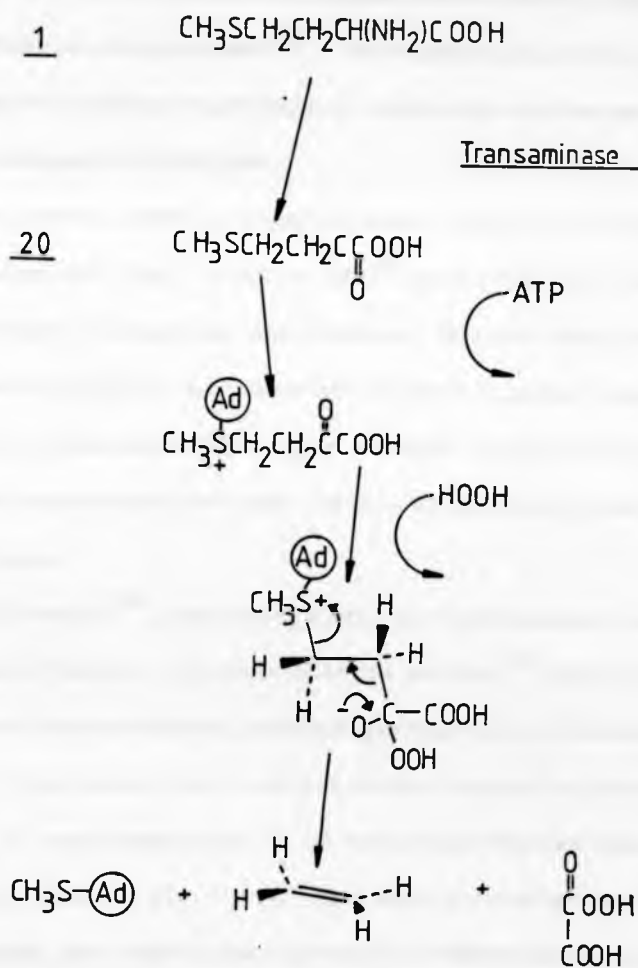


FIG. 7-A-3

FORMATION OF KMBA, AND ITS FRAGMENTATION TO ETHYLENE,
AFTER ACTIVATION

the fragmenting species. These require the "leaving" groups to be oriented trans to each other²⁵³. This geometry is clearly not attainable by ACC. Step wise fragmentations, which proceed via either C^+ or C^- species, have only been observed in systems which contain groups capable of stabilising the charge formed²⁵³. The fragmentation of ACC to ethylene via a step wise process would require considerably stabilisation of the charge developed to be available.

In contrast, KMBA is a good substrate, after activation (e.g. adenosylation of S, Fig. 7-A-3), for HOO^{\ominus} attack followed by concerted fragmentation to ethylene and other products. This is in direct analogy to the concerted ~~hex~~^eolytic fragmentations observed in normal organic systems²⁵³. The features which make KMBA a good substrate, would also be expected to render an intermediate from which KMBA is formed during isolation, a good substrate also.

Our results²⁴⁵, implicating KMBA as an intermediate in the production of ethylene by bacteria, and those of Adams and Yang⁶⁶ implicating ACC in plants, could reflect different pathways operating in the two biochemical systems. Alternatively they could both reflect facets of the same overall pathway. A simple mechanism for the formation of ethylene from methionine, via KMBA is shown in Fig. 7-A-3. Methionine is converted into KMBA by a transaminase, this KMBA is then activated by S-adenosylation, and then reacts with H_2O_2 to give ethylene via a concerted fragmentation (trans elimination).

By proposing a more complex pathway to that of Fig. 7-A-3, it is possible to account for both our own observations²⁴⁵, and those of others^{66, 254}, to a large extent. Such a pathway is shown in Fig. 7-A-4.

