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SOME ASPECTS OF THE SYNTHESIS, ANALYSIS AND DECOMPOSITION OF LABELLED AMINO ACIDS AND PEPTIDES.

A thesis submitted to the Department of Chemistry of the University of Surrey in partial fulfilment of the requirements for the degree of Master of Philosophy.

by

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Summer 1991.

Abstract.

The work herein describes an investigation into the synthesis, analysis and decomposition of some labelled amino acids and peptides. The work was carried out to investigate the potential of a novel biochemical method for labelling amino acids with hydrogen isotopes and to study the effects of pH on the storage of tritiated amino acid derivatives in aqueous solutions.

Chapter One describes the synthesis, analysis and radiation decomposition of tritiated amino acids and peptides. Methods for the preparation of N-acyl-2,3-didehydroamino acids and N-acyl-2,3-didehydropeptides via azlactone and azido carboxylic acid intermediates are given. The catalytic reduction of the unsaturated precursors using hydrogen-tritium mixtures has been investigated. The radiation decomposition of N-acetyl-[4,5-$^{3}H_{2}$]-leucine (3.75Ci/mmol), N-acetyl-[2,3-$^{3}H_{2}$]-phenylalanine (18.2Ci/mmol) and N-benzoyl-[2,3-$^{3}H_{2}$]-phenylalanine (12Ci/mmol) in buffer solutions are reported. Analysis of the samples by tritium nmr spectroscopy and radio-tlc analysis revealed that in all cases the majority of radiation decomposition was not at the site of tritium labelling.

Chapter Two describes the deuteration of amino acids using Pseudomonas putida cells as a catalyst. Attempts to use semi-purified extracts of methionine-γ-lyase as a catalyst for the deuterium labelling of amino acids were unsuccessful. The immobilization of the bacteria cells using Biofix C2 support is reported. A wide range of amino acids have been investigated and the results of labelling studies using free and immobilized cells are compared. Labelling studies using semi-aqueous and non-aqueous solvent media, including the deuteration of glycine esters in dimethylsulphoxide and dimethylformamide, have been reported.

Dedication.

This thesis is dedicated to my parents in acknowledgement of their support and encouragement over the many years I have spent in pursuance of an education.

Acknowledgements.

I would like to express my sincere thanks to Professor J. R. Jones for his supervision and encouragement over the last three years. I am also indebted to Dr. R. Wade of Ciba-Geigy Pharmaceuticals Division, Horsham, Sussex, whose contributions to this work are gratefully appreciated. My thanks also go to Ciba-Geigy Pharmaceuticals Division and the Science and Engineering Research Council for providing a CASE studentship for this work.

I would also like to thank Drs. M. O. Moss and A. Chamberlain of the School of Biological Sciences for many useful conversations about the biological work undertaken. A debt of gratitude is due to Mrs. Leonor Carroll, Mr. Jim Bloxsidge and other technical staff of the Chemistry Department for their help and friendship. My sincere thanks also go to Mrs. Theresa Amor, Mrs. Sue Wall and Mr. Tim Baker of the School of Biological Sciences for their help and interest in this work.

Finally, I would like to thank my friends and colleagues in the Chemistry Department and the School of Biological Sciences for making my three years at Surrey University an enjoyable and memorable experience.

Contents.

CHAPTER ONE

The Synthesis, Analysis and Radiation Decomposition of Tritiated Amino Acids and Peptides.

1.1 Introduction.

1.1.1 General.

Amino acids and their polymeric derivatives peptides and proteins play an important role in biology. They are essential to all life and are involved in a wide variety of functions. Attempts to understand their action have led to chemists and biochemists using a range of labels to mark these compounds. The label that is chosen must be easy to detect, easy to incorporate and preferably chemically stable. The labelled molecule should under ideal circumstances mimic the behaviour of the unmarked compound it is representing. Indeed, it is only isotopes of hydrogen, carbon, nitrogen, oxygen and sulphur that can be incorporated into amino acids and peptides without altering their chemical structure.

There are two types of isotopes, namely those that are stable and those that are radioactive. A wide range of spectroscopic and other techniques can be used to detect both categories of isotopes. However, the major advantage of radioisotopes over their stable counterparts is that they can be detected at tracer level. The most commonly used radioisotopes used for labelling amino acids and peptides are tritium, carbon-14, iodine-125 and sulphur-35.

Only deuterium and tritium have been used in the present research. Both these hydrogen isotopes are

relatively cheap compared to isotopes of other elements. In addition, they have several advantages that make them more attractive than others to use. Since the isotopes are simply being used to replace hydrogen in a molecule then they can be used as tracers for both hydrogen and carbon.

The aims of the work described herein were:

(a) To prepare some N-acyldidehydroamino acids and N-acyldidehydropeptides.

(b) The optimisation of the catalyst: substrate ratio for the reduction of N-acyldidehydroamino acids with hydrogen-tritium mixtures.

(c) To study the effects of varying the solvent for the labelling of N-acyldidehydroamino acids by catalytic reduction.

(d) The catalytic reduction of some N-acyldidehydropeptides using hydrogen-tritium mixtures.

(e) To study the radiation decomposition of tritiated N-acylamino acids by tritium nuclear magnetic resonance (^{3}H nmr) spectroscopy, liquid scintillation counting and radio thin layer chromatography (tlc).

1.1.2 Properties of tritium.

Tritium is a radioactive isotope of hydrogen and has an atomic mass of three [1, 2]. The general properties of tritium are shown in Table 1.1. The decay process is shown in Figure 1.1. The β^--particle (or negatron as it is sometimes called) is an energetic electron that migrates from the nucleus and leaves behind an isotope with an increased atomic number. β^--Particles are caused by the instability of the nucleus due to the presence of too many neutrons.

$$^{3}_{1}H \rightarrow {}^{3}_{2}He^{+} + \beta^{-}$$

Figure 1.1 Decay process of ^3H atoms.

Tritium has a natural abundance of less than 10^{-16} and is produced in the upper atmosphere as a result of nuclear reactions caused by cosmic radiation. Tritium can be artificially prepared by several methods, as shown in Figure 1.2. Tritium may be incorporated into water by oxidation or reduction. Tritium is used for synthesis either in the form of a diatomic gas, as a pure isotope or as a hydrogen isotopomeric mixture, tritiated water or as a metal tritide. Hence, there is a range of methods that can be employed to incorporate tritium into molecules. Some of these will be discussed in section 1.1.6.

Table 1.1: Some properties of tritium.

Mass	3
Radiation emitted	β^--Particle
Half-life	12.43 years
Maximum energy of particle	18.6 Kev
Decay product (stable)	Helium (^{3}He)
Maximum specific activity	28.76 Ci/mA
Method of measurement (efficiency)	Liquid scintillation (40%)
Range of particles:	
in air	4.5-6mm
in water	6nm
Volume of $^3H_2$ gas at STP	0.385ml

Tracer use for both hydrogen and carbon

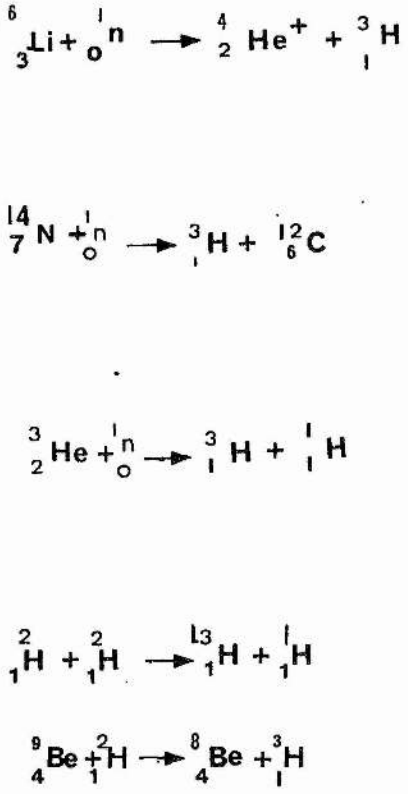


Figure 1.2 Methods of preparing tritium atoms.

Since tritium is one of the least toxic radisotopes, and because it is a weak ß⁻-particle emitter, it is often handled in large quantities with samples containing curies frequently being used. The properties of tritium make it difficult to detect in the laboratory and so must be handled in accordance with stringent safety regulations[3]. Therefore, the handling of tritium in the laboratory needs to be carried out using the following precautions:

(a) Always wear rubber gloves when handling tritiated compounds.

(b) Work should preferably be carried out in a fumehood.

(c) Work over a spill tray.

(d) Wear a face mask when handling fine powders.

(e) Always wash off any spills with water.

Monitoring of the laboratory should be carried out by using swabs and counting the activity of the samples by liquid scintillation counting. The routine monitoring of laboratory workers should also be carried out by taking urine samples, since the low energy of the β^--particles produced by tritium mean that film badges cannot be used.

1.1.3 Liquid scintillation counting.

The measurement of tritium is most conveniently made using liquid scintillation counting [4-8] and the principles of the technique are briefly outlined here. The method can be used to detect α-particle, β-particle and τ-ray emitters in a variety of sample forms. The radioactive sample is dissolved in a liquid scintillation cocktail, which consists of a solvent system and one or more scintillator compounds.

The phenomenon of fluorescence is used for the process of liquid scintillation counting. As the emitted α-particle, β-particle or τ-ray passes through the scintillant solution it loses energy to the solvent, which is elevated to an excited state. Energy is transferred to the

scintillant solutes from the excited solvent molecules and is then emitted as photons due to the process of fluorescence, which is detected by a photomultiplier tube. The solvent chosen must efficiently transfer the energy to the scintillant molecule. Aromatic solvents such as toluene or xylene are most favoured though for aqueous environments 1, 4-dioxane may be used.

The efficiency of the counting process will vary from sample to sample. Quenching is a phenomenon which interferes with the creation or transmission of light. The effects of quenching will mar the measurement of fluorescence during the counting process. There are three types of quenching:

(a) Colour quenching.
(b) Photon quenching.
(c) Chemical quenching.

Quench correction can be achieved by the methods of internal standards, channels ratio method and / or external standards.

1.1.4 Tritium nmr spectroscopy.

The development of tritium nmr spectroscopy [2, 7, 8] in the late 1960's and early 1970's revolutionalized the use of tritium as a tracer for hydrogen and carbon in biological and chemical molecules. This was due to the fact that for

the first time it was possible to determine the distribution of tritium in a compound by a non destructive technique. This meant that tritium had a distinct advantage over other isotopes such as carbon-14 and sulphur-35 in that its position of label could be determined with very little sample preparation and no laborious chemical degradation processes.

The nmr properties of tritium are compared to those of other isotopes in Table 1.2. It will be noticed that there is a close similarity between ^{1}H and ^{3}H nuclei. This implies that it is possible to relate the chemical shifts of the tritium spectra to the corresponding shifts in the proton spectra. The high resonance frequency of tritium coupled with the fact that it has a higher detection sensitivity than even hydrogen makes it a suitable isotope for nmr study. The chemical shifts of proton and tritium signals are similar and are related by the ratio of the Larmor frequencies of the nuclei. Hence, it is possible to relate the tritium nmr directly with that of the proton nmr. The tritium nmr spectra can be 'ghost' referenced to the proton nmr by multiplying the chemical shift of the proton reference signal of tetramethylsilane (tms) or 2, 2-dimethylsilapentane-5-sulphonic acid (dss) by the magnetogyric ratio.

In addition, the tritium coupling constants are related to those of the proton values by the expression shown in Equation 1.3.

Table 1.2: Nuclear properties of the hydrogen isotopes.

Isotope	^{1}H	^{2}H	^{3}H
Natural abundance (%)	99.984	0.0156	$<10^{-16}$
Nuclear spin	1/2	1	1/2
Magnetic moment μ/μ_N	4.8371	1.2125	5.1594
Magnetogyric ratio ($\tau/10^7$ radians $T^{-1}s^{-1}$)	26.7519	4.1064	28.5336
Resonance frequency (MHz at 7.0463T)	300.00	46.05	320.13
Relative sensitivity for equal number of nuclei at constant field	1.0	9.65×10^{-3}	1.21
Radiation	Stable	Stable	β^--Particle

$$J_{T,T} = J_{H,T} (\tau_T/\tau_H) = J_{H,H} (\tau_T/\tau_H)^2 \qquad \text{Eqn. 1.3}$$

where:

$J_{T,T}$ is the ^3H-^3H spin coupling constant

$J_{H,H}$ is the ^1H-^1H spin coupling constant

$J_{H,T}$ is the ^1H-^3H spin coupling constant

τ_H/τ_T is the magnetogyric ratio and has the value of 1.06663974

Also the Nuclear Overhauser Effect from concomitant proton irradiation is small and no tritium standard is required for field-frequency locked spectrometers.

1.1.5 Radiochromatography.

Radio thin layer chromatography (tlc) has been used for this reseach to analyse radiation decomposition of tritiated amino acids. Inactive carrier material should be added to the sample of radiolabelled compound to reduce the formation of sources of error during the elution and analysis of the tlc plate [9]. Sources of error can be due to many causes such as poor sample loading, decomposition of the sample during loading, elution, and analysis of the tlc plate, and the existence of inter-equilibrating forces.

1.1.6 Tritium labelling.

There are four main ways hydrogen isotopes can be incorporated into a molecule [1, 10]:

(i) Isotope exchange reactions
(ii) Direct chemical synthesis
(iii) Biochemical methods
(iv) Recoil labelling

Recoil labelling, although of interest to physical chemists due to mechanistic aspects, is seldom used for preparative purposes. The method involves the reaction of recoil tritium atoms from nuclear reactors with compounds. The products tend to have low specific activities. Radiochemical and chemical yields of the compounds are often poor due to the formation of labelled impurities during the reaction. Therefore, the technique will not be discussed any further. The biochemical methods are discussed in Chapter 2.

1.1.6.1 Isotope exchange reactions.

These reactions involve the reversible process of exchange of two isotopes of the same element. The technique is useful for labelling complex molecules but often leads to a general distribution of the isotope. There are two procedures used to exchange label compounds with tritium: (a) gas exposure method; (b) catalytic exchange in solution.

The gas exposure method is often referred to as the Wilzbach method [11] after the scientist who first published details of the method. The method involves allowing a compound to remain in contact with tritium gas for several days or weeks. During this time the radiation induces exchange reactions between the hydrogen atoms of the compound and the tritium gas. There are several limitations to the method. The radiochemical purity is low due to the formation of tritiated impurities, which arise either because of direct damage to the target material or by decomposition of the product and the labelling pattern of the product is non specific. Improvements to the method include: (i) activating the tritium gas with high energy sources; (ii) absorption of the substrate on a suitable support, such as charcoal; (iii) using a catalyst, such as platinum or palladium black.

Most of the proteinogenic amino acids have been labelled using improved Wilzbach methods [1]. The specific activities obtained varied from 0.2-29 mCi/mmol. The racemization of some L-amino acids has been reported to occur during Wilzbach labelling [12, 13].

The tritiation of peptides have been carried out by using modified Wilzbach methods. Peng [14] has studied the tritiation of the simple peptides glycylglycyl-L-leucine and glycyl-L-leucine by a microwave discharge technique. Adrenocorticotrophic hormone [15] and Gonadotropin releasing hormone[16] have also been labelled by the Wilzbach technique.

Catalytic exchange in solution is used to label compounds in non-labile positions. The method is useful for preparing compounds of high radiochemical purity but has the disadvantage in that labelling patterns are often non-specific. Most of the common amino acids have been labelled by tritiated water in the presence of platinum catalysts [1]. The specific labelling of amino acids in the α-position has been achieved by refluxing the N-acetyl derivative with tritiated acetic acid and anhydride.[17]

The labelling of amino acid and their derivatives have been used to study potential models for the tritiation of peptides. Woodworth and Dobson[18] have selectively deuterated aromatic amino acids at specific sites, using Raney nickel as a catalyst and ambient temperatures, and Oehlke[19] has investigated the conditions of deuteration of N-acetyl-L-histidine amide.

Catalytic exchange tritiation of peptides has been carried out by several workers. Brundish and Wade[20] have labelled ß-corticotrophin-(1-24)-tetracosapeptide to a level of 0.48Ci/mmol with 80% of the tritium incorporated into the histidine residue. The catalytic exchange labelling of L-cysteine containing peptides was reported to give the corresponding tritiated L-alanine containing peptides[20.21]. Bienert et. al.[22] tritiated histidine residues of analogues of luteinizing hormone-releasing agonists at high specific activities.

1.1.6.2 Direct chemical synthesis.

There are three general methods used for chemical synthesis of a labelled molecule: (i) reduction of an unsaturated precursor; (ii) catalytic halogen-tritium replacement and (iii) reduction of compounds with tritiated metal hydrides.

The catalytic reduction of unsaturated compounds is one of the most convenient ways of introducing tritium into a compound. The types of reactions that can be envisaged are shown in Figure 1.4.

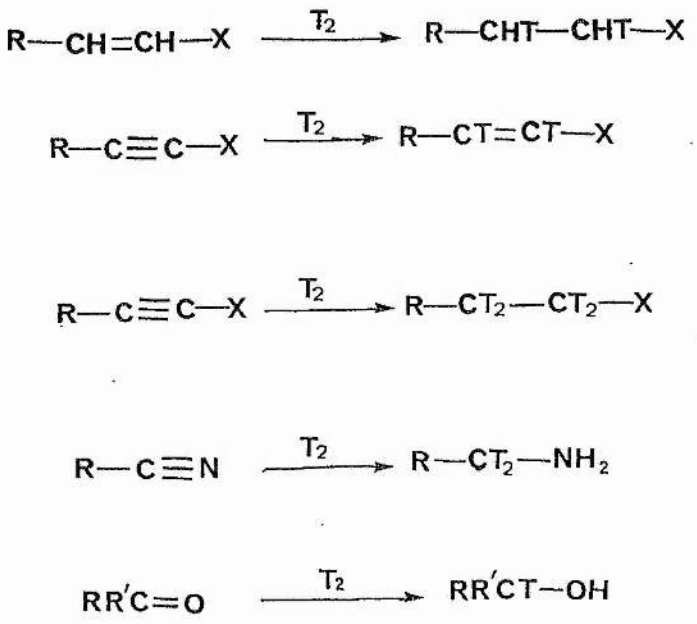


Figure 1.4 Catalytic reduction of unsaturated compounds.

The catalytic tritiation of most of the common amino acids has been carried out [1]. Labelled amino acids have also been obtained by the reduction of unsaturated compounds such as azlactones, acetamidoacrylic acid derivatives, α-keto acids and hydoxyiminocarboxylic acids. These tritiated intermediates are converted to the corresponding amino acid derivatives by a range of synthetic methods. Reduction of α-hydroxyiminopropionic acid and ß-phenyl-α-hydroxypropionic acid with tritium gave DL-[4,5-$^{3}H_2$]-leucine [23], [2,3-$^{3}H_2$]-alanine [24] and [2,3-$^{3}H_2$]-phenylalanine [25]. 2-amino-DL-[4, 5-$^{3}H_2$]-adipic acid [26], and DL-[4, 5-$^{3}H_2$]-lysine [27] by reduction of unsaturated malonates.

Several workers have reported the labelling of peptides containing didehydroamino acid residues. A protected gastrin tetrapeptide amide containing a 4,5-didehydronorleucyl residue was tritiated to a specific activity of 0.14Ci/mmol [28]. The [allyl-glycine]-ß-corticotrophin-(1-24)-tetracosapeptide gave upon labelling the corresponding [4,5-$^{3}H_2$]-norvaline containing peptide with a specific activity of 7.42Ci/mmol [29]. Felix [30] reported the labelling of 3, 4-didehydroproline residue in a tripeptide analog of thyroliberin to a specific activity of 58.4Ci/mmol. The asymmetric tritiation of N-acetyl-2,3-didehydrophenylalanyl-S-phenylalanine methyl ester using tritium gas in the presence of rhodium-(+)diop catalyst was reported by Levine-Pinto [31]. The ^{3}H nmr spectrum of N-acetyl-[2,3-$^{3}H_2$]-R-phenylalanyl-S-phenylalanine showed the distribution of tritium to be 46% : 54% C_α: C_β respectively.

In the case of N-acetyl-[2,3-$^{3}H_2$]-S-phenylalanyl-S-phenylalanine an even distribution of tritium in the C_α and C_β positions was observed. The difference in the distribution of the tritium for the former diastereomer was interpreted as being due to exchange with the reaction solvent. Sheppard et. al.[32] have tritiated Locust adipokinetic hormone containing either a 4,5-didehydroleucine residue or 3, 4-didehydroproline residues. In both cases over 95% of the tritium was found to be present in the expected residues with specific activities of 115Ci/mmol and 12Ci/mmol being obtained respectively for the peptides. Oehlke et. al.[33] studied the conditions of catalytic deuteration of N-acetyl-3,4-didehydroproline amide as a model for the tritiation of peptides.

Catalytic halogen-tritium exchange is a useful synthetic reaction that enables labelling of compounds under similar conditions to those for hydrogenations. Several reports have been made about the tritiation of amino acids by dehalogenation [1, 2].

Several reports have been published concerning the preparation of tritiated peptides by dehalogenation. Arvis et. al[34] have used flash photolysis in the tritiation of peptides containing an iodotyrosine residue. Irradiation of the peptide at 304nm was found to lead to the formation of a tyrosyl free radical, which can abstract a tritium atom from tritiated alcohol to give a product of high radiochemical purity. Tritiation by the dehalogenation technique has been

used to label analogues of the peptides corticotrophin[35], somatostatin [36] and calcitonin [37]. Kaspersen et. al [38] labelled two neuropeptides, Org 2766 (an ACTH (4-9) analogue) and Org GK 78 (des-Tyr-γ-endorphin) by catalytic deiodination of the (p-iodophenyl)alanine precursors using tritium gas. Exchange labelling into histidine residues has also been observed by several workers [39] - [41]. Toth et. al[42] prepared ^{3}H-D-ala-leu-enkephalin-chloromethyl ketone at high specific activity from dehalogenation of Boc-3, 5-diiodotyr-D-ala-gly with tritium gas. Oehlke et. al.[43] have studied the effects of catalyst pretreatment on the labelling of N-acetyldiiodotyrosine amide for optimization of the conditions of tritiation of peptides.

Several other methods of preparing tritiated amino acids and peptides include: (i) chain elongation by the Arndt-Eistert reaction, which was used to label the α-position of β-amino acids [44] - [46]; (ii) decarboxylation of acetamidomalonic acid derivatives to obtain amino acids specifically labelled in the α-position[1]; (iii) the preparation of [6-^{3}H]-Lysine and [5-^{3}H]-ornithine by reduction of α-amino-ω-cyanocarboxylic acids [1] and (iv) the reduction of didehydropyrroline carboxylic acids with sodium borotritide [47].

1.1.7 Synthesis of didehydroamino acids and didehydropeptides.

The lack of a wide range of commercially available didehydroamino acids and didehydropeptides made it necessary to carry out the preparation of suitable unsaturated precursors prior to any tritiation studies being undertaken. Two methods were used for the synthesis of didehydroamino acid and didehydropeptides: (i) synthesis via azlactone route and (ii) synthesis via an azide intermediate Previous reports of synthesis using these methods are described briefly below.

1.1.7.1 Synthesis via azlactone intermediates.

The chemistry of azlactones have been reviewed by Baltazzi[48]. Azlactones can be categorised as being saturated or unsaturated. The synthesis and properties of unsaturated azlactones have only been described here because they are the group that is used as intermediates for the preparation of didehydroamino acids and didehydropeptides.

The Erlenmeyer reaction, as it is sometimes called after the scientist who developed the synthesis of azlactones, is believed to proceed via a mechanism which can be considered to be a special case of the Perkin condensation. The acylglycine is firstly converted into its saturated azlactone, which contains a reactive methylene group. Condensation then occurs between the intermediate and

the carbonyl compound. The Erlenmeyer reaction occurs under milder conditions than those required for Perkin condensation. There are four ways in which unsaturated azlactones can be prepared:

(a) by the reaction of an aldehyde with an acylglycine.
(b) by the reaction of an acetic anhydride with an α-haloacylamino acid in the presence of pyridine.
(c) by dehydration of acyldehydroamino acids with acetic anhydride.
(d) by the action of acetic anhydride, or an acid chloride, with an α-amino-ß-alkoxy acid.

Synthesis of the azlactones by method (a) usually involves refluxing an N-acylglycine with a carbonyl compound and acetic anhydride in the presence of an anhydrous salt, typically sodium acetate (see Figure 1.5). Improvements in the method, which enabled the preparation of azlactones from aliphatic or arylaliphatic aldehydes and ketones, can be made by carrying out the reaction in boiling tetrahydrofuran and in the presence of lead acetate. Baltazzi and Robinson have prepared several 4-alkylidene azlactones [48]. Finar and Libmann [50] used copper acetate for the azlactonization of thioaldehydes.

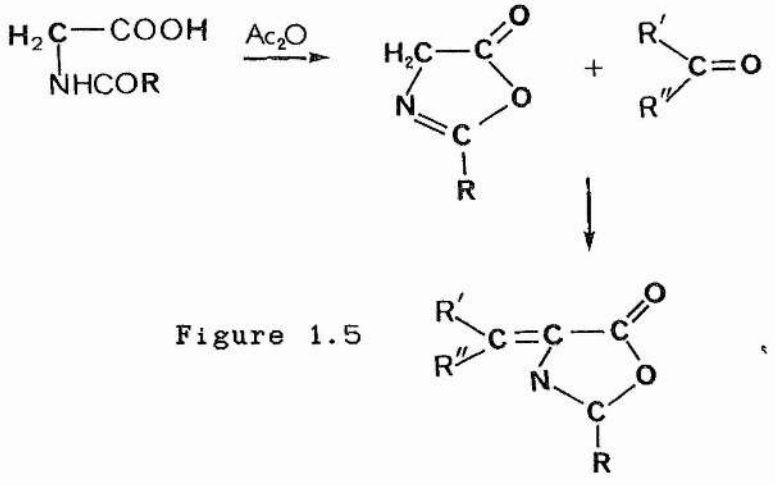


Figure 1.5

The preparation of unsaturated azlactones by method (b), which involves the reaction of an α-(α'-acyloxyacyl)-or an α-(α'-halogenoacyl) amino acid with acetic anhydride and pyridine at 0°C or room temperature , is useful for the synthesis of α-keto acids. The mechanism, which is shown in Figure 1.6, involves a 'pseudo azlactone'[51]. The pseudo azlactone is considered to be in dynamic equilibrium with the unsaturated azlactone. The pseudo azlactone would seem to be stabilized when R' is an aryl group because of its greater degree of conjugation.

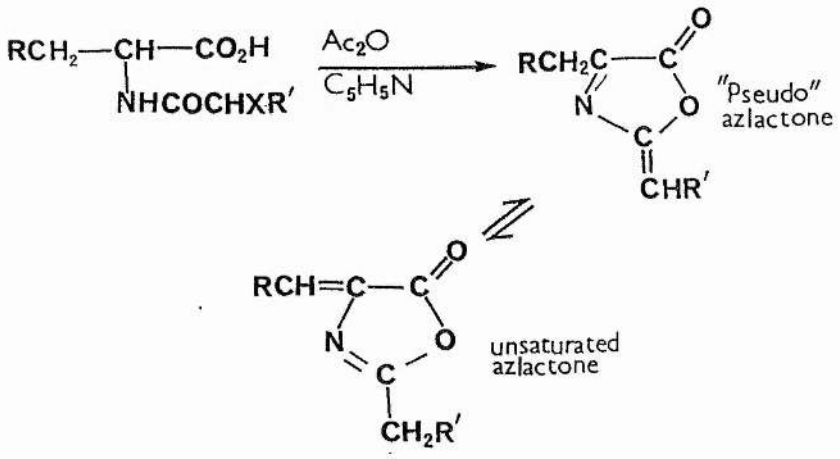


Figure 1.6

The preparation of unsaturated azlactones by reaction of an acid chloride, or an acid anhydride, with an α-acylamino-ß-hydroxy acid, method (d), has been reported by several authors [52-53]. Figure 1.7 shows the reaction mechanism. The saturated azlactone intermediate formed contains a very reactive α-hydrogen and this reacts with the ß-hydroxyl group and by elimination leads to the formation of the unsaturated azlactone.

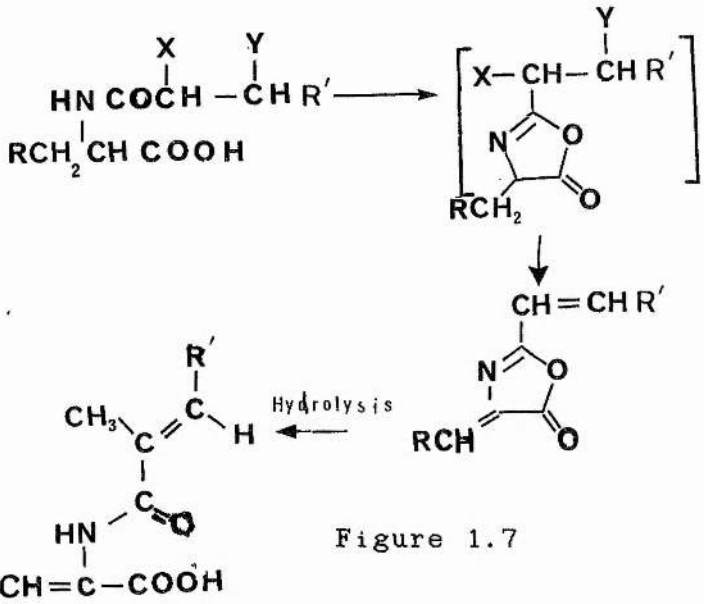


Figure 1.7

In the case of sensitive aldehydes, it is advisable to prepare the saturated azlactone intermediate first, perhaps by the reaction of the acylglycine with ethyl chlorocarbonate and triethylamine in the cold. Subsequent condensation could then be carried out in the presence of diazabicycloundicene or an ion exchanger [64,65]. The (Z)-azlactones formed during the reaction can be converted to the E-isomers by reaction with hydrogen bromide.

Unsaturated azlactones undergo aminolysis, alcoholysis and hydrolysis. The latter reactions are very slow and therefore unsaturated azlactones can be recrystallized from alcohol. Hydrolysis can be carried out in the presence of acidic or alkaline conditions (see Figure 1.8). The product of hydrolysis is a N-acyl-2, 3-didehydroamino acid. Sodium hydroxide in aqueous methanol is convenient for this synthesis since it proceeds via the formation of the N-

acylamino ester, which is subsequently hydrolysed. Mild hydrolysis can be achieved by refluxing the azlactone in aqueous acetone solution. Prolonged hydrolysis with aqueous alkali can lead to formation of the α-keto acid. Ramage and Simonsen[56], in studying the oxidation products of didehydronorcaryophyllenic acid, prepared the azlactone 2-phenyl-4-isopropylidene azlactone. This azlactone was converted into N-benzoyl-2, 3-didehydrovaline by refluxing with potassium hydroxide solution.

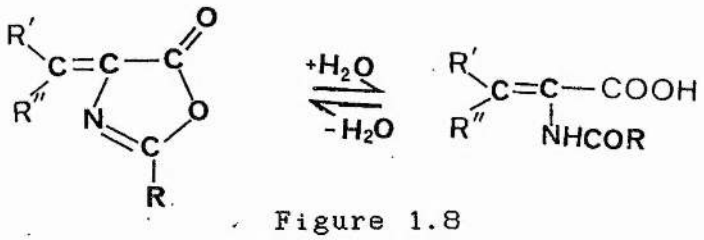


Figure 1.8

Unsaturated azlactones are relatively stable in alcohols. However, in the presence of an acid or base ring opening occurs and N-acyl-2, 3-didehydroamino acid esters are formed[48]. Higher alcohols react readily with azlactones in the presence of an acid or base. The reaction of azlactones with methanol have been reported to result in the formation of peptides as well as the desired esters. The synthesis of N-acyl-2, 3-didehydropeptides can be obtained by aminolysis of the unsaturated azlactones. Bergmann has reported the preparation of several N-acyldidehydropeptides[57]. The preparation of simple dipeptides containing didehydroamino acid residues can be achieved by reacting the azlactone with an amino acid or amino acid salts under very mild conditions, see Figure 1.9.

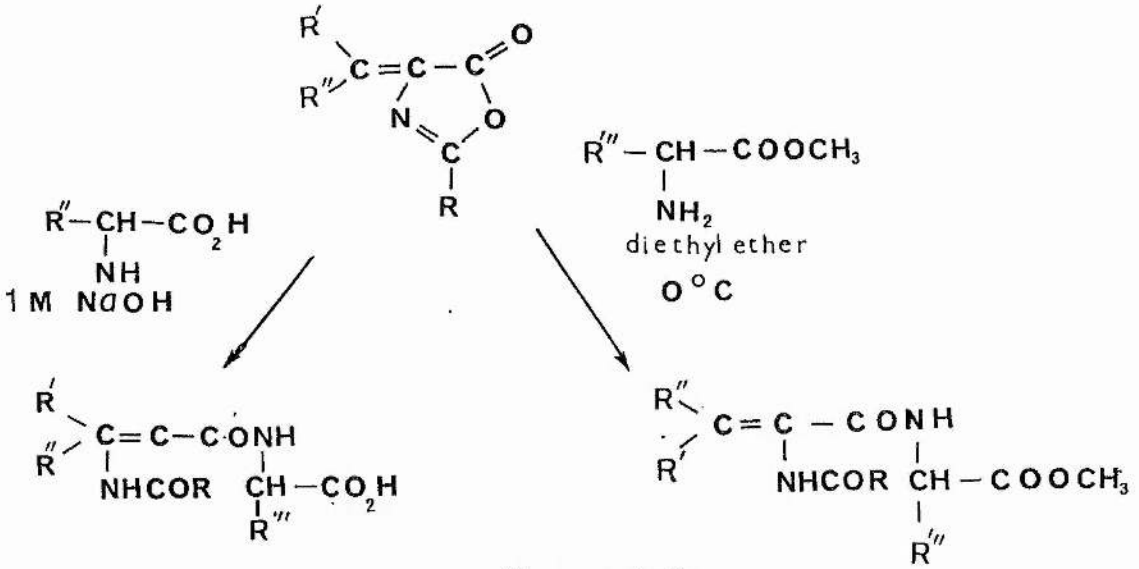


Figure 1.9

Condensation of peptides having a C-terminal glycine with an aldehyde results in the formation of an azlactone. Ring cleavage of this azlactone with an amino acid salt leads to the formation of a peptide containing a central didehydroamino acid residue. Di-, tri-, tetra and penta-peptides containing one or more didehydroamino acid residues have been prepared [60-61].

1.1.7.2 Synthesis via an azide intermediate.

α-Azidocarboxylic acids and aziridines are useful intermediates for the synthesis of didehydroamino acid derivatives. The preparation of α-azidocarboxylic acids has been reported by several authors [62-64]. The azido compounds can be obtained by reaction of an inorganic azide with an α-bromocarboxylic acid, usually in the presence of a phase transfer catalyst. It is necessary to protect the carboxyl group by esterification so that no acyl azides are formed

during the reaction. Effenberger [62] reacted sodium azide with α-bromocarboxylic acid esters in an aqueous system and in the presence of Aliquat 336 (tricaprylmethylammonium chloride). The azido products were obtained in quantitative yields. Manis and Rathke [63] carried out the reaction by stirring the α- bromocarboxylic ester with sodium azide in dimethylformamide at 25°C. Nakajima et. al. [64] reported the azidation of α-bromocarboxylic acids to occur quantitatively in the presence of 18-crown-6 and tetrabutylammonium bromide but virtually no reaction was observed in the absence of phase transfer catalysts.

The α-azidocarboxylic acids readily undergo reduction to give the corresponding amino acids by hydrogenation in the presence of palladium catalysts[64,65]. The synthesis of N-protected didehydroamino acids from azido intermediates has been achieved in several ways. Manis and Rathke[63] formed iminocarboxylates by reaction of the azide with lithium ethoxide in tetrahydrofuran and ethanol. The iminocarboxylate was converted to the N-acyl-2, 3-didehydroamino acid by reaction with triethylamine and acetyl chloride at 0°C.

Effenberger and Beisswenger [62, 66, 67] have reported the synthesis of several N-acetyl-2, 3-didehydroamino acid derivatives from their corresponding α-azidocarboxylic acid esters. Nitrogen elimination was achieved by reaction of the azido compounds with acetic anhydride/ acetic acid in the presence of dirhenium heptasulphide and sometimes

hydrochloric acid. The diacetyl product was found to be formed in some circumstances. α-N-Acetyl-α, β-didehydro-ω aminocarboxylic acid lactam was prepared from its corresponding azido lactam. The reaction is believed to proceed via a nitrene, from which an imine is formed by hydride shift or through an insertion reaction to give an aziridine and subsequent ring opening.

The reactions were carried out at high temperatures and often for a long period of time. The yield of the monoacetylated and diacetylated products was dependent upon the reaction conditions used. Only the monoacetylated compound was obtained by carrying out the reaction in a solution of 4:1 ratio of acetic acid: acetic anhydride. The monoacetyldidehydroamino acid ester was formed exclusively if water was added to the reaction mixture before work up. Since harsh reaction conditions were used, it was necessary to add a small amount of quinol to the reaction mixture to prevent polymerisation of the unsaturated product.

The preparation of N-acetyl-2, 3-didehydroamino acid esters from α-azidocarboxylic acid esters were carried out using the following scheme. α-Bromocarboxylic acids were obtained from the carboxylic acids by the Hell-Volhard-Zelinsky reaction. The corresponding α-azidocarboxylic acids were obtained by the method of Nakajima [64] and were converted to the didehydroamino acids by reaction with acetic anhydride/ acetic acid and dirhenium heptasulphide[62]. The overall reaction scheme is shown in Figure 1.10.

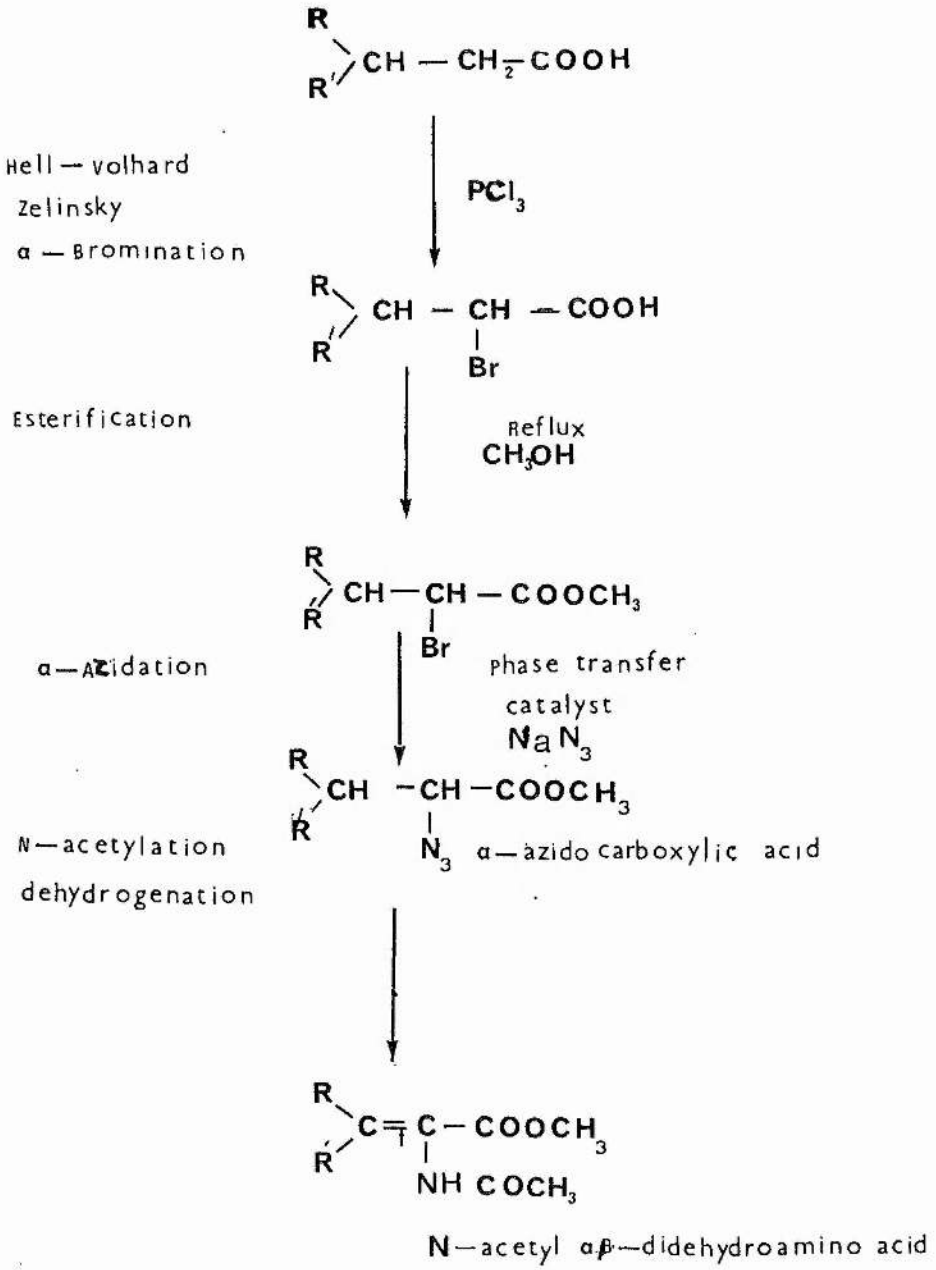

R
R'
CH — CH2 — COOH
Hell — volhard
Zelinsky
α — Bromination
PCl3
CH — CH — COOH
Br
Esterification
Reflux
CH3OH
CH — CH — COOCH3
Br
α — Azidation
Phase transfer
catalyst
Na N3
CH — CH — COOCH3
N3
α — azido carboxylic acid
N — acetylation
dehydrogenation
C = C — COOCH3
NH COCH3
N — acetyl αβ — didehydroamino acid

Figure 1.10

1.1.8 Radiation decomposition.

The stability of compounds labelled with radioisotopes must be carefully considered when trying to evaluate the suitability of tracers for biological and chemical studies. The decomposition of labelled molecules has been studied in relation to their biological stability and storage under various conditions [1, 68, 69]. Hence, there is a need to monitor regularly the condition of the radiolabelled sample.