Methionine reacts firstly with pyridoxal phosphate, to give the intermediate shown (54). Hydrolysis of this intermediate gives KMBA, and pyridoxamine. In the presence of O_2 this pyridoxamine would be converted into pyridoxal, and thus render the above process cyclic, accounting for the observed need for O_2 for KMBA formation by bacterial cultures⁷². Adenylation of intermediate (54) would give the intermediate proposed by Adams and Yang (53). In the presence of O_2 this intermediate could react with H_2O_2 (formed by a peroxidase) and fragment to give ethylene, 5' methylthio adenosine, and (55). Degradation of this (55) would give the other observed products i. e. $HCOOH$ and CO_2 , as well as pyridoxal. The formation of ethylene from an intermediate such as (53) via a concerted elimination, has been proposed previously by Murr and Yang²⁵⁴. In the absence of O_2 , no H_2O_2 would be available, and thus no breakdown of intermediate (53) would be observed (or formation of KMBA, see above). However the fragmentation of (53) to ACC does not require O_2 , as the products are ACC and pyridoxal, and so formation of ACC would be observed under these conditions. The observations of Adams and Yang⁶⁶, that ACC is converted to ethylene in the presence of oxygen could reflect either the reversibility of the reactions which form ACC (53), (attack of nucleophiles at activated cyclopropane carbons is well documented²⁵⁵), or break down of ACC to ethylene. The latter process would require the intervention of some stabilising factor, e.g. a metal ion, to delocalise the charge formed in this necessarily step wise reaction. The observation⁶⁶ that inhibitors of pyridoxal phosphate mediated reactions inhibit the formation of ethylene from ACC suggest that the latter process may be operating.

In conclusion, it is possible that both KMBA (i.e. intermediate 54) and ACC are intermediates in the formation of ethylene by bacteria and plants, under different conditions. More experiments are necessary to verify the existence of a pathway of the type outlined in Fig. 7-A-4. For example, analysis of bacterial cultures grown in the absence of O_2 (and therefore not producing KMBA or ethylene) for ACC⁶⁷, analysis of apple tissues producing ethylene for KMBA by autoradiography, investigations of the possible relationship between ACC and KMBA, via intermediate (53) etc. The results of experiments of this type could lead to an elucidation of the mechanisms involved in the biosynthesis of ethylene from methionine, in natural systems.

7-B EXPERIMENTAL

Great care was taken to avoid acetone contamination at all times.

1. Microorganisms and growth conditions

All microorganisms and fungi were from the culture collection maintained in the Department of Biological Sciences, at the University of Warwick. All organisms were grown in the defined medium of Brown and Dilworth²⁵⁶, supplemented with (S)methionine at 500 $\mu\text{g}/\text{cm}^3$ unless otherwise stated. Cultures were incubated until the stationary phase of growth was reached (2 days, 30°C), and to prevent photochemical degradation of KMBA, were incubated in the dark.

2. 24 DNP Reagent; 0.4%w/v solution in sulphuric acid (ca 2 N)

2,4-dinitrophenylhydrazine (recrystallised twice from AR ethyl acetate, 2.0 g, 0.01 mol) was dissolved in AR sulphuric acid (50 cm^3) by stirring. The resulting solution was diluted to 400 cm^3 with distilled water and stored in a dark bottle.

3. Authentic KMBA DNP's

a) To a solution of commercial KMBA (0.045 g, 0.2 mol) in double distilled water (10 cm^3) was added 24 DNP reagent (30 cm^3). After standing for 30 min, the precipitated yellow solid was filtered off, washed with double distilled water (3 x 10 cm^3) and dried in vacuo to give KMBA DNP (a) as an orange solid (0.056 g, 67%); mp 121-123°C, 90 MHz ¹H n.m.r. ([²H₆] DMSO, TMS), δ 2.12 (3 H, s), 2.17 (3 H, s), 2.82 (8 H, s), 8.01 (1 H, d), 8.08 (1 H, d),

8.4 (1 H, q), 8.5 (1 H, q), 8.86 (2 H, d), 11.09 (1 H, broad s) and 11.50 (1 H, broad s) p.p.m. ca 1:1 mixture of E and Z isomers. (Fig. 7-A-1), t.l.c. see Table 7-A-1, i.r. (CH_2Cl_2 solution) 1760 (m), 1620 (s), 1590 (s), 1500 (s), 1420 (m), 1340 (m), 1250 (m) and 1120 (m) cm^{-1} .