The study of radiation decomposition previously undertaken has often been carried out in an empirical manner. The advances made in ^{2}H nmr spectroscopy now mean that the re-evaluation of such studies can be undertaken. The formation of new chemical shifts in the product may give some idea of the type of decomposition products formed. The coupling of the method with radio tlc and liquid scintillation counting makes it possible to identify a number of decomposition products.

There are four types of decomposition that have been identified by Bayly and Wiegel [70] which constitute the phenomena of self-irradiation decomposition. These are:

(a) Primary (internal) radiation decomposition
(b) Primary (external) radiation decomposition
(c) Secondary radiation decomposition
(d) Chemical effects

Primary (internal) radiation decomposition is caused by the transmutation of a disintegrating radioisotope. In the case of tritium the product of the decomposition is the β^--particle and a helium atom. The energy that is imparted to the helium nuclide is very small, on average 0 - 3 ev, and is not sufficient to induce chemical degradation. Indeed, it is the change in charge of the resulting molecule that is likely to lead to decomposition. The transmutation effect is related to the half-life. In the case of radioisotopes with short half-lives, such as fluorine-18, the effect becomes very important whilst for isotopes with long half-lives, such as carbon-14, the effect will be virtually insignificant.

Primary (external) radiation decomposition has a more significant role than the transmutation effect in the degradation of tritiated molecules. This mode of decomposition is that which results from the effect of the β^--particle interacting with the other molecules in the system. The molecule will absorb some of the energy of the β^-particle during the collision. The molecule will then become excited or form an ion by the loss or gain of a charged particle due to the collision. Hence, it immediately becomes apparent that there is a large number of resulting possibilities that can occur due to primary (external) radiation decomposition. Some of these will be discussed below with specific reference to amino acids and results reported in the literature.

Secondary radiation decomposition occurs due the interaction of excited molecules, which are caused mainly by the primary (external) effects, with other molecules or radicals. The products of the primary effects may undergo fragmentation, rearrangement, chain reaction or even interact with other molecules or ions in the system. Consequently, this type of decomposition can be the most damaging since it leads to a greater number of possible reactions occurring within the system.

Chemical and enviromental effects should also be taken into account when trying to understand radiation decomposition. The way in which a radioisotopically labelled molecule is stored will affect its stability. Hence, labelled compounds have been stored in various polar or non-polar solutions or solid form, either on a support or in a free form. The temperature at which the labelled molecule has been stored will also affect the rate of decomposition. Low temperature will make the molecules more crowded. This increases the direct and indirect effects unless the molecules are frozen. Also pH, photochemical or microbiological effects could lead to decomposition of labelled molecules.

The amount of decomposition can be expressed in terms of the G (-M) value, sometimes referred to as the destruction coefficient[1]. Equation 1.11 shows the relationship between the percentage decomposition (Pd) and the G(-M) value. It is obvious from simple evaluation of

this expression that the amount of radiation decomposition that is observed increases with an increase in the specific activity of the compound.

$$Pd = (1-e^{-FEG(-M)\,St.6 \times 10^{-16}})100 \qquad \text{Eqn. 1.11}$$

where:

F is the fraction of its own radiation energy absorbed by the compound. The value of F is often taken as unity for tritium because of the low penetrating power of the weak β^--particles.

S is the specific activity of the compound (Ci mol^{-1})

t is the time in seconds

E is the average energy of the tritium radiation (5.7 Kev)

G* (-M) is the number of molecules irreversibly damaged per 100ev of absorbed energy.

There are several ways in which radiation decomposition can be reduced. The primary (external) radiation effects can be reduced by diluting the active compound with its inactive counterpart and /or by immersing it in a solution. The effect may also be reduced by thinly spreading the labelled material over a solid support, such as paper or clay. Secondary radiation decomposition may also be reduced by dilution. Radical scavengers, such as alcohols or thiols, will also reduce the damage caused by secondary effects. The effects of chemicals and the environment can be best reduced by keeping samples at low temperatures.

Although the storage of tritiated compounds in solution can reduce decomposition by self-irradiation, the type of solvent used will have a profound effect on the stability of the labelled compound. Those solvents which readily produce free radicals due to radiolysis are not usually recommended. Organic hydrocarbons, such as benzene, would be the most suitable solvents to use since they only form radicals in small yields. Unfortunately most biologically important compounds, including amino acids, are only soluble in aqueous or alcoholic solvent systems. The ionization of the solvent occurs in 'spurs' along the paths of the radiation[1]. The weaker the β^--radiation the closer together are the 'spurs' and in the case of tritiated compounds will have a profound effect on the stability of tritiated compounds in aqueous solution.

Evans[1, 66] has reported radiation decomposition of tritiated amino acids in aqueous solution and solid form at various temperatures. Willix and Garrison [71] reported that hydrated electrons react with both zwitterionic and cationic forms of amino acids by a reductive deamination. The reaction of radicals, produced by the radiolysis of water, with L-[methyl-^{3}H]-methionine has been studied by Evans[1]. The labelled amino acid is vulnerable to decomposition by the reaction with hydroxyl radicals. The reaction of aromatic amino acids with hydroxyl radicals has been reported to occur at a faster rate than with aliphatic amino acids, with the exception of methionine [72]. The radiation decomposition of tritiated amino acids in aqueous solution

would seem to be related to the reactivity of hydroxyl radicals with amino acids. The effects of radiolysis on amino acids and peptides using external radiation sources have been studied by several authors. These studies can be considered to be complementary to the study of self-irradiation of labelled compounds.

In the case of tritiated compounds labelled at high specific activity the amount of substrate that is studied is often in such a small quantity that full interpretation of decomposition is not possible. However experiments using external radiation sources to study the radiolysis of non-labelled compounds may reveal some possible decomposition processes that could also possibly occur with self-irradiation of tritiated compounds. Garrison[73] has reviewed the effects of γ-radiation on amino acids and their N-acylated derivatives. The decomposition of amino acids has been shown to occur mainly by secondary effects. Deamination of simple amino acids has been proposed to occur by reaction of hydrated electrons and hydroxyl radicals to yield keto- and alkanoic acids. Increasing the length of the aliphatic side chain has been reported to lead to a decrease in deamination [74]. In the case of N-acylated amino acids no deamination is reported to occur in oxygen free systems. Also Panim[75] has used ^{1}H nmr spectroscopy to follow the build up of decomposition products of simple amino acids and peptides due to γ-radiolysis.

The racemization of tritiated optically active amino acids has been reported by Evans et. al. [1]. The exact mechanism of the racemization is not known, though it is considered to be caused by chemical effects rather than due to the ß-radiation because similar results were not observed for carbon-14 labelled amino acids. The use of neutral radical scavengers, such as ethanol, would seem to minimize the racemization. The use of basic salts, such as sodium formate, although reducing the amount of radiation decomposition was found to increase racemization[7e]. In the case of L-[3, 5-^{3}H]-tyrosine 88% of the tritium was found to be present in the DL-form of the amino acid after storage for 4.5 months at +2°C. A solution of the amino acid containing 0.1% sodium formate was found to contain 100% of the tritium in the DL-form but a solution containing 1% ethanol showed no signs of racemization.

Most studies of radiation decomposition have been carried out using labelled compounds with specific activities of less than 12Ci/mmol. However, Hempel [7e] reported the radiation decomposition of some amino acids with specific activities of 12 Ci/mmol or higher for ten different storage conditions. It was found that tritiated lysine stored as a solid adsorbed on paper or sand was relatively stable but rapidly decomposed when adsorbed on NaCl. The storage of tritiated lysine in various solutions at -15°C and +20°C were also observed. The tritiated lysine was found to be most stable in 80% ethanol at -15°C.

Similar studies were carried out for tritiated samples of phenylalanine, dopa, leucine and α-aminoadipic acid. In all cases the labelled amino acids were found to be, more stable stored in solution than in solid form. The impurities formed upon storage of the tritiated amino acids were identified by high voltage electrophoresis. The formation of ethyl esters were found to be the predominant labelled impurities formed during storage in 80% ethanol solutions. Phenylpyruvic acid, phenylethylamine and an electrically neutral compound were found to be present after storage of tritiated phenylalanine as a solid adsorbed on sodium chloride.

Evans[1] recommended that the following storage conditions be used for amino acids:

(a) For low specific activities they should be stored as freeze dried solids at +2°C.

(b) For high specific activities they should be stored at +2°C in aqueous solution, ideally containing up to 2% ethanol as a radical scavenger. with a maximum sample concentration of 1mCi/ml.

1.2 Experimental.

1.2.1 General.

1.2.1.1 Nuclear Magnetic Resonance Spectroscopy.

^{1}H and ^{3}H nmr spectra were recorded at 25°C on a Bruker AC 300 E(300.13 MHz) Fourier Transform nuclear magnetic resonance spectrometer using deuterated solvents as indicated below and referenced to either tetramethylsilane (tms) or 2, 2-dimethylsilapentane-5-sulphonic acid (DSS). Some ^{1}H nmr were recorded using a Varian EM360 (60 MHZ) continuous wave nuclear magnetic resonance spectrometer.

Tritium nmr samples were prepared as follows: A known quantity of the tritiated compound was dissolved in 100µl of deuterated solvent or aqueous buffer solution. The radioactivity of the sample was checked by liquid scintillation counting. Then the sample was placed in a Wilmad 3mm wide necked nmr tube. The solution was frozen in liquid nitrogen and evacuated. The nmr tube was then narrowed and sealed in a bunsen flame. In general the nmr samples were stored at -20°C prior to analysis.

1.2.1.2 Infrared Spectroscopy.

IR spectra were recorded using a Perkin Elmer grating infrared spectrophotometer model 577 instrument. The spectra were compared, where possible, with those reported

in the Sadtler index.

1.2.1.3 Thin Layer Chromatography.

Thin layer chromatography (tlc) was run using conditions as outlined under particular experiments where it was required. In all cases fluorescent silica gel plates were used.

1.2.1.4 Radioactive Thin Layer Chromatography.

Radio tlc analyses were carried out using a Berthold LB2842 automatic tlc linear analyser fitted with an LB2821 detector. Samples were run on 20cm x 20cm fluorescent backed silica gel plates.

1.2.1.5 Gas Chromatography.

Gas chromatography (GC) was carried out using a Sigma 3B gas chromatography instrument fitted either with a Carbowax 20M or OV1 column.

1.2.1.6 High Performance Liquid Chromatography.

High performance liquid chromatography (HPLC) was carried out using a Kontron instrument model Uvikon 735LC fitted with a Ubondapak 10C18 30cm x 3.9mm column.

1.2.1.7 Elemental Microanalysis.

CHN elemental analysis was carried out by Mrs N. Walker using a Carlo Erba elemental analyzer model 1106.

1.2.1.8 Melting Points.

Melting points and decomposition temperatures were recorded using a Gallenkamp apparatus.

1.2.1.9 Liquid Scintillation Counting.

The radioactivity of tritiated samples were counted using a Beckman LS 1800 liquid scintillation counter. Samples were prepared using a solution of 2,5-diphenyloxazole (PPO) in toluene as the scintillant ($3.4 g l^{-1}$), unless otherwise stated.

1.2.1.10 Reagents.

The reagents used throughout this work were all analytical grade, unless stated otherwise, and were either used directly or purified as described for each experiment.

1.2.1.11 Solvents.

Pure tetrahydrofuran was prepared for use by leaving HPLC grade solvent over lithium aluminium hydride and distilling over the dry fraction at 65-66°C. The purified

tetrahydrofuran was used immediately or stored over calcium hydride.

1.2.1.12 Buffer Solutions.

Buffer solutions were prepared as described below, in accordance with the methods outlined by Perrin and Dempsey[77].

(a) Acetate buffer (pH 4.0): 0.21M acetic acid ($80cm^3$), 0.19M sodium hydroxide ($22cm^3$).

(b) Phosphate buffer (pH 6.8): 0.025M anhydrous potassium dihydrogenphosphate ($3.425g/l^{-1}$), 0.025M anhydrous dipotassium hydrogenphosphate ($4.4g/l^{-1}$).

(c) Borate buffer (pH 9.0): 0.5M anhydrous tetrasodium borate ($11.3g/100cm^3$).

1.2.2 Synthesis of didehydroamino acids via azide intermediates.

1.2.2.1 2-Bromopropionic acid.

Pure propionic acid was prepared by distilling a sample of the general purpose reagent at 140-141°C, after pre-drying by standing over 4A molecular sieve. Dry bromine was prepared, in accordance with the instructions of Vogel [78] by shaking bromine with an equal volume of sulphuric acid and separating off the halogen layer.

Freshly distilled dry propionic acid (30mls, 0.40 moles) and dry bromine (15mls, 0.29 moles) were placed in a three necked flask, fitted with a thermometer and condenser, to which a sodium hydroxide trap was attached. Phosphorus trichloride (1.5mls) was slowly added to the mixture and the reaction heated at 50°C for two hours. 2-Bromopropionic acid was collected by distillation at 162°C / 30mmHg. Yield 67% (84g, 0.55 moles). A yield of 45% (53.9g, 0.37 moles) was obtained using unpurified reagents. ^{1}H nmr (solvent $CDCl_3$) δ ppm: 11.7 (1H, broad s, ^{1}H of carboxylic acid group proton); 4.4 (1H, q, CH group); 1.8 (3H, d, CH_3 group). IR (Nujol mull, NaCl plates) cm^{-1}: 3100-3000 broad sh (carboxyl OH stretch), 2980-2700 v. sh, 1720 sh (C=O stretch), 1420 sh (C-O and OH coupled vibrations). CHN (for C3, H5, O2, Br): calculated C 23.70%, H 3.30%; found C 23.58%, H 3.27%.

1.2.2.2 2-Bromo-4-methylpentanoic acid.

4-Methylpentanoic acid (25g, 0.21 moles) was mixed with dry bromine (15mls, 0.29 moles) and phosphorus trichloride (4mls) was added slowly. The reaction mixture was heated at 75°C for four hours. 2-Bromo-4-methylpentoic acid was collected by distillation at 64°C. Yield 57% (25.7g, 0.122 moles). ^{1}H nmr (solvent $CDCl_3$) δ ppm: 4.1 (1H, m, α-CH group); 1.7 (1H, m, CH group); 1.4 (2H, m, CH_2 group); 1.0 (6H, d, δ-CH_3 groups). IR (Nujol mull, NaCl plates) cm^{-1}: 3100 broad m (carboxyl OH stretch), 3000-2900 sh, 1750 m (Carboxyl C=O stretch). CHN (for C6, H11, O2, Br): calculated C 36.92%, H 5.64%; found C 37.17%, H 5.76%.

1.2.2.3 2-Bromopropionic acid methyl ester.

2-Bromopropionic acid (20mls, 0.13moles), 2, 2-dimethoxypropane (10mls, 0.1moles), methanol (5mls, 0.13moles) and toluene-p-sulphonic acid (0.1g) were added together. The mixture was refluxed for two hours at 60°C. The mixture was washed with sodium bicarbonate (20mls) to neutralise any unreacted 2-bromopropionic acid. The organic layer was extracted with dichloromethane (3 x 20mls). 2-Bromopropionic acid methyl ester was collected by distillation at 143-5°C. Yield 75% (16mls, 0.096 moles). ^{1}H nmr (solvent $CDCl_3$) δ ppm: 4.3 (1H, q, CH group); 3.7 (3H, s, CH_3 of methyl ester group); 1.8 (3H, d, CH_3 group). IR (Nujol mull, NaCl plates) cm^{-1}: 3000-2800 sh, 1750 sh (C=O stretch), 1180 m (C-O stretch). CHN (for C4, H7, O2, Br): calculated C 28.74%, H 4.19%; found C 30.58%, H 4.42%.

1.2.2.4 2-Bromo-3-methylbutanoic acid methyl ester.

2-Bromo-3-methylbutanoic acid (10g, 0.055moles), methanol (3mls, 0.075moles), 2, 2-dimethoxypropane (12mls, 012moles) and toluene-p-sulphonic acid (0.1g) were refluxed together for three hours at 70°C. The reaction mixture was washed with sodium bicarbonate (10mls). The organic layer was extracted with dichloromethane (3 x 20mls). The dichloromethane was evaporated off and 2-bromo-3-methylbutanoic acid methyl ester collected at 80°C / 20mmHg. Yield 54%. (5ml, 0.025 moles). ^{1}H nmr (solvent $CDCl_3$) δ ppm: 4.0 (1H, d, α-CH group); 3.7 (3H, s, CH_3 of methyl ester group); 2.1 (1H, m, β-CH group); 1.1 (6H, dd, γ-CH_3

groups). IR (Nujol mull, NaCl plates) cm^{-1}: 3000-2900 sh, 1740m and 1620m (C=O stretch), 1160m (C-O stretch). CHN (for C6, H11, O2, Br): calculated C 36.92%, H 5.64%; found C 36.85%, H 5.93%.

1.2.2.5 2-Bromo-4-methylpentanoic acid methyl ester.

Methanol (5mls, 0.13moles), toluene-p-sulphonic acid (0.1g), 2-bromo-4-methylpentanoic acid methyl ester (8g, 0.044moles) and 2, 2-dimethoxypropane (10mls, 0.1moles), methanol (5mls) and toluene-p-sulphonic acid (0.1g) were refluxed for three hours at 70°C. The reaction mixture was washed with sodium bicarbonate (10mls) and then the organic layer extracted with dichloromethane (3 x 20mls). The volume of the solvent was reduced by rotary evaporation. 2-Bromo-4-methylpenatanoic acid methyl ester was collected at 94°C / 25mmHg. Yield 58% (5ml, 0.025 moles). ^1H nmr (solvent $CDCl_3$) δ ppm: 4.1 (1H, m, CH group); 3.7 (3H, s, CH_3 of methyl ester group); 1.5 (1H, m, CH group); 1.3 (2H, m, CH_2 group); 1.0 (6H, d, δ-CH_3 groups). IR (Nujol mull, NaCl plates) cm^{-1}: 3000-2900 sh, 1740 m (C=O ester carbonyl), 1170 (CO stretch). CHN (for C7, H13, O2, Br): calculated C 40.19%, H 6.22%; found C 40.67%, H 6.08%.

1.2.2.6 2-Azidopropionic acid methyl ester.

Three experiments were carried out to optimise the reaction conditions. These are described below.

(a) Small scale preparation: 2-Bromopropionic acid methyl ester (2mls, 0.012moles), water (4mls), sodium azide (0.96g)

and tetrabutylammonium bromide (150mg) were stirred for five hours. The product was then extracted with dichloromethane (3 x 10mls). The solution was dried over sodium sulphate and then the solvent removed by rotary evaporation. Yield 89% (1.4g, 0.011moles). 93% pure by GC.

(b) Twelve hour preparation: 2-Bromopropionic acid methyl ester (9mls, 0.054moles), water (20mls), sodium azide (4g) and tetrabutylammonium bromide (0.5g) were stirred for twelve hours at room temperature. The reaction mixture was worked up as above. Yield 92% (6.5g, 0.05moles). 96% pure by GC.

(c) Short reaction times: The above reaction was repeated but the time was reduced to one and a half hours. In the second experiment the weight of the phase transfer catalyst was halved. The samples were worked up as previously described. The yields were 82% (5.7g, 0.044moles) and 70% (4.9g, 0.038moles) respectively. 94% pure by GC.

^{1}H nmr (solvent $CDCl_3$) δ ppm: 3.9 (1H, m, CH group); 3.7 (3H, s, CH_3 of methyl ester group); 1.4 (3H, d, CH_3 group). IR (Nujol mull, NaCL plates) cm^{-1}: 3000-2900 sh, 2100 sh (C-N stretch azido group), 1750 m (C=O ester carbonyl), 1625 m (N=N stretch), 1360 m (C-N stretch), 1160 w (C-O stretch propionate). CHN (for C4, H7, O2, N3): calculated C 37.20%, H 5.43%, N 32.56%; found C 37.46%, H 5.54%, N 32.78%.

1.2.2.7 2-Azido-3-methylbutanoic acid methyl ester.

2-Bromo-3-methylbutanoic acid (8g, 0.4moles), sodium azide (4g), water (20mls) and tetrabutylammoniun bromide (0.4g) were stirred for six hours. 2-Azido-3-methylbutanoic acid methyl ester was collected by vacuum distillation at 76°C / 20mmHg. Yield was 67% (4mls, 0.026moles). 96% pure by GC. ^{1}H nmr (solvent $CDCl_3$) δ ppm: 4.0 (1H, d, CH group); 3.7 (3H, s, CH_3 of methyl ester group); 2.2 (1H, m, CH group); 1.2 (3H, d, τ-CH_3 group); 1.1 (3H, d, τ-CH_3 group). IR (Nujol mull, NaCl plates) cm^{-1}: 3000-2900 sh, 2100 sh (C-N stretch azido group), 1740 m (C=O stretch ester carbonyl), 1620 m (N=N stretch), 1160 w (C-O stretch). CHN (for C6, H11, N3, O2): calculated C 45.86%, H 7.00%, N 26.75%; found C 45.65%, H6.86%, N 26.34%.

1.2.2.8 2-Azido-4-methylpentanoic acid methyl ester.

2-Bromo-4-methylpentanoic acid methyl ester (4g, 0.022moles), sodium azide (2g), distilled water (8mls) and tetrabutylammonium bromide (0.4g) were stirred for six hours. The product was purifed as above. Yield 78% (3ml, 0.017moles). 97% pure by GC. ^{1}H nmr (solvent $CDCl_3$) δ ppm: 4.0 (1H, m, CH group); 3.7 (3H, s, CH_3 of methyl ester group); 1.7 (1H, m, τ-CH group); 1.3 (2H, m, β-CH_2 group); 1.0 (6H, d, δ-CH_3 groups). IR (Nujol mull, NaCl plates) cm^{-1}: 3000-2900 sh, 2080 sh (C-N stretch azido group), 1740 m (C=O stretch ester carbonyl), 1630 (N=N stretch), 1160 (C-O stretch). CHN (for C7, H13, O2, N3): calculated C 49.12%, H

7.60%, N 24.56%; found C 49.45%, H 7.34%, N 24.25%.

1.2.2.9 Chromatographic analysis of 2-azidopropionic acid methyl ester.

(a) Tlc analysis of the 2-azidocarpropionic acid methyl ester was carried out using the following solvent systems: chloroform, 1:1 (v/v) hexane: chloroform, 1:1 (v/v) hexane: ethanol. Samples were referenced to 2-bromopropionic acid methyl ester and propionic acid. Tlc plates were air dried and visualized by exposure to iodine and by spraying with sulphuric acid. Similar analysis was carried out for 2-azido-3-methylbutanoic acid methyl ester and 2-azido-4-methylpentanoic acid methyl ester.

(b) Gas chromatography analysis of 2-azidopropionic acid methyl ester was carried out using the following procedure. 0.5µl of 2-azidopropoinic acid methyl ester (50µl/ml dichloromethane) was injected onto the column (OV1), which was set at 100°C. Reference samples of propionic acid and 2-bromopropionic acid were prepared. The purity of 2-azidopropionic acid methyl ester was determined by measuring the amount of 2-bromopropionic acid methyl ester and propionic acid present in the sample compared to the reference samples. Both Carbowax 20M and OV1 columns were used. In the case of carbowax 20M insufficient resolution of the product was obtained. Attempts were made to carry out analysis at higher column temperatures but it was found that the azide decomposed. Similar analysis was

carried out for samples of 2-azido-3-methylbutanoic acid methyl ester and 2-azido-4-methylpentanoic acid methyl ester.

1.2.2.10 2-Acetamidoprop-2-enoic acid methyl ester. (N-acetyl-2, 3-didehydroalanine methyl ester)

2-Azidopropionic acid methyl ester (4g, 0.03moles), acetic anhydride (50mls) and glacial acetic acid (24mls) were refluxed for sixteen hours at 80°C in the presence of quinol (0.1g) and rhenium heptasulphide (0.1g). Acetic acid and acetic anhydride were removed by distillation at 30°C/20mmHg. The residue was taken up in 1:1 (v/v) petroleum ether: diethyl ether (100mls). The solvents were removed by rotary evaporation. The crude product was recrystallised from a minimum volume of 1:1 (v/v) petroleum ether: diethyl ether after the insoluble matter had been removed by filtration. Yields 10% (.48g, 0.003moles) and 35% (1.68g, 0,012moles). M.pt 51-52°C found, 52-54°C literature [62]. ^{1}H nmr (solvent d_6-DMSO) δ ppm: 9.3 (1H, s, NH group); 6.1 (1H, s, ß-CH_2 group); 5.6 (1H, s, ß-CH_2 group); 3.7 (3H, s, CH_3 of methyl ester group); 2.0 (3H, s, CH_3 of acetyl group). IR (Nujol mull, NaCl plates) cm^{-1}: 3400-3200 broad m (amide I), 3000-2900 sh, 1780-1760 m (C=O stretch of methyl ester group), 1650 m (C=O, amide II/ C=C stretch). CHN (for C5, H7, O3, N): calculated C 50.35%, H 6.29%, N 9.80%; found C 50.04%, H 10.01%, N 6.42%.

1.2.2.11 Attempted preparation of 2-acetamido-3-methylbut-2-enoic acid methyl ester (N-acetyl-2, 3-didehydrovaline methyl ester).

2-Azido-3-methylbutanoic acid methyl ester (1g, 0.005moles) was reacted with acetic anhydride (5mls) in the presence of quinol (10mg) and rhenium heptasulphide (42mg) for thirty five hours at 90°C. Water (3mls) was added to the mixture. The reaction mixture was heated for a further thirty hours at 90°C. The product was extracted using 1:1 (v/v) petroleum ether : diethyl ether (100mls). A black residue was obtained after evaporating off the solvents. Attempts to isolate a crystalline product from the crude mixture was unsuccessful.

1.2.2.12 First attempted preparation of 2-acetamido-4-methylpent-2-enoic acid methyl ester (N-acetyl-2, 3-didehydroleucine methyl ester).

2-Azido-4-methylpentanoic acid methyl ester (4g, 0.023moles), acetic anhydride (56mls) and acetic acid (24mls) were refluxed in the presence of rhenium heptasulphide (0.1g) and quinol (0.1g) at 90°C. The reaction was monitored by following the disappearance of the azido stretching frequency at 2100cm^{-1} in the infrared spectrum. After thirty hours the reaction was stopped. Excess acetic acid and acetic anhydride were removed by vacuum distillation at 30°C/20mmHg. The residue was taken up in 1:1 (v/v) petroleum ether: diethyl ether (80mls). The

solvents were removed by rotary evaporation. Attempts to recrystallise the product from a minimum volume of 1:1 (v/v) petroleum ether: diethyl ether was unsuccessful. The product of the reaction was a black tarry solid.

1.2.2.13 Second attempted preparation of 2-acetamido-4-methylpent-2-enoic acid methyl ester.

2-Azido-4-methylpentanoic acid methyl ester (1g, 0.006moles) and acetic anhydride (5mls) was refluxed in the presence of quinol (40mg) and rhenium heptasulphide (40mg) for thrty five hours at 90°C. Water (4mls) was added to the system and the reaction mixture refluxed for a further thirty hours at 90°C. The product was extracted with 1:1 (v/v) petroleum ether: diethyl ether (100mls). The solvents were removed by evaporation and the crude residue recrystallised from a minimum volume of 1:1 (v/v) petroleum ether: diethyl ether. A dirty white powder was obtained. Subsequent analysis showed that the reaction had been unsuccessful.

1.2.3 Synthesis of didehydroamino acids via azlactone intermediates.

1.2.3.1 Preparation of 4-isopropylidene-2-phenyloxazol-5-one (N-benzoyl-2, 3-didehydrovaline azlactone).

The following synthesis was based on the method of Ramage and Simonsen [66]. Powdered benzoylglycine (40g),

acetic anhydride (90g), acetone (120g) and sodium acetate (24g), which had been pre-dried at 100°C, were placed in a dry 500ml round bottomed flask. The reaction mixture was then refluxed at 90°C for six hours. The solution was allowed to cool and then poured into distilled water (300mls). The resulting crystalline solid was collected by vacuum filtration. The crude product was washed with a dilute solution of sodium bicarbonate (3 x 30 mls). Recrystallization from ethanol gave a pink needle crystalline product. M.pt. 103-4°C found, 100-102°C literature [66]. Yield 40% (17.4g). ^{1}H nmr (solvent $CDCl_3$) δ ppm: 8.0 (2H, dd, ortho protons of 2-phenyl group); 7.3 (3H, complex, meta and para protons of 2-phenyl group); 3.4 (6H, d, CH_3 groups).

1.2.3.2 Attempted preparation of 4-isopropylidene-2-methyloxazol-5-one (N-acetyl-2, 3-didehydrovaline azlactone).

N-Acetylglycine (40g); acetic anhydride (90g); acetone (120g) and sodium acetate, pre-dried at 100°C, were added to a dry 500ml round bottomed flask. The reaction mixture was refluxed at 85°C for six hours. Initially a pasty solid formed and this eventually dissolved to give a green solution. The solution turned orange on prolonged heating. After cooling, the solution was poured into distilled water (400mls). Attempts to isolate a product were unsuccessful.

1.2.3.3 Preparation of 4-benzylidene-2-methyloxazol-5-one (N-acetyl-2, 3-didehydrophenylalanine azlactone).

N-Acetylglycine (5g), benzaldehyde (8mls), anhydrous sodium acetate (3g) and acetic anhydride (10mls) were liquified. The solution was boiled for two hours. After cooling, it was kept in a refrigerator overnight. The crystalline product was stirred with water (30mls) and filtered under suction. The product was washed with cold water (2 x 25mls) and ether (10mls). The product was dried at 100°C. The pale yellow crystalline product had a yield of 83% (4.68g). M.pt 148-9°C found, 150°C literature [78]. ^1H nmr (solvent $CDCl_3$) δ ppm: 8.0 (2H, dd, ortho protons of 2-phenyl group); 7.4 (3H, m, meta and para protons of 2-phenyl group); 4.4 (2H, s, CH_2 group).

1.2.3.4 Preparation of 4-benzylidene-2-phenyloxazol-5-one (N-benzoyl-2, 3-didehydrophenylalanine azlactone).

N-Benzoylglycine (5g), freshly distilled benzaldehyde (12mls), acetic anhydride (10mls) and anhydrous sodium acetate (5g) were added to a conical flask and liquified on a hot plate. The solution was boiled at 110°C for one hour. Absolute ethanol (50ml) was added slowly to the solution. Cooling the solution in ice resulted in the formation of a pale green crystalline product. The product was collected by filtration under suction and washed with cold ethanol (2

x 10mls), and then with boiling water. The product was recrystallised from benzene. Yield 89% (6.33g). M.pt 166-167°C found, 167-168°C literature [7]. ^{1}H nmr (solvent $CDCl_3$) δ ppm: 8.1 (4H, overlapping dd, ortho protons of both the 2-phenyl and 4-phenyl groups); 7.4 (6H, complex, overlapping meta and para protons of both the 2-phenyl and 4-phenyl groups); 7.2 (1H, s, CH group).

1.2.3.5 2-acetamido-3-phenylprop-2-enoic acid (N-acetyl-2, 3-didehydrophenylalanine).

4-Benzylidene-2-methyloxazol-5-one (4g), water (18mls) and acetone (45mls) were refluxed for four hours at 98°C. The acetone was evaporated off and water (40mls) added to the solution. The solution was boiled for ten minutes and then filtered through a hot funnel. Decolourising charcoal was added to the filtrate and the mixture boiled for ten minutes. The mixture was filtered through a hot funnel and the filtrate stood in a refrigerator overnight. The resulting crystalline product was collected by vacuum filtration and washed with water (2 x 10mls). The product was dried at 100°C. Yield 79% (3.78g). M.pt. 191-192°C found, 192°C literature [7]. ^{1}H nmr (solvent d_6-DMSO) δ ppm: 9.5 (1H, s, NH group); 7.6 (2H, d, ortho protons of phenylalanine aromatic ring); 7.4 (3H, m, meta and para protons of phenylalanine aromatic ring); 7.2 (1H, s, ß-CH group); 1.9 (3H, s, CH_3 of acetyl group). CHN (for C11,H11,N, O3): calculated C 64.39%, H 5.36%, N 6.82%; found C 64.03%, H 5.28%, N 6.82%.

1.2.3.6 2-benzamido-3-phenylprop-2-enoic acid (N-benzoyl-2, 3-didehydrophenylalanine).

4-Benzylidene-2-phenyloxazol-5-one (1g) and potassium hydroxide (0.5g/ 10mls water) were refluxed at 100°C for two hours. Excess concentrated hydrochloric acid was added to the solution. The white needle crystalline product was collected by vacuum filtration. Yield 93% (0.94g). M.pt 198-199°C found, 199-200°C literature [57]. ^{1}H nmr (solvent d_6-DMSO) δ ppm: 9.9 (1H, s, NH group); 8.0 (2H, d, ortho protons of phenylalanine aromatic ring); 7.6 (5H, complex, overlapping meta and para protons of phenylalanine aromatic ring and ortho protons of benzoyl group aromatic ring); 7.3 (4H, m, overlapping meta and para protons of benzoyl group aromatic ring and ß-CH group). CHN (for C16, H15, N, O3): calculated C 71.91%, H 4.86%, N 5.24%; found C 70.45%, H 4.67%, N 5.53%.

1.2.3.7 N-benzoyl-3-methylbut-2-enoic acid (N-benzoyl-2, 3-didehydrovaline).

2-Phenyl-4-isopropylideneoxazol-5-one (1.21g) and potassium hydroxide (0.5g/ 10mls distilled water) were heated on a water bath for two hours. Excess concentrated hydrochloric acid was added and the reaction solution stood over ice for four hours. The resulting crystalline product was collected by vacuum filtration and washed with cold water (3 x 30mls). Yield 98% (1.45g). M.pt 215°C (decomposition). ^{1}H nmr (solvent d_6-DMSO) δ ppm: 9.5 (1H, s,

NH group); 7.9 (2H, d, ortho protons of benzoyl group aromatic ring); 7.5 (3H, m, meta and para protons of benzoyl group aromatic ring); 2.1 (3H, s, τ-CH_3 group); 1.8 (3H, s, τ-CH_3 group). CHN (for C7, H13, N, O3): calculated C 52.83%, H 8.17.%, N 8.80.%; found C 53.26%, H 8.37%, N 8.56%.

1.2.3.8 N-acetyl-4, 5-didehydroleucine.

N-acetyl-4, 5-didehydroleucine was supplied by Dr. Wade of Ciba-Geigy pharmaceuticals. M. pt. 158-159°C found, 158-159°C literature [78]. ^{1}H nmr (solvent d_6-DMSO) δ ppm: 8.0 (1H, d, NH group); 4.8 (1H, s, δ-CH_2 group); 4.6 (1H, s, δ-CH_2); 4.3 (1H, m, α-CH group); 2.4 (1H, m, β-CH_2 group); 2.2 (1H, m, β-CH_2 group); 1.8 (3H, s, CH_3 of acetyl group); 1.6 (3H, s, δ-CH_3).

1.2.4 Synthesis of didehydropeptides via azlactone intermediates.

1.2.4.1 Synthesis of N-acetyl-2, 3-didehydrophenylalanyl-L-alanine.

L-Alanine (0.9g), acetone (10mls), 1M sodium hydroxide (10mls) and 4-benzylidene-2-methyloxazol-5-one (2.0g) were stirred for several hours until a clear solution was formed. 1M hydrochloric acid (11 mls) was added to the solution. The solvents were evaporated off to leave a creamy white residue. Recrystallization from 60% methanol by the addition

of water gave a creamy crystalline product. The recrystallization of the product was carried out three times. Yield 54% (1.6g). M.pt 193-194°C (decomposition) found; 195-196°C (decomposition, corrected) literature [57]. ^{1}H nmr (solvent d_6-DMSO) δ ppm: 9.4 (1H, s, NH of acetyl group); 8.1 (1H, d, NH of peptide bond group); 7.5 (2H, d, ortho protons of phenylalanine aromatic ring); 7.4 (3H, m, meta and para protons of phenylalanine aromatic ring); 7.0 (1H, s, ß-CH group); 4.3 (1H, m, α-CH group of alanine); 1.9 (3H, s, CH_3 of acetyl group); 1.3 (3H, d, ß-CH_3 group). CHN (for C14, H16, N2, O4): calculated C 60.87%, H 5.79%, N 10.14%; found C 61.26%, H 5.56%, N 10.13%.

1.2.4.2 Attempted synthesis of N-benzoyl-2, 3-didehydrophenylalanyl-L-glutamic acid.

L-Glutamic acid (3g) was stirred in a solution of 1M sodium hydroxide (10mls) and acetone (5mls) for ten minutes and then 4-benzylidene-2-phenyloxazol-5-one (5g) was added. The mixture was stirred for several hours until a clear solution formed. Then 1M hydrochloric acid (10mls) was added and the resulting precipitate collected by vacuum filtration. Recrystallization from 60% methanol by subsequent addition of water gave a white crystalline product. Analysis of the product by ^{1}H nmr showed that several compounds were present. Attempts to isolate the desired compound by repeated recrystallization and preparative tlc using a 5:2:3 (v/v) butan-1-ol: acetic acid: water system were unsuccessful.

1.2.4.3 Attempted preparation of N-acetyl-2, 3-phenylalanyl-L-tyrosine.

L-Tyrosine (3g), 4-benzylidene-2-phenyloxazol-5-one (5.5g), acetone (38mls) and 1M sodium hydroxide (17mls) were stirred for five hours. The mixture was filtered and washed with water (3 x 10mls). The combined filtrate and washings were collected and acidified with 1M hydrochloric acid (18mls). The solution was stood in ice and a thick white precipitate formed. The product was collected and recrystallised from methanol. Analysis of the product showed it to contain predominantly N-benzoyl-2,3-phenylalanine. The reaction was repeated but at temperature of 0°C, 30°C and 50°C. In all cases the reaction was unsucessful. The reaction was then carried out with the 1M sodium hydroxide replaced by 2M sodium hydroxide (17mls) and 2M potassium hydroxide (17mls) respectively. In both cases no coupling of the azlactone and L-tyrosine was observed.

1.2.4.4 Preparation of N-acetyl-2,3-didehydrophenylalanylglycine.

Glycine (3g) was suspended in acetone (40mls) and 1M sodium hydroxide (40mls) was added slowly. After stirring the mixture for several minutes, 4-benzylidene-2-methyl oxazol-5-one (7.5g) was added. The mixture was stirred until a clear solution formed and then 1M hydrochloric acid (40mls) was added. The solution was stood in a refrigerator for two days. The resulting crystalline product were

filtered under suction. The crude product was dissolved in 10% aqueous potassium bicarbonate (20mls) and reprecipitated by the addition of 1M hydrochloric acid. The product was washed and dried at 60°C. Yield 91% (9.6g). M.pt 193-194°C found; 194-195°C literature [57]. ^{1}H nmr (d_6-DMSO) δ ppm: 9.4 (1H, s, NH of acetyl group); 8.2 (2H, d, NH of peptide bond group); 7.5 (2H, d, ortho protons of phenylalanine aromatic ring); 7.4 (3H, m, meta and para protons of phenylalanine aromatic ring); 7.1 (1H, s, ß-CH group); 3.8 (2H, s, CH_2 group); 1.9 (3H, s, CH_3 of acetyl group). CHN (for C13, H14, N2, O4): calculated C 59.54%, H 5.34%, N 10.68%; found C 59.67%%, H 5.63%%, N 10.59%.

1.2.4.5 Attempted synthesis of N-benzoyl-2, 3-didehydrovalinylglycine ethyl ester.

4-Isopropylidene-2-phenyloxazol-5-one (1g) was stirred at 0°C in diethyl ether (15mls) until a clear solution formed. After one hour freshly prepared glycine ethyl ester, dissolved in diethyl ether (0.5mls, 0.5g/ml diethyl ether. The mixture was stirred overnight but no product precipitated out. The ether was evaporated off and the residue recrystallized from ethyl acetate. Analysis of the residue by ^{1}H nmr showed a mixture of compounds to be present. Attempts to isolate the desired compound by repeated recrystallization and preparative tlc using a 12:3:4 butan-1-ol: acetic acid: water system and a 9:1 chloroform: methanol system were unsuccessful. The reaction was repeated using glycine methyl ester instead of glycine

ethyl ester but the reaction was also unsuccessful.

1.2.4.6 Attempted synthesis of N-benzoyl-2, 3-didehydrovalinyl-L-alanine.

4-Isopropylidene-2-phenyloxazol-5-one (4g) and L-alanine (2.5g) were stirred in 1M sodium hydroxide (15mls) for ten minutes and then acetone (8mls) was added. The mixture was stirred for several hours until a clear solution formed. 1M hydrochloric acid (18mls) was added and the resulting precipitate collected by filtration under vacuum. The product was recrystallized twice from 60% methanol by addition of water. The product was dried at 60°C. Analysis of the product by ^{1}H nmr spectroscopy showed the major component to be N-benzoyl-2, 3-didehydrovaline.

1.2.4.7 Synthesis of N-benzoyl-2, 3-didehydrophenylalanylglycine ethyl ester.

4-Benzylidene-2-phenyloxazol-5-one (1g) was dissolved in diethyl ether (20mls) and stirred for half an hour at 0°C. Freshly prepared glycine ethyl ester, dissolved in diethyl ether (0.5mls) was added. The mixture was stirred at 0°C and after one hour a white crystalline product was formed. The product was filtered and dried at 60°C. Yield 76% (1.07g). M.pt 138-9°C found, 135-136°C literature[80]. ^{1}H nmr (solvent d_6-DMSO) δ ppm: 9.9 (1H, s, NH of benzoyl group); 8.3 (2H, d, NH of peptide bond group); 8.0 (2H, d, ortho protons of phenylalanine aromatic ring); 7.6 (5, m,

overlapping meta and para protons of phenylalanine aromatic ring and ortho protons of benzoyl group aromatic ring); 7.3 (4H, m, overlapping meta and para protons of benzoyl group aromatic ring and ß-CH group); 3.8 (2H, d, CH_2 group of glycine). CHN (for C20, H20, N2, O4): calculated C 68.18%, H 5.68%, N 7.95%; found C 68.26%, H 5.73%, N 8.17%.

1.2.4.8 Synthesis of N-benzoyl-2, 3-didehydrophenylalanylglycine (first method).

N-Benzoyl-2, 3-didehydrophenylalanylglycine ethyl ester (1g) was suspended in 1M sodium hydroxide (10mls) and stirred. After half an hour a clear solution formed. The solution was neutralized by the addition of 1M hydrochloric acid. The solvent was removed by freeze drying. The residue was taken up in hot 60% methanol (10ml) and filtered through a hot funnel to remove the insoluble matter. Decolourising charcoal was added to the solution and the mixture was boiled for five minutes. The charcoal was removed by filtration through a hot funnel. The methanol was removed by rotary evaporation. Recrystallization from acetone by the addition of water gave a white needle crystalline product. Yield 98% (1.07g). M.pt 203-204°C found; 208-209°C literature[57]. ^1H nmr (solvent d_6-DMSO) δ ppm: 9.9 (1H, s, NH of benzoyl group aromatic ring); 8.3 (1H, s, NH of peptide bond group); 7.9 (2H, d, ortho protons of benzoyl group aromatic ring); 7.6 (5H, m, overlapping meta and para protons of benzoyl group aromatic ring and ortho protons of phenylalanine aromatic ring); 7.2 (4H,

complex, overlapping meta and para protons of phenylalanine aromatic ring and ß-CH group); 3.7 (2H, d, CH_2 of glycine). CHN (for C18, H16, N2, O4): calculated C 66.67%, H 4.94%, N 8.64%; found C 66.45%, H 5.02%, N 8.44%.

1.2.4.9 Synthesis of N-benzoyl-2, 3-didehydrophenylalanylglycine (second method).

Glycine (3g) was dissolved in acetone (40mls) and 1M sodium hydroxide (40mls) and stirred for twenty minutes. 4-Benzylidene-2-phenyloxazol-5-one (8g) was added to the solution. The mixture was stirred for several hours until a clear solution formed. 1M hydrochloric acid (40mls) was added to the solution and a precipitate formed immediately. The product was collected by vacuum filtration and recrystallized from 60% methanol by the addition of water. The white crystalline product was dried at 60°C. Yield 81% (8.41g). M.pt 208-209°C found; 208-209°C literature [57]. ^1H nmr (solvent d_6-DMSO) δ ppm: 9.8 (1H, s, NH of benzoyl group); 8.3 (1H, s, NH of peptide bond group); 8.0 (2H, d, ortho protons of benzoyl group aromatic group); 7.6 (5H, complex, overlapping meta and para protons of benzoyl group and ortho protons of phenylalanine aromatic ring), 7.2 (4H, complex, overlapping protons of meta and para protons of phenylalanine aromatic ring and ß-CH group); 3.8 (2H, complex d, CH_2 of glycine). CHN (for C18, H16, N2, O4): calculated C 66.67%, H 4.94%, N 8.64%; found C 66.76%, H 5.23%, N 8.88%.

1.2.4.10 Synthesis of N-acetyl-2, 3-didehydrophenylalanyl-L-phenylalanine.

L-Phenylalanine (0.9g), acetone (10mls), 1M sodium hydroxide (5mls) and 4-benzylidene-2-methyloxazol-5-one (0.9g) were stirred together until they formed a clear solution. 1M hydrochloric acid (5mls) was added and a crystalline product formed immediately. The product was recrystallized three times from methanol by the subsequent addition of water. Yield 62% (1.05g). M.pt 213-214°C (decomposition) found; 213-215°C (decomposition) literature [57]. ^{1}H nmr (solvent d_6-DMSO) δ ppm: 9.4 (1H, s, NH of acetyl group); 8.1 (1H, d, NH of peptide bond group); 7.6 (2H, ortho protons of didehydrophenylalanine aromatic ring); 7.3 (8H, complex, meta and para protons of didehydrophenylalanine aromatic ring and phenylalanine aromatic ring); 7.0 (1H, s, ß-CH group); 4.5 (1H, m, α-CH group of phenylalanine); 3.0 (2H, complex, ß-CH_2 of phenylalanine); 1.9 (3H, s, CH_3 of acetyl group). CHN (for C20, H20, N2, O4): calculated C 68.18%, H 5.68%, N 7.95%; found C 67.96%, H 5.34%, N 7.76%.

1.2.4.11 Attempted synthesis of N-benzoyl-2, 3-didehydrophenylalanyl-L-proline.

L-Proline (4g) was dissolved in acetone (25ml) and 1M sodium hydroxide (27ml). 4-Benzylidene-2-phenyloxazol-5-one (8.6g) was added to the solution and the mixture was stirred until a clear solution formed. After stirring the solution

for two hours. 1M sodium hydroxide (26mls) was added and the resulting precipitate was collected. Recrystallization of the product from carbon tetrachloride gave a white powder. Analysis of the product showed it to be a mixture of compounds, of which N-benzoyl-2, 3-didehydrophenylalanine was the major component.

1.2.4.12 Attempted synthesis of N-acetyl-2, 3-didehydrophenylalanyl-L-lysine.

L-Lysine (5g) was dissolved in 1M sodium hydroxide (18mls) and acetone (8mls) was added. The solution was stirred for ten minutes and then 4-benzylidene-2-methyl oxazol-5-one (6g) was added slowly. The mixture was stirred overnight and the residual azlactone removed by filtration. 1M Hydrochloric acid (25mls) was added and the resulting precipitate collected. The product was recrystallized from methanol. Analysis of the product by ^1H nmr showed it contained mainly N-acetyl-2, 3-didehydrophenylalanine and that coupling of the azlactone and amino acid had not occured.

1.2.5 Miscellaneous synthesis.

1.2.5.1 Preparation of N-acetylglycine.

Glycine (50g, 0.67mol) was dissolved in distilled water (100ml) and acetic anhydride (130ml, 140g, 1.4mol) was added in three equal portions. The solution was cooled and the

resulting crystalline product collected by vacuum filtration. The product was recrystallised from boiling water. Yield of acetylglycine was 96% (75g). M.pt 206-207°C found, 207-208°C literature [7e].

1.2.5.2 Preparation of N-benzoylglycine.

Glycine (50g, 0.67mol) was dissolved in 1M sodium hydroxide solution (550mls) and benzoylchloride (90mls, 108g, 0.77mol) was added in five portions with shaking. After cooling, the solution was acidified with concentrated hydrochloric acid. The resulting precipitate was collected under vacuum. The product was boiled in carbon tetrachloride. Recrystallisation from boiling water (600mls) gave a white crystalline product. Yield was 88g (75%). M.pt 186-7°C found, 187°C literature [7e].

1.2.5.3 Preparation of glycine methyl ester.

Glycine methyl ester was prepared by dissolving glycine methyl ester hydrochloride in 0.1M NaOH. The free ester was extracted with diethyl ether (3 x 20ml). The solvent was removed by rotary evaporation. The product was used directly to prevent the formation of diketopiperazone.

1.2.5.4 Preparation of alanine methyl ester.

Alanine methyl ester was prepared from alanine methyl ester hydrochloride by using the same procedure as outlined above for obtaining glycine methyl ester.

1.2.5.5 Preparation of glycine ethyl ester.

Glycine ethyl ester was obtained from glycine ethyl ester hydrochloride by using the procedure outlined for glycine methyl ester.

1.2.5.6 Preparation of leucine ethyl ester.

Leucine ethyl ester was prepared from leucine ethyl ester hydrochloride by using the same method as outlined for glycine

1.2.5.7 Preparation of [4, 5-^{3}H]-leucine.

N-Acetyl-[4,5-^{3}H]-leucine (specific activity = 943mCi/mmol) was refluxed with 3M hydrochloric acid for six hours. The product was lyophilized by freeze drying and washed with ether (3 x 5ml). The product was dissolved in water (1ml) and lyophilized by freeze drying. The sample was weighed and its radioactivity determined. The product of [4, 5-^{3}H]-leucine was found to have a specific activity of 897mCi/mmol. Analysis of the product was by ^{1}H and ^{3}H nmr spectroscopy. ^{1}H nmr (solvent D_2O) δ ppm: 4.1 (1H, m,

α-CH group); 1.7 (1H, m, τ-CH); 1.5 (2H, m, β-CH_2); 0.9 (6H, dd, δ-CH_3 groups). ^{1}H nmr (solvent D_2O) δ ppm: 1.5 (τ-CH group, 15.1% T); 0.9 (δ-CH_3 groups, 84.9% T)

1.2.5.8 Preparation of N-acetyl-2, 3-didehydroalanine.

N-acetyl-2,3-didehydroalanine methyl ester (0.5g) was stirred with hot 1M sodium hydroxide (10mls) for half an hour. The solution was acidified with 1M hydrochloric acid. The solvent was evaporated off and the residue taken up in ethyl acetate (20mls). The insoluble matter was filtered off and the solvent removed by rotary evaporation. ^{1}H nmr (solvent d_6-DMSO) δ ppm: 9.3 (1H, s, NH group); 6.1 (1H, s, β-CH_2 group); 5.6 (1H, s, β-CH_2 group); 1.9 (3H, s, CH_3 of acetyl group).

1.2.6 Hydrogenation of didehydroamino acids.

1.2.6.1 General hydrogenation method.

N-acyldidehydroamino acid or peptide (6-12mg) and 5% Pd/C catalyst (3-6mg) were suspended in tetrahydrofuran (1ml). The reaction flask was attached to the hydrogenation line and its contents frozen in liquid nitrogen. The hydrogenation line was evacuated and helium swept through it (3 times) to remove any air. Hydrogen (10ml) was transferred to the mercury burette, which had been pre-evacuated. The hydrogen gas was then transferred to the reaction flask. The reaction mixture was thawed out and

stirred for two hours. The catalyst was removed by filtration. The product was lyophilized by freeze drying. The product was analysed by ^1H nmr. The ^1H nmr spectroscopy data of the hydrogenation products of N-acyldidehydroamino acids is given below and the corresponding information for the hydrogenation of didehydropeptides is found in section 1.2.7.

1.2.6.2 Hydrogenation of N-acetyl-2, 3-didehydroalanine methyl ester.

^1H nmr (solvent d_6-DMSO) δ ppm: 8.2 (1H, d, NH group); 4.2 (1H, m, α-CH group); 3.7 (3H, s, CH_3 of methyl ester group); 1.8 (3H, s, CH_3 of acetyl group); 1.2 (3H, d, β-CH_3 group).

1.2.6.3 Hydrogenation of N-benzoyl-2, 3-didehydrovaline.

^1H nmr (solvent d_6-DMSO) δ ppm: 8.4 (1H, d, NH group); 7.9 (2H, d, ortho protons of benzoyl group aromatic ring); 7.5 (3H, m, meta and para protons of benzoyl group aromatic ring); 4.3 (1H, m, α-CH group); 2.2 (1H, m, β-CH group); 1.0 (6H, dd, γ-CH_3 groups).

1.2.6.4 Hydrogenation of N-acetyl-2, 3-didehydrophenylalanine.

^{1}H nmr (solvent d_6-DMSO) δ ppm: 8.1 (1H, d, NH group); 7.2 (5H, m, Phenylalanine aromatic ring); 4.4 (1H, m, α-CH group); 3.0 (1H, m, ß-CH_2 group); 2.8 (1H, m, ß-CH_2 group); 1.8 (3H, s, CH_3 of acetyl group).

1.2.6.5 Hydrogenation of N-acetyl-4, 5-didehydroleucine.

^{1}H nmr (solvent d_6-DMSO) δ ppm: 8.1 (1H, d, NH group); 4.2 (1H, m, α-CH group); 1.8 (3H, s, CH_3 of acetyl group); 1.6 (1H, m, τ-CH group); 1.4 (2H, m, ß-CH_2 group); 0.9 (6H, dd, δ-CH_3 groups).

1.2.6.6 Hydrogenation of N-benzoyl-2, 3-didehydrophenylalanine.

^{1}H nmr (solvent d_6-DMSO) δ ppm: 8.6 (1H, d, NH group); 7.8 (2H, d, ortho protons of phenylalanine aromatic ring); 7.5 (3H, m, meta and para protons of phenylalanine aromatic ring); 7.3 (5H, m, benzoyl group aromatic ring); 4.6 (1H, m, α-CH group); 3.1 (2H, m, ß-CH_2 group).

1.2.6.7 Hydrogenation of N-acetyl-2, 3-didehydroalanine.

^{1}H (solvent D_2O) δ ppm: 8.1 (1H, d, NH group); 4.3 (1H, m, α-CH group); 1.9 (3H, m, CH_3 of acetyl group); 1.2 (3H, d, ß-CH_3 groups).

1.2.7 Hydrogenation of didehydropeptides.

1.2.7.1 Hydrogenation of N-acetyl-2, 3-didehydrophenylalanyl-L-alanine.

^{1}H nmr (solvent d_6-DMSO) δppm: 8.2 (2H, cd, NH of acetyl and peptide bond groups); 7.3 (5H, complex, phenylalanine aromatic ring); 4.6 (1H, m, α-CH group of phenylalanine); 4.2 (1H, m, α-CH group of alanine); 3.0 (1H, m, ß-CH_2 group of phenylalanine); 2.8 (1H, m, ß-CH_2 group of phenylalanine); 1.8 (3H, s, CH_3 of acetyl group); 1.2 (3H, d, ß-CH_3 group of alanine).

1.2.7.2 Hydrogenation of N-benzoyl-2, 3-didehydrophenylalanylglycine.

^{1}H (solvent d_6-DMSO) δ ppm: 8.6 (1H, d, NH of benzoyl group); 8.4 (1H, d, NH of peptide bond group); 7.8 (2H, d, ortho protons of benzoyl group aromatic ring); 7.5 (5H, complex, overlapping meta and para protons of benzoyl group and ortho protons of phenylalanine aromatic rings); 7.2 (3H, meta and para protons of phenylalanine aromatic ring); 4.7 (1H, m, α-CH group); 3.8 (2H, m, CH_2 of glycine); 3.2 (1H, m, ß-CH group); 3.0 (1H, m, ß-CH group).

1.2.7.3 Hydrogenation of N-benzoyl-2, 3-didehydrophenylalanylglycine ethyl ester.

^{1}H (solvent d_6-DMSO) δ ppm: 8.6 (1H, d, NH of benzoyl group); 8.4 (1H, d, NH of peptide bond group); 7.8 (2H, d, ortho protons of benzoyl group aromatic ring); 7.5 (5H, complex, overlapping meta and para protons of benzoyl group and ortho protons of phenylalanine aromatic rings); 7.2 (3H, meta and para protons of phenylalanine aromatic ring); 4.7 (1H, m, α-CH group); 3.8 (2H, m, CH_2 of glycine); 3.2 (1H, m, ß-CH group); 3.0 (1H, m, ß-CH group); 2.0 (2H, m, CH_2 of ethyl ester group); 1.3 (3H, t, CH_3 of ethyl ester group).

1.2.7.4 Hydrogenation of N-acetyl-2, 3-didehydrophenylalanyl-L-phenylalanine.

^{1}H nmr (solvent d_6-DMSO) 8.3 (1H, d, NH of acetyl group); 8.1 (1H, d, NH of peptide bond group); 7.7 (2H, d, ortho protons of N-acetylphenylalanine aromatic ring); 7.5 (3H, m, meta and para protons of N-acetylphenylalanine aromatic ring); 7.2 (5H, m, phenylalanine aromatic ring); 4.6 (1H, m, α-CH of N-acetylphenylalanine group); 4.3 (1H, m, α-CH of phenylalanine); 3.0 (4H, cm, overlapping ß-CH_2 of N-acetylphenylalanine and phenylalanine); 2.0 (3H, s, CH_3 of acetyl group).

1.2.8 Tritiation of didehydroamino acids.

1.2.8.1 General tritiation method.

N-Acyldidehydroamino acid or peptide (6-12mg) and 5% Pd/C catalyst (6-12mg) were suspended in redistilled tetrahydrofuran (1ml) and reacted with a hydrogen-tritium mixture (20ml H_2, 1Ci T_2) and stirred for two hours. Then the catalyst was removed by filtration. The solvent was removed by evaporation. Labile tritium was removed from the product by repeatedly dissolving the product in methanol (1ml) and evaporating off the solvent until the radioactivity of the product was a constant value. The product was analysed by ^1H and ^3H nmr spectroscopy. The specific activities and ^3H nmr spectroscopy data for the tritiated amino acids is given below while the corresponding data for tritiated peptides is found in section 1.2.9.

1.2.8.2 N-Acetyl-[2, 3-^{3}H]-alanine methyl ester.

specific activity = 635mCi/mmol.

^3H nmr (solvent d_6-DMSO) δ ppm: 4.2 (α-CHT, 4.6% T); 1.2 (β-CH_2T, 95.4% T).

1.2.8.3 N-Acetyl-[4, 5-^{3}H]-leucine.

specific activity = 943mCi/mmol.

^3H nmr (solvent d_6-DMSO) δ ppm: 1.6 (γ-CT, 13% T); 0.9 (δ-CH_2T, 87% T).

1.2.8.4 N-Acetyl-[2, 3-^{3}H]-phenylalanine.

specific activity = 827mCi/mmol.

^{3}H nmr (solvent d_6-DMSO) δ ppm: 4.4 (α-CT, 67.2% T); 3.0 (ß-CHT, 32.8% T).

1.2.8.5 N-Benzoyl-[2, 3-^{3}H]-valine.

specific activity = 612mCi/mmol.

^{3}H nmr (solvent d_6-DMSO) δ ppm: 4.3 (α-CT, 50% T); 2.2 (ß-CT, 50% T).

1.2.8.6 N-Benzoyl-[2, 3-^{3}H]-phenylalanine.

specific activity = 733mCi/mmol.

^{3}H nmr (solvent d_6-DMSO) δ ppm: 4.6 (α-CT, 58.6% T); 3.1 (ß-CHT, 41.4% T).

1.2.9 Tritiation of didehydropeptides.

1.2.9.1 N-Acetyl-[2, 3-^{3}H]-phenylalanyl-L-alanine.

specific activity = 2.7 Ci/mmol.

^{3}H nmr (solvent d_6-DMSO) δ ppm: 4.5 (α-CH phenylalanine, 60.4% T); 3.0 (ß-CH_2 phenylalanine, 16.6% T); 2.7 (ß-CH^2 phenylalanine, 14.7% T); 1.2 (ß-CH_3 alanine, 8.3% T).

1.2.9.2 N-Acetyl-[2, 3-^{3}H]-phenylalanyl-L-phenylalanine.

specific activity = 2.1 Ci/mmol.

^{3}H nmr (solvent d_6-DMSO) δ ppm: 4.5 (α-CH first isomer, 34.3% T); 4.4 (α-CH second isomer, 30.9% T); 3.0 (ß-CH_2 second isomer, 15.2% T); 2.9 (ß-CH_2 first isomer, 19.5% T).

^{3}H nmr (considering first isomer only) δ ppm: 4.5 (α-CH, 61.1%T); 2.9 (ß-CH_2, 39.9%T).

^{3}H nmr (considering second isomer only) δ ppm: 4.4 (α-CH, 62.8% T); 3.0 (ß-CH_2, 37.2% T).

Diastereoisomer ratio first: second = 52.6: 47.3

1.2.9.3 N-Benzoyl-[2, 3-^{3}H]-phenylalanylglycine.

specific activity = 1.8 Ci/mmol.

^{3}H nmr (solvent d_6-DMSO) δ ppm: 4.7 (α-CH phenylalanine, 50.6% T); 3.2 (49.4%T).

1.2.10 Optimisation of the labelling of N-acetyl-[4, 5-^{3}H]-leucine.

1.2.10.1 Variation of catalyst: substrate ratio.

N-Acetyl-4, 5-didehydroleucine (7.5mg) and a known quantity of 5% Pd/C catalyst (0.2 to 5 x weight of the substrate) were suspended in glacial acetic acid (1ml). The

mixture was reacted with hydrogen (30ml) and tritium (1Ci) for two hours. The product was worked up and analysed as described under the general tritiation procedure (see part 1.2.8.1). The catalyst: substrate (w/w) ratio was varied in the following proportions 1:5, 1:4, 1:3, 1:2, 1:1, 2:1, 3:1, 4:1, 5:1. Investigations were carried out for both 5% Pd/C type B and C catalysts (Johnson Matthey). Analysis of the products was by ^{1}H and ^{3}H nmr. Results for the catalyst: substrate ratio of 5:1, which resulted in a partially hydrogenated product are given below.

5:1 (w/w) catalyst: substrate ratio.

^{1}H nmr, result for both 5% Pd/C types B and C (solvent d_6-DMSO) δ ppm: 8.0 (broad s, NH group); 4.8 (d, δ-CH_2 group of N-acetyl-4, 5-didehydroleucine); 4.3 (m, α-CH group); 2.4 (m, β-CH_2 group of N-acetyl-4, 5-didehydroleucine); 2.3 (m, τ-CH group of N-acetyl-4, 5-didehydroleucine); 1.8 (s, CH_3 of acetyl group); 1.6 (δ-CH_3 group of N-acetyl-4, 5-didehydroleucine); 1.5 (m, τ-CH group); 0.9 (dd, δ-CH_3 group)

^{3}H nmr, result for 5% Pd/C type B (solvent d_6-DMSO) δ ppm: 4.8 (δ-CH_2 group, 65.0% T); 1.6 (δ-CH_3 group, 10.7%T); 1.5 (τ-CH, 2.3% T); 0.9 (δ-CH_3 groups, 21.8% T).

^{3}H nmr, result for 5% Pd/C type C (solvent d_6-DMSO) δ ppm: 4.8 (δ-CH_2 group, 68.4% T): 1.6 (δ-CH_3 group, 8.4% T); 1.5 (τ-CH, 2.5% T); 0.9 (δ-CH_3 groups, 20.7% T).

1.2.10.2 Variation of solvent.

N-Acetyl-4, 5-didehydroleucine (7.5mg) and 5% Pd/C type B or C (7.5mg) were suspended in solvent (1ml) and reacted with a mixture of hydrogen (10mls) and tritium (1Ci) for two hours. The samples were worked up as described above under the general tritiation procedure. The solvents studied were tetrahydrofuran, ethyl acetate and glacial acetic acid. Analysis was by ^{1}H and ^{3}H nmr spectroscopy.

1.2.11 Radiation decomposition studies of tritiated amino acids.

1.2.11.1 General method of sample preparation.

^{3}H Nmr samples were prepared for the tritiated N-acylamino acids in the usual manner with the compounds dissolved in acetate, phosphate and borate buffer solutions (100µl) respectively. The ^{3}H nmr samples were stored at room temperature and analysed by nmr spectroscopy at known time intervals. Subsequent analyses of the samples by radio tlc analyses and by liquid scintillation counting was carried out at a known time interval after the decomposition studies were concluded. These latter analyses were carried ou after the ^{3}H nmr studies were completed. The methods of analysis are described in detail below. The following compounds were studied:

(a) N-acetyl-[4, 5-^{3}H]-leucine (specific activity 3.78 Ci/mmol) with a ^{3}H nmr sample concentration of 150 mCi/ml.
(b) N-acetyl-[2, 3-^{3}H]-phenylalanine (specific activity 18.2 Ci/mmol) with a ^{3}H nmr sample concentration of 400 mCi/ml.
(c) N-benzoyl-[2, 3-^{3}H]-phenylalanine (specific activity 12.0 Ci/mmol with a ^{3}H nmr sample concentration of 400 mCi/ml.

1.2.11.2 ^{3}H nmr studies.

^{3}H nmr spectra were obtained at known time intervals for the labelled samples in buffer solutions. In all cases deuterated water was used as an external lock solvent. Buffer solutions were referenced to the same tritiated compound in d_6-DMSO. In all cases ^{3}H nmr spectra were run for 1000 scans (acquisition time of 1.6s per scan) with proton decoupling. Some proton coupled ^{3}H nmr spectra were also run. The formation of new chemical shifts in the ^{3}H nmr spectra were measured as a function of time.

1.2.11.3 ^{1}H nmr studies

Since the decomposition of tritiated compounds in buffer solutions was being investigated, the presence of a large water signal meant that direct ^{1}H nmr spectra gave little information about the decomposition products being formed. Some presaturation experiments were carried out but these did not reveal much information due to the close proximity of the chemical shift of the water signal and some

of the chemical shifts of the amino acids. However, some detail was obtained by simple expansion of the ^1H nmr spectra.

1.2.11.4 Analysis of decomposition products by radio tlc.

The tritium nmr sample of the decomposition product (80µl) was added to methanol (5ml). Portions (10µl) of the solution were spotted onto tlc plates. Reference samples of N-acylated and amino acids and free amino acids were spotted over the tritiated samples and, after drying the spots in air, the plates were run in a variety of solvent systems. Radio tlc were run for all buffer solutions of tritiated compounds. The solvent systems used were 5: 2: 3 butan-1-ol: acetic acid: water, 1:1 ethyl acetate: diethyl ether, 9:1 chloroform: methanol, 12:4:5 butan-1-ol: acetic acid: water, 1:1:1 pyridine: butan-1-ol: water and 8:1 hexane: ethyl acetate.

A variety of compounds have been run in the solvent systems to elucidate potential decomposition products by comparison of R_f values to those obtained by radio tlc. Some classes of compounds considered were N-acylamino acids, amino acids, pyruvic acids and carboxylic acids.

1.2.11.5 Analysis of labile tritium.

An aliquot (0.5mls) of the tritiated sample (80µl buffer solution dissolved in 5mls of methanol) was freeze dried. 10µl of the distillate was counted in Unisolve liquid scintillation solvent. This was assumed to represent the total tritium present in a labile form plus any volatile material formed on storage. ^{3}H nmr spectra were obtained for the residue and showed a disappearance of the chemical shift at δ4.8 ppm. These observations were in agreement with the analysis of labile and volatile tritium by liquid scintillation counting.

1.3 Results.

1.3.1 General.

The results for the work described in this chapter are shown below. ^{1}H and ^{3}H nmr spectra of tritiated amino acids and peptides are illustrated in sections 1.3.3 and 1.3.4.

^{3}H Nmr spectra for the radiation decomposition of tritiated N-acylamino acids have been illustrated here so as to show the formation of new chemical shifts with time (see section 1.3.6). Several possible radiation decomposition products have been identified by radio-tlc. These results are shown in sections 1.3.7 and 1.3.8. A comprehensive list of all the reference compounds used for radio-tlc analysis has not been given, although those compounds which had Rf values that were similar to those of radiation decomposition products are shown in Table 1.28

Several conclusions can be drawn from the synthesis of N-acyl-2,3-didehydroamino acids and peptides. The synthesis of N-acetyl-2,3-didehydroamino acid esters via azido intermediates was generally unsuccessful. The α-bromo- and α-azidocarboxylic acid precursors were prepared in reasonable yields. N-acetyl-2,3-didehydroalanine methyl ester was successfully prepared from 2-azidopropanoic acid methyl ester using the method of Effenberger[82]. The low yields obtained were probably due to the vigorous reaction conditions, which may have caused some polymerisation of the

product. Attempts to prepare N-acetyl-2,3-didehydrovaline methyl ester and N-acetyl-2,3-didehydroleucine methyl ester from their corresponding 2-azidocarboxylic acids were unsuccessful. Black tarry residues were formed instead of the desired products.

N-acyl-2,3-didehydroamino acids were prepared in good yields from azlactone intermediates. The preparation of N-acyl-2,3-didehydrophenylalanyl dipeptides containing L-alanine, L-phenylalanine and glycine residues were successfully carried out. Attempts to prepare N-acyl-2,3-didehydrophenylalanine dipeptides containing L-tyrosine, L-proline, L-glutamic acid and L-lysine residues were unsuccessful. The major product, in these latter cases, was N-acetyl-2,3-didehydrophenylalaninine and no peptides were observed to have been formed. In the case of reactions involving L-glutamic acid and L-lysine, two other products were found to be present but could not be properly identified. Attempts to synthesis N-benzoyl-2,3-didehydrovaline dipeptides were also unsuccessful and the major product was N-benzoyl-2,3-didehydrovaline.

1.3.2 Optimisation of the catalytic tritiation of N-acetyl-4, 5-didehydroleucine

Table 1.3: Variation of the catalyst: substrate ratio

Catalyst: Substrate Ratio (w/w)	Specific Activity for 5% Palladium on Charcoal	
	type B (mCi/mmol)	type C (mCi/mmol)
5:1	23	14
4:1	118	97
3:1	421	448
2:1	854	908
1:1	1246	1171
1:2	1156	1080
1:3	980	1000
1:4	780	831
1:5	580	497

Table 1.4: Variation of the solvent

Solvent	Radioactivity (mCi)	
	sample A	sample B
Glacial acetic acid	29	35
Ethyl acetate	43	54
Tetrahydrofuran	66	59

1.3.3 Nmr. spectra of tritiated amino acids.

The following pages contain ^{1}H nmr spectra of N-acyldidehydroamino acids and the ^{1}H and ^{3}H nmr spectra of the corresponding tritiated amino acids.

Figure 1.12 ^{1}H Nmr spectrum of N-acetyl-2, 3-didehydroalanine methyl ester.

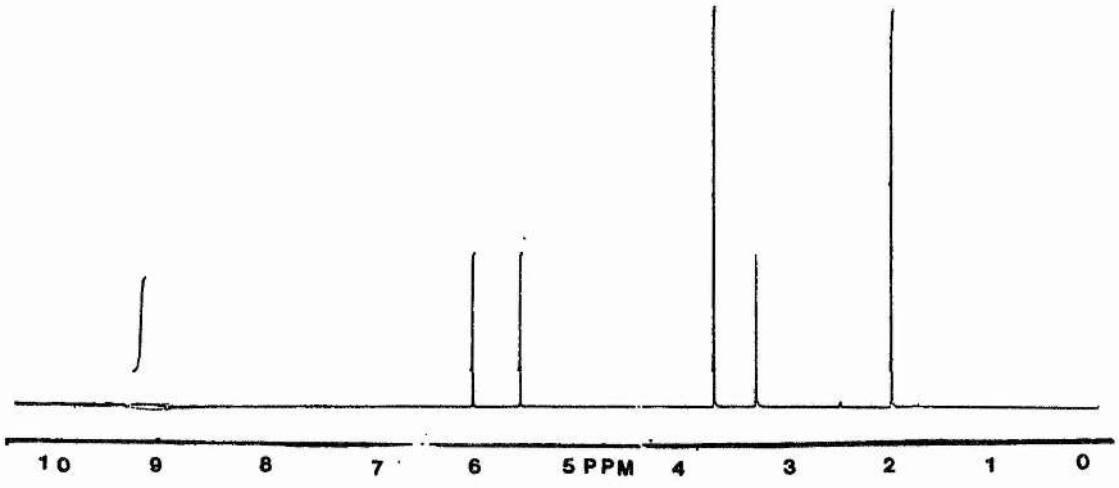


Figure 1.13 ^{1}H Nmr spectrum of N-acetyl-[2, 3-^{3}H]-alanine methyl ester.

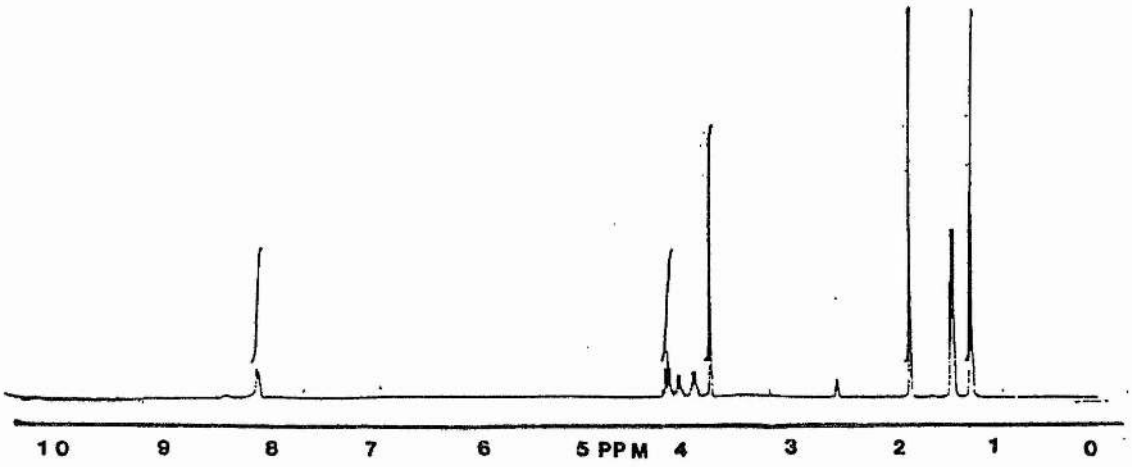


Figure 1.14 ^{3}H Nmr spectrum of N-acetyl-[2, 3-^{3}H]-alanine methyl ester.

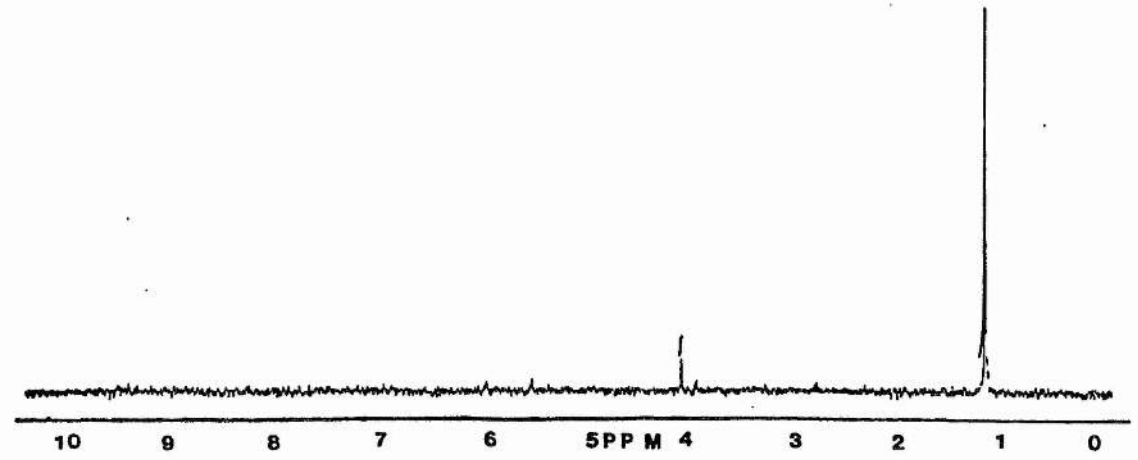


Figure 1.15 ^{1}H Nmr spectrum of N-benzoyl-2, 3-didehydrovaline.

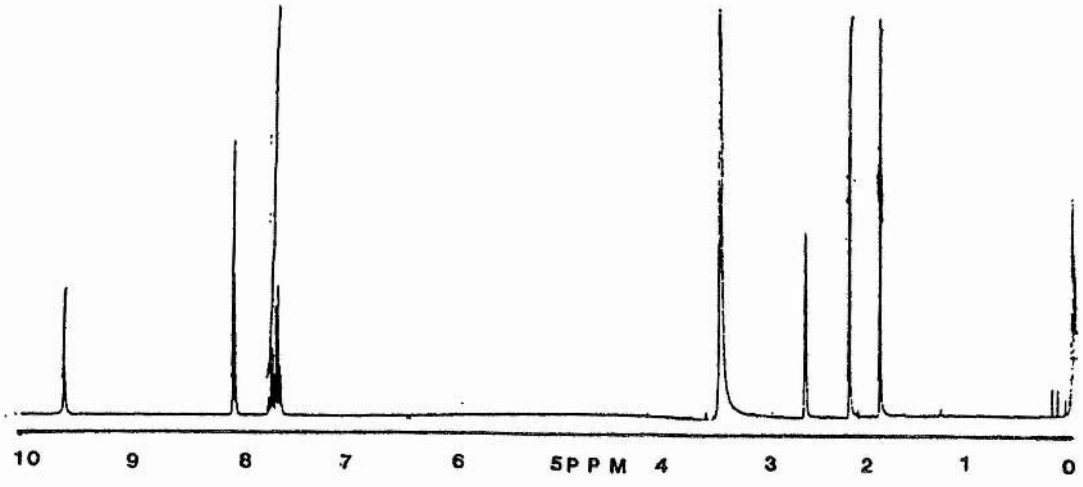


Figure 1.16 ^{1}H Nmr spectrum of N-benzoyl-[2, 3-^{3}H]-valine

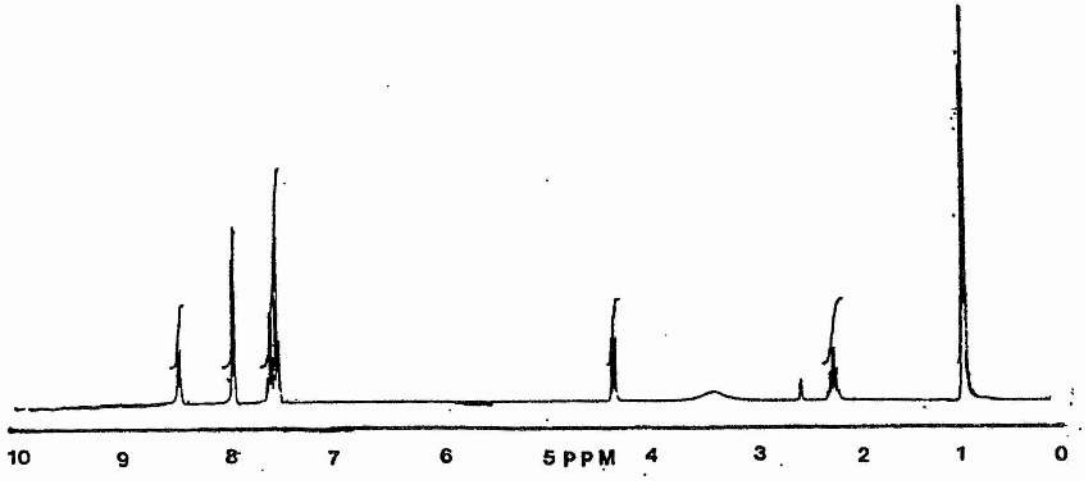


Figure 1.17 ^{3}H Nmr spectrum of N-benzoyl-[2, 3-^{3}H]-valine

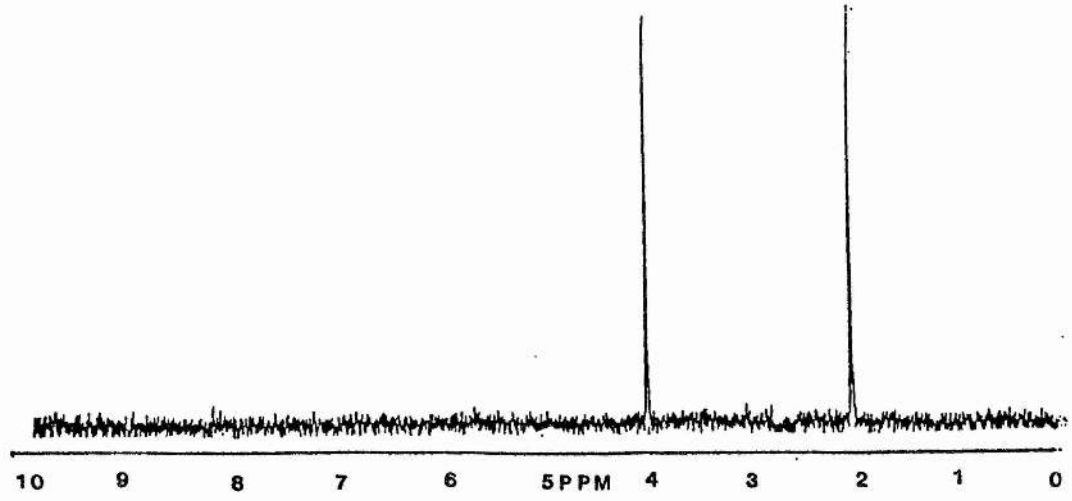


Figure 1.18 ^{1}H Nmr spectrum of N-acetyl-4, 5-didehydroleucine.