b)²⁴⁶ To rac-methionine (9 g, 0.06 mol) dissolved in sodium hydroxide solution (60.3 cm^3 of a 1M solution) at 0°C, was added 2-bromopropionyl bromide (12.96 g, 0.06 mol) and sodium hydroxide solution (60.3 cm^3 of a 1 M solution) in aliquots, at 5 min intervals over 1 h. After standing at room temperature for 30 min the pH of the solution was reduced to 5 by addition of hydrochloric acid (6M) and the solution was evaporated to dryness under reduced pressure, to give 2-bromopropionyl methionine (16.4 g, 96%) as white crystals mp 113-118 (δ ($^2\text{H}_6$] acetone, TMS) 1.75 (3 H, d), 2.1 (3 H, s), 2.5 (5H, m), 4.6 (1 H, q), and 8.2 (1 H, broad s) p.p.m.

To 2-bromopropionyl methionine (2.9 g, 0.01 mol) and freshly fused sodium acetate (1.65 g, 0.02 mol) was added acetic anhydride (9.2 cm^3 , 9.2 g, 0.09 mol) and the mixture was stirred for 10 min. Hydrochloric acid (23.2 cm^3 of a 1 M solution) was added, and the reaction heated at 100°C for 5 min. After cooling to 25°C, 24 DNP reagent (400 cm^3) was added and the solution stored at 0°C for 4 h. The precipitated yellow solid was filtered off, washed with double distilled water (3 x 30 cm^3) and dried in vacuo to give KMBA DNP(b) (2.26 g, 69%) as a yellow/orange solid, mp 122-124°C, 90 MHz ^1H n.m.r. ($^2\text{H}_6$] DMSO, TMS) 2.11 (3 H, s), 2.16 (3 H, s), 2.82 (8 H, s), 8.01 (1 H, d), 8.08 (1 H, d), 8.4 (1 H, q), 8.5 (1 H, q), 8.86 (2 H, d) 11.1 (1 H, broad s) and 11.52 (1 H, broad s) p.p.m., ca 1:1 mixture of E and Z isomers (Fig. 7-A-1), t.l.c. See Table 7-A-1, i.r. (CH_2Cl_2 , solution),

1760 (m), 1620 (s), 1590 (s), 1420 (m), 1340 (m), 1250 (m), and 1120 (m) cm^{-1} ,
 m/e 328 (M^+ , 15%), 281 (60%), 237 (30%), and 61 (100%). M^* at m/e 240.7
 (calculated m^* for 328-281 = 240.7) and 200.0 (calculated m^* for 281 \rightarrow 237 =
 199.9).

c) The above procedure (b) was followed, except hot (ca 70°C) 24 DNP
 reagent was added to the hot (ca 70°C) solution of KMBA. Isolation of the
 precipitate as above gave KMBA DNP as a bright yellow solid (2.3 g, 70%)
ca 90% Z isomer. Chromatography on a column of silica gel (200-400 mesh)
 with solvent system (A) as eluant gave pure (Z)-KMBA DNP (c), m.p. 148.5-
 149°C, 90 MHz ^1H n.m.r. ($[\text{}^2\text{H}_6]$ DMSO, TMS) δ 2.12 (3 H, s), 2.81 (4 H, s),
 7.95 (1 H, d), 8.37 (1 H, q), 8.81 (1 H, d), and 11.53 (1 H, broad s), p.p.m.,
 t.l.c. see Table 7-A-1, (CH_2Cl_2 solution) 1610 (m), 1590 (m), 1420 (m),
 1340 (m), 1250 (m), 1120 (m), and 1100 (m), m/e 328 (M^+ , 10%), 281 (55%),
 237 (28%), and 61 (100%), M^* at m/e 240.6 (calculated M^* for 328-281 =
 240.7) and 200.0 (calculated M^* for 281-237 = 199.9).