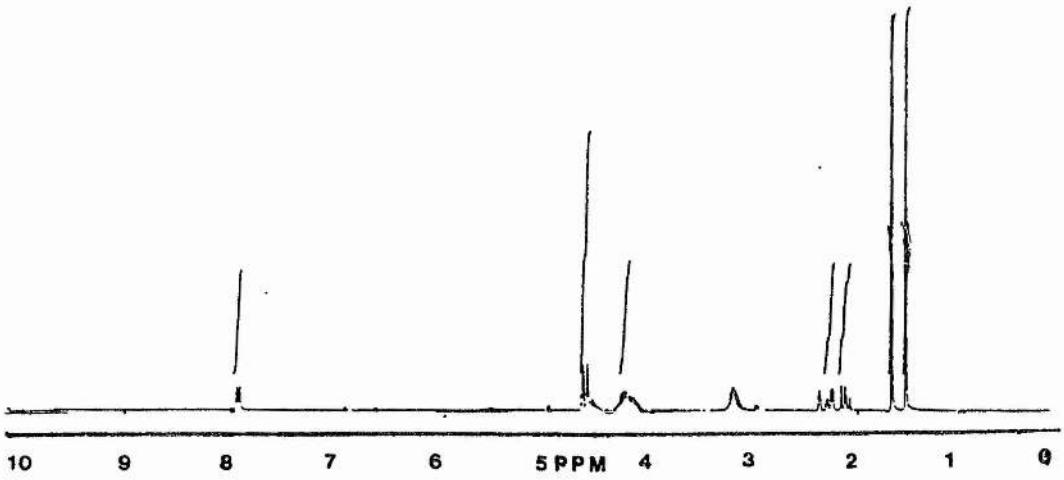


Figure 1.19 ^{1}H Nmr spectrum of N-acetyl-[4, 5-^{3}H]-leucine.

Figure 1.20 ^{3}H Nmr spectrum of N-acetyl-[4, 5-^{3}H]-leucine.

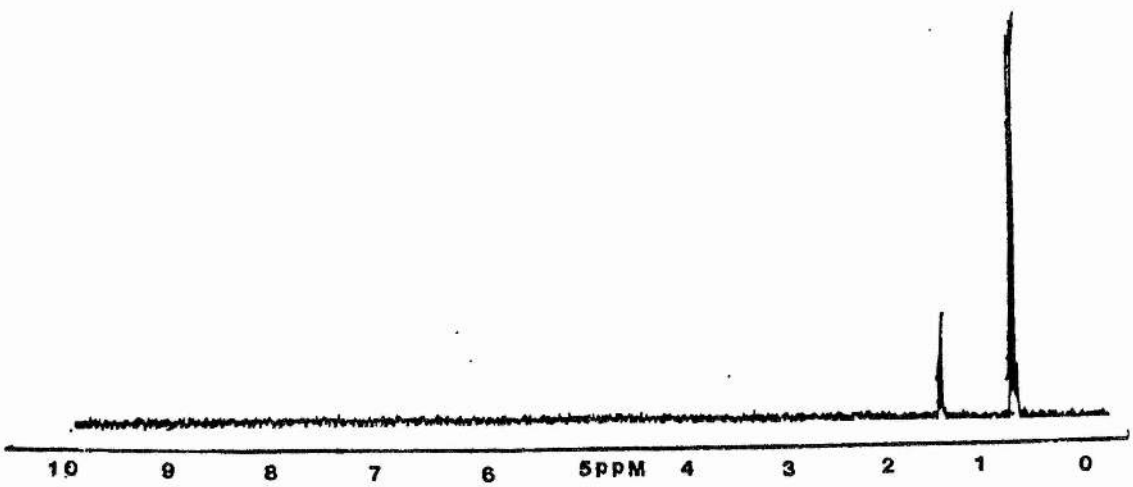


Figure 1.21 ^{1}H Nmr spectrum of N-acetyl-2, 3-didehydrophenylalanine.

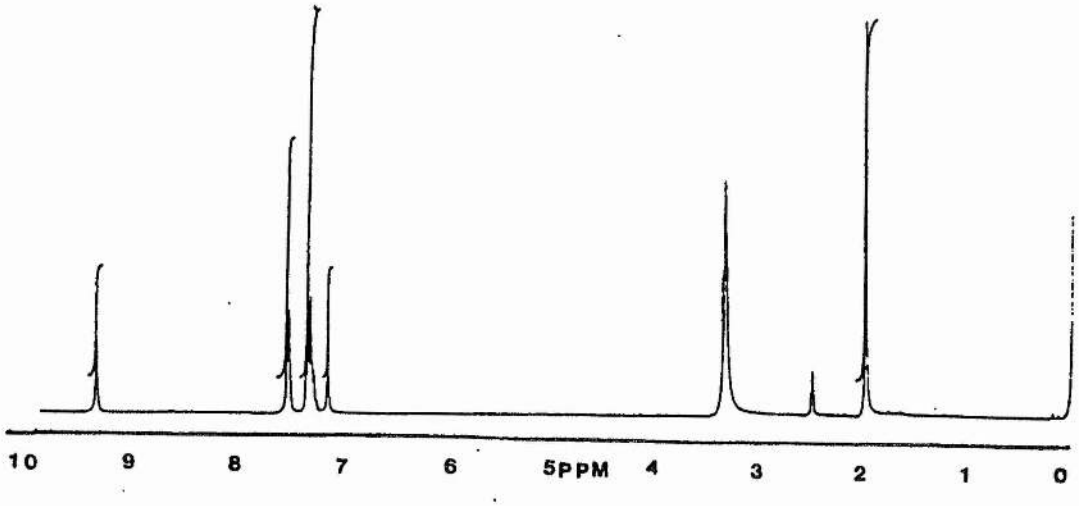


Figure 1.22 ^{1}H Nmr spectrum of N-acetyl-[2, 3-^{3}H]-phenylalanine.

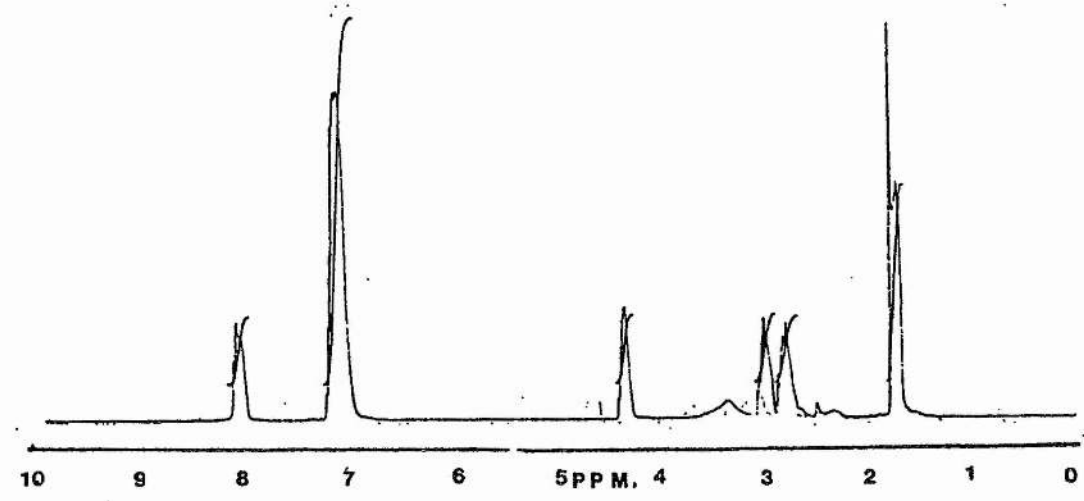


Figure 1.23 ^{3}H Nmr spectrum of N-acetyl-[2, 3-^{3}H]-phenylalanine.

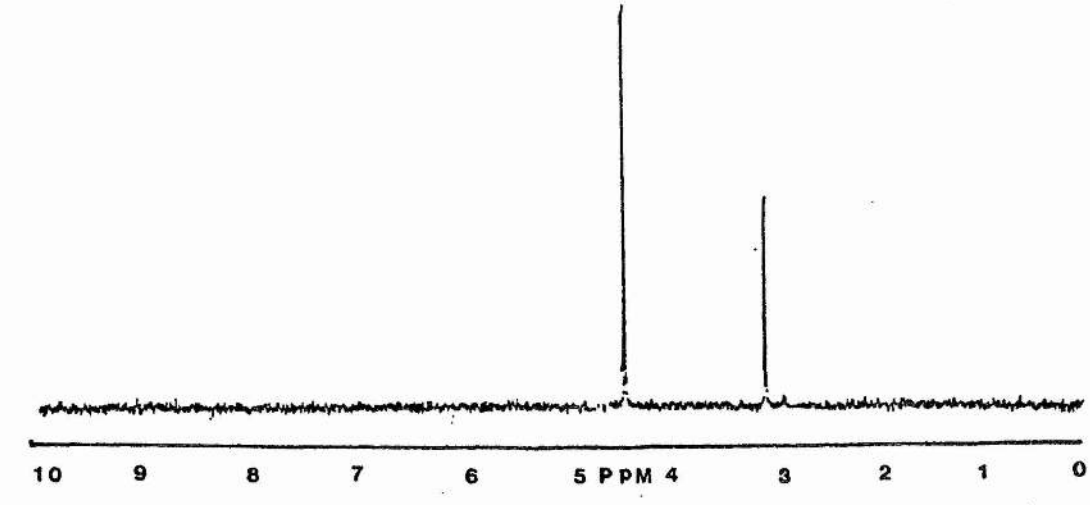


Figure 1.24 ^{1}H Nmr spectrum of N-benzoyl-2, 3-didehydrophenylalanine.

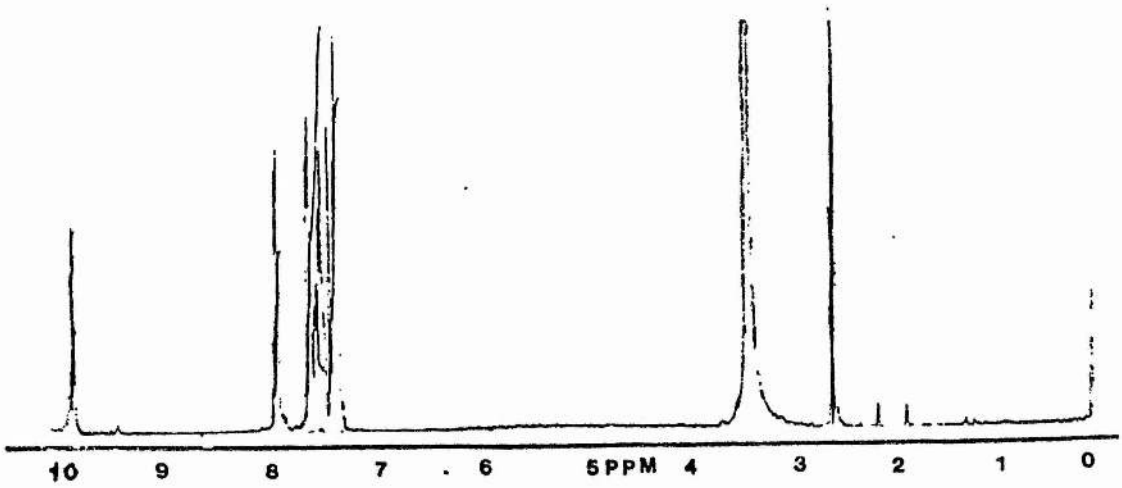


Figure 1.25 ^{1}H Nmr spectrum of N-benzoyl-[2, 3-^{3}H]-phenylalanine.

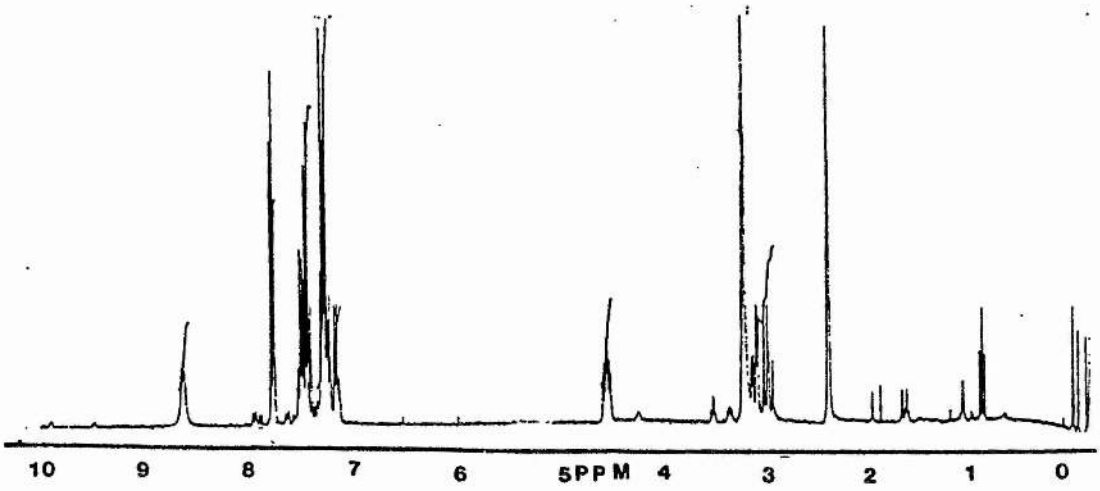
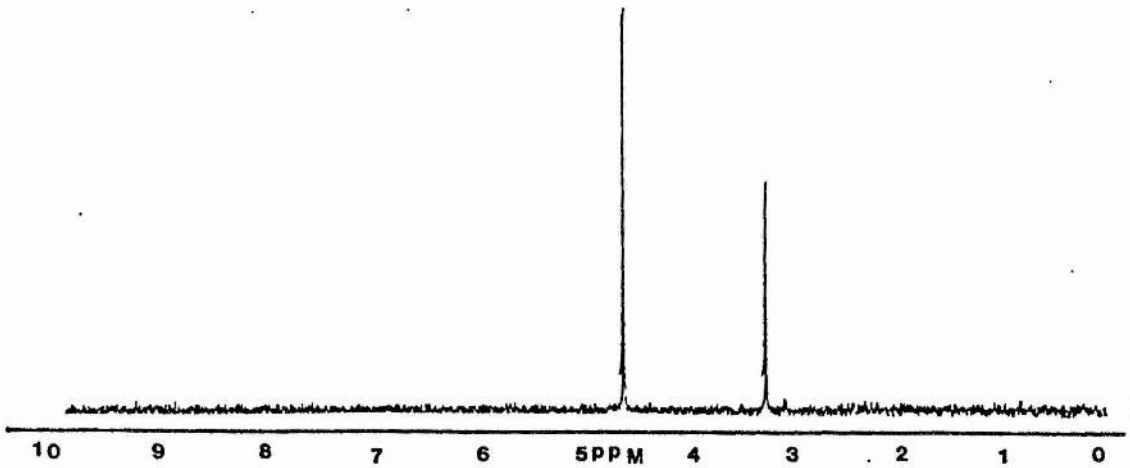


Figure 1.26 ^{3}H Nmr spectrum of N-benzoyl-[2, 3-^{3}H]-phenylalanine.



1.3.4 Nmr spectra of tritiated peptides.

^{1}H and ^{3}H nmr spectra associated with the tritiation of N-acyl-2, 3-didehydrophenylalanine peptides are shown on the following pages.

Figure 1.27 ^{1}H Nmr spectrum of N-benzoyl-2, 3-didehydrophenylalanylglycine.

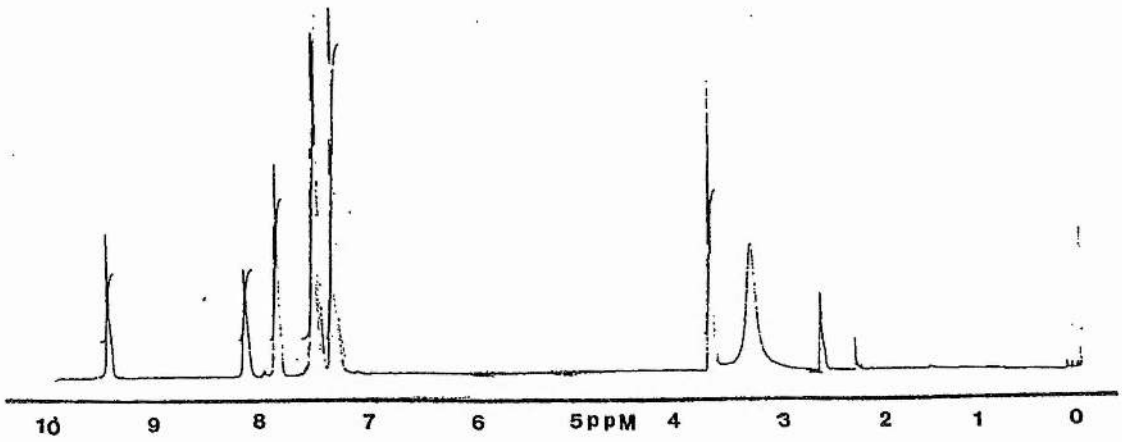


Figure 1.28 ^{1}H Nmr spectrum of N-benzoyl-[2, 3-^{3}H]-phenylalanylglycine.

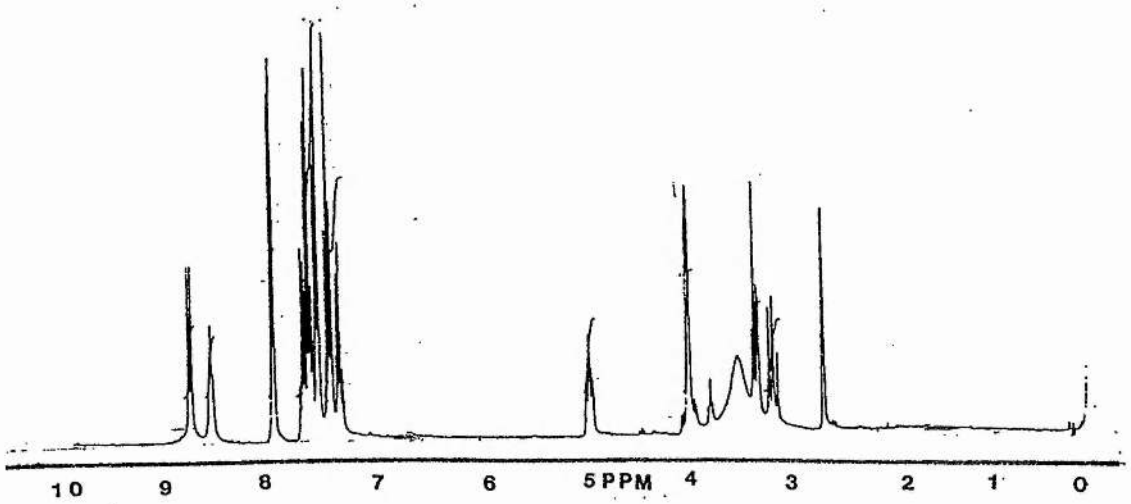


Figure 1.29 ^{3}H Nmr spectrum of N-benzoyl-[2, 3-^{3}H]-phenylalanylglycine.

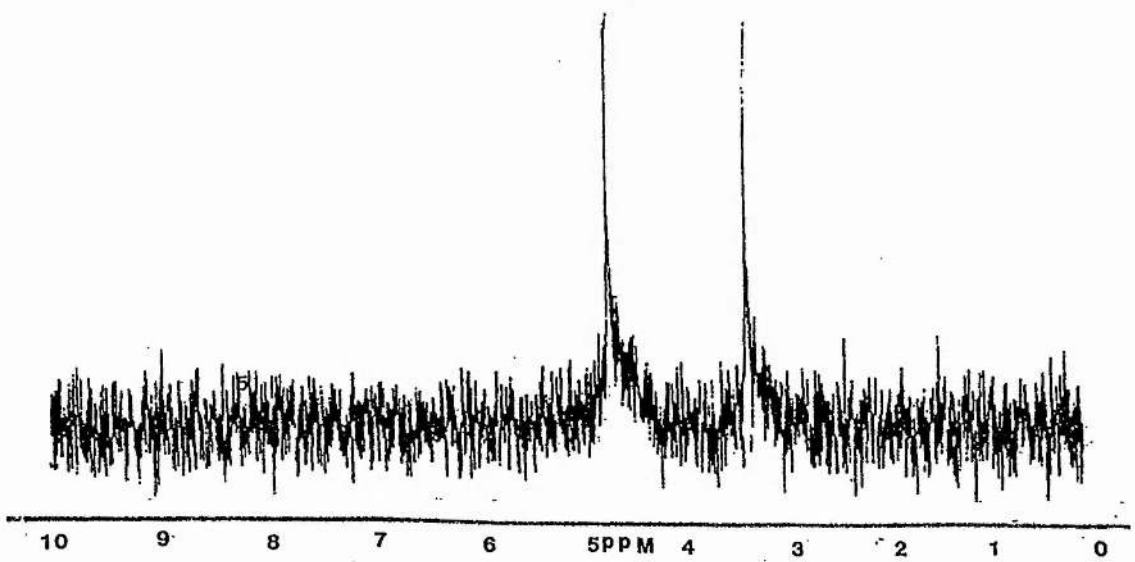


Figure 1.30 ^{1}H Nmr spectrum of N-acetyl-2, 3-didehydrophenylalanylalanine.

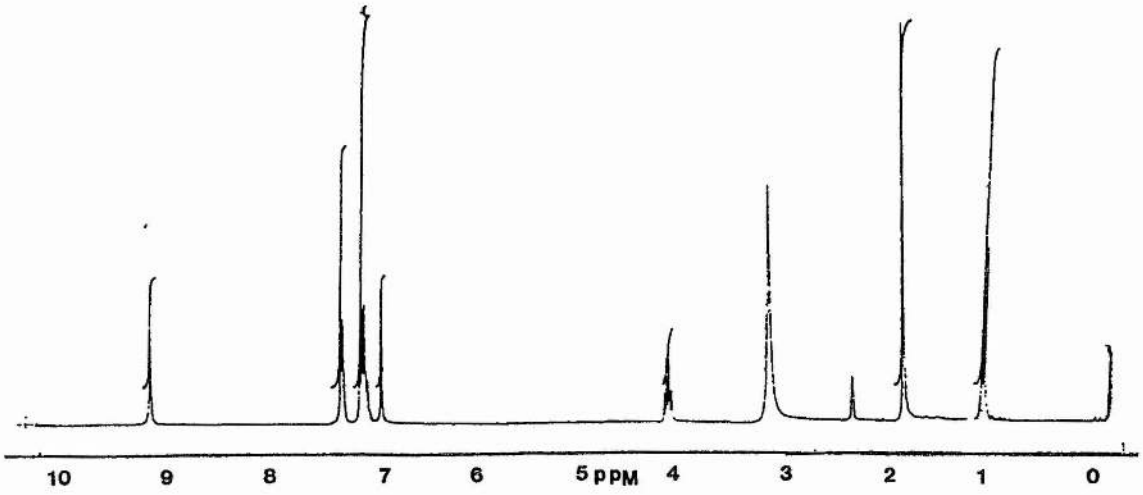


Figure 1.31 ^{1}H Nmr spectrum of N-acetyl-[2, 3-^{3}H]-phenylalanylalanine.

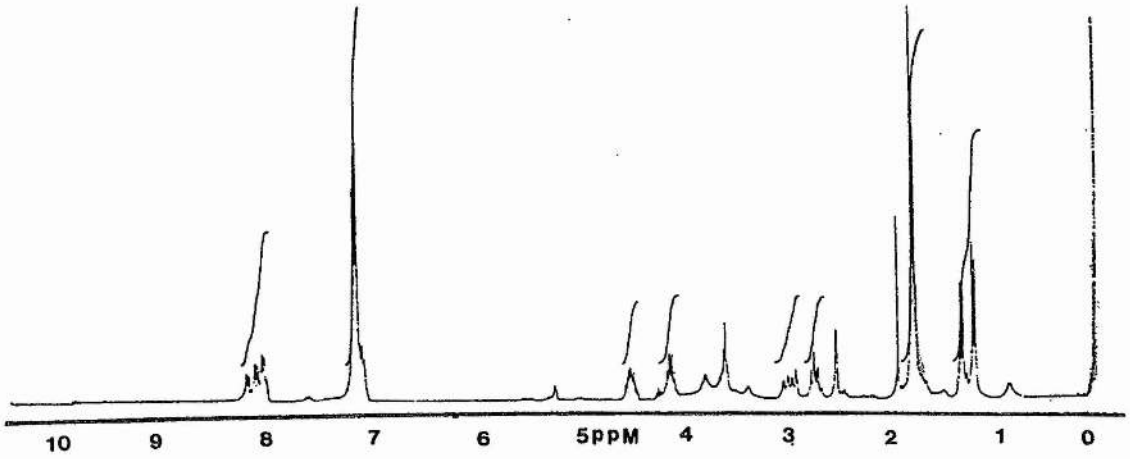


Figure 1.32 ^{3}H Nmr spectrum of N-acetyl-[2, 3-^{3}H]-phenylalanylalanine.

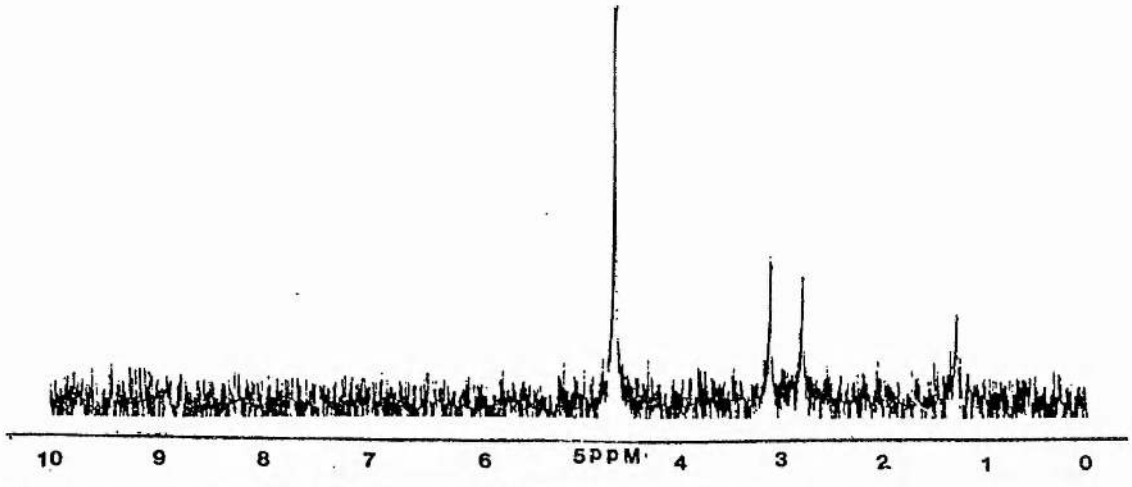


Figure 1.33 ^{1}H Nmr spectrum of N-acetyl-2, 3-didehydrophenylalanylphenylalanine.

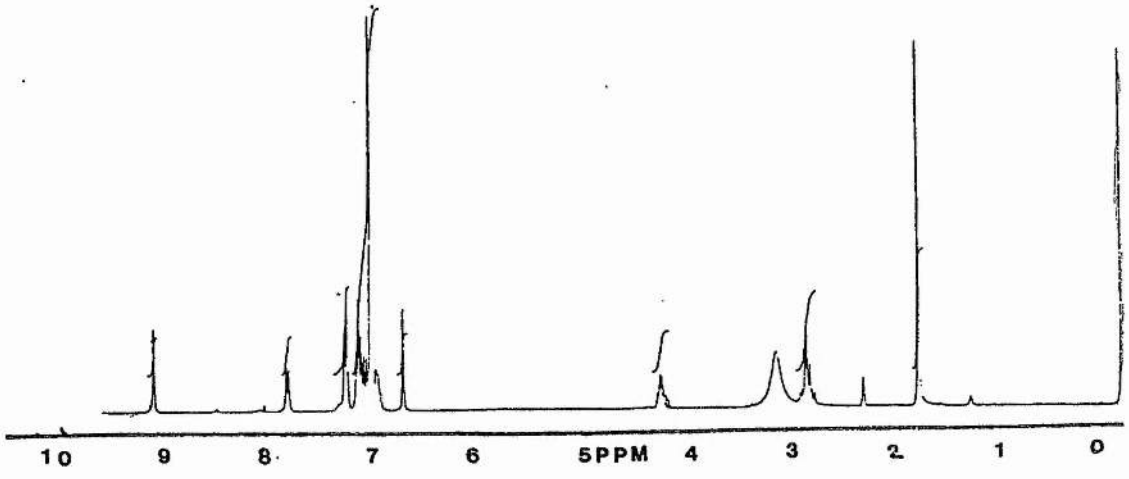


Figure 1.34 ^{1}H Nmr spectrum of N-acetyl-[2, 3-^{3}H]-phenylalanylphenylalanine.

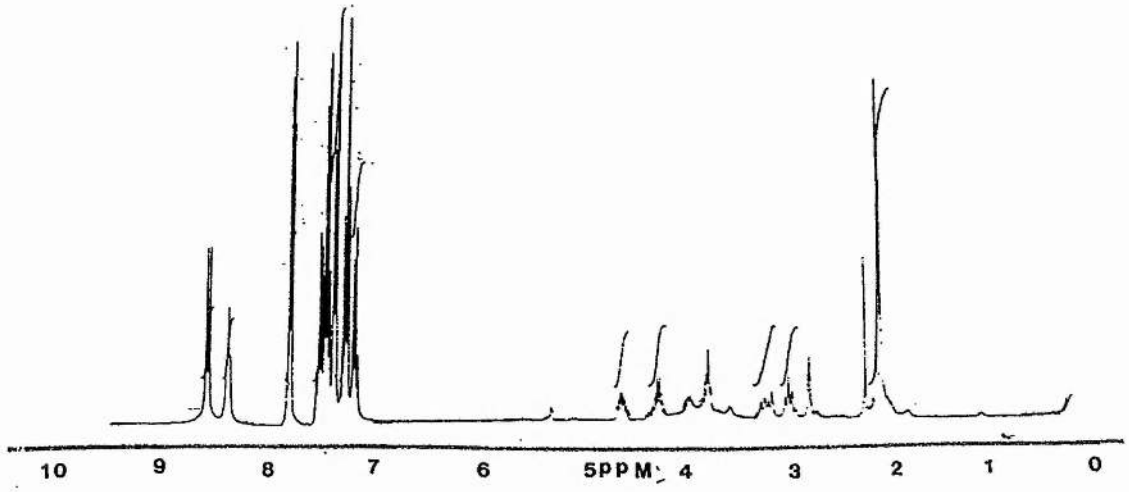
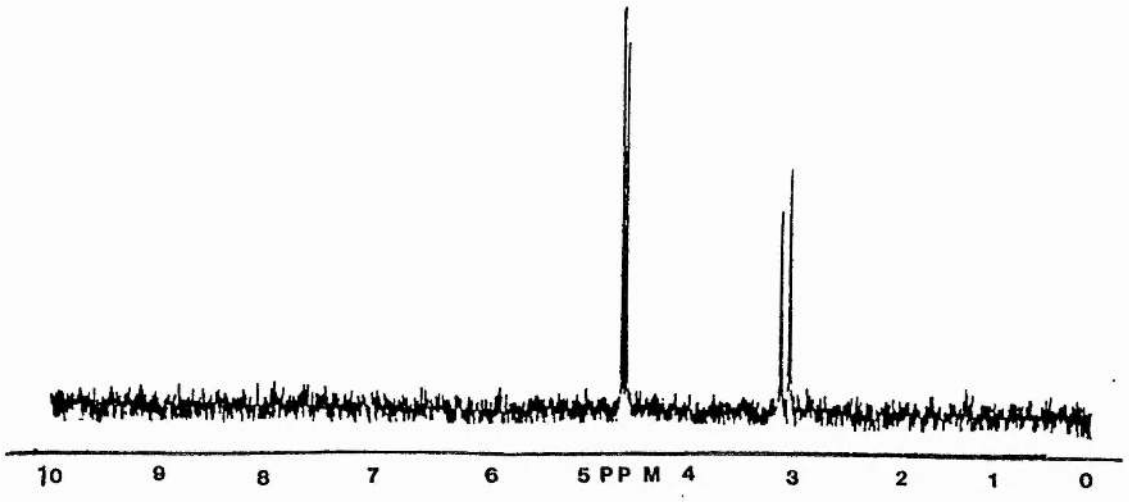


Figure 1.35 ^{3}H Nmr spectrum of N-acetyl-[2, 3-^{3}H]-phenylalanylphenylalanine.



1.3.5 Radiation decomposition analysis by ^{3}H nmr

The following tables contain information about the radiation decomposition of tritiated N-acylamino acids, which were stored in buffer solutions. The formation of new ^{3}H chemical shifts in the tritium spectra of the tritiated N-acylamino acids were monitored. Changes in the integrals of the new ^{3}H chemical shifts with time have been measured and expressed as a percentage of the total tritium in the system.

Table 1.5: N-Acetyl-[4, 5-^{3}H]-leucine (3.75 Ci/mmol) in acetate buffer (pH 4)

No. of days	% decomposition at δ 2.2 ppm	% decomposition at δ 4.8 ppm	total % decomposition
30	1.1		1.1
36	1.7		1.7
43	1.5	0.9	2.4
50	1.6	1.4	3.0
73	2.7	2.5	5.2
112	4.1	4.9	9.0

Table 1.6: N-Acetyl-[4, 5-^{3}H]-leucine (3.75 Ci/mmol) in phosphate buffer (pH 7)

No. of days	% decomposition at δ 2.2 ppm	% decomposition at δ 4.8 ppm	total % decomposition
19	0.8		0.8
30	1.8		1.8
36	0.7	1.4	2.1
43	2.0	0.6	2.6
50	1.8	1.4	3.2
73	2.9	1.9	4.8
112		5.5	nd

Table 1.7: N-Acetyl-[4, 5-^{3}H]-leucine (3.75 Ci/mmol) in borate buffer (pH 9)

No.of days	% decomposition at δ 2.2 ppm	% decomposition at δ 4.8 ppm	total % decomposition
43	2.7		2.7
50	2.6	0.6	3.2
73	4.8	0.4	5.2
112	4.4	3.4	7.8

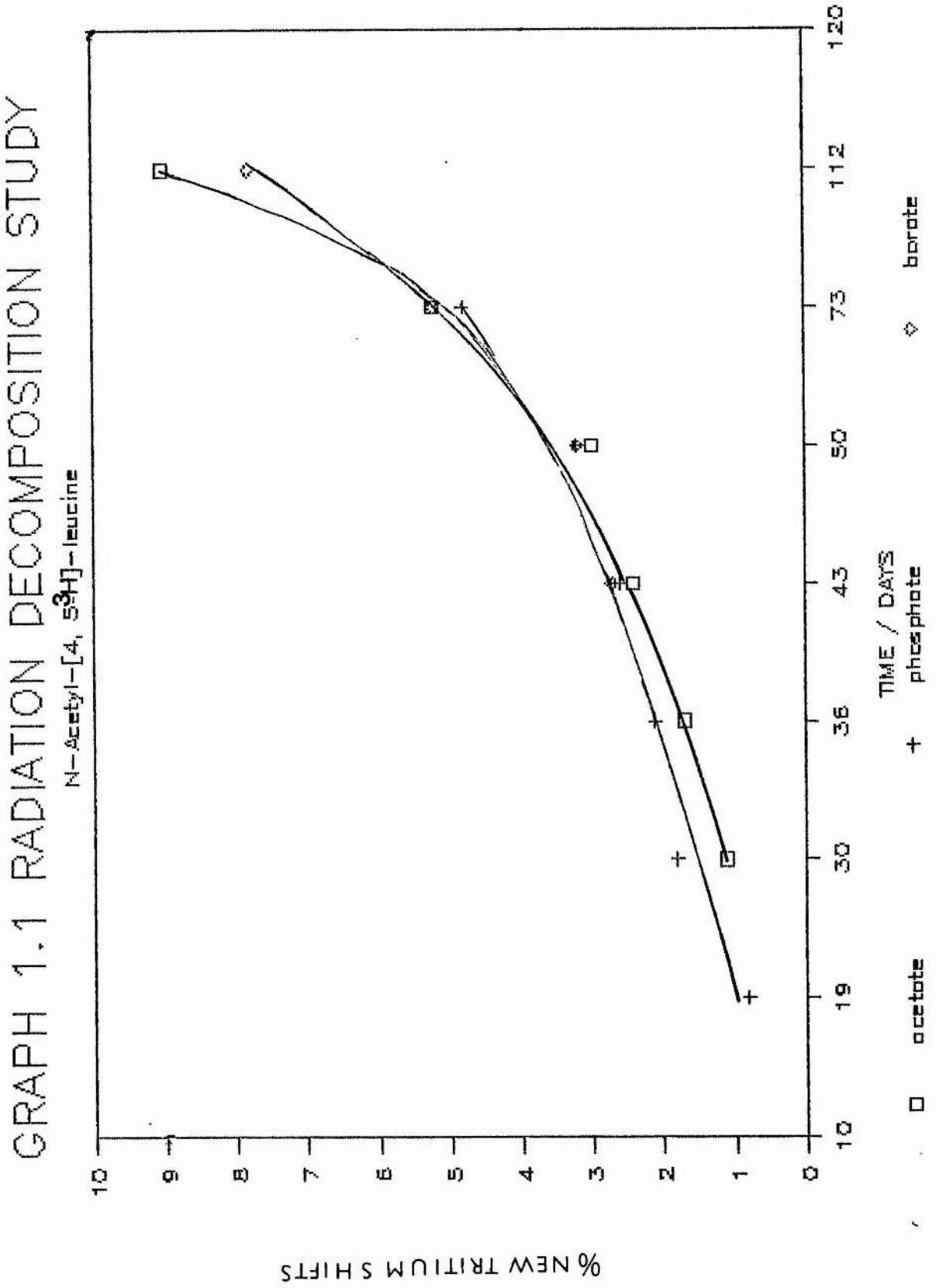

GRAPH 1.1 RADIATION DECOMPOSITION STUDY
N-Acetyl-[4, 5-3H]-leucine
% NEW TRITIUM SHIFTS
0
1
2
3
4
5
6
7
8
9
10
10
19
30
36
43
50
73
112
120
TIME / DAYS
□ acetate
+ phosphate
◇ borate

Table 1.8: N-Acetyl-[2, 3-^{3}H]-phenylalanine (18 Ci/mmol) in acetate buffer (pH 4)

No. of days	% decomposition at δ 2.9 ppm	% decomposition at δ 4.8 ppm	total % decomposition
38	1.5	1.2	2.7
41	1.3	1.8	3.1
72	1.9	3.3	5.2
100	2.1	3.6	5.7
136	3.7	4.0	7.7

Table 1.9: N-Acetyl-[2, 3-^{3}H]-phenylalanine (18 Ci/mmol) in phosphate buffer (pH 7)

No. of days	% decomposition at δ 2.9 ppm	% decomposition at δ 4.8 ppm	total % decomposition
25	1.7		1.7
38	1.7	0.4	2.1
41	1.5	0.6	2.1
72	2.3	1.2	3.7
100	2.4	1.6	4.0
136	3.5	2.4	5.9

Table 1.10: N-Acetyl-[2, 3-^{3}H]-phenylalanine (18 Ci/mmol) in borate buffer (pH 9)

No. of days	% decomposition at δ 2.9 ppm	% decomposition at δ 4.8 ppm	total % decomposition
25	1.1	0.5	1.6
38	1.4	0.6	2.0
41	1.5	1.1	2.6
72	2.3	1.4	3.7
100	2.7	2.3	5.0
136	3.2	3.4	6.6

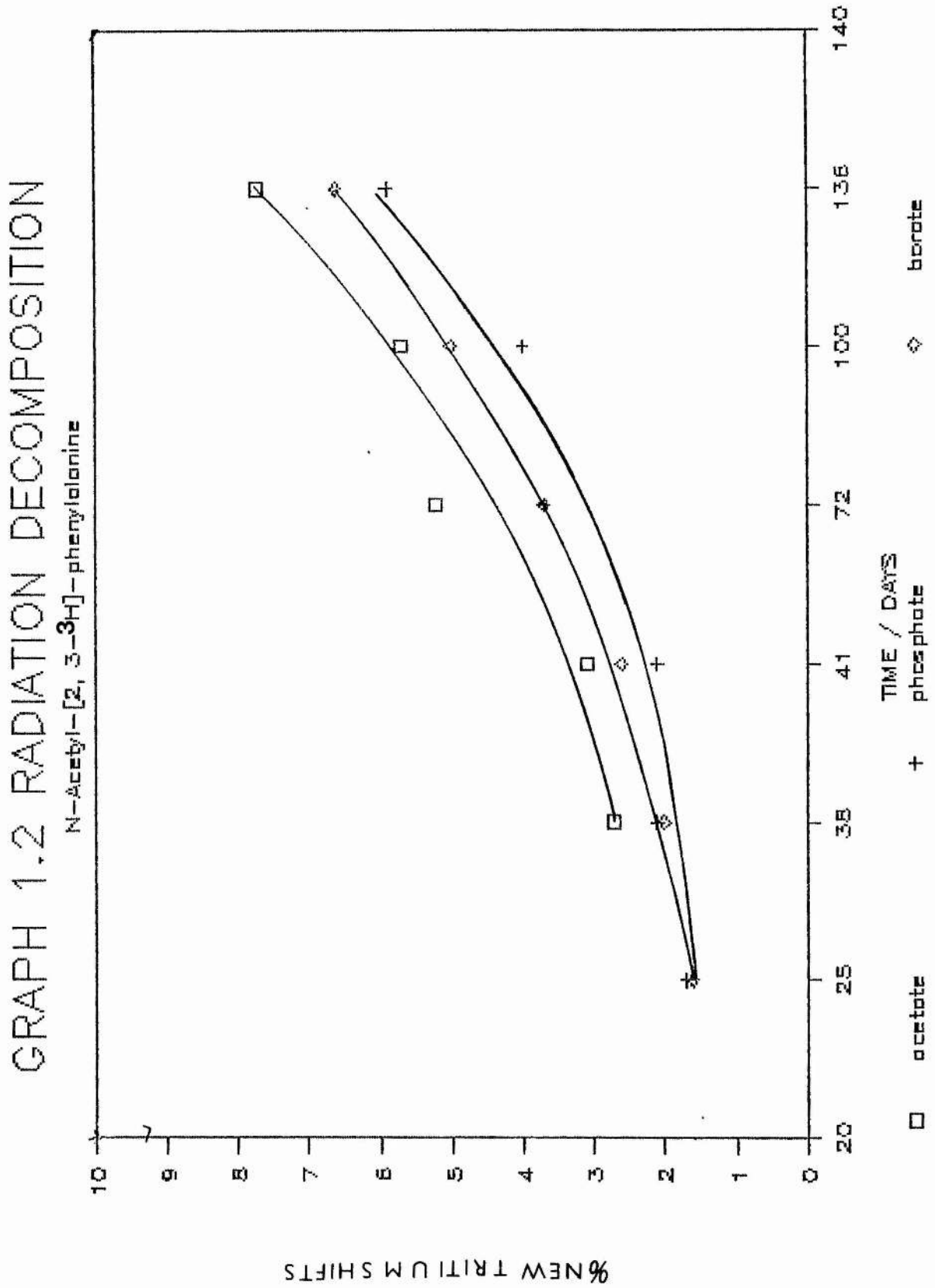

GRAPH 1.2 RADIATION DECOMPOSITION
N-Acetyl-[2, 3-3H]-phenylalanine
%NEW TRITIUM SHIFTS
0
1
2
3
4
5
6
7
8
9
10
20
25
38
41
72
100
136
140
TIME / DAYS
acetate
phosphate
borate

Table 1.11: N-Benzoyl-[2, 3-^{3}H]-phenylalanine (12Ci/mmol) in acetate buffer (pH 4).