4. Isolation of DNP's from culture fluids of D7F and B12E

24 DNP reagent (150 cm^3) was added to the supernatant fluids
 obtained from the low speed centrifugation of 2 cultures (each 500 cm^3) of
 D7F and 2 cultures (each 500 cm^3) of B12 E incubated in the presence of
 (S)-methionine. After standing for 30 min the precipitated DNP's were
 recovered and examined by t.l.c., see Table 7-A-1. The precipitates
 (0.46 g, 42% D7F; and 0.47 g, 43% B12E) were identical by t.l.c., ^1H n.m.r.,
 i.r. and m.s. m.p. 120-124°C, 90 Mhz ^1H n.m.r. ($[\text{}^2\text{H}_6]$ DMSO, TMS)

δ 2.12 (3 H, s), 2.17 (3 H, s), 2.83 (8 H, s), 8.3 (1 H, d), 8.09 (1 H, d), 8.39 (1 H, q), 8.48 (1 H, q), 8.68 (2 H, d), 11.10 (1 H, broad s) and 11.49 (1 H, broad s), t.l.c., see table 7-A-1, i.r. (CH_2Cl_2 solution), 1760 (m), 1520 (m), 1590 (s), 1500 (s), 1420 (m), 1340 (m), 1250 (m), and 1120 (m) cm^{-1} , m/e 328 (M^+ , 20%), 281 (65%), 237 (35%), and 61(100%), m^* at m/e 240.7 (calculated m^* for 328-281 = 240.7) and 200.0 (calculated for 281-327 = 199.9).

5. Isolation of [methyl- $^2\text{H}_3$] KMBA DNP from a culture of B12E grown in the presence of (S)-[methyl- $^2\text{H}_3$] methionine

24 DNP reagent (75 cm^3) was added to the supernatant obtained from the low speed centrifugation of a culture (500 cm^3) of B12 E grown in the presence of (S)-[methyl- $^2\text{H}_3$] methionine. After washing and drying the precipitate was examined by t.l.c. and shown to be >95% KMBA DNP, t.l.c. identical to authentic KMBA DNP (a), m/e 331 (M^+ , 20%), 328 (4 %) and 61 (100%).

6. Attempts to detect methional (19) as its DNP in culture fluids

After isolation of KMBA DNP, the culture fluids were extracted with CH_2Cl_2 until colourless. Drying and evaporation of these organic extracts gave orange solids, which were examined by t.l.c. Only in the culture fluid of B12E was a spot corresponding to methional DNP observed, at very high loading, and it was estimated as ca 1% of the yield of KMBA DNP from the same culture.

7. Isolation of KMBA DNP from the culture fluids of other bacteria and fungi

24 DNP reagent (150 cm^3) was added to the supernatant fluids obtained by low speed centrifugation of cultures (500 cm^3) of Aeromonas hydrophilla B12E (a) and B19E (b), Coryneform bacteria D7F (c) and D19E (d), Pseudomonas Areaginsesa PAT (e), Escherichas coli C603 (d) and SPAO (g), Saccharaomyces Cervisaie DD1 (h) and Penicillium digitatum (i). Examination of the precipitates obtained showed the presence of KMBA DNP (by t.l.c.) but not methional DNP for orgnaisms a-d. The other organisms did not produce sufficient KMBA to be detected by this method.

8. Detection of KMBA DNP by Autoradiography

Cultures (20 cm^3) of organisms (e) - (i) were grown in the presence of rac-methionine ($500 \mu\text{g}/\text{cm}^3$) and [^{35}S] methionine ($50 \mu\text{Ci}$, sp. activity $300 \text{ Ci}/\text{mmol}$) for 2 days at 30°C . KMBA (0.01 g , $68 \mu\text{mol}$) was added followed by 24 DNP reagent (10 cm^3). After standing for 30 min the precipitates were collected as above, and were run on t.l.c. using solvent system (A) (20×20 plate). The plate was dried, and overlaid with Kodak Xlomat film, and stored in the dark for 7 days. The film was developed (Kodak DX80, Fixer Kodak FX40) to reveal fogged areas corresponding to the t.l.c. spots of KMBA DNP for all organisms.