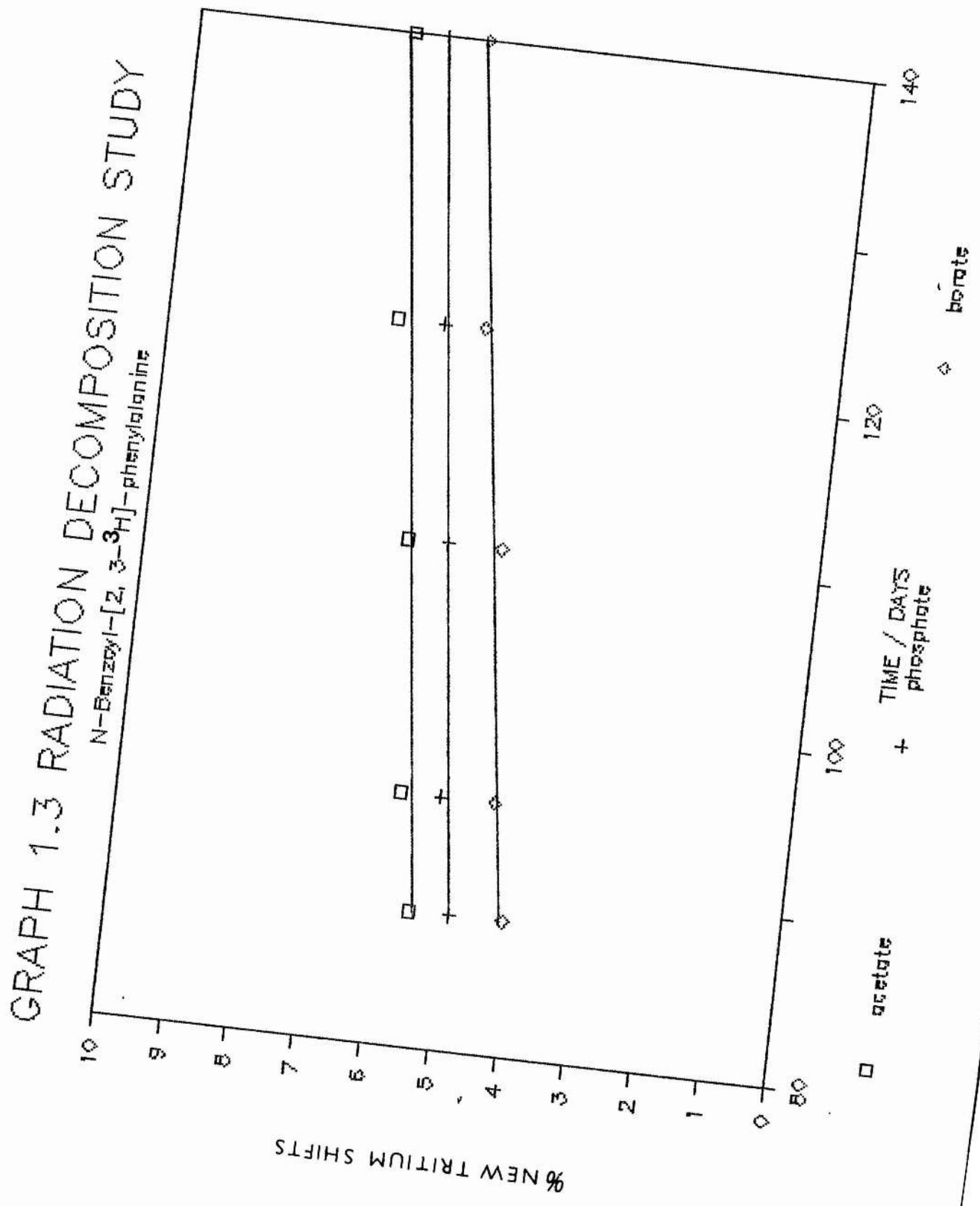
No. of days	% decomposition for chemical shifts at 3.1ppm	at 3.2ppm	at 3.7ppm	at 4.8ppm	total % decomposition
88	1.1	0.9	1.2	2.3	5.5
95	0.9	1.3	1.4	2.2	5.8
110	0.5	0.9	1.9	2.8	6.1
123	1.0	0.7	1.7	3.2	6.6
140	1.3	0.8	1.5	3.4	6.8

Table 1.12: N-Benzoyl-[2, 3-^{3}H]-phenylalanine (12Ci/mmol) in phosphate buffer (pH 7).

No. of days	% decomposition for chemical shifts at 3.1ppm	at 3.2ppm	at 3.7ppm	at 4.8ppm	total % decomposition
88	0.4	1.1	1.3	2.1	4.9
95	0.7	0.9	1.8	1.8	5.2
110	1.0	1.2	1.6	1.7	5.5
123	1.1	1.4	1.6	1.8	5.9
140	1.3	1.3	1.8	1.9	6.3

Table 1.13: N-Benzoyl-[2, 3-^{3}H]-phenylalanine (12Ci/mmol) in borate buffer (pH 9).

No. of days	% decomposition for chemical shifts at 3.1ppm	at 3.2ppm	at 3.7ppm	at 4.8ppm	total % decomposition
88	1.2	0.7	1.5	0.7	4.1
95	1.1	1.2	1.4	0.9	4.4
110	1.2	1.4	1.4	0.7	4.7
123	1.4	1.4	1.5	1.0	5.3
140	1.1	1.5	1.7	1.4	5.7

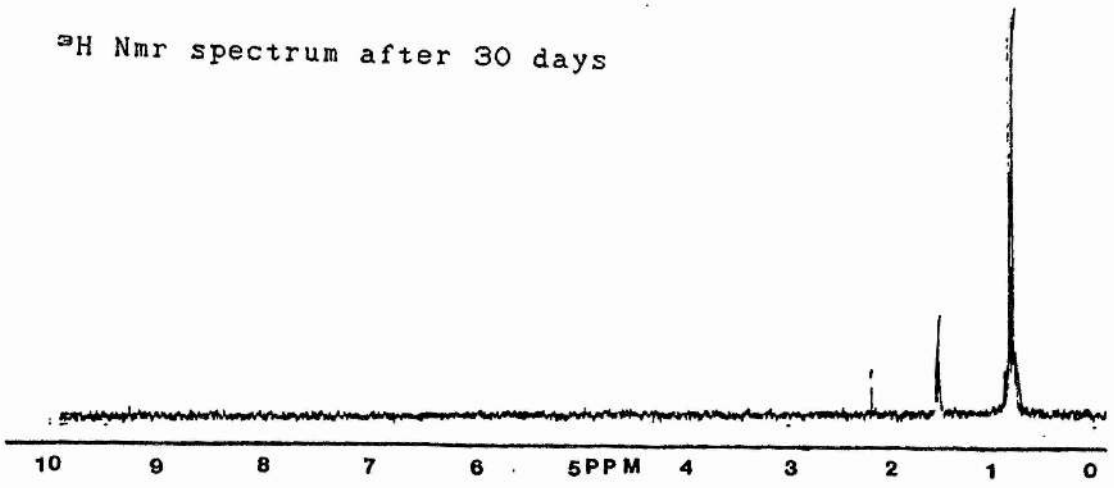

GRAPH 1.3 RADIATION DECOMPOSITION STUDY
N-Benzoyl-[2, 3-3H]-phenylalanine
% NEW TRITIUM SHIFTS
TIME / DAYS
acetate
phosphate
borate
0
1
2
3
4
5
6
7
8
9
10
80
100
120
140

1.3.6 ^{3}H Nmr spectra of radiation decomposition studies.

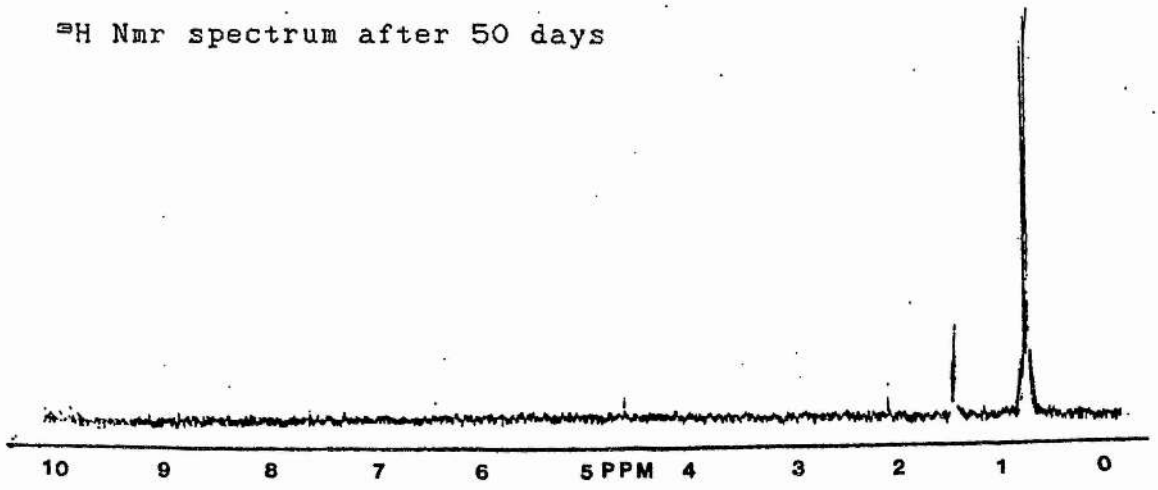
Some of the ^{3}H nmr spectra for the radiation decomposition of tritiated N-acylamino acids are shown on the following pages. The spectra have been selected to illustrate the different stages of radiation decomposition for the tritiated N-acylamino acids.

Figure 1.36 ^{3}H Nmr spectra of N-acetyl-[4, 5-^{3}H]-leucine.
(Radiation decomposition of phosphate buffer sample)

^{3}H Nmr spectrum after 30 days



^{3}H Nmr spectrum after 50 days



^{3}H Nmr spectrum after 112 days

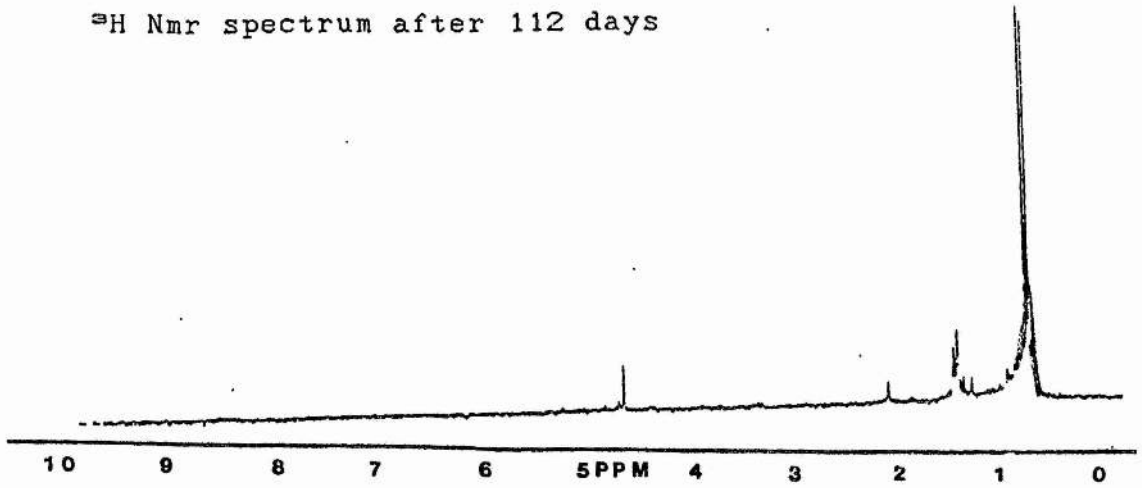
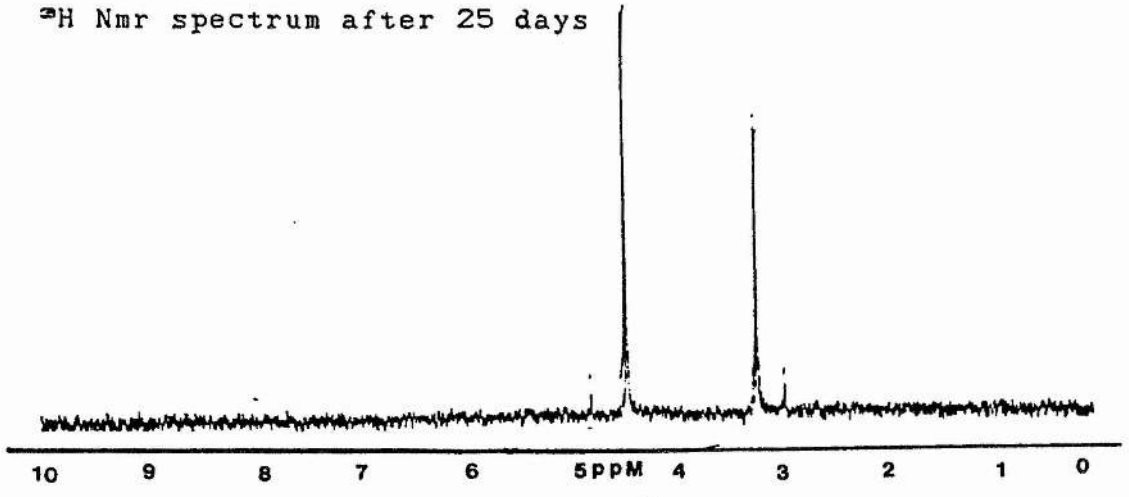
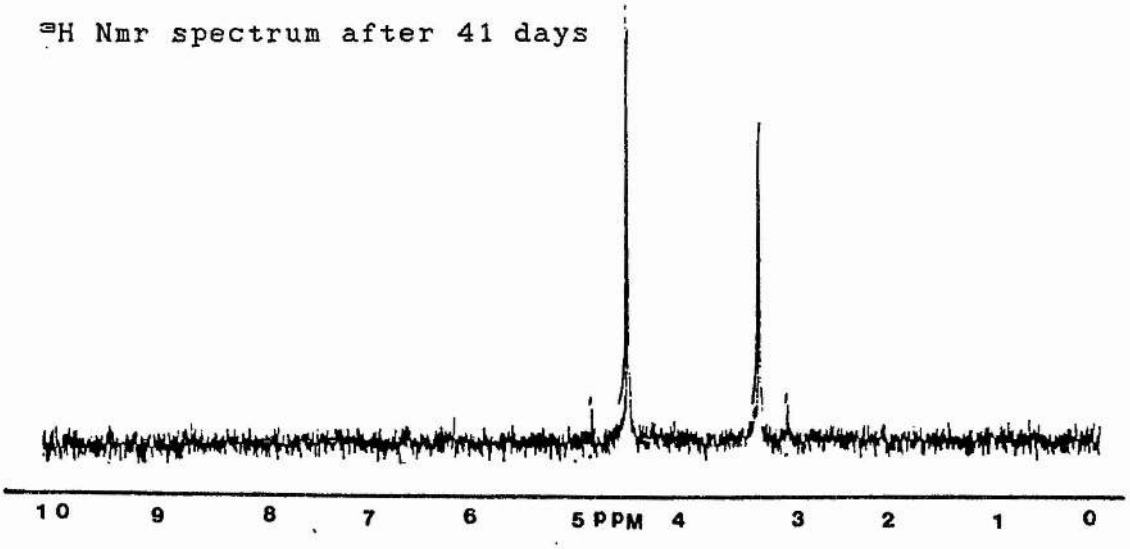


Figure 1.37 ^{3}H Nmr spectra of N-acetyl-[2, 3-^{3}H]-phenylalanine.
(Radiation decomposition of acetate buffer sample)





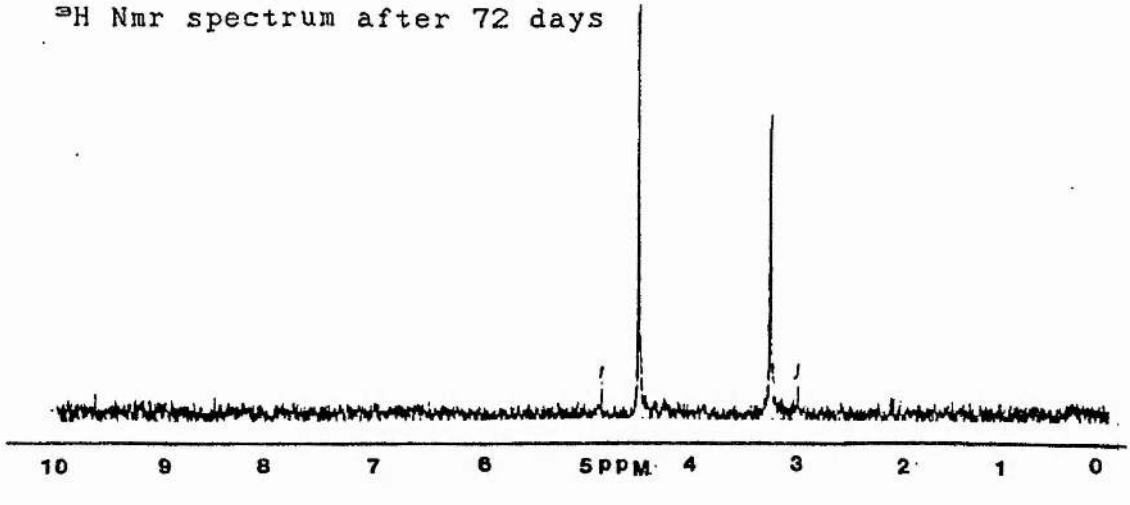
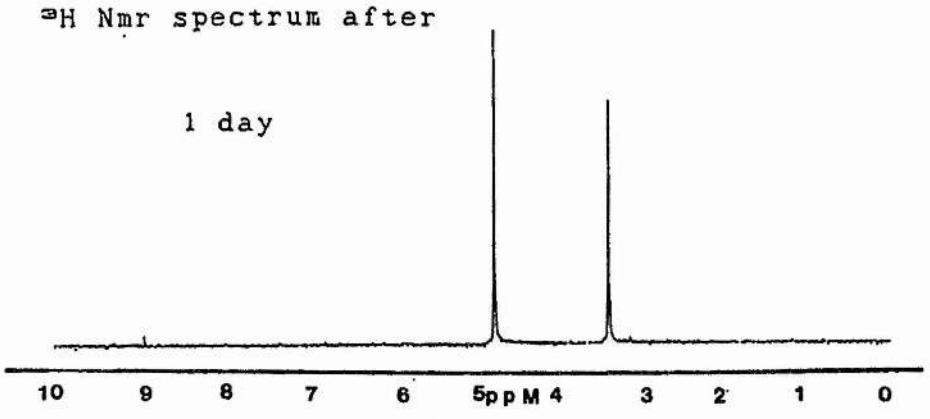
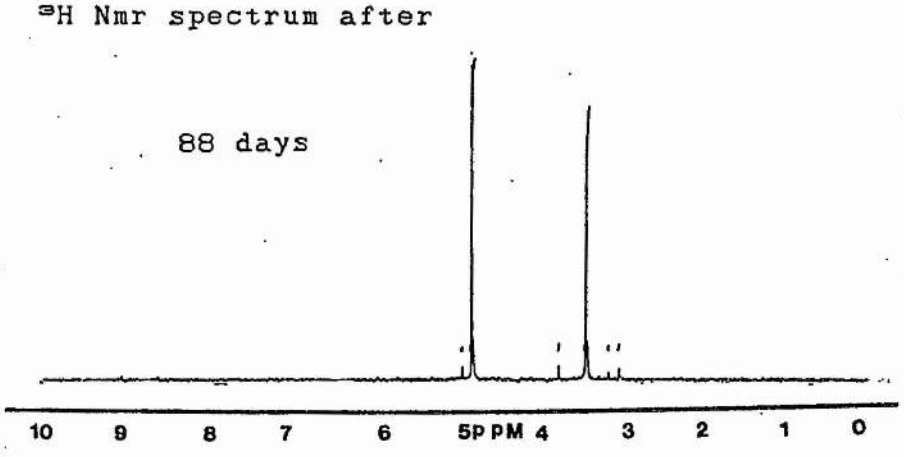
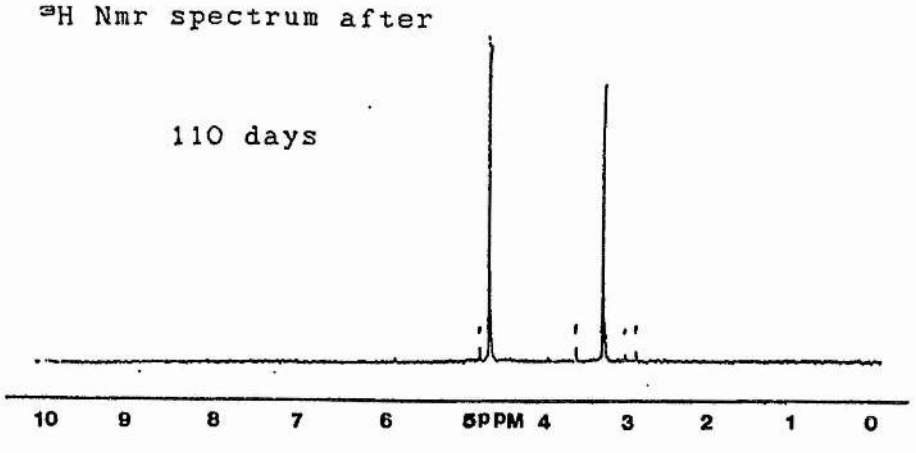


Figure 1.38 ^{3}H Nmr spectra of N-benzoyl-[2, 3-^{3}H]-phenylalanine.
(Radiation decomposition of borate buffer sample)







1.3.7 Radiation decomposition analysis by radio tlc

The following solvent systems were used for radio-tlc analysis of the radiation decomposition of tritiated amino acids:

A) 5:2:3 Butan-1-ol: acetic acid: water

B) 1:1 Ethyl acetate: diethyl ether

C) 1:1:1 Pyridine: butan-1-ol: water

D) 12:4:5 Butan-1-ol: acetic acid: water

E) 9:1 Chloroform: methanol

F) 8:1 Hexane: ethyl acetate

Tritiated samples were prepared and analysed as described in section 1.2.11.4. A variety of methods were used to analyse the tlc plates including: (i) Ninhydrin spray; (ii) UV/ fluorescence and (iii) sulphuric acid spray.

Some reference samples which had R_f values that corresponded to those of radiation decomposition products are shown in Table 1.28.

Table 1.14: N-Acetyl-[4, 5-^{3}H]-leucine (3.75 Ci/mmol) acetate buffer sample (solvent system A)

Peak	R_f value	Sample A % Tritium	Sample B % Tritium	Ninhydrin test
1	0	0.36	0.47	negative
2	0.37	5.69	8.06	positive
3	0.47	19.32	21.73	positive
4	0.54	12.40	9.21	negative
5	0.62	62.16	60.54	negative

Table 1.15: N-Acetyl-[4, 5-^{3}H]-leucine (3.75 Ci/mmol) phosphate buffer sample (solvent system A)

Peak	R_f value	Sample A % Tritium	Sample B % Tritium	Ninhydrin test
1	0	0.06	0.57	negative
2	0.37	5.24	7.61	positive
3	0.47	18.42	22.31	positive
4	0.54	13.56	6.75	negative
5	0.62	61.73	62.76	negative

Table 1.16: N-Acetyl-[4, 5-^{3}H]-leucine (3.75 Ci/mmol) borate buffer sample (solvent system A)

Peak	R_f value	Sample A % Tritium	Sample B % Tritium	Ninhydrin test
1	0	0.22	0.02	negative
2	0.37	9.38	6.61	positive
3	0.47	23.83	20.03	positive
4	0.54	11.84	11.70	negative
5	0.62	54.73	61.64	negative

Table 1.17: N-acetyl-[4, 5-^{3}H]-leucine buffer samples (other solvent systems)

Solvent	R_f value	Ninhydrin test	Inference
B	0	positive	Single peak at baseline.
C	0.60	positive	Broad single peak with some forward tailing.
D	0	positive	Single peak at baseline.
E	0	positive	Major peak at baseline with very minor peaks.

Table 1.18: N-Acetyl-[2, 3-^{3}H]-phenylalanine (18 Ci/mmol) acetate buffer sample (solvent system A)

Peak	R_f value	Sample A % Tritium	Sample B % Tritium	Ninhydrin test
1	0	0.23	0.27	negative
2	0.38	30.29	33.87	positive
3	0.62	55.15	54.38	negative
4	0.70	11.80	11.48	negative

Table 1.19: N-Acetyl-[2, 3-^{3}H]-phenylalanine (18 Ci/mmol) phosphate buffer sample (solvent system A)

Peak	R_f value	Sample A % Tritium	Sample B % Tritium	Ninhydrin test
1	0	0.45	0.65	negative
2	0.38	33.16	32.67	positive
3	0.61	55.53	54.89	negative
4	0.68	10.86	11.79	negative

Table 1.20: N-Acetyl-[2, 3-^{3}H]-phenylalanine (18 Ci/mmol) borate buffer sample (solvent system A)

Peak	R_f value	Sample A % Tritium	Sample B % Tritium	Ninhydrin test
1	0	0.63	0.30	negative
2	0.39	33.19	36.75	positive
3	0.61	55.97	55.96	negative
4	0.70	10.22	6.98	negative

Table 1.21: N-Acetyl-[2, 3-^{3}H]-phenylalanine (18 Ci/mmol) acetate buffer sample (solvent E)

Peak	R_f value	Sample A % Tritium	Sample B % Tritium	Ninhydrin test
1	0	0.03	0.51	negative
2	0.25	11.83	11.76	negative
3	0.32	42.18	41.96	negative
4	0.43	6.34	6.31	negative
5	0.49	39.61	39.41	positive

Table 1.22: N-Acetyl-[2, 3-^{3}H]-phenylalanine (18 Ci/mmol) phosphate buffer sample (solvent E)

Peak	R_f value	Sample A % Tritium	Sample B % Tritium	Ninhydrin test
1	0	0.26	0.15	negative
2	0.25	11.94	11.80	negative
3	0.32	42.36	42.13	negative
4	0.43	8.26	6.35	negative
5	0.49	37.18	39.57	positive

Table 1.23: N-Acetyl-[2, 3-^{3}H]-phenylalanine (18 Ci/mmol) borate buffer sample (solvent E)

Peak	R_f value	Sample A % Tritium	Sample B % Tritium	Ninhydrin test
1	0	0.24	0.15	negative
2	0.25	11.14	11.78	negative
3	0.32	42.58	42.15	negative
4	0.43	9.21	6.31	negative
5	0.50	36.83	39.61	positive

Table 1.24: N-acetyl-[2, 3-^{3}H]-phenylalanine buffer samples (other solvent systems)

Solvent	R_f value	Ninhydrin test	Inference
B	0	positive	Single peak at baseline
C	0.60	positive	Single peak with tailing
D	0	positive	Single peak at baseline

Table 1.25: N-Benzoyl-[2, 3-^{3}H]-phenylalanine (12 Ci/mmol) acetate buffer sample (solvent system A)

Peak	R_f value	Sample A % Tritium	Sample B % Tritium	Ninhydrin test
1	0	0.27	0.10	negative
2	0.69	48.45	50.82	negative
3	0.72	51.28	49.08	negative

Table 1.26: N-Benzoyl-[2, 3-^{3}H]-phenylalanine (12 Ci/mmol) phosphate buffer sample (solvent A)

Peak	R_f value	Sample A % Tritium	Sample B % Tritium	Ninhydrin test
1	0	0.24	0.33	negative
2	0.69	49.86	50.58	negative
3	0.72	50.14	49.42	negative

Table 1.27: N-Benzoyl-[2, 3-^{3}H]-phenylalanine (12 Ci/mmol) borate buffer sample (solvent A)

Peak	R_f value	Sample A % Tritium	Sample B % Tritium	Ninhydrin test
1	0	0.40	0.31	negative
2	0.69	50.09	43.98	negative
3	0.72	49.51	55.71	negative

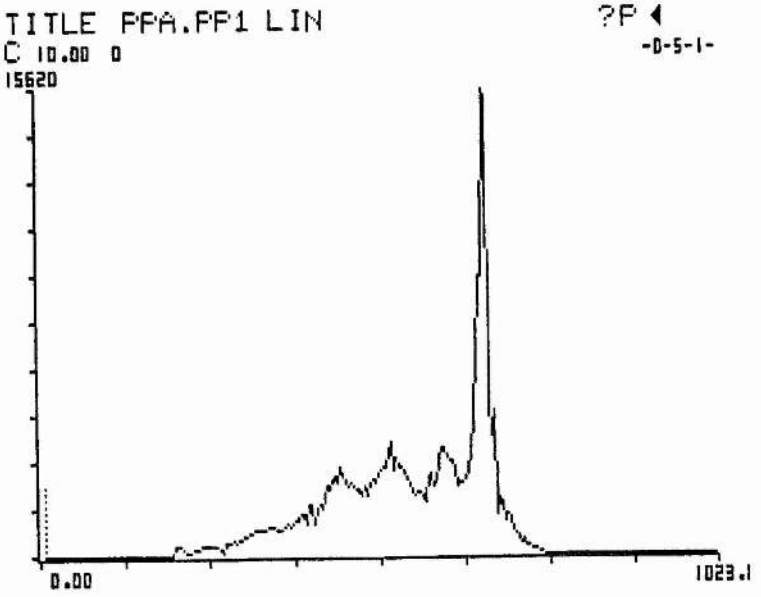
Table 1.28: Tlc analysis of some reference compounds.

Compound	Solvent system	R_f value
Leucine	A	0.47
	C	0.60
Phenylalanine	A	0.45
	C	0.60
	E	0.19
Tyrosine	A	0.37
	E	0.46
3, 4-dihydroxy phenylalanine	A	0.38
	E	0.48
Norvaline	A	0.31
	C	0.59
N-acetylnorvaline	A	0.56
	C	0.57
N-acetylleucine	A	0.62
	C	0.61
N-acetylphenylalanine	A	0.62
	C	0.60
	E	0.32
N-acetyltyrosine	A	0.66
	C	0.60
	E	0.40
N-benzoylphenylalanine	A	0.70

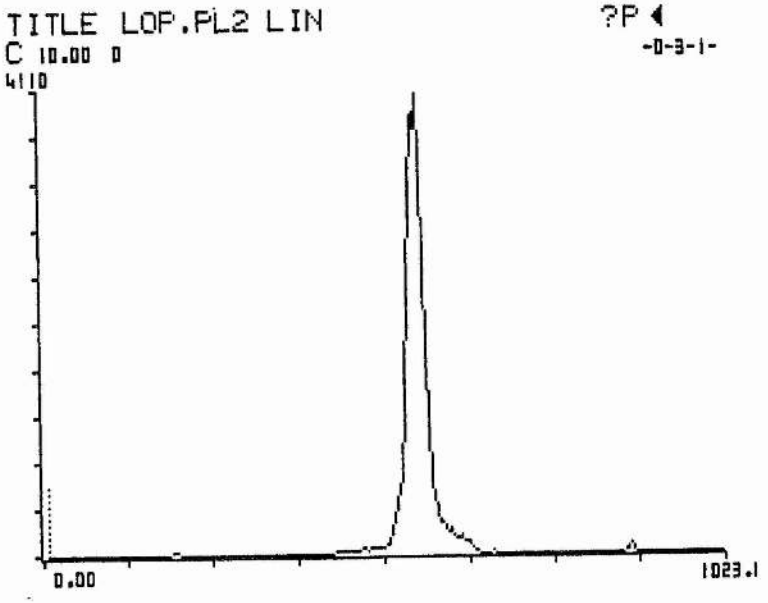
1.3.8 Examples of radio-tlc of radiation decomposition samples.

Examples of the radio-tlc for the radiation decomposition studies are shown over. Chromatograms for some of the different solvent systems are illustrated.

Figure 1.39 Radio tlc analyses of N-acetyl-[4, 5-^{3}H]-leucine

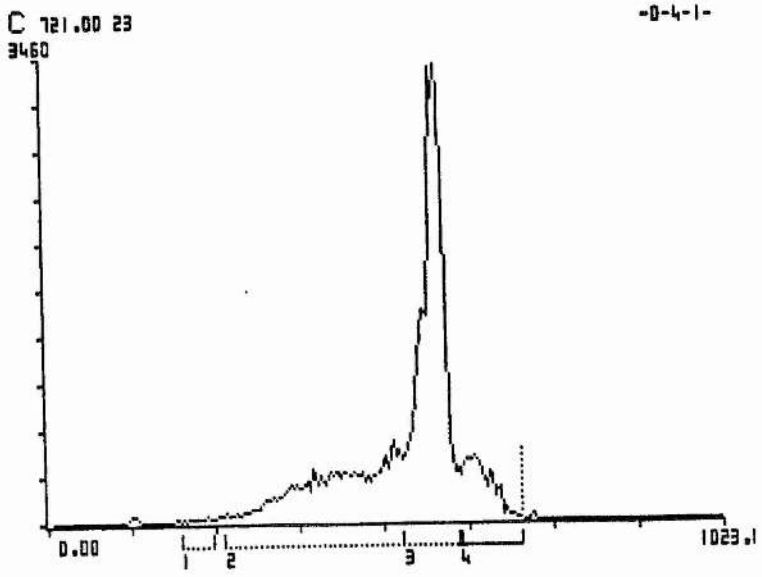


(a) Tlc plate ran in 5:2:3(v/v) butanol: acetic acid: water

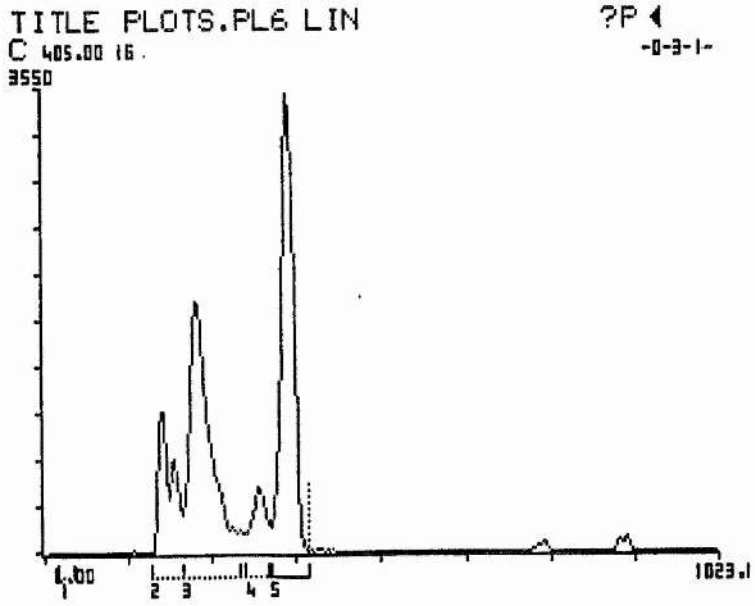


(b) Tlc plate ran in 1:1:1(v/v) pyridine: acetic acid: water

Figure 1.40 Radio tlc analyses of N-acetyl-[2, 3-^{3}H]-phenylalanine.

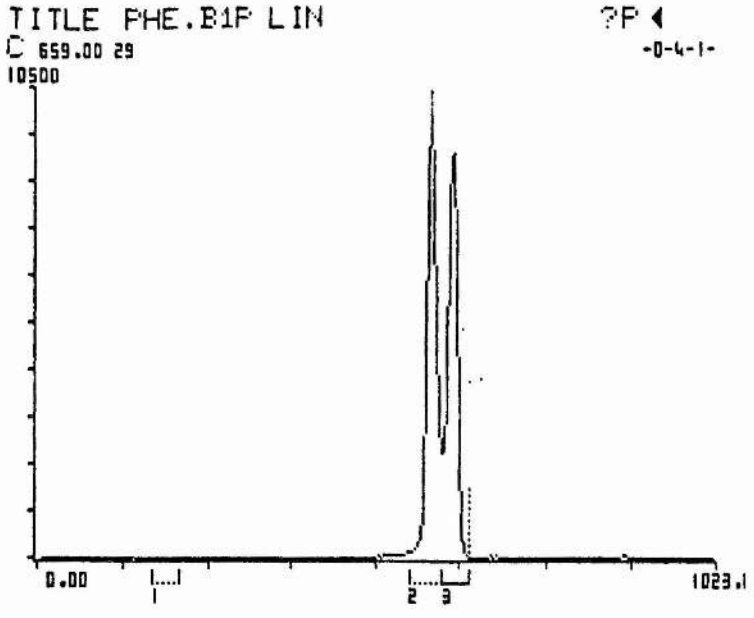


(a) Tlc plate ran in 5:2:3(v/v) butanol: acetic acid: water



(b) Tlc plate ran in 9:1(v/v) chloroform: methanol

Figure 1.41 Radio tlc analyses of N-benzoyl-[2, 3-^{3}H]-phenylalanine.



(a) Tlc plate ran in 5:2:3(v/v) butanol: acetic acid: water

1.3.9 Analysis of labile and volatile components of radiation decomposition samples.

Table 1.29 Labile tritium and tritiated water produced by radiation decomposition of N-acetyl-[4,5-^{3}H]-leucine (240 days)

Buffer solution	Activity of sample (mCi)			% THO
	A	B	average	average
acetate	0.57	0.64	0.61	4
phosphate	0.73	0.65	0.69	5
borate	0.55	0.53	0.54	4

Table 1.30: N-acetyl-[2,3-^{3}H]-phenylalanine (240 days)

Buffer solution	Activity of sample (mCi)			% THO
	A	B	average	average
acetate	0.97	0.90	0.94	2
phosphate	1.10	1.20	1.15	3
borate	0.60	0.70	0.65	2

Table 1.31: N-benzoyl-[2,3-^{3}H]-phenylalanine (240 days)

Buffer solution	Activity of sample (mCi)			% THO
	A	B	average	average
acetate	0.72	0.80	0.76	2
phosphate	1.27	1.21	1.24	3
borate	0.98	1.00	0.99	3

1.4 Discussion.

In order to prepare N-acetyl-2, 3-didehydroamino acid esters a reaction scheme involving azido intermediates and based upon the work of Effenberger et. al[82] had been proposed. It was hoped to prepare methyl esters of N-acetyl-2, 3-didehydroalanine, N-acetyl-2, 3-didehydrovaline and N-acetyl-2, 3-didehydroleucine by this route.

The first stage of the synthesis involved the conversion of carboxylic acids into their corresponding 2-bromocarboxylic acids. The Hell-Volhardt-Zelinsky reaction was used to carry out the synthesis. The 2-bromocarboxylic acids were then converted into the corresponding methyl esters by refluxing in a mixture of 2, 2-dimethoxypropane and methanol and catalytic amounts of toluene-p-sulphonic acid. The reaction involves the regeneration of methanol by degradation of 2, 2-dimethoxypropane into acetone and methanol. 2-Bromopropionic acid methyl ester was prepared in a reasonable yield of 75%. The yields of 2-bromo-3-methylbutanoic acid methylester and 2-bromo-4-methylpentanoic acid methyl ester were much lower at 54% and 58% respectively. One reason for this could be that the reflux temperature of the latter compounds were higher than that for the case of the esterification of 2-bromopropionic acid. This may have led to a more rapid degradation of 2, 2-dimethoxypropane than the reaction required.

The preparation of 2-azidocarboxylic acid methyl ester from the 2-bromo derivatives was carried out using the method of Nakajima [84]. The reaction involves stirring the bromo compound with sodium azide in water and in the presence of tetrabutylammonium bromide. The product was recovered from the reaction mixture by solvent extraction with dichloromethane. The reaction proceeds efficiently at room temperature and good yields, in excess of 80%, were obtained. The purity of the azides were determined by GC analysis and were found to be greater than 97%.

The reaction for converting the 2-azidocarboxylic acid esters into N-acetyl-2,3-didehydroamino acid esters involves refluxing the azide in acetic acid/ glacial acetic acid in the presence of rhenium heptasulphide as a catalyst. A long time and high temperatures are required for the reaction to be successful. Since such harsh conditions are used, it is necessary to protect the alkene group of the didehydroamino acid product from polymerisation by the addition of quinol. The reaction can lead to the formation of a mixture of both mono- and diacetylated products. The latter can be prevented from forming by adding water to the reaction medium. Effenberger and Beisswenger [62] have reported the synthesis of several N-acetyl-2,3-didehydroamino acid methyl esters using various conditions. The reported yields varied from about 20% to 98% and were found to be dependent upon the reaction conditions used.

N-acetyl-2,3-didehydroalanine methyl ester was successfully prepared in a yield of 30%. N-acetyl-2,3-didehydroalanine was prepared by stirring the ester in a hot solution of potassium hydroxide. However, the preparations of N-acetyl-2,3-didehydrovaline methyl ester and N-acetyl-2,3-didehydroleucine methyl ester were unsuccessful. Tarry residues were formed when the attempted synthesis of the latter compounds were carried out. In these cases polymerisation seems to have occured.

The attempted synthesis of several N-acyl-2,3-didehydroamino acids and peptides have been carried out using azlactones as intermediates. The azlactones of N-acetyl-2,3-didehydrophenylalanine and N-benzoyl-2,3-phenylalanine were prepared in good yields by condensation of the corresponding acylglycine with benzaldehyde. The preparation of N-benzoyl-2,3-didehydrovaline azlactone was carried out using the method of Ramage and Simonsen [66].

The azlactones were converted into the N-acyl-2,3-didehydroamino acids in good yields either by refluxing in an acetone-water mixture or with aqueous potassium hydroxide. N-Acyl-2,3-didehydropeptides have been prepared by coupling the azlactones with amino acids or amino acid esters. The syntheses of simple N-acyl-2,3-didehydrophenylalanine peptides containing glycine, glycine ethyl ester, L-alanine and L-phenylalanine have been carried out using the methods of Bergmann et. al [67]. However, attempts to couple L-glutamic acid, L-tyrosine, L-proline

and L-lysine with the azlactones were unsuccessful. In these cases the reactions may not have proceeded due to improper neutralisation of the amino acids with sodiumm hydroxide.

The optimum conditions for the labelling of N-acetyl-4,5-didehydroleucine using tritium-hydrogen mixtures were studied. The highest incorporation of tritium occurred when the tritiation reaction was carried out in THF. Glacial acetic acid as the reaction solvent was found to give the lowest levels of tritium incorporation. In the latter case, there are 1.75×10^{-2} moles (1.053×10^{22} molecules) of glacial acetic acid and 4.38×10^{-6} moles (2.64×10^{18} molecules) of N-acetyl-4,5-didehydroleucine in the reaction system. Hence, there is a total of 1.056×10^{22} carboxyl group protons which can undergo labile isotope exchange with tritium atoms. However, there are only 1.724×10^{-6} moles (2.07×10^{18} atoms) of tritium in the reaction system. Hence, there are approximately 500 times more carboxyl group protons as there are tritium atoms in the reaction system. It can be concluded that there may be a reduction in the effective amount of tritium available for reduction of the didehydroamino acid substrate because of the large excess of carboxyl group protons, which can can take part in labile isotope exchange reactions.

The variation of catalyst: substrate ratio has been studied for two 5% Pd/C catalysts (see Table 1.3). Incomplete tritiation of N-acetyl-4,5-didehydroleucine

occurred with the 5:1 catalyst: substrate ratio. Analysis by ^{1}H and ^{3}H nmr spectroscopy showed a mixture of products had been formed. The majority of the tritium was present at a chemical shift of 4.6ppm, which corresponded to the δ-methylene chemical shift of the unsaturated precursor. This would seem to imply that the labelling occured mainly via an isotope exchange process at a 5:1 catalyst: substrate ratio since tritium was not present at a chemical shift of 0.9ppm. Similar results have been reported by Tang [81] for the partial tritiation of N-acetyl-4,5-didehydroleucine with a small volume of a hydrogen-tritium mixture.

Complete hydrogenation occured at all other catalyst: substrate ratios. In all cases ^{3}H nmr spectra of the products showed that the majority of the tritium was found in the terminal methyl groups and the remainder was present in the γ-methine group.

As can be seen from Table 1.3 the optimum tritium incorporation was found to occur with a 1:1 catalyst: substrate ratio. There was no significant difference between the amount of tritium incorporated when carrying out the labelling reactions in the presence of 5% Pd/C type B and C catalysts.

The tritiation of N-acyldidehydroamino acids were carried out using a mixture of hydrogen (20mls) and tritium (1Ci). The labelling of didehydroamino acids containing terminal vinylic methylene groups, such as N-acetyl-2,3-

didehydroalanine methyl ester and N-acetyl-4,5-didehydroleucine, lead to products where the majority of the tritium was incorporated in the terminal methyl groups. This implied that isotopic exchange with the vinylic methylene protons occurred as well as the reduction of the alkene bond. This observation was in agreement with work reported by Sheppard et. al.[32].

In the case of N-acyl-[2,3-^{3}H]-phenylalanine compounds about 60% of the tritium was found in the 2-position with the remainder in the 3-position of the aliphatic side chain. No tritiation of the aromatic ring occurred with 5% Pd/C as the catalyst. In the case of N-benzoyl-2,3-didehydrovaline even distribution of tritium across the alkene bond occured.

The tritiation of N-acetyl-2,3-didehydrophenylalanine peptides was carried out using a mixture of hydrogen (2mls) and tritium (1Ci). The tritiation of N-benzoyl-2,3-didehydrophenylalanylglycine resulted in a product with a labelling pattern that was in accordance with that found for N-benzoyl-[2,3-^{3}H]-phenylalanine and no tritium was found to be present in the methylene protons of the glycine residue.

The tritiation of N-acetyl-2,3-didehydrophenylalanyl-L-alanine resulted in a tritiated product with a specific activity of 2.7 Ci/mmol. Analysis by ^{3}H Nmr spectroscopy showed that tritium was also incorporated into the ß-CH_3 group, δ1.2ppm, of the alanine residue as well as the α- and ß-positions of phenylalanine side chain. The

additional presence of tritium in the alanine residue at a level of 8% implied that an isotope exchange process had occurred in addition to the reduction of the didehydrophenylalanine residue. The observation is supported by result obtained for the tritiation of N-acetyl-2,3-didehydroalanine methyl ester. In the case of N-acetyl-[2,3-^{3}H]-alanine methyl ester 95% of the tritium was found in the ß-position while the remainder was in the α-position. This result indicates that isotope exchange labelling is occurring in addition to the reduction of the vinylic methylene group.

Diasteroisomers were formed when N-acetyl-2,3-didehydrophenylalanyl-L-phenylalanine was tritiated. Tritium was found in the side chain of the N-acetylphenylalanine but not in the L-phenylalanine residue. The methine group was found to contain 61% of the tritium while the remainder was found in the methylene group. The ratio of the diastereoisomers was determined from the ^{3}H nmr spectrum by measuring the ratio of the two methine chemical shifts and was found to be 53:47.

Although several authors have studied the radiation decomposition of tritiated amino acids no similar investigations have been reported for labelled amino acid derivatives. N-Acylated amino acids are important biological molecules in their own right and understanding of the radiation decomposition of the radiolabelled compounds is desirable. The studies described herein are possibly the

first systematic investigation of radiation decomposition of such compounds.

The pH of the solvent in which tritiated N-acylamino acids were stored would seem to affect their stability. The radiation decomposition of all compounds studied were found to be greatest in acidic and basic solvent systems (see Tables 1.5-1.13).

There would seem to be an induction period before new ^{3}H chemical shifts were observed. N-acetyl-[4,5-^{3}H]-leucine (3.75 Ci/mmol), stored in phosphate buffer solution, was found to have the shortest induction period (19 days) while N-benzoyl-[2, 3-^{3}H]-phenylalanine (18.2 Ci/mmol) had the longest period (88 days). Evans[1] has reported induction periods for the radiation decomposition of samples of tritiated compounds, including [G-^{3}H]-phenylalanine. The reasons for this apparent period of stability are not fully understood. The induction period could be due to the build up of reactive radical species or ions being necessary before any radiation decomposition of the radiolabelled compounds can occur. This hypothesis may be applicable to the observations for the tritiated amino acid derivatives in buffer solutions.

Radio-tlc analysis of N-acetyl-[4,5-^{3}H]-leucine (3.75 Ci/mmol) samples showed that decomposition had occured. The major tlc component was found to be N-acetyl-[4,5-^{3}H]-leucine. Comparison of the decomposition products with

suitable reference samples indicated that tritiated leucine and N-acetylnorvaline may be present. Tlc analysis of the tritiated compounds run in 5:2:3 BuOH:AcOH:H_2O gave a component at a R_f value of 0.47 (Ninhydrin +ve), which corresponded to that for a reference sample of leucine. A second component at a R_f value of 0.54 (Ninhydrin -ve) was found to match with the reference sample of N-acetylnorvaline. The third radiation decomposition product was not clearly identified. No significant difference was observed for the tlc analysis of the radiation decomposition products of the three buffer samples of N-acetyl-[4,5-^{3}H]-leucine.

Radio-tlc analysis of buffer solutions of N-acetyl-[2, 3-^{3}H]-phenylalanine (18.2 Ci/mmol) indicated the presence of at least three decomposition products. Tritiated hydroxy substituted phenylalanine derivatives have been shown to possibly occur by running the radiation decomposition samples against reference compounds. Tritiated phenylalanine was shown not to be present. Tlc analysis of samples run in 5:2:3 BuOH:AcOH:H_2O gave three components at: (i) a R_f value of 0.38 (Ninhydrin +ve), which corresponded to reference samples of 3,4-dihydroxyphenylalanine and tyrosine; (ii) a R_f Value of 0.61 (Ninhydrin -ve), which corresponded to a reference sample of N-acetylphenylalanine and (iii) a R_f value of 0.68 (Ninhydrin ve), that matched with a reference sample of N-acetyltyrosine. Tlc analysis of samples run in 9:1 $CHCl_3$:MeOH gave four components at: (i) a R_f value of 0.25 (Ninhydrin -ve), which was

unidentified; (ii) a R_f value of 0.32 (Ninhydrin +ve), which matched with phenylalanine; (iii) a R_f value of 0.43 (Ninhydrin -ve), which corresponded to a reference sample of N-acetyltyrosine and (iv) a R_f value of 0.49 (Ninhydrin +ve), which matched with a reference sample of tyrosine.

The radio-tlc analysis of the samples of N-benzoyl-[2, 3-^{3}H]-phenylalanine (12 Ci/mmol) produced a double peak when the tlc plate was run in 5:2:3 BuOH:AcOH:H_2O. This phenomenon was observed with and without the addition of carrier material. The presence of a double peak should be considered as an undesirable effect of the tlc system used. The validity of the results obtained using the above solvent system should be treated with sceptism. All other solvent systems indicated that only the starting material was present.

Tables 1.32, 1.33 and 1.34 show the total tritium present in a labile form and any volatile components formed in storage. Buffer solutions of N-acetyl-[4,5-^{3}H]-leucine (3.75 Ci/mmol) were found to contain the highest percentage of tritium in a labile form and/ or as volatile compounds. The lowest values were found for buffer solutions of N-benzoyl[2,3-^{3}H]-phenylalanine (12 Ci/mmol).

Several points have emerged in carrying out the radiation decomposition studies. ^{3}H Nmr spectroscopy can be used to successfully follow the fate of the labelled positions of tritiated N-acylamino acid. The labelled

positions in the tritiated N-acylamino acids would seem to be fairly stable. This observation is in agreement with the reports of several authors [1, 78].

Radio-tlc analysis showed that several radiation decomposition products were formed upon storage of tritiated N-acylamino acids in buffer solutions. The method of analysis will only allow the determination of non-volatile compounds and, to obtain a better picture of the radiation decomposition processes, it is also necessary to determine the total tritium present in a labile form or as volatile materials.

Secondary effects, due to the interaction of reactive species in the solvent system and the labelled compoumd, would thus seem to be the predominant cause of radiation decomposition. The actual site of labelling is not directly involved in the radiation decomposition of the compound. The products formed in storage would probably support this hypothesis.

Hydroxyl radicals have been reported to exchange with tritium in the aromatic rings of labelled phenylalanine and tyrosine compounds. Indeed evidence for the presence of labelled hydroxyl substituted phenylalanine derivatives has been shown by radio tlc. Both mono- and dihydroxyphenylalanine derivatives are formed. Radio-tlc analysis of buffer solutions of N-acetyl-[2,3-^{3}H]-phenylalanine upon storage showed components that matched

with reference samples of tyrosine (R_f 0.38 solvent 5:2;3 BuOH:AcOH:H_2O R_f 0.49 solvent 9:1 $CHCl_3$:MeOH), N-acetyltyrosine (R_f 0.68 solvent 5:2;3 BuOH:AcOH:H_2O) and 3,4-dihydroxyphenylalanine (R_f 0.38 Solvent 5:2;3 BuOH:AcOH:H_2O) to be present as radiation decomposition products.

Tritiated leucine has been shown to be formed during storage of N-acetyl-[4,5-^{3}H]-leucine. Radio-tlc analysis of samples of N-acetyl-[4,5-^{3}H]-leucine run in 5:2;3 BuOH:AcOH:H_2O showed a component to be present at a R_f value of 0.47 (Ninhydrin + ve) whiched matched with the reference sample of leucine. There is no indication that further reaction by deamination of either the trititiated acylated or free amino acid had occured. Supportive evidence for this observation comes from the work of Garrison [73], who found that deamination did not occur during the radiolysis of N-acetylleucine with an external ^{32}P source. The formation of any tritiated 2-keto-4-methylpentanoic acid during the storage period would thus seem to be at the most a minor product.

Similar deacetylation of tritiated N-acylphenylalanine compounds may not occur, since no tritiated phenylalanine or phenylpyruvic acid was found to be present by radio-tlc analysis. Radio-tlc analysis of tritiated N-acetylphenyalanine samples which had been stored in buffer solutions showed components that matched with reference samples of tyrosine (R_f 0.38 solvent 5:2:3 BuOH:AcOH:H_2O),

N-actyltyrosine (R_f 0.68 solvent 5:2:3 BuOH:AcOH:H_2O) and 3,4-dihydroxyphenylalanine (R_f 0.38 solvent 5:2:3 BuOH:AcOH:H_2O) were present.

The radio-tlc analysis indicated the possible formation of tritiated N-acetylnorvaline occured during storage of N-acetyl-[4,5-^{3}H]-leucine. This observation implies loss of one of the terminal methyl groups would have happened. The process would most likely result from a secondary mode of radiation decomposition. It can be envisaged that in an aqueous environment this may lead to the formation of an alcohol or carbonyl compound as a by-product. It is likely that the process may not involve the simple interaction of N-acetyl-[4,5-^{3}H]-leucine with reactive solvent species but could be brought about by fragmentation of the starting material into radicals or ions.

The identification of the third radiation decomposition product of N-acetyl-[4,5-^{3}H]-leucine by radio-tlc analysis was not possible. The R_f value of the decomposition product was 0.37 (solvent 5:2:3 BuOH:AcOH:H_2O) and it gave a positive ninhydrin test. The product was unidentified due to the fact that none of the reference compounds selected had R_f values that exactly correlated with the radiation decomposition product. The reference compounds that had similar R_f values to the tritiated unknown were found to be those without an acetyl group. This observation would imply that the unknown may be an amino acid derivative.

The formation of a chemical shift at δ 4.8ppm was observed to occur in the ^{3}H nmr spectra of all buffer solutions of the tritiated N-acylamino acids. ^{3}H Nmr spectra of the samples after lyophiphilisation showed that the chemical shift had disappeared. Therefore, the chemical shift at δ 4.8ppm may indicate the presence of tritiated water. Tritiated water may be produced by loss of tritium from the labelled site in the molecule. Interaction of a reactive solvent species with the tritiated compound at a labelled site could bring about this situation. The amount of tritium associated with the chemical shift was found to be less than 10% for all tritiated N-acylamino acids.

Evidence for the formation of a volatile radiation decomposition products comes from the ^{3}H nmr studies of the storage of N-acetyl-[4,5-^{3}H]-leucine. The formation of a chemical shift at δ 2.2ppm was observed for samples of N-acetyl-[4,5-^{3}H]-leucine in buffer solutions. The new ^{3}H chemical shift could be associated with the change in enviroment of the tritiated methine group due to the cleavage of the C_{2}-C_{3} bond. The change in chemical shift of the tritiated methine group is therefore a result of molecular fragmentation and not due to the effect of the instassing the radiation decomposition of tritiated N-acylamino acids no attempt has been made to propose any reaction mechanisms. This is because several reactive species may be produced by the radiolysis of water. Hence the environment in which radiation decomposition of the labelled amino acid derivatives occurs is a complex system

in which several competing reaction processes could possibly be envisaged. It is considered that in carrying out these studies the identification of the radiation products was of primary importance. However, in explaining the formation of the products the most likely mode of radiation decomposition and type of reaction have been suggested.

There are several conclusions that can be drawn from the study of tritiated N-acylamino acids and peptides. The reduction of compounds that contain terminal vinyl groups, such as N-acetyl-4,5-didehydroleucine and N-acetyl-2,3-didehydroalanine methyl ester, with hydrogen-tritium mixtures yield products where the majority of tritium is found in the methyl group. The tritiation of other N-acyldidehydroamino acids led to products where the addition of tritium to the alkene group was more evenly distributed.

The labelling of N-acetyl-2,3-didehydrophenylalanine peptides has been investigated. In the case of N-acetyl-[2,3-^{3}H]-phenylalanyl-L-alanine (2.7Ci/mmol) about 8% of the tritium was found to be present in the ß-methyl group of the alanine residue. This implied that a hydrogen isotope exchange reaction was competing with the reduction of the alkene group. The tritiation of N-acetyl-2,3-didehydrophenylalanyl-L-phenylalanine gave the corresponding diastereomers. The tritiation of N-benzoyl-2,3-didehydrophenylalanylglycine gave a product that had a similar pattern to that of N-benzoyl-[2,3-^{3}H]-phenylalanine.

The radiation decomposition of tritiated N-acylamino acids in aqueous buffer solutions would seem to occur by secondary effects rather than by the direct radioactive disintegration of the tritium label. Evidence for decomposition by secondary modes comes from the discovery of tritiated hydroxyphenylalanine derivatives in samples of N-acetyl-[2,3-^{3}H]-phenylalanines after a period of storage. Deacetylation of the tritiated amino acid derivatives has been shown to occur by radio tlc analyses of buffer solutions.

The radiation decomposition of tritiated N-acylamino acids have been successfully followed by ^{3}H nmr spectroscopy. In all cases there is an induction period before any radiation decomposition is observed. This period may be due to a build up of solvent radicals. The greatest decomposition were observed for buffer solutions of N-acetyl-[4,5-^{3}H]-leucine while much slower rates were observed with samples of N-acyl-[2,3-^{3}H]-phenylalanines.

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CHAPTER TWO

The Development of a Biological Method for Labelling Amino Acids with Deuterium.

2.1 Introduction.

2.1.1 General.

The synthesis of compounds by chemical methods may sometimes result in a mixture of stereochemical products. Stereochemical mixtures are a major problem to the pharmaceutical industry, where current practice often demands that a drug should not only be chemically pure but also present only in one stereochemical form. In an attempt to overcome these problems chemists have used biochemical methods to synthesize desired products [1, 2].

The use of isotope exchange labelling to introduce deuterium or tritium into a molecule by chemical methods can lead to a mixture of generally labelled products. This can be a nuisance if the labelled molecule is to be used as a tracer or for studying reaction mechanisms. For these cases the desired compound would be of more value if it was labelled specifically and in one stereochemical form. Several attempts have been made to use biochemical methods to prepare specifically labelled compounds [3-5].

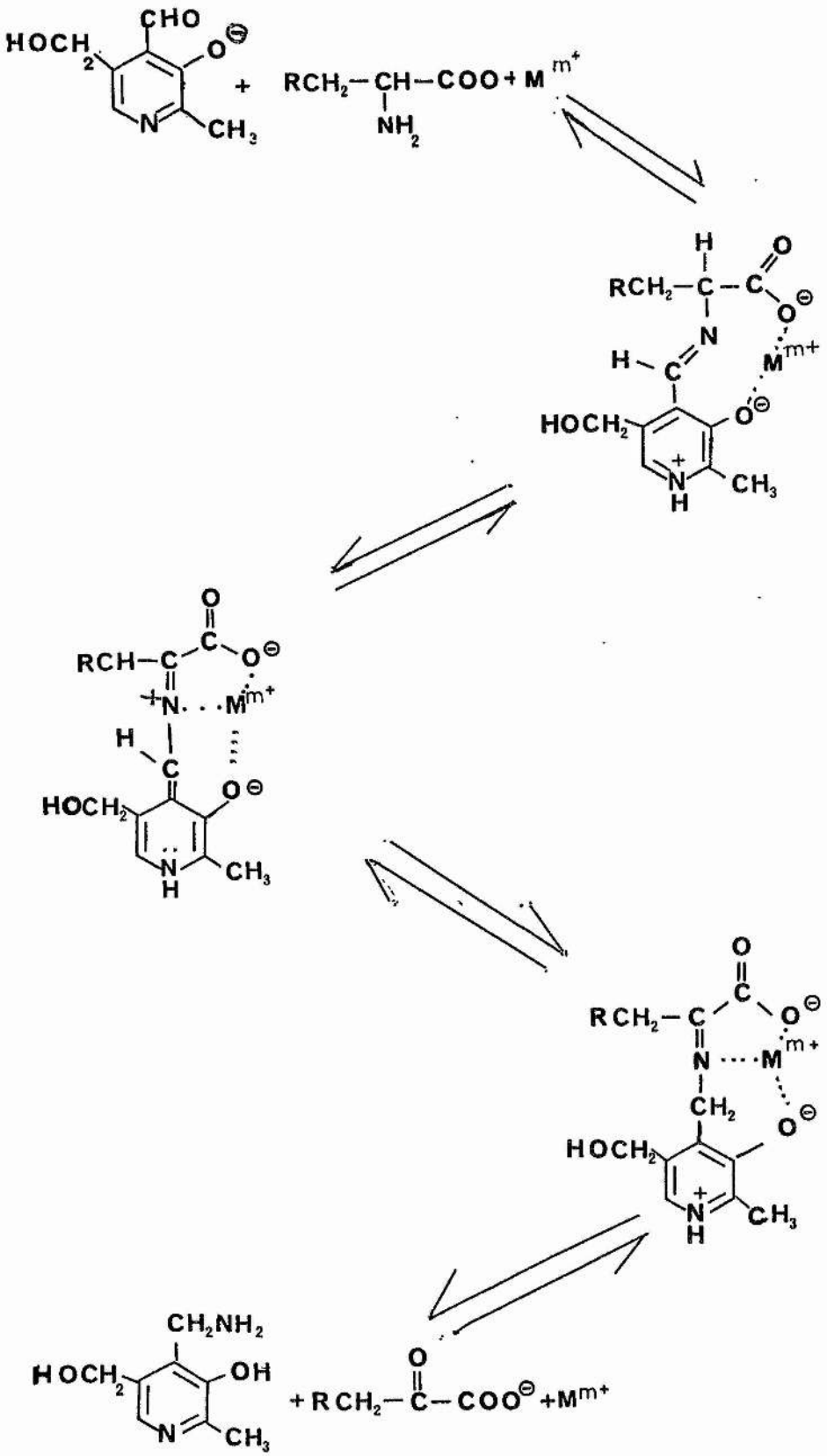
The work described in this chapter is concerned with the development of a biochemical method for labelling amino acids. The transaminase methionine-γ-lyase and bacteria Pseudomonas putida have been used as catalysts for the hydrogen isotope exchange of the α- and β-positions of amino acids.

2.1.2 The transamination process.

The transamination reaction was discovered by Braunstein in 1937[6] when L-glutamic acid and pyruvic acid were incubated with minced pigeon breast muscle to generate alanine and α-ketoglutaric acid. The reaction involves transfer of an amine group from an amino acid or an amine to a keto acid. The reaction is catalysed by enzymic [7-13] or non-enzymic processes [14-17]. Indeed the transamination reaction has been shown to be important in the synthesis of amino acids in plants, animals and microorganisms [18] with keto acids often being prepared as precursors.

The presence of pyridoxal phosphate as a coenzyme would seem to be essential to the transamination reaction. A non-enzymic model with copper, iron or zinc ions replacing the apoenzyme showed this certainly to be the case [19, 20]. The study of vitamin B_6 deficient rats revealed that the catalytic activity of the heart and kidney transaminases was increased by the addition of pyridoxal phosphate [21].

The mechanism of enzymatic transamination for α-amino acids was proposed by Braunstein et. al. [22] and Snell et. al. [23]. The reaction is shown in equation 2.1 and involves the formation of a Schiff's base between the pyridoxal coenzyme and the amino acid. This results in the labilisation of the α-hydrogen of the amino acid. The pyridoxal coenzyme is converted into pyridoxamine by transfer of the nitrogen of the amino acid and results in



Eqn. 2:

the formation of a keto acid. In the case of diamines or ω-amino acids the corresponding aldehydes are formed [8, 12].

In order to try to understand and utilise the transamination reaction, several substitutes for the pyridoxal coenzymes have been used. The stereochemistry of the reaction was studied by Dunathan et. al [24], who prepared both enantiomers of pyridoxamine in a monodeuterated form at the 4-methylene group. Kuzuhara [25] studied the transamination reaction using pyridoxamine analogs with planar chirality that were capable of enantio-face differentiation. Murakami et. al. [26] studied the effects of a pyridoxamine analog in vesicular and micellar phases. They found that the transamination of L-phenylalanine proceeded slowly in these phases but was greatly increased by the presence of metal ions, especially copper.

2.1.3 Classification of transaminases.

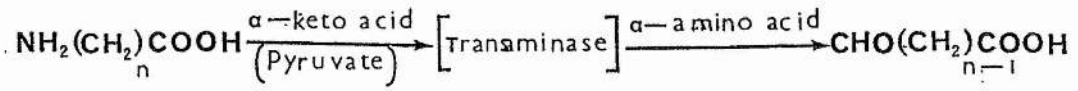
Transaminase enzymes are conveniently classified into three groups [12], as shown in equations 2.2, 2.3 and 2.4, according to the type of amino acid and/ or amine that they react with. However, it is important to appreciate that often the transaminases cannot be classified so clearly. The transaminases may promote more than one type of transamination process as well as other reactions [12]. Also they will often act as catalysts for a wide range of amino acids, amines and carbonyl compounds [12, 13].

Type 1 transaminases which convert α-amino acids into their corresponding keto acids

Eqn. 2.2

examples are methionine-γ-lyase and serine glyoxalate transaminase

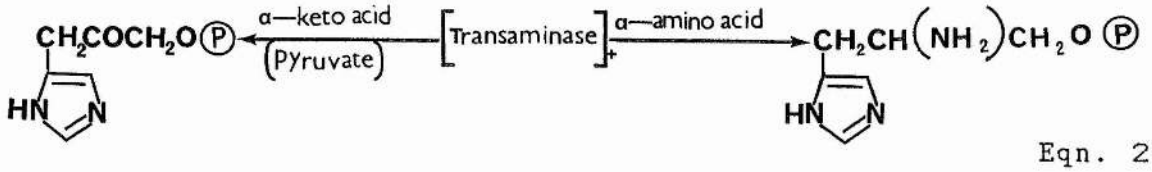
Type 2 transaminases which convert ω-amino acids and amines into their corresponding aldehydes.



Eqn. 2.3

examples are δ-aminovalerate, GABAT, diamine aminotransferase

Type 3 transaminases which are a miscellaneous group of enzymes that promote transamination for one substrate.

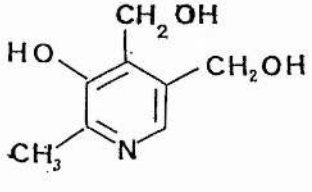


Eqn. 2.4

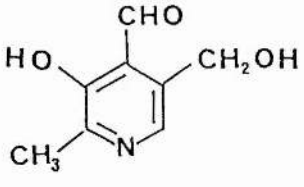
Transamination using whole cells has not been fully investigated[12]. It is considered that several transaminases may be present in cells. The enzymes may be multispecific and their roles could overlap several biochemical pathways. Two groups of aromatic transaminases which showed an overlap of different biochemical pathways were found to occur in Escherichia coli [12] and in Pseudomonas aeruginosa [27] respectively. In the latter case the biosynthesis of L-phenylalaine and L-tyrosine was shown to occur by catalysis of phenylpyruvate with five transaminases.

2.1.4 Pyridoxine coenzymes.

Pyridoxine, or vitamin B_6 as it is more commonly known, is one of the most important biochemicals [28, 29]. The derivatives of pyridoxine, pyridoxal and pyridoxamine, particularly in the form of phosphate esters, as discussed above are important coenzymes in the biological process. The structures of pyridoxine, pyridoxal and pyridoxamine are shown in figure 2.1.



pyridoxine



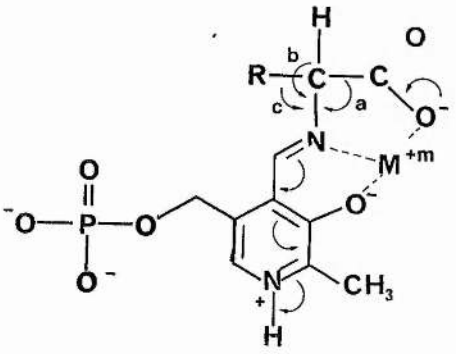
pyridoxal

pyridoxamine

Figure 2.1

The pyridoxine derivatives also act as coenzymes for several other enzymatic reactions [28, 29]. Some of these

reactions are summarised in equations 2.5-2.10. The pyridoxine derivatives act as "electron sinks" as electrons are delocalised away from the substrate into the aromatic ring of the Schiff's base. It is the direction of delocalisation that dictates the type of reaction that will occur (see figure 2.2).



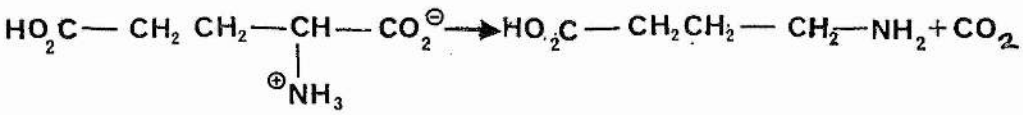
process a: leads to decarboxylation
process b: leads to transamination
process c: leads to aldol-type condensation and retrocondensation

Figure 2.2

The labilisation of the α-hydrogen of the substrate in the Schiff's base complex during transamination makes it possible to use the enzymes as catalysts for the labelling of amino acids with hydrogen isotopes. The study of hydrogen isotope exchange reactions of transaminases is briefly reviewed below.

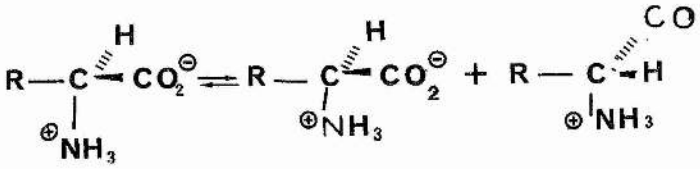
2.1.5 Hydrogen isotope exchange by transaminase enzymes.

The ability of some pyridoxal dependent enzymes to catalyse hydrogen isotope exchange with amino acids is the key feature to work described in this chapter. Grisolia [30] and Hilton et. al. [31] were among the first workers to demonstrate this phenomenon when they showed that glutamic-oxalacetic transaminase will catalyse the α-hydrogen exchange of glutamate. Evidence for ß-hydrogen exchange was reported by Oshima and Tamiya [32] when they labelled L-



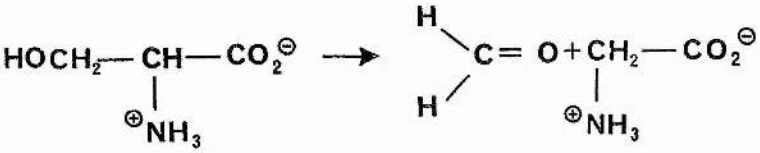
Eqn. 2.5

decarboxylation



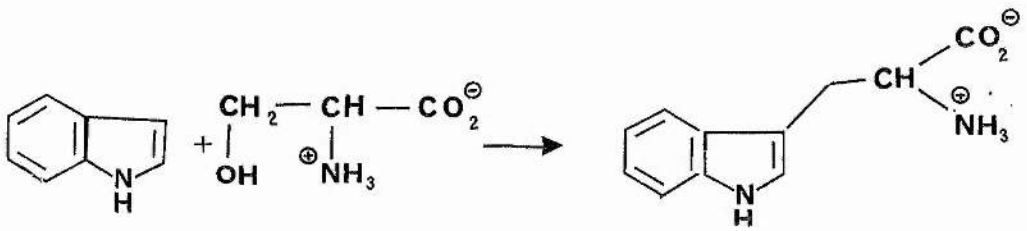
Eqn. 2.6

racemization



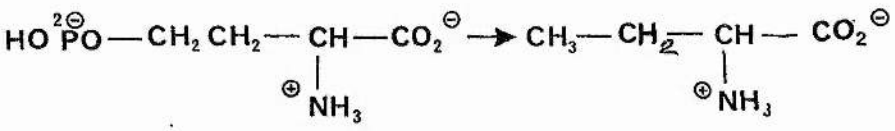
Eqn. 2.7

reverse condensation



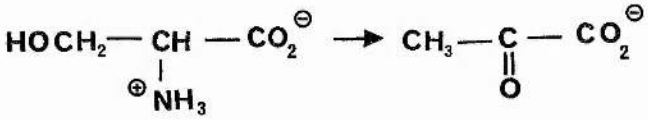
Eqn. 2.8

tryptophan synthesis



Eqn. 2.9

phosphate elimination-hydration



Eqn. 2.10

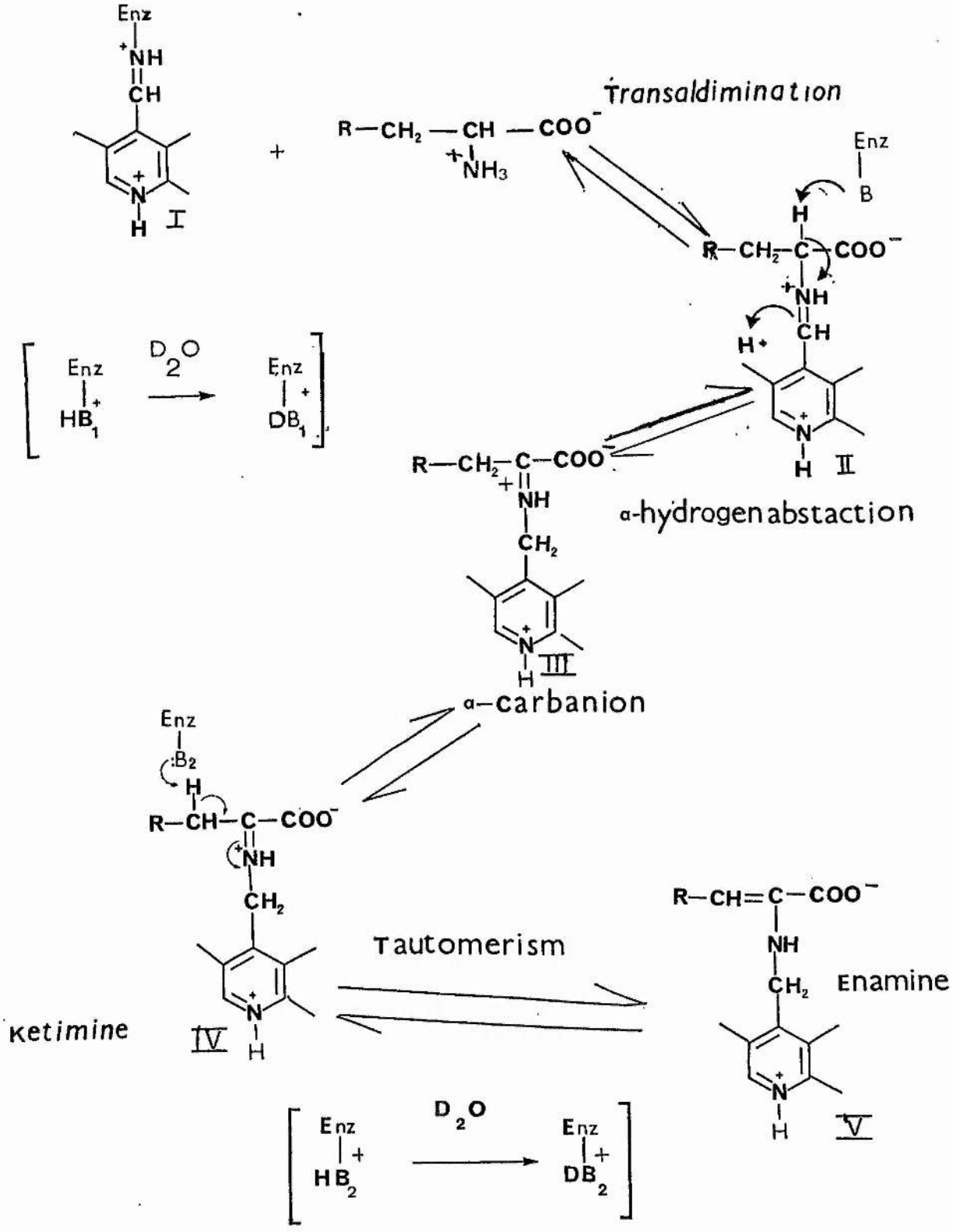
elimination-hydration

phenylalanine.

Esaki et. al [5] showed that methionine-γ-lyase was able to catalyse both the α-hydrogen and ß-hydrogen exchange of L-methionine and L-S-methylcysteine. They also found that the L-isomers of linear aliphatic α-amino acids underwent isotope exchange at the α- and ß-positions but no reaction was observed for branched chain α-amino acids. In the case of glycine, L-phenylalanine and L-tryptophan slow α-hydrogen exchange was observed.

The mechanism proposed by Esaki et. al [5] for the hydrogen isotope exchange labelling of α- and ß-positions of α-amino acids by transaminases is shown in equation 2.11. The first stage of the reaction involves the transaldimination of the coenzyme-enzyme Schiff's base (I) to the coenzyme-substrate Schiff's base (II). This is followed by the abstraction of the α-hydrogen by another enzyme-coenzyme Schiff's base to give an α-carbanion (iminium ion) intermediate (III). The protonated enzyme-coenzyme Schiff's base undergoes rapid isotope exchange in the presence of deuterated (or tritiated) solvent. Reversal of the reaction at this stage leads to the formation of an α-amino acid labelled in the α-position with deuterium (or tritium).

The labelling of the ß-position occurs via the tautomerism of the ketimine (IV) to enamine (V), after the ß-hydrogen is abstracted by an enzyme-coenzyme Schiff's


Transaldimination
α-hydrogen abstaction
α-carbanion
Tautomerism
Ketimine
Enamine

Eqn. 2.11

alanine at the α- and ß-positions using semi-purified glutamate-alanine transaminase. Nmr spectroscopy has been used by several authors [33-35] to study the latter reaction. Evidence from this work suggested that ß-hydrogen exchange occurred via tautomerism of a pyridoxamine-keto acid Schiff's base rather than by enolisation of the keto acid. Indeed kinetic studies by Cooper [34] showed that the rate limiting step of the reaction was the abstraction of the α-hydrogen from the amino acid. This implied that one of the protons of the amino acid could be exchanged from the amino acid onto the active site of the enzyme and back to the substrate while the other position undergoes isotopic exchange at the active site.

Several other reports about the enzymatic exchange labelling of amino acids have been made. Babu and Johnston[35] found that glutamic-oxalacetic transaminase also catalysed the α- and ß-positions of L-alanine. The same authors also found that glutamate-alanine transaminase exchanges one hydrogen of glycine. Both enzymes were found to label the α-positions of glutamate and aspartate with deuterium.

There is considerable confusion as to whether the ß-hydrogens of glutamate and aspartate are exchanged in the presence of α-keto acids by these enzymes [36-38]. Also a study by Gout et. al. [39] showed that aspartate transaminase, isolated from Escherichia coli, was able to catalyse the α-hydrogen but not the ß-hydrogen exchange of aspartate and

base. The protonated enzyme-coenzyme Schiff's base undergoes isotope exchange with the solvent deuterium (or tritium). Reversal of the reaction at this stage results in deuterium (or tritium) being added to the ß-position.

The above reports show that transaminases are useful catalysts for labelling amino acids by the hydrogen isotope exchange reaction. The enzymes are usually able to catalyse the labelling of more than one amino acid.