9. Isolation of the ethylene produced by cultures of D7F grown in the presence of stereochemically labelled rac-[3,4- $^2\text{H}_2$] methionine

40 cultures (20 cm^3 , in 250 cm^3 flask with suba-seal caps) of D7F were grown for 48 h in the presence of rac-(3R,4R)methionine ($500 \mu\text{g}/\text{cm}^3$),

and in the absence of light. The atmosphere was removed from each flask in turn by pumping through a series of 4 traps cooled in liquid nitrogen, to a pressure of <2 mm Hg. The flasks were then vented to atmospheric pressure, and the culture fluids used for the riboflavin assay. The isolated frozen gas (CO_2 + ethylene) was allowed to expand to a stirred solution of sodium hydroxide (25 g in 25 cm^3 water) in order to remove the CO_2 . After 30 min the remaining gas was frozen in a liquid nitrogen cooled trap, and then expanded through a dry ice/acetone cooled trap into a 10 cm path length gas i.r. cell. i. r. 3080 (m, broad), 2300 (m, broad), 1400 (w, pqr), 1000 (m), 990(w), 940 (m), 840 (w), 800 (m), and $725 \text{ (vw), cm}^{-1}$. The above 40 flasks were subjected to the riboflavin assay conditions described in Ref. 245, and the ethylene thus produced was isolated and examined as detailed above. i. r. 3080 (m, broad), 2300 (m, broad), 1400 (w, pqr), 1000 (m), 980 (vw), 940 (m), 840 (vw), and 800 (m), cm^{-1} . The above experiment was repeated and analogous result obtained (i. e. isolation of $[1\text{-}^2\text{H}_1]$ ethylene containing a small quantity of E and $Z[1, Z\text{-}^2\text{H}_2]$ ethylene.

10. The exchange of KMBA in $^2\text{H}_2\text{O}$

Commercial KMBA sodium salt (70 mg, 0.4 mmol) in $^2\text{H}_2\text{O}$ (0.5 cm^3 final pH 6.3) was stored at 37°C and ^1H n. m. r. spectra recorded at intervals. Comparison of integrals gave a measure of the $\% ^2\text{H}$ present at C-3, which was used to estimate graphically the $t_{\frac{1}{2}}$ of the reaction as ca 12 h. The above experiment was repeated, at pH 4.3 (obtained by the addition of $[^2\text{H}]$ hydrochloric acid to the solution) and a $t_{\frac{1}{2}}$ of ca 8 h was obtained. Each of the above experiments was repeated, in the presence of magnesium ions (MgCl_2 , 10 mg,

0.1 mmol, 0.25 mol. equiv.) and the estimated $t_{1/2}$'s for exchange were ca 1 h at pH 6.2 and ca 1.5 h at pH 4.3.

11. Preparation of [3-²H₂] KMBA

Commercial KMBA sodium salt (100 mg, 0.58 mmol) was incubated in ²H₂O (1 cm³) at 37°C for 60 h, when ¹H n.m.r. analysis showed ≥ 97% ²H at the 3 position (maximum possible incorporation of ²H = 98%, see chapter 3). This solution was used directly as a source of [3-²H₂] KMBA.

12. Modified 24 DNP reagent

24 DNP (recrystallised twice from ethyl acetate, estimated 1.5 mol. excess over KMBA present in sample) was dissolved in hydrochloric acid (38 cm³ of a 2M solution per 500 cm³ of sample solution) by boiling. This solution was used at once.

13. Isolation of KMBA DNP from [3-²H₂] KMBA

To [3-²H₂] KMBA 97 atom % (0.5 cm³ of the solution described above, 50 mg, 0.3 mmol) in double distilled water (500 cm³) was added a solution of 24 DNP (0.083 g, 0.4 mmol) in boiling hydrochloric acid (38 cm³ of a 2 M solution). After standing for 15 min, the solution was extracted until colourless with CH₂Cl₂. The combined, dried, extracts were evaporated to dryness under reduced pressure. The resulting red solid was extracted with 2% sodium carbonate solution, until no red colouration was observed in the extract (ca 200 cm³, in 30 cm³ portions). The combined extracts were acidified to pH 4 by the addition of hydrochloric acid (5 M). The precipitated

yellow solid was isolated at the pump, washed with double distilled water ($3 \times 30 \text{ cm}^3$) and dried in vacuo to give KMBA DNP, (48 mg, 50%), t.l.c. shows KMBA DNP only, ^1H n.m.r. identical to authentic KMBA DNP, comparison of 220 MHz ^1H n.m.r. integrals gives a ^2H content at C-3 of $\geq 90\%$.