2.1.6 Pseudomonas putida.

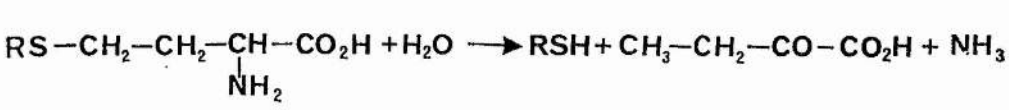
Pseudomonas putida [40, 41] has been used for this research to obtain the enzyme methionine-γ-lyase. The bacterium is found naturally in soil and water. The taxonomy of Pseudomonas putida is summarised in Table 2.1. The bacteria is fairly versatile and can be grown on a wide range of substrates, including most of the proteinogenic amino acids.

Pseudomonas putida whole cells have been used for several biochemical processes. The preparation of cis-diols from benzene and other aromatic compounds by catalytic action of Pseudomonas putida is carried out on an industrial scale [42]. This is a reaction that has yet to be successfully carried out using conventional chemistry methods. The cis-diol can be used to prepare inositol and polyphenylene.

Recent studies have been carried out on the adhesion of Pseudomonas bacteria to soil particles [43]. Evidence suggested that Pseudomonas fluorescens and Pseudomonas putida strains adhere strongly to sand and may involve the flagella. The results imply that Pseudomonas putida is suitable for whole cell immobilization.

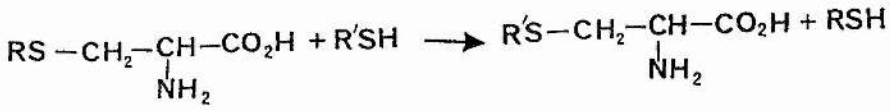
2.1.7 Transaminases of Pseudomonas putida.

There are two important transaminase enzymes that can be found in Pseudomonas putida. These are methionine-γ-lyase, which is a potential anti-tumor enzyme [44], and a branched-chain transaminase. The former is a type 1 transaminase, found in the cytoplasmic region of the bacteria. The enzyme has been purified by several authors [45-48]. Its ability to label the α- and ß-carbon positions of amino acids by hydrogen isotope exchange [5] has already been discussed. The enzyme is also capable of promoting several other reactions [49-51] such as α, γ-elimination, γ-replacement, α, ß-elimination and ß-replacement with sulphur containing amino acids and thiols, as shown in equations 2.12-2.13. The reactions were also shown to be possible when selenium was used in place of sulphur [52].



2.12

α, γ-elimination



Eqn. 2.13

The branched-chain transaminase belongs to the type 2 category and has been studied because of its ability to catabolise both L-and D-lysine [63-65]. The potential of the enzyme for catalysing hydrogen isotope exchanges with amino acids has not yet been assessed.

2.1.8 Whole cell and enzyme immobilization.

One of the major problems in carrying out reactions using biochemical processes is the recovery and reuseability of the biological catalyst [58]. Immobilization of the biological catalyst is a technique which overcomes these problems. The immobilized biocatalyst is physically confined or localized in a defined region of space but still retains its catalytic activity. Several reviews have been written about the methods of immobilization of cells and enzymes [57-58].

The most common method of cell immobilization is entrapment. The whole cells are physically confined to the pores of a polymeric carrier [60, 61]. Agar, polyacrylamides, cellulose and epoxides have all been used as support matrices. The method has the drawback in that often cell loading is poor and the support material may be sensitive to chemicals. Methods employing adsorption, flocculation,

covalent bonding and encapsulation have also been used to immobilize whole cells. The preferred methods of immobilization of enzymes involve covalent attachment or adsorption to a support.

The immobilization matrix should, in addition to being economically priced, meet the following criteria:

1. Loss in catalytic activity on immobilization should be kept low.
2. High cell or enzyme loading should be obtained.
3. Substrates and products should be able to diffuse freely through the biosupport.
4. The biosupport should have high mechanical strength and show negligible abrasion.
5. The biosupport should be thermally and chemically stable.
6. Regeneration of the biocatalyst should be possible.
7. The biosupport should have a high contact area with the surrounding medium.

2.1.9 Biofix biosupports.

Biofix is the name given to a range of ceramic biosupports[82] developed by English China Clays international limited (ECC) from the mineral kaolinite ($Al_2O_3.2SiO_2.2H_2O$). There are two grades of biofix designed for whole cell and enzyme immobilization respectively. The cell supports consist of hollow porous microspheres of interconnecting ceramic needles, which resemble birdsnests in appearance,

Table 2.1: Taxonomy of Pseudomonas Putida.

1. Member of the pseudomonas genus
2. A gram negative bacteria
3. Bacteria are rod shaped, non-sporulating and have polar flagella
4. Grows on a wide range of substrates
5. Does not hydrolyse gelatin
6. Incapable of denitrification
7. Forms fluorescent pigments
8. No strains grow above 41°C
9. Most strains do no not grow below 4°C
10. Maximum growth between 25-30°C
11. Subdivided into two biotypes- only biotype B utilizes L-tryptophan for growth
12. Dimensions: Length 2-4μm; width 0.1-0.2μm

Table 2.2: Properties of biofix C2 biosupport (ECC).

Hollow porous microspheres

Pore diameter	= 20μm
Mean pore size	= 900Å
Particle size	= 50-75μm
Surface area	= 9.5m^2/g
Bulk density	= 0.50g/cm
pH stability	= 1-14
Thermal stability	= 1000°C

that allow substrate and nutrients to flow freely in and out of the entrapped cell. Whereas the enzyme supports consist of porous microspheres that allow the ingress of enzymes and substrates.

Biofix cells supports have been used for the immobilization of Saccharomyces cerevisiae while the enzyme support has been used for immobilzation of trypsin and urease [82]. The supports give high levels of biocatalyst loading. In the case of the cell support uniform loading down a column can be achieved. Hence, biofix supports would seem to be suitable for the immobilization of methionine-γ-lyase and Pseudomonas putida, which as has been mentioned above shows good adherence properties to silicates. The immobolization of Pseudomonas putida to biofix C2 support has been studied in this work. The properties of Biofix C2 are shown in Table 2.2.

2.2 Experimental.

2.2.1 General.

2.2.1.1 Reagents.

All chemicals and solvents used for growth of Pseudomonas putida were autoclaved at 140°C prior to use. The exception to this was L-methionine which was sterilized by filtration through a millipore filter.

2.2.1.2 Apparatus.

All glassware and plastic apparatus used for the growth, cultivation and storage of Pseudomonas putida and methionine-γ-lyase were either autoclaved at 140°C prior to use or purchased in pre-sterilized packages.

2.2.1.3 Enzyme assay.

The activity of the enzyme methionine-γ-lyase was determined using the method of Ito et. al[46]. The method utilizes the reaction of methanethiol with 5, 5'- dithiobis-(2-nitrobenzoic) acid to form thionitrobenzoic acid. The formation of thionitrobenzoic acid can be followed conveniently by uv spectroscopy.

A fresh solution containing 5mM of L-methionine, 50μM of pyridoxal phosphate and 0.1M potassium pyrophosphate (pH

8.5) was prepared and designated the reaction solution. The enzyme assay reagent was prepared by dissolving 4mM of 5, 5'-dithiobis-(2-nitrobenzoic) acid in 50mM potassium phosphate buffer (pH 7).

The reaction solution (1ml) was incubated at 30°C for ten minutes. The enzyme sample (1ml) was added to the reaction solution. The methanethiol released from the reaction solution was collected in the enzyme assay reagent (1ml). After thirty minutes, 60% perchloric acid (0.1ml) was added to the reaction solution. The reaction solution was stood for a further twenty minutes at 30°C to allow complete absorption of all the methanethiol by the enzyme assay reagent.

The uv absorption of the enzyme sample was measured at 30°C using a fixed wavelength of 412nm. The sample was referenced to a blank solution of the enzyme assay reagent. The number of moles of methanethiol released by the enzyme was determined using the Beer-Lambert law and a molar extinction coefficient of $12000 l mol^{-1} cm^{-1}$ for thionitrobenzoic acid. One unit of enzyme activity for methionine-γ-lyase was defined as being equivalent to one μ mole of methanethiol [46].

2.2.1.4 Protein assay.

The bicinchoninic acid (BCA) protein assay kit [83] was used to determine protein concentration during the

purification of methionine-γ-lyase. The protein assay kit consists of two ready made reagents: Reagent A contains sodium carbonate, sodium bicarbonate, BCA detection reagent and sodium tartrate in 0.2M sodium hydroxide; Reagent B contains a 4% copper sulphate solution.

A working reagent was prepared by adding 50 parts of reagent A to 1 part of reagent B. Protein standards were prepared using albumin standard (BSA, 2mg/ml) at concentrations of 200-1200μg/ml inclusively. The working reagent (2mls) was added to each of the protein standards (1ml). The assay sample was prepared by taking a known volume of the extract of methionine-γ-lyase (25-1000μl) and making up to 1ml with doubly distilled water. Working reagent (2mls) was added to the assay sample. The solutions of the protein standards and assay sample were left for a minimum period of two hours and then their uv absorptions were measured at 562nm. The protein concentration of methionine-γ-lyase was determined from a plot of the uv absorption against concentration for the protein standards.

2.2.1.5 Plate counting.

The number of colonies of Pseudomonas putida were determined using a plate counting method [84]. The suspension of Pseudomonas putida in 50mM of potassium phosphate buffer (1ml) was added to a 10% peptone solution (9mls). A portion (1ml) of this solution was diluted by adding it to 10% peptone (9mls). Further samples were prepared in a likewise

manner until a final solution containing a 10^{8} fold dilution of the original bacteria cell concentration had been made. Six spots of the sample (20µl) were plated out onto a dry nutrient agar plate using a 50 dropper pipette. Four plates were used per sample. The process was repeated for all solutions of Pseudomonas putida.

The plates of Pseudomonas putida were stored at 30°C for one day. The colonies of bacteria per spot were counted for each sample. Only plates that contained spots with between 30 and 100 colonies were deemed accurate enough for counting. The concentation of Pseudomonas putida was determined as the number of bacteria colonies/ ml.

2.2.1.6 Centrifuge.

Bacteria cells and enzyme extracts were centrifuged at 4°C using a MSE HS18 high speed centrifuge.

2.2.1.7 Nmr spectroscopy.

^{1}H and ^{2}H Nmr spectra were run on a Bruker AC300E spectrometer. In some cases, particularly the kinetic studies, a water suppression method was used to improve the FID of ^{1}H spectra of the labelled amino acids. In the case of glycine and alanine the change in the integrals of the ^{1}H chemical shifts for the α- and/ or ß-positions were measured relative to an external standard of sodium acetate (6mg), which was added to the sample prior to analysis.

2.2.2 Growth and cultivation of Pseudomonas putida.

Pseudomonas putida bacteria were grown using the method of Ito et. al [48]. Pseudomonas putida cells (ATCC 2032) suspended in a 10% peptone solution were streaked onto horse blood enriched agar (Oxoid) plates. The plates were incubated at 30°C overnight. The Pseudomonas putida cells were scraped off the agar plates and grown in a culture medium of the following composition (w/v): 2.0% glycerol, 0.015% L-methionine, 0.001% $FeCl_3.6H_2O$, 0.17% $MgCl_2.6H_2O$, 0.01% $CaCl_2.2H_2O$, 0.35% $NaNH_4HPO_4.4H_2O$; in potassium phosphate buffer (pH7.0, 50mM). The bacteria cells were grown in 600 mls of the medium (6x100mls portions) on a platform shaker at 30°C for a period of 24 hours. The bacteria cells were centrifuged down at 9000rpm and the culture medium decanted off. The bacteria cells were then washed with buffered saline solution (pH7, 3x20mls) and then either used directly or stored at -20°C.

2.2.3 Cell disruption and enzyme extraction.

A suspension of Pseudomonas putida cells in potassium phosphate buffer solution (pH7, 50mM) were stood over ice and disrupted using a polytron shearing instrument (4x30s bursts). The cell debris was centrifuged down at 5000rpm. The crude extract of the enzyme was collected by decanting off the supernatent. The crude enzyme was either semi-purified or used directly in labelling studies.

2.2.4 Semi purification of methionine-γ-lyase.

2.2.4.1 Enzyme precipitation with ammonium sulphate.

20Mls of the crude extract of methionine-γ-lyase was stirred at 0°C for ten minutes. 4Mls of 20% streptomycin sulphate solution, adjusted to pH 7 with NaOH, was added dropwise. The precipitate that formed was removed by centrifugation. Solid $(NH_4)_2SO_4$ was added slowly to the supernatant. The solution was stirred for thirty minutes. The resulting precipitate was collected.

2.2.4.2 Enzyme purification by DEAE cellulose chromatography.

A DE32 cellulose ion exchange column was prepared as follows. The dry cellulose (30g) was pretreated by stirring with 0.5M HCl (450mls) and leaving for one hour. The supernatant was filtered off and the filtrate was washed with doubly distilled water until an intermediate pH 4 was obtained. The cellulose was stirred with 0.5M NaOH (450mls) and left for thirty minutes. The supernatant was removed and the cellulose was stirred with a second amount of 0.5M NaOH (450mls) and left for thirty minutes. The mixture was filtered and the filtrate washed with doubly distilled water until neutral pH was obtained.

The cellulose was stirred with 0.2M potassium phosphate for three minutes. The slurry was allowed to settle and the supernatant decanted off. The ion exchange material was

redispersed in buffer solution (1000mls) and allowed to settle for eighteen hours. The slurry was packed into the column and was washed with 0.2M potassium phosphate buffer at a flow rate of 45mls/hour until the column bed height was constant. The column was then washed with a 0.5M KCl buffer until the pH of the solution at the top and bottom of the column was equal.

The ammonium sulphate precipitate of the enzyme was dissolved in 0.2M potassium phosphate buffer and applied to the DE32 column without predialysis. The enzyme was eluted with 50ml of KCl buffer gradient from 0 to 0.5M, in steps of 10ml of 0.1M, at a flow rate of 40mls/hour. The eluent was collected in 10ml fractions. The fractions were combined and reduced in volume by evaporation to 5mls. The total protein content and enzyme activity were determined for the semi-purified methionine-γ-lyase.

2.2.5 Labelling studies using methionine-γ-lyase.

2.2.5.1 Attempted deuteration of amino acids using crude extracts of methionine-γ-lyase.

Amino acid (30mg), pyridoxal phosphate (1mg), methionine-γ-lyase (enzyme activity of 87units/mg) in 2mls of potassium phosphate buffer solution (pH7, 50mM) and 0.5mls of D_2O were left at 37°C for seven days. The product was freeze dried. The residue was repeatedly dissolved in water and freeze dried to remove the labile deuterium. The

product was then analysed by ^{1}H and ^{2}H nmr spectroscopy. Attempts were made to label glycine, L-alanine and L-methionine by this method. In all cases no incorporation of deuterium was observed.

2.2.5.2 Attempted deuteration of amino acids using semi-purified extracts of methionine-γ-lyase.

The method described above for the attempted deuteration of amino acids using a crude extract of the enzyme was repeated, with the exception that a post cellulose column extract of methionine-γ-lyase (enzyme activity of 1443units/mg) was used. The samples were worked up as above. Analysis by nmr spectroscopy showed the reactions were unsuccessful.

2.2.6 Labelling studies using Pseudomonas putida.

2.2.6.1 Deuteration of amino acids using D_2O.

Initial deuteration studies using Pseudomonas putida were carried out in the absence of a cofactor. However, it was found that insufficient incorporation of deuterium occured in these cases. The method below was used for further studies.

A mixture of amino acid (30mg), pyridoxal phosphate (5mg), Pseudomonas putida (approx. $3x10^{4} \pm 0.5x10^{4}$ bacteria colonies) and 50mM potassium phosphate in D_2O (0.5mls, pH 7)

were stored at 37°C for seven days. The bacteria cells were removed by filtration and the sample freeze dried. The sample was successively dissolved in water and freeze dried until all the labile deuterium had been removed. ^{1}H and ^{2}H nmr spectra of the sample was obtained.

2.2.6.2 Deuteration of amino acids using D_2O-H_2O mixtures.

After establishing that the deuteration of amino acids could be carried out in the presence of Pseudomonas putida and a pyridoxine coenzyme, it was decided to investigate the effect of using a D_2O-H_2O mixture as the solvent medium rather than D_2O.

Amino acid (30mg), pyridoxal phosphate (5mg), Pseudomonas putida (approx. $3x10^{4} \pm 0.5x10^{4}$ bacteria cells) and 50mM potassium phosphate (pH 7.0) in a mixture of H_2O (0.4mls)- D_2O (0.1mls) were stood at 37°C for seven days. The sample was worked up and analysed as described in section 2.2.6.1.

2.2.6.3 Labelling studies using different batches of Pseudomonas putida.

In order to verify the reproducibility of the labelling studies, it was decided to compare the deuteration of amino acids using different batches of Pseudomonas putida. Three batches of newly grown Pseudomonas putida were used to deuterate L-methionine using a H_2O-D_2O mixture as described

above in section 2.2.6.2.

2.2.7 Immobilization of Pseudomonas putida cells.

A biofix C2 column (5x1.5cm) was prepared by suspending the biosupport material (5g) in potassium phosphate buffer solution (pH7, 50mM, 20mls). The biofix C2 column was washed with potassium phosphate buffer solution (pH 7, 50mM, 200mls).

Pseudomonas putida cells were suspended in potassium phosphate buffer solution (pH7, 50mM, 100mls) and loaded onto the biofix C2 column at a flow rate of 5mls/min. The column was then washed with potassium phosphate buffer solution (2x200mls). The biocatalyst was stored at -20°C until required.

The efficiency of cell loading was measured by plate counting by taking colony counts for: (i) the initial and final cell concentrations for the loading solution; (ii) the eluted solution during loading at known time intervals and (iii) the eluent from washing the biofix column with buffer solution.

2.2.8 Labelling studies using immobilized *Pseudomonas putida* cells.

Since the deuteration of amino acids using whole cells had been shown to be clearly possible, then the next stage of the studies involved trying to recover and reuse the biocatalyst. Successful immobilization of *Pseudomonas putida* enabled labelling studies using bound cells to be undertaken. The following method was used.

Amino acid (30mg), pyridoxal phosphate (5mg), immobilized *Pseudomonas putida* (0.2g) and 50mM potassium phosphate in a mixture of H_2O (0.4mls)-D_2O (0.1mls) were stored at 37°C for seven days. The biocatalyst was removed by filtration. Labile deuterium was removed by several cycles of dissolving the sample in water followed by freeze drying. The product was analysed by ^1H and ^2H nmr spectroscopy.

2.2.9 Biocatalyst regeneration.

Biocatalyst, which had been previously used for labelling studies, was collected on a sterilized sintered funnel by gravity filtration. The biocatalyst (1g) was suspended in 10% peptone solution (10mls) and stood at 30°C overnight. The peptone solution was removed by gravity filtration. The biocatalyst was washed with potassium phosphate buffer (pH7, 50mM, 50mls).

In order to assess the viability of the regeneration process, the deuteration of L-methionine was carried out respectively in the presence of freshly prepared biocatalyst, regenerated biocatalyst and previously used biocatalyst. The standard labelling method, as described above, for immobilized Pseudomonas putida was followed.

2.2.10 Labelling studies using different coenzymes.

The effects of different coenzymes on the efficiency and labelling pattern of deuteration have been studied. Pyridoxine, pyridoxal phosphate and pyridoxal hydrochloride were used as coenzymes. The same labelling studies for amino acids deuterated with Pseudomonas putida, both as a free and immobilized cell, and pyridoxal phosphate were repeated but using the other two coenzymes instead.

2.2.11 Labelling studies using sugar solutions.

Sugar solutions were used to investigate the effect of cell dehydration on the ability of the bacteria to deuterate amino acids. A change in the osmotic pressure, due to dehydration of the bacteria, may result in disruption of the cell wall and could even inhibit some of the reactions catalysed by the bacteria's enzymes. Hence, these experiments were used to determine the suitability of Pseudomonas putida as a catalyst for isotope exchange studies in semi- and non-aqueous solvent systems. The deuteration of amino acids in 40% (w/v) sugar solutions were

studied by the following method. Solutions of sucrose, fructose and glucose were used for the study.

A mixture of amino acid (30mg), pyridoxal phosphate (5mg), immobilized Pseudomonas putida (0.1g), sugar solution (0.4mls) and D_2O (0.1ml) were stood at 37°C for seven days. The sample was worked up and analysed as described under section 2.2.8.

A Dowex 50 W column (15cm) was prepared by washing through with citric acid buffer (pH 3.4; 40mls of 0.1M citric acid and 10mls of 0.1M sodium citrate diluted to 100mls). The crude sample of deuterated amino acid was dissolved in formic acid-acetic acid buffer (pH 1.88, 5mls). The buffer solution was applied to the column. The column was washed with further portions of the formic acid-acetic acid buffer (3x10mls). The amino acid was eluted with pyridine-acetic acid buffer (pH6.5). The buffer solution was freeze dried and the sample analysed by ^1H and ^2H nmr spectroscopy.

2.2.12 Labelling studies using semi- and non-aqueous solvent systems.

The following semi- and non-aqueous solvent systems have been investigated as potential media for the deuteration of amino acids and their derivatives: 10% DMSO, 40% glycerol, 10% DMF, 10% ethanol, 50% ethanol, ethanol, chloroform, tetrahydrofuran, DMSO and DMF.

A mixture of amino acid (30mg), pyridoxal phosphate (5mg), immobilized Pseudomonas putida (0.1g), solvent (0.5mls) and D_2O (50µl) were stood at 37°C for seven days. The sample was worked up and analysed as described under section 2.2.8.

2.2.13 Qualitative assessment of the effects of inorganic salts on the deuteration of amino acids.

The effect of metal ions on the amino acid-enzyme and amino acid-coenzyme Schiff's bases has been assessed in terms of the labelling pattern and efficiency of deuteration of amino acids. The following inorganic salts were used for the study: iron (III) chloride, calcium chloride, magnesium chloride, copper (II) chloride and zinc acetate.

Amino acid (30mg), pyridoxal phosphate (5mg), D_2O (0.1ml), 10% (w/v) inorganic salt solution (0.4ml) and biocatalyst (0.1g) were stood at 37°C for seven days. The sample was worked up and analysed as described in section 2.2.8.

2.2.14 Kinetic studies.

The deuteration of glycine esters by Pseudomonas putida has been studied by ^1H nmr spectroscopy. The loss in the ^1H chemical shifts of the α-positions of the amino acids were followed at 37°C as a function of time. Glycine methyl ester and glycine ethyl ester have been studied. The method below

was used.

Amino esters (20mg), pyridoxal phosphate (5mg), 50mM potassium phosphate in d_6-dmso (0.5mls), D_2O (50µl) and Pseudomonas putida (approx. $3x10^4 \pm 0.5x10^4$ bacteria colonies) were placed in a 5mm nmr tube. The ^1H nmr spectra were obtained at 37°C using an automated solvent suppression technique. ^1H nmr spectra were recorded at ten minute intervals for 3 hours. Subsequent ^1H nmr spectra were obtained for samples that were seven days old and had been stored at 37°C.

The integrals of the chemical shifts for the α-positions of the amino acids were measured. The change in the integrals were plotted as a function of time and the first order rate constants have been determined for the amino acids.

2.3 Results.

2.3.1 General.

The results obtained for protein and enzyme assays of the crude and semi-purified extracts of methionine-γ-lyase are summarised in sections 2.3.2. The results of plate counting experiments for determining the number of bacteria colonies are summarised in section 2.3.3.

The deuteration of amino acids by using free and immobilized whole cells of Pseudomoas putida are summarised in sections 2.3.4-2.3.9. The results of the labelling studies have been tabulated in terms of the percentage distribution of deuterium in the amino acids and the degree of incorporation at the sites of isotope exchange. The former results were obtained directly from the integration of the ^{2}H nmr spectra of the deuterated amino acids. The amount of deuterium incorporated was determined from the integrals of the ^{1}H nmr spectra of the deuterated amino acids and has been expressed as a percentage of the total hydrogen atoms at a particular position.

The following amino acids and amino acid derivatives were found not to undergo isotope exchange labelling with deuterium using the methods described in section 2.2.

(i) L-α-amino acids: valine, isoleucine, tyrosine, serine, threonine, tryptophan, proline, hydroxyproline, aspartic

acid, glutamic acid, β-hydroxyglutamic acid, ornithine, histidine, arginine and lysine.

(ii)ω-amino acids: β-alanine, γ-aminobutyric acid and 6-aminohexanoic acid.

(iii) Sulphur-containing L-α-amino acids: cysteine and cystine.

(iv) D-α-amino acids: alanine, leucine and methionine.

(v) DL-amino acids: alaninine, leucine, norleucine, phenylalanine and methionine.

(vi) N-substituted amino acids: N-acetylalanine, N-acetylvaline, N-acetylleucine, N-acetylphenylalanine, N-benzoylvaline, N-benzoylphenylalanine, N-methylalanine and N-methylmethionine.

(vii) Dipeptides: glycylglycine and glycylalanine.

2.3.2 Enzyme specific assay results for crude and semi-purified extracts of methionine-γ-lyase.

Table 2.3: Enzyme specific assay results

Sample no.	1	2	3	4	5	6
Crude enzyme						
Absorbance (562nm)	1.12	1.18	1.23	1.20	1.31	1.20
Protein conc. (mgl^{-1})	410	460	500	410	550	410
Absorbance (412nm)	1.41	1.35	1.39	1.41	1.37	1.43
Total enzyme activity /batch of Ps. putida (units x 10^{-3})	117	112	115	117	114	119
Enzyme specific activity (units 10^{-3}/mg Protein)	285	243	230	285	207	291
Semi-purified enzyme						
Absorbance (562nm)	0.58	0.57	0.59	0.57	0.58	0.61
Protein conc. (mgl^{-1})	207	206	208	206	207	215
Absorbance (412nm)	0.41	0.42	0.57	0.47	0.51	0.40
Total enzyme activity /batch of Ps. putida (units x 10^{-3})	138	140	188	156	171	133
Enzyme specific activity (units 10^{-3}/mg Protein)	667	680	904	761	830	619

2.3.3 Plate counting results for samples of *Pseudomonas putida*.

Table 2.4: Bacterial colonies of P. putida cultures

Batch No.	Average Colony count / ml of peptone solution	Number of bacterial colonies /600ml of culture media
A	$32 \pm 8 \times 10^3$	$19.2 \pm 4.8 \times 10^6$
B	$46 \pm 15 \times 10^3$	$27.6 \pm 9.0 \times 10^6$
C	$36 \pm 6 \times 10^3$	$21.6 \pm 3.6 \times 10^6$

Table 2.5: Efficiency of bacteria cell immobilization

Sample	Colony count/ml	Sample volume / ml	Total number of bacteria colonies
Initial load solution concentration	44×10^4	100	44×10^6
1st eluent sample	32×10^4	50	16×10^6
2nd eluent sample	36×10^4	50	18×10^6
1st wash sample	78×10^3	100	78×10^5
2nd wash sample	2×10^3	100	2×10^5
Immobilized bacteria concentration / 4.5g biofix			2.2×10^6
Immobilized bacteria concentration / g biofix			5×10^5
Efficiency of bacteria cell immobilization	=	5%	

2.3.4 Deuteration of amino acids using Pseudomonas putida and pyridoxal phosphate.

Table 2.6: Studies using sulphur-containing L-α-amino acids (Labelling pattern of amino acids from ^{2}H nmr spectra)

Amino acid	position	Chemical shift δ ppm	Distribution of deuterium (%)
Methionine	α	3.9	64
	β	2.2	36
Ethionine	α	3.9	64
	β	2.2	36
S-Methylcysteine	α	3.9	64
	β	3.1	36

Table 2.7: Studies using sulphur-containing L-α-amino acids (Degree of ^{2}H incorporation)

Amino acid	position	% of ^{2}H incorporated into stable positions by P. putida in the form of	
		free cells	bound cells
Methionine	α	26	76
	β	7	21
Ethionine	α	18	67
	β	5	19
S-Methylcysteine	α	13	43
	β	4	12

Table 2.8: Studies using other L-α-amino acids

(Labelling pattern of amino acids from ^{2}H nmr spectra)

Amino acid	position	Chemical shift δ ppm	Distribution of deuterium (%)
Glycine	α	3.5	100
Alanine	α	3.6	83
	ß	1.4	17
Norvaline	α	4.0	87
	ß	2.6	13
Leucine	α	4.0	100
Norleucine	α	4.0	100
Phenylalanine	α	4.0	100
Glycine methyl ester	α	3.8	100
Glycine ethyl ester	α	3.8	100

Table 2.9: Studies using other L-α-amino acids

(Degree of ^{2}H incorporation)

Amino acid	position	% of ^{2}H incorporated into stable positions by *P. putida* in the form of free cells	bound cells
Glycine	α	18	79
Alanine	α	20	63
	ß	1	5
Norvaline	α	12	57
	ß	1	5
Leucine	α	17	46
Norleucine	α	21	41
Phenylalanine	α	13	41
Glycine methyl ester	α	11	63
Glycine ethyl ester	α	11	58

Table 2.10: Biocatalyst regeneration studies

(Deuteration of methionine)

Sample	Methionine position	% of ^{2}H incorporated into stable positions	Description of the immobilized *Pseudomonas putida*
1	α	77	Freshly
	ß	21	prepared
2	α	14	Previously
	ß	3	used
3	α	48	Regenerated
	ß	11	

Table 2.11: Labelling studies using different batches of Pseudomonas putida

(Deuteration of methionine)

Sample	Methionine position	% of ^2H incorporated into stable positions	Batch of Pseudomonas putida
1	α	28	A
	ß	8	
2	α	33	B
	ß	9	
3	α	25	C
	ß	7	
Average	α	29	
	ß	8	

2.3.5 Deuteration of amino acids using Pseudomonas putida and pyridoxal hydrochloride.

Table 2.12: Studies using sulphur-containing L-α-amino acids

(Labelling pattern of amino acids from ^2H nmr spectra)

Amino acid	position	Chemical shift δ ppm	Distribution of deuterium (%)
Methionine	α	3.9	70
	ß	2.2	13
	ß (Keto acid)	2.0	17
Ethionine	α	3.9	64
	ß	2.2	36
S-Methylcysteine	α	3.9	64
	ß	3.1	36

Table 2.13: Studies using sulphur-containing L-α-amino acids

(Degree of ^{2}H incorporation)

Amino acid	position	% of ^{2}H incorporated into stable positions by P putida in the form of	
		free cells	bound cells
Methionine	α	9	18
	β	2	4
Ethionine	α	16	35
	β	4	9
S-Methylcysteine	α	7	27
	β	2	8

Table 2.14: Studies using other L-α-amino acids.

(Labelling pattern of amino acids from ^{2}H nmr spectra)

Amino acid	position	Chemical shift δ ppm	Distribution of deuterium (%)
Glycine	α	3.5	100
Alanine	α	3.6	21
	β	1.4	79
Norvaline	α	4.0	7
	β	2.6	93
Leucine	α	4.0	100
Norleucine	α	4.0	100
Glycine methyl ester	α	3.8	100
Glycine ethyl ester	α	3.8	100

Table 2.15: Studies using other L-α-amino acids

(Degree of ^{2}H incorporation)

Amino acid	position	% of ^{2}H incorporated into stable positions by *P. putida* in the form of	
		free cells	bound cells
Glycine	α	11	22
Alanine	α	3	4
	β	4	4
Norvaline	α	1	1
	β	6	8
Leucine	α	3	5
Norleucine	α	3	9
Glycine methyl ester	α	7	14
Glycine ethyl ester	α	4	19

2.3.6 Deuteration of amino acids using Pseudomonas putida and pyridoxine.

Table 2.16: Studies using sulphur-containing L-α-amino acids

(Labelling pattern of amino acids from ^2H nmr spectra)

Amino acid	position	Chemical shift δ ppm	Distribution of deuterium (%)
Methionine	α	3.9	66
	β	2.2	34
Ethionine	α	3.9	62
	β	2.2	38
S-Methylcysteine	α	3.9	67
	β	3.1	33

Table 2.17: Studies using sulphur-containing L-α-amino acids

(Degree of ^2H incorporation)

Amino acid	position	% of ^2H incorporated into stable positions by P. putida in the form of	
		free cells	bound cells
Methionine	α	23	67
	β	6	17
Ethionine	α	34	63
	β	10	17
S-Methylcysteine	α	14	52
	β	4	13

Table 2.18: Studies using other L-α-amino acids

(Labelling pattern of amino acids from ^2H nmr spectra)

Amino acid	position	Chemical shift δ ppm	Distribution of deuterium (%)
Glycine	α	3.5	100
Alanine	α	3.6	85
	ß	1.4	15
Norvaline	α	4.0	89
	ß	2.6	11
Leucine	α	4.0	100
Norleucine	α	4.0	100
Phenylalanine	α	4.0	100
Glycine methyl ester	α	3.8	100
Glycine ethyl ester	α	3.8	100

Table 2.19: Studies using other L-α-amino acids

(Degree of ^{2}H incorporation)

Amino acid	position	% of ^{2}H incorporated into stable positions by *P. putida* in the form of	
		free cells	bound cells
Glycine	α	15	57
Alanine	α	31	45
	β	2	3
Norvaline	α	22	43
	β	2	3
Leucine	α	25	46
Norleucine	α	21	39
Phenylalanine	α	23	41
Glycine methyl ester	α	18	47
Glycine ethyl ester	α	23	54

2.3.7 Deuteration of amino acids in sugar solutions.

Table 2.20: Deuteration of methionine in sugar solutions

Sample	Methionine position	% of ^{2}H incorporated into stable positions	40%(w/v) sugar solution
1	α	53	Fructose
	β	14	
2	α	44	Sucrose
	β	12	
3	α	47	Glucose
	β	10	

2.3.8 Labelling studies in semi- and non-aqueous solvents.

Table 2.21: Deuteration of glycine esters in non-aqueous solvents

Amino acid	Position	% of ^{2}H incorporated into stable positions	Non-aqueous solvent
Glycine methyl ester	α	negative	chloroform
Glycine ethyl ester	α	negative	"
Glycine methyl ester	α	75	DMSO
Glycine ethyl ester	α	68	"
Glycine methyl ester	α	67	DMF
Glycine ethyl ester	α	68	"
Glycine methyl ester	α	negative	ethanol
Glycine ethyl ester	α	negative	ethanol
Glycine methyl ester	α	negative	THF
Glycine ethyl ester	α	negative	THF

Table 2.22: Deuteration of amino acids in semi-aqueous solutions

Amino acid	Position	% of ^{2}H incorporated into stable positions	Semi-aqueous solution
Glycine	α	47	10% DMSO
Alanine	α	61	"
	β	4	
Methionine	α	56	"
	β	18	
Glycine	α	53	10% DMF
Alanine	α	52	"
	β	4	
Methionine	α	60	"
	β	16	
Glycine	α	35	40% Glycerol
Alanine	α	54	"
	β	4	
Methionine	α	71	"
	β	19	
Glycine	α	negative	10% Ethanol
Alanine	α	negative	"
	β	negative	
Methionine	α	negative	"
	β	negative	
Glycine	α	negative	50% Ethanol
Alanine	α	negative	"
	β	negative	
Methionine	α	negative	"
	β	negative	

2.3.9 Deuteration of amino acids in 10% inorganic salt solutions.

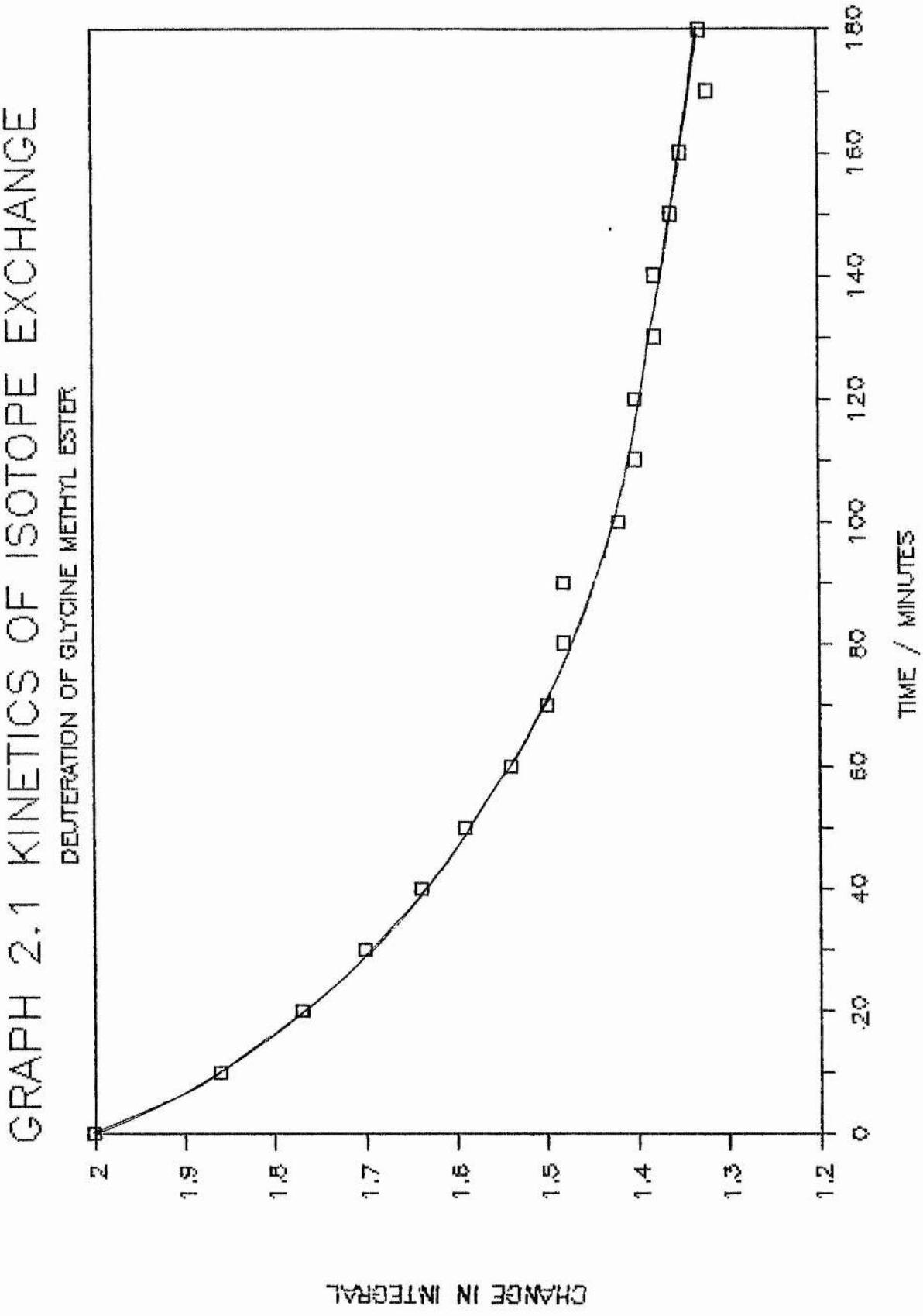
Table 2.23: Deuteration of amino acids in inorganic salt solutions

Amino acid	Position	% of ^{2}H incorporated into stable positions	10%(w/v) inorganic salt solution
Glycine	α	55	$FeCl_3$
Alanine	α	63	"
	β	5	
Methionine	α	68	"
	β	18	
Glycine	α	63	$CaCl_2$
Alanine	α	54	"
	β	3	
Methionine	α	65	"
	β	18	
Glycine	α	63	$MgCl_2$
Alanine	α	57	"
	β	3	
Methionine	α	68	"
	β	20	
Glycine	α	62	$CuCl_2$
Alanine	α	50	"
	β	4	
Methionine	α	73	"
	β	16	
Glycine	α	59	$ZnAc_2$
Alanine	α	56	"
	β	4	
Methionine	α	64	"
	β	19	

2.3.10 Some kinetic data for the deuteration of amino acids by Pseudomonas putida.

Table 2.24: Studies using glycine methyl ester in d_6-DMSO

Time (min)	Change in the ^1H chemical shift of the α-position integral (I)	Log_e (I-I∞)
0	2.00	0.418
10	1.86	0.322
20	1.77	0.255
30	1.70	0.199
40	1.64	0.148
50	1.59	0.104
60	1.54	0.058
70	1.50	0.020
80	1.48	0.000
90	1.48	0.000
100	1.42	-0.062
110	1.40	-0.083
120	1.40	-0.083
130	1.38	-0.105
140	1.38	-0.105
150	1.36	-0.128
160	1.35	-0.139
170	1.32	-0.174
180	1.33	-0.162
10000 (I∞)	0.48	


GRAPH 2.1 KINETICS OF ISOTOPE EXCHANGE
DEUTERATION OF GLYCINE METHYL ESTER
CHANGE IN INTEGRAL
2
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
0
20
40
60
80
100
120
140
160
180
TIME / MINUTES