14. Exchange of KMBA in growth medium

$[3\text{-}^2\text{H}_2]$ KMBA, 97 atom % (50 mg, see above) was incubated in sterile growth medium (500 cm^3) for 3.5 h at 30°C . 24 DNP reagent was added and KMBA DNP isolated as above. T.l.c. and ^1H n.m.r. identical to authentic KMBA DNP, comparison of 220 MHz ^1H n.m.r. integrals gives a ^2H content at C-3 of $\geq 90\%$.

15. Isolation of KMBA DNP from cultures of D7F supplemented with $\text{rac-}[3\text{-}^2\text{H}_2]$ methionine (see chapter 4)

KMBA DNP was isolated from the supernatant fluid obtained from low speed centrifugation of a culture of D7F (500 cm^3) grown for 3.5 h at 30°C , in the absence of light, and in the presence of $\text{rac-}[3\text{-}^2\text{H}_2]$ methionine (see chapter 4) ($500 \mu\text{g}/\text{cm}^3$), as described above (13). T.l.c. and ^1H n.m.r. identical to authentic KMBA DNP, exact comparison of 220 MHz ^1H n.m.r. integrals gave a ^2H content of C-3 of ca 20%.

7-C STUDIES ON THE BIOSYNTHESIS OF SPERMIDENE

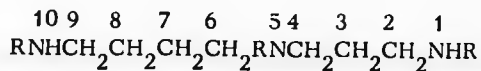
1. Introduction

The overall pathway involved in the biosynthesis of spermidene (6) from putrescine (8) and decarboxylated S-adenosyl methionine (5a) has been demonstrated using radiolabelling techniques (chapter 1). However the mechanism involved in the formation of (6) from (8) and (5a) is unknown, and the incorporation of the intact methionine alkyl chain into (6) has not been demonstrated. It was considered that ^{13}C and ^2H C-3, C-4 labelled methionines could be of value in studies of the mechanism of this reaction, and these materials were synthesised as described in chapter 4. The results of experiments (performed by Mr. I. K. Nassereddin) on the incorporation of (S)-[3, 4- $^{13}\text{C}_2$] methionine into spermidene by E. Coli are reported. Future work in this area involving the use of the ^2H labelled methionines is outlined.

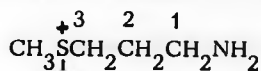
2. Possible mechanisms for the formation of polyamine (6)

There are three plausible alternative mechanisms for the formation of polyamine (6) from diamine (8) and decarboxylated S-adenosylmethionine (5a) (cf Fig. 1-B-3):

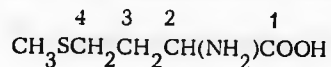
- (i) Enzyme mediated $\text{S}_{\text{N}}2$ attack of a nitrogen atom of putrescine (8) on C-3 of (5a) leading directly to (6). Thus C-3 of (5a) becomes C-4 of (6), and the configuration at this carbon is inverted in (6) relative to (5a).
- (ii) $\text{S}_{\text{N}}2$ attack at C-3 of (5a) by a nucleophilic group of aminopropyl transferase, giving an aminopropylated enzyme, which then reacts



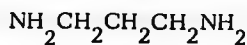
6 R = H ; derivative R = PhNHCS



5a



1



8

with diamine (8) also via an S_N2 mechanism. Thus C-3 of (5a) becomes C-4 of (6), and the configuration at this carbon atom is retained in (6) relative to (5a).

- (iii) Enzyme mediated intramolecular closure of (5a) to azetidene, which reacts via attack of diamine (8) on either of the homotropic carbons adjacent to nitrogen. Thus either C-1 or C-3 of (5a) becomes C-4 of (6).

Mechanisms (i) and (ii) may be distinguished from mechanism (iii) by the fate of C-3, C-4 of (5a). Mechanism (i) may be distinguished from mechanism (ii) by the overall stereochemical course of the reaction with regard to the relative configuration of C-3 of (5a) (C-4 of (6)).

3. The incorporation of (S)-[3,4- $^{13}\text{C}_2$] methionine into polyamine (6) by E. Coli (Ref. 257)

The incorporation of [3,4- $^{13}\text{C}_2$] methionine into spermidene would

allow the distinction of mechanism (i) or (ii) from mechanism (iii). Thus mechanism (i) or (ii) would lead to $[3,4-^{13}\text{C}_2]$ spermidene, and mechanism (iii) to a mixture of $[3,4-^{13}\text{C}_2]$ and $[2,3-^{13}\text{C}_2]$ spermidenes. Following the successful synthesis of rac- $[3,4-^{13}\text{C}_2]$ methionine, studies on the incorporation of this material into polyamine (6) were conducted (by I.K.N.). An E. Coli (S)-methionine requiring mutant was grown in a medium supplemented with (S)- $[3,4-^{13}\text{C}_2]$ methionine (obtained from the rac material by a literature resolution procedure). Spermidene (6) and putrescine (8) were isolated as their phenyl thiourea derivatives, purified, and examined by ^{13}C n.m.r. spectroscopy. As expected (cf chapter 1) the ^{13}C n.m.r. spectrum of the derivative of diamine (8) did not show the presence of ^{13}C above natural abundance.

The ^{13}C n.m.r. spectrum of an authentic sample of the phenylthiourea derivative of (6) shows inter alia peaks at δ ($[^2\text{H}_6]$ DMSO) 24.7 (C-8), 26.1 (C-7), 26.9 (C-3), 41.7 (C-2), 43.7 (C-9), 48.6 (C-4) and 50.2 (C-6) p.p.m., assigned by comparison with spectra of model compounds. The sample of the phenylthiourea derivative of polyamine (6) isolated from the culture of E. Coli grown in the presence of (S)- $[3,4-^{13}\text{C}_2]$ methionine shows in its ^{13}C n.m.r. spectrum an intense AX system (doublets, $J = 35$ Hz astride singlets at δ 26.8 (C-3) and 48.5 (C-4) p.p.m.), in addition to signals at natural abundance (cf the ^{13}C n.m.r. spectrum of $\text{CH}_3\text{S}^{13}\text{CH}_2^{13}\text{CH}_2\text{Cl}$ Fig. 4-B-6). The intensities of the signals from C-3 and C-4 indicated that the ^{13}C methionine had been incorporated into polyamine (6) without significant dilution. This result proves that the C-3, C-4 unit of methionine is the precursor of C-3, C-4 of polyamine (6). It also excludes the operation

of mechanism (iii) above because the intermediacy of azetidene (homotropic C-2 and C-4) would have led to a ca 1:1 mixture of C-2, C-3 and C-3, C-4 labelled material, and no enrichment at C-2 is observed. The labelled phenylthio urea derivative of (6) can easily be converted back to [3,4-¹³C] polyamine (6) and thus double labelled (6) is now readily available for ¹³C n.m.r. binding studies, and studies on the metabolism of (6).

4. Future Work

The mechanistic possibilities (i) and (ii) above may be distinguished by determination of the relative configuration of C-3 of (5a), before and after its incorporation into polyamine (6). For this purpose the stereospecifically labelled methionine whose syntheses and stereochemical identity is described and demonstrated in chapters 4 and 6 could be used. The relative configurations of these materials (i. e. rac-(3R, 4R)-methionine and rac-(3R, 4S)-methionine) has been established. Once the incorporation of each of these methionines into polyamine (6) has been achieved, the relative configuration at C-4 could be determined by ¹H n.m.r. spectroscopy (cf the analysis of dehydromethionines derived from rac-(3R, 4R) and (3R, 4S)-methionine, chapters 4 and 6. To this end it would be necessary to render the diastereotopic protons on C-3 and C-4 of the derivative of (6)(or (6) itself) distinguishable by ¹H n.m.r. This could be possible by reducing the number of conformations available to the molecule, by incorporation of (6) into a cyclic system (e.g. complexation to a metal ion, or reaction with a di-ketone).

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to

CHAPTERS 1 - 7

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