GRAPH 2.2 KINETICS OF ISOTOPE EXXCHANGE

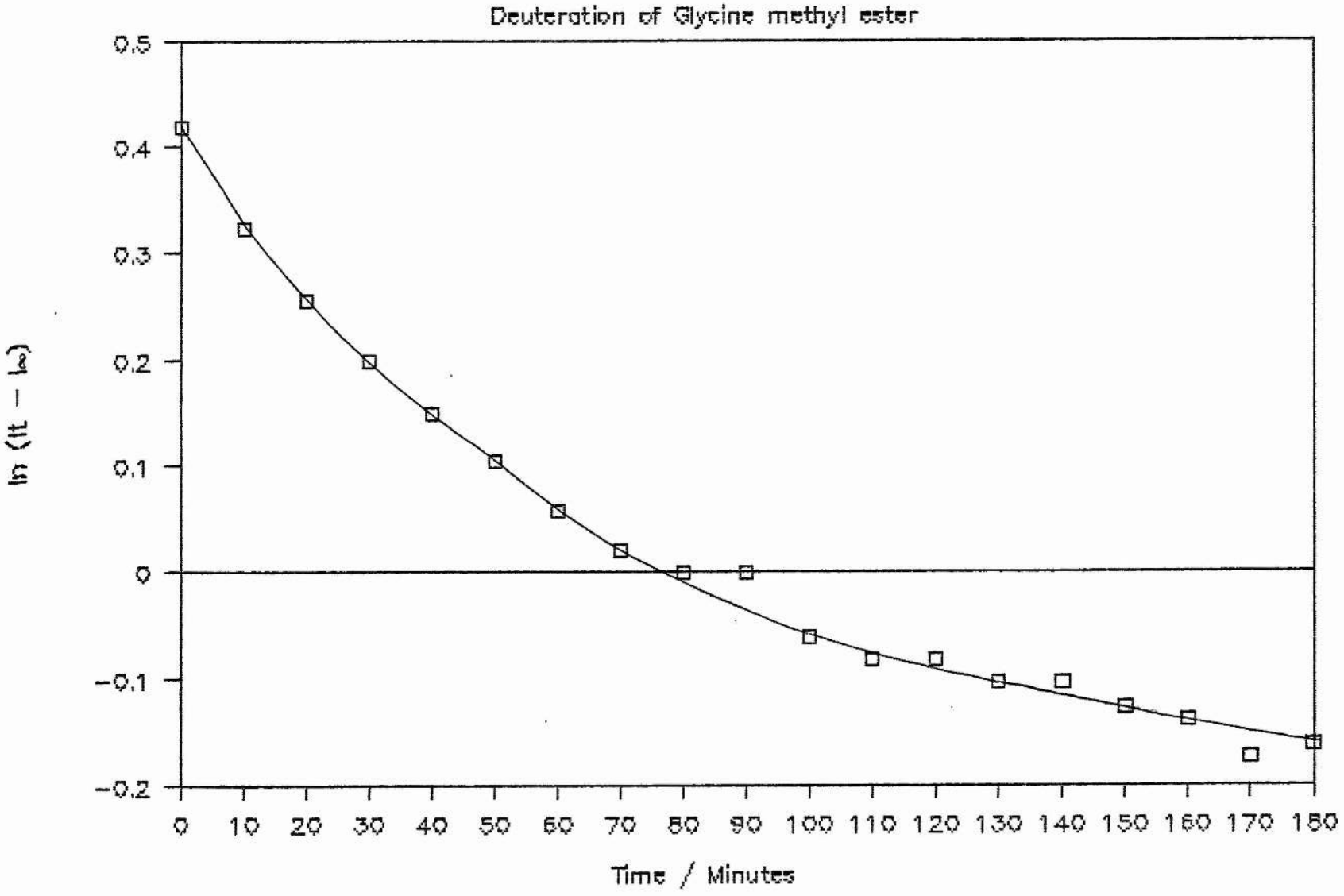
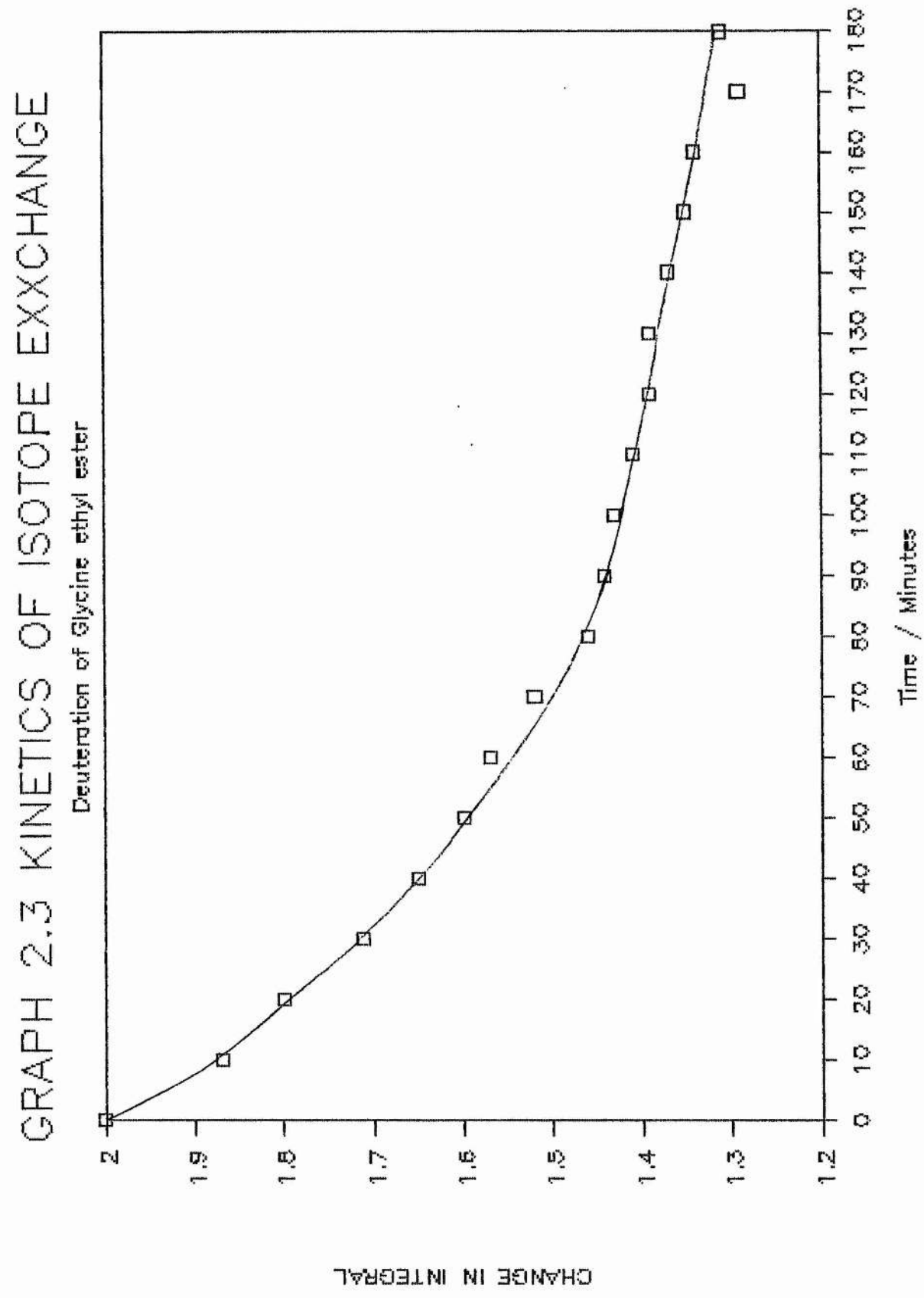


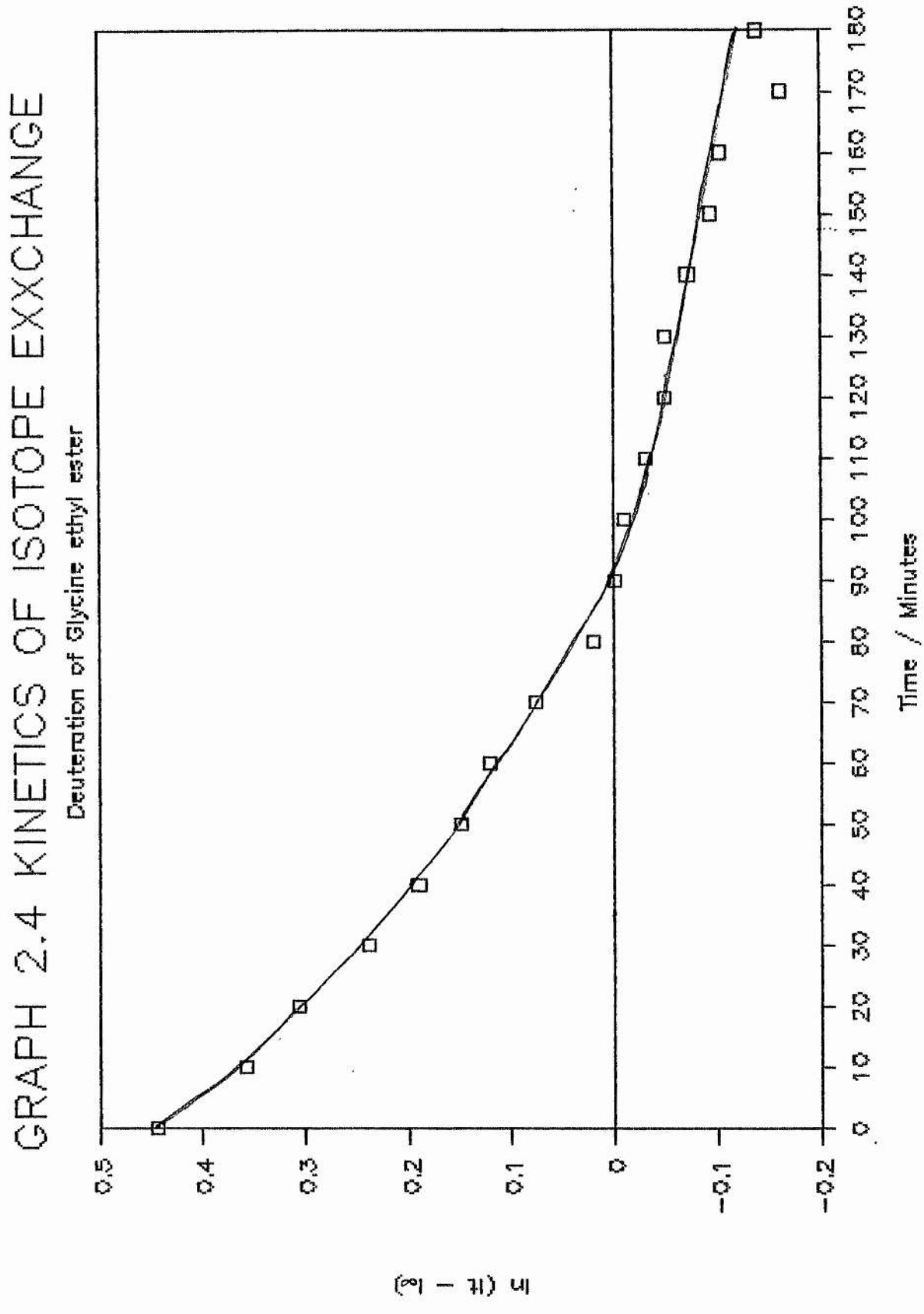
Table 2.25: Studies using glycine ethyl ester in d_6-DMSO

Time (min)	Change in the ^1H chemical shift of the α-position integral (I)	Log_e $(I-I_\infty)$
0	2.00	0.444
10	1.87	0.357
20	1.80	0.307
30	1.71	0.239
40	1.65	0.190
50	1.60	0.148
60	1.57	0.122
70	1.52	0.077
80	1.46	0.020
90	1.44	0.000
100	1.43	-0.010
110	1.41	-0.030
120	1.39	-0.051
130	1.39	-0.051
140	1.37	-0.072
150	1.35	-0.094
160	1.34	-0.105
170	1.29	-0.162
180	1.31	-0.139
10000 (7 days)	0.44	

Table 2.26: Rates of deuteration of amino acids

Amino acid	First Order Rate constant (k) (min^{-1})	(s^{-1})
Glycine methyl ester	5.21×10^{-3}	8.68×10^{-5}
Glycine ethyl ester	4.64×10^{-3}	7.73×10^{-5}


GRAPH 2.3 KINETICS OF ISOTOPE EXXCHANGE
Deuteration of Glycine ethyl ester
CHANGE IN INTEGRAL
2
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180
Time / Minutes


GRAPH 2.4 KINETICS OF ISOTOPE EXXCHANGE
Deuteration of Glycine ethyl ester
ln (lt − l∞)
0.5
0.4
0.3
0.2
0.1
0
−0.1
−0.2
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180
Time / Minutes

2.3.11 Some examples of the ^{1}H and ^{2}H nmr spectra obtained for deuterated amino acids.

The following pages contain some examples of the ^{1}H and ^{2}H nmr spectra that have been obtained for the deuterated amino acids. Although a complete collection of spectra is not shown, it is hoped that those chosen will illustrate the results that were obtained using the deuteration methods employed.

Figure 2.3 ^{1}H nmr spectrum of methionine.
(reference sample)

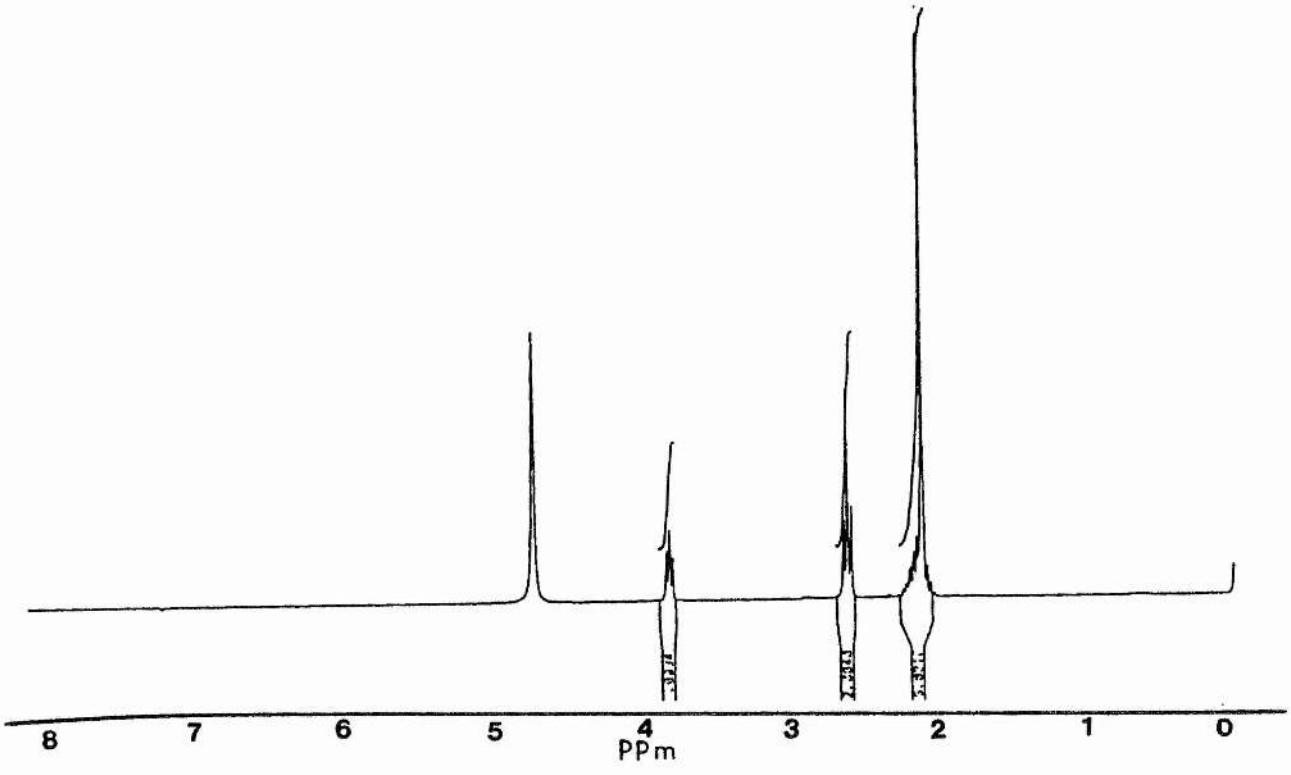


Figure 2.4 ^{1}H nmr spectrum of [2, 3-^{2}H]-methionine.
(pyridoxal phosphate used as coenzyme)

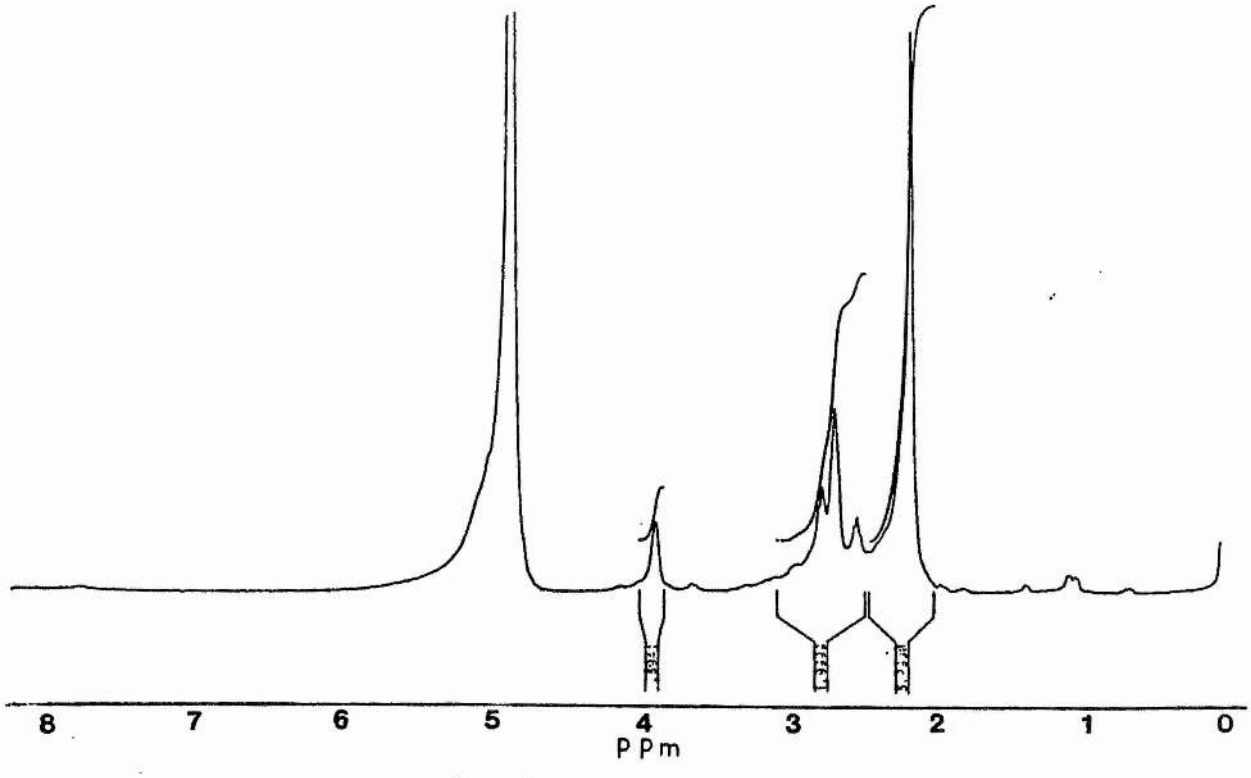


Figure 2.5 ^{2}H nmr spectrum of [2, 3-^{2}H]-methionine.
(pyridoxal phosphate used as coenzyme)

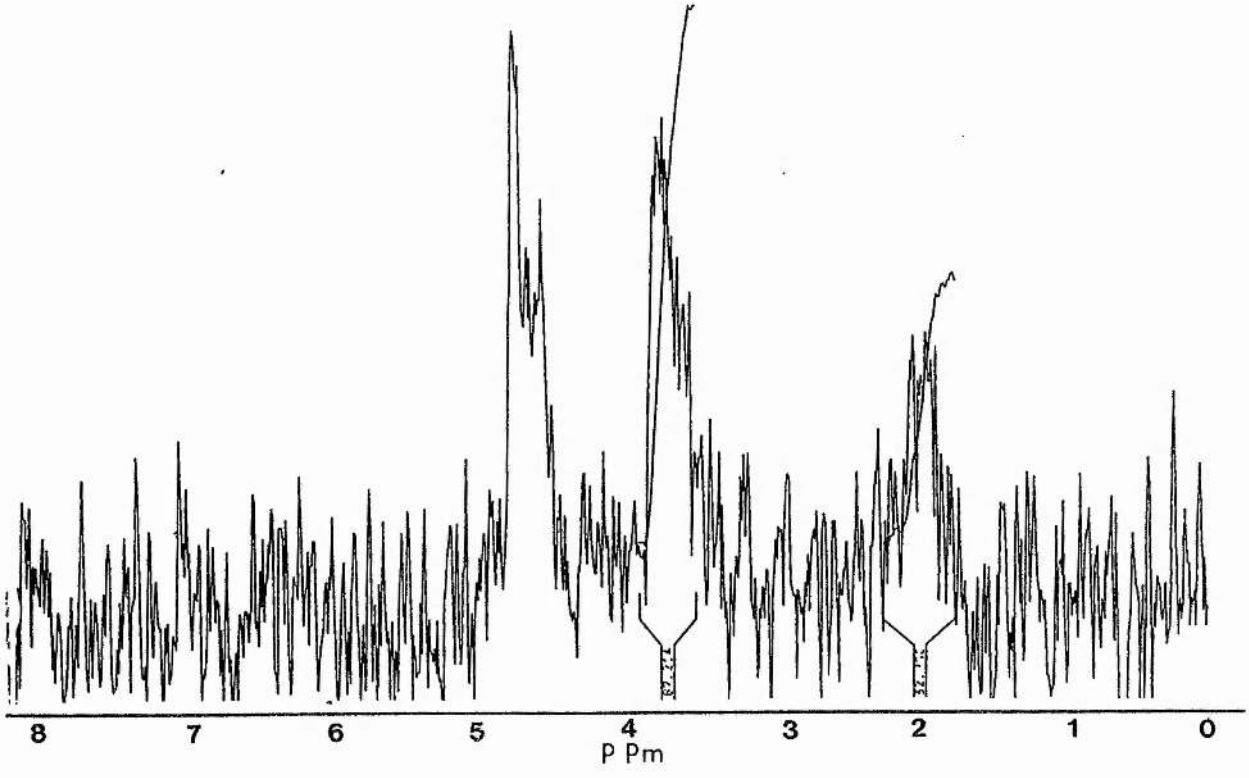


Figure 2.6 ^{2}H nmr spectrum of [2, 3-^{2}H]-methionine.
(pyridoxal hydrochloride used as coenzyme)

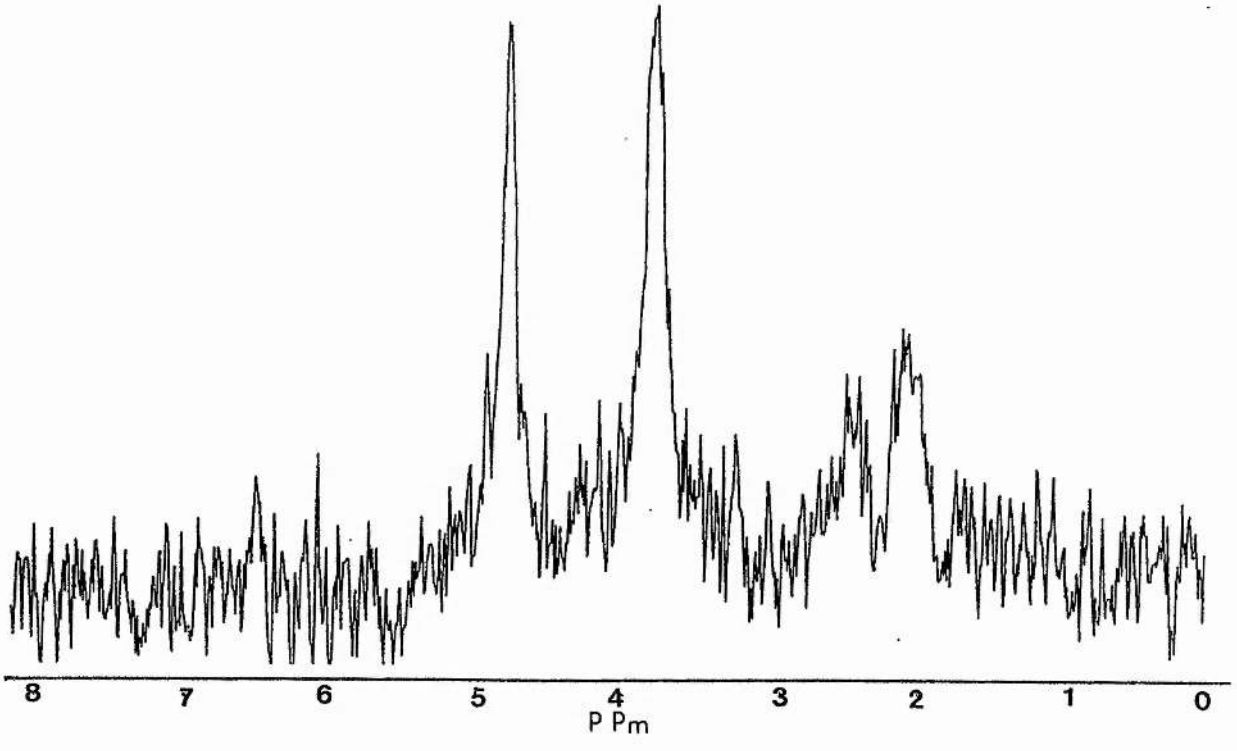


Figure 2.7 ^{2}H nmr spectrum of [2, 3-^{2}H]-methionine.
(labelled in 40% glucose solution)

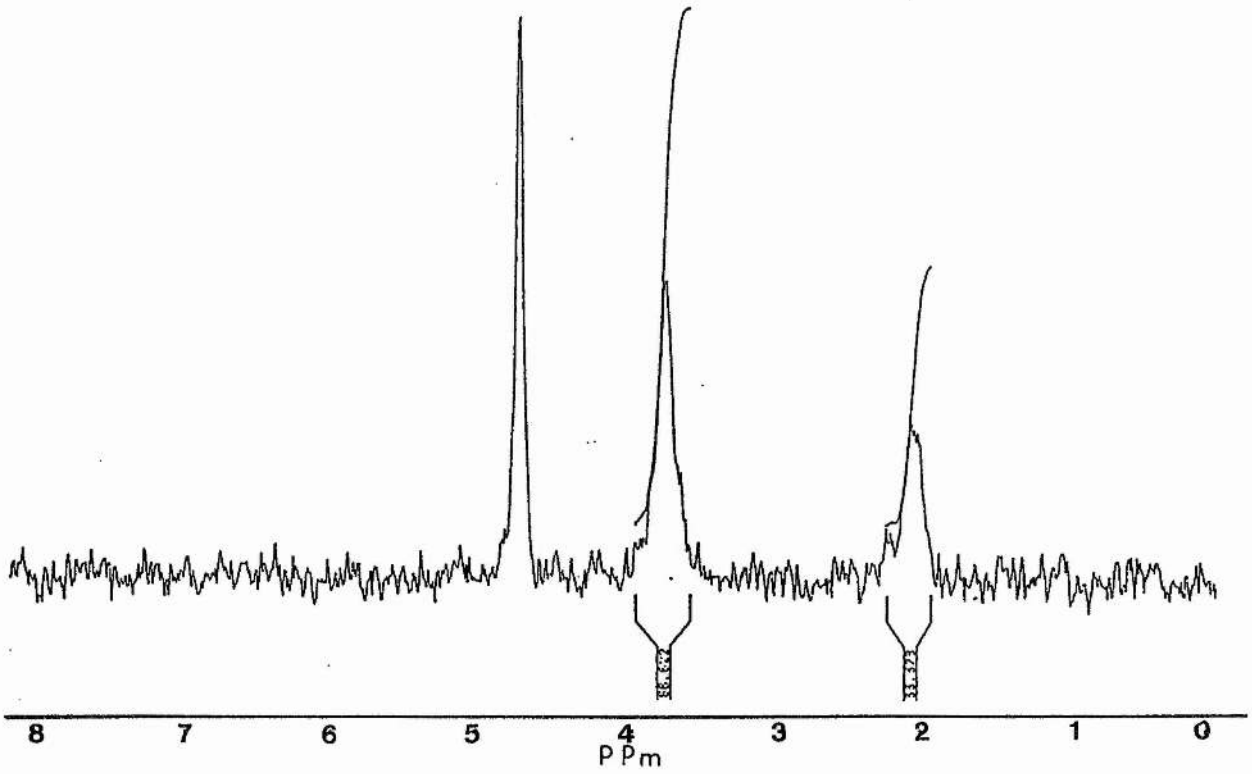


Figure 2.8 ^{2}H nmr spectrum of [2, 3-^{2}H]-methionine
(labelled in 10% $FeCl_3$ solution)

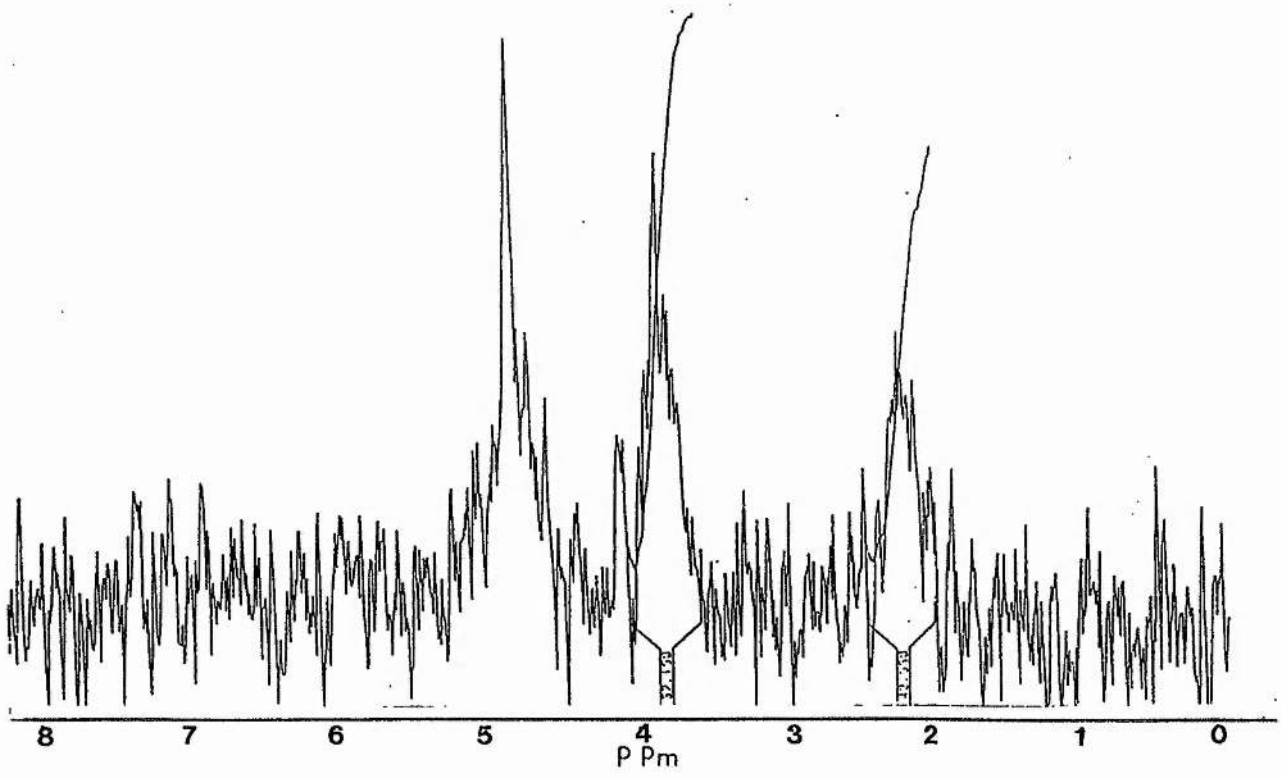


Figure 2.9 ^{1}H nmr spectrum of ethionine.
(referece sample)

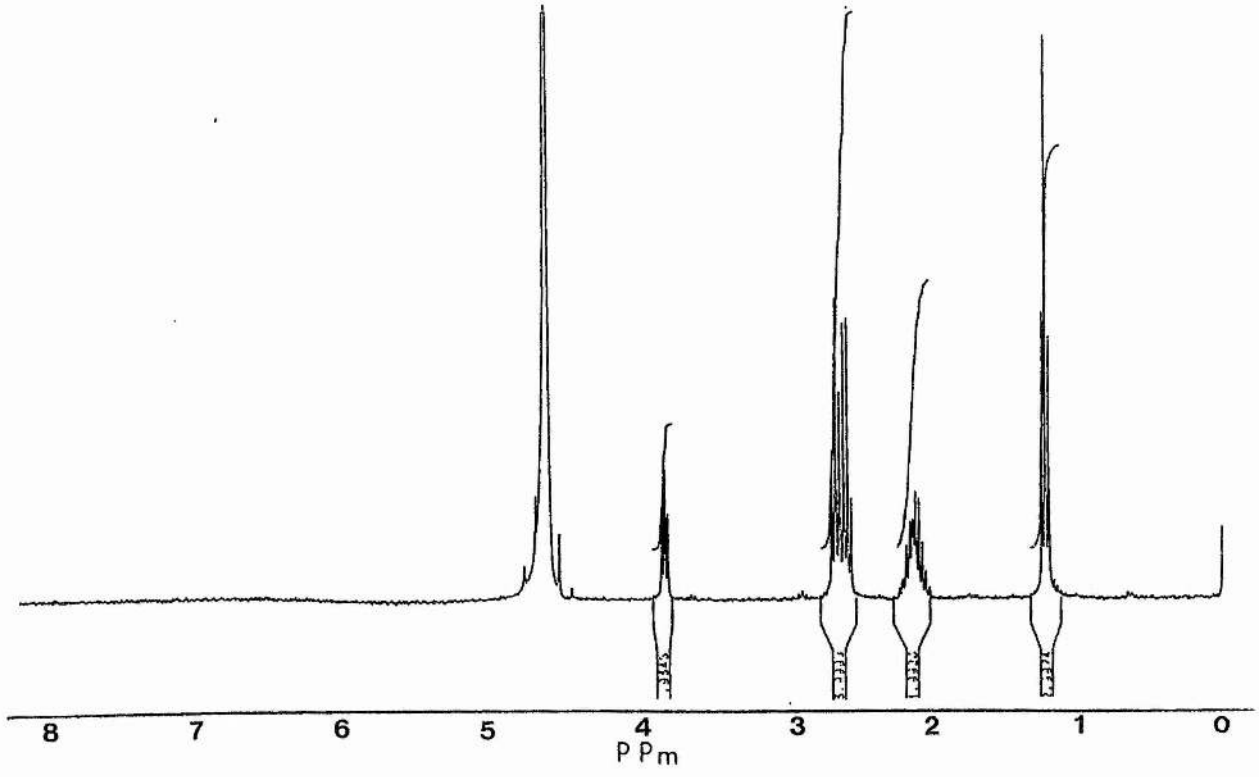


Figure 2.10 ^{1}H nmr spectrum of [2, 3-^{2}H]-ethionine.
(pyridoxal phosphate used as coenzyme)

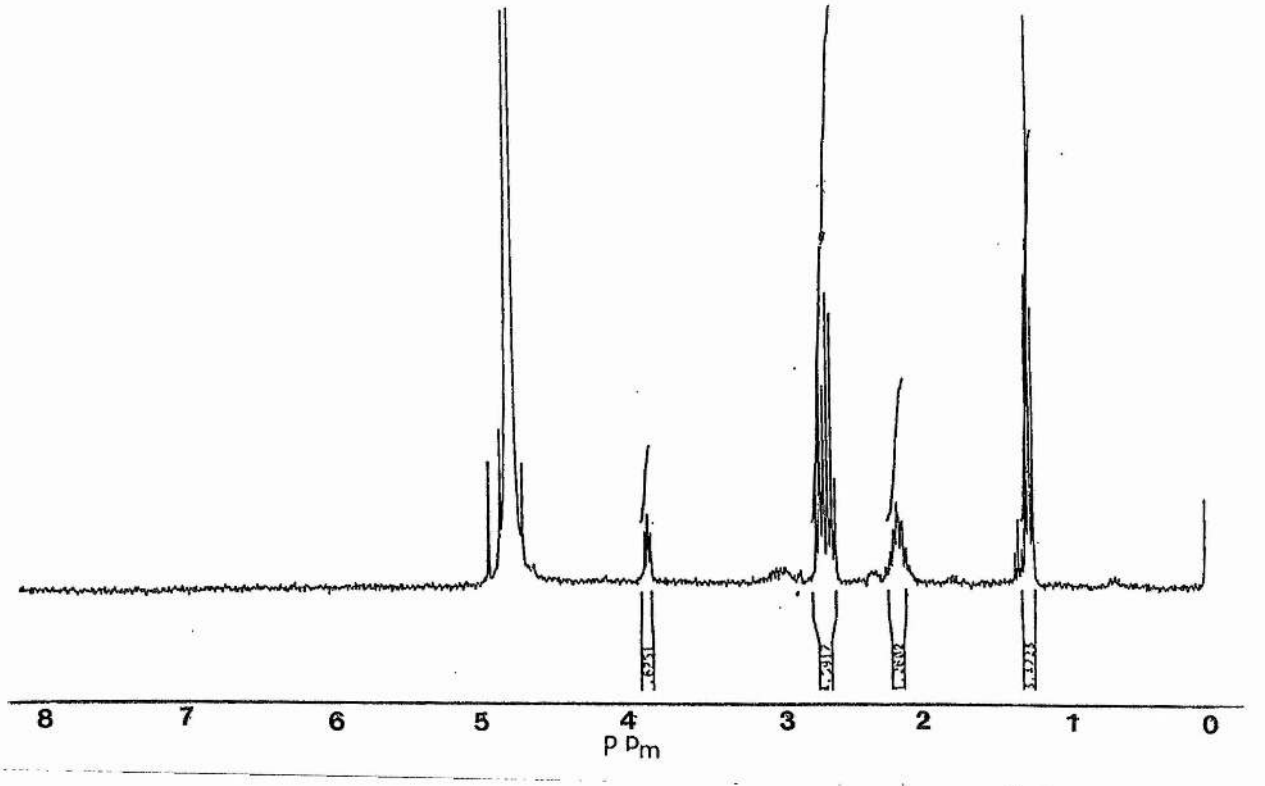


Figure 2.11 ^{2}H nmr spectrum of [2, 3-^{2}H]-ethionine.
(pyridoxal phosphate used as coenzyme)

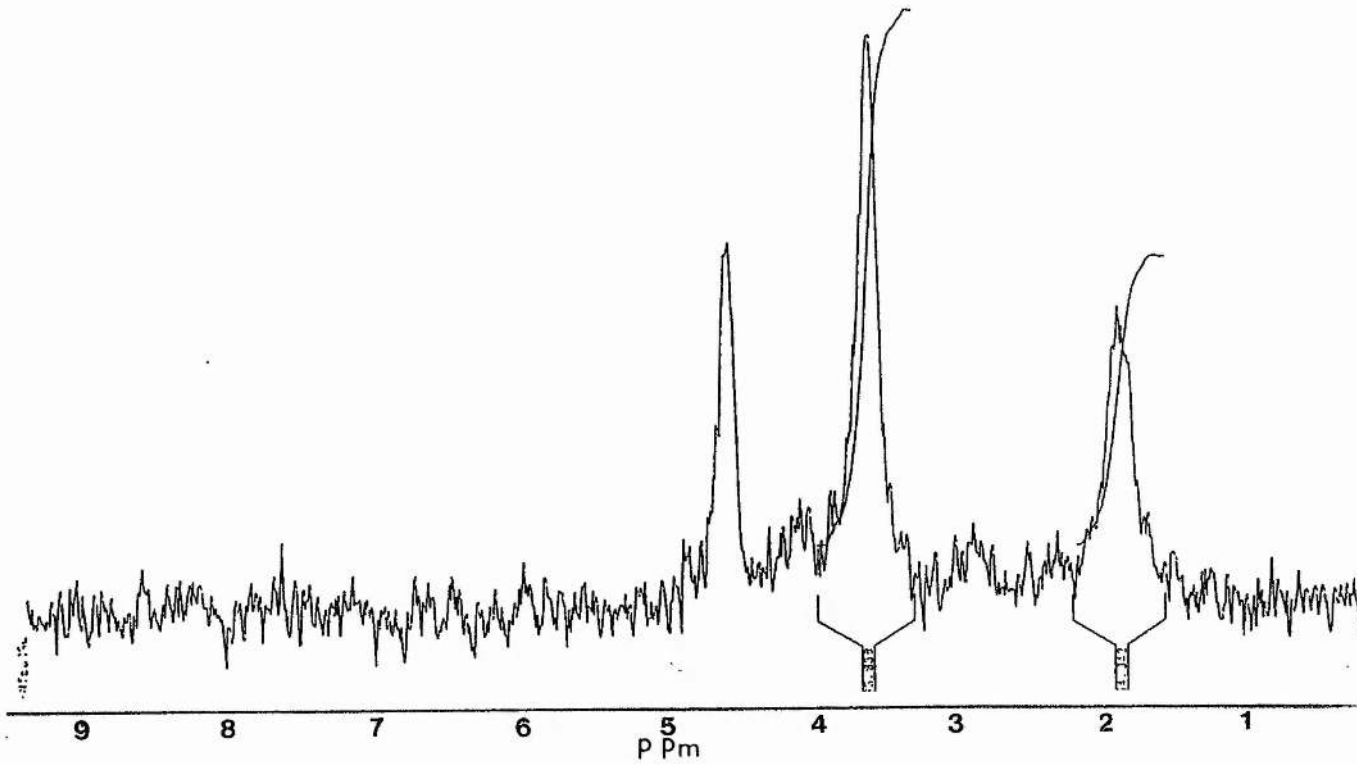


Figure 2.12 ^{2}H nmr spectrum of [2, 3-^{2}H]-ethionine.
(pyridoxine used as coenzyme)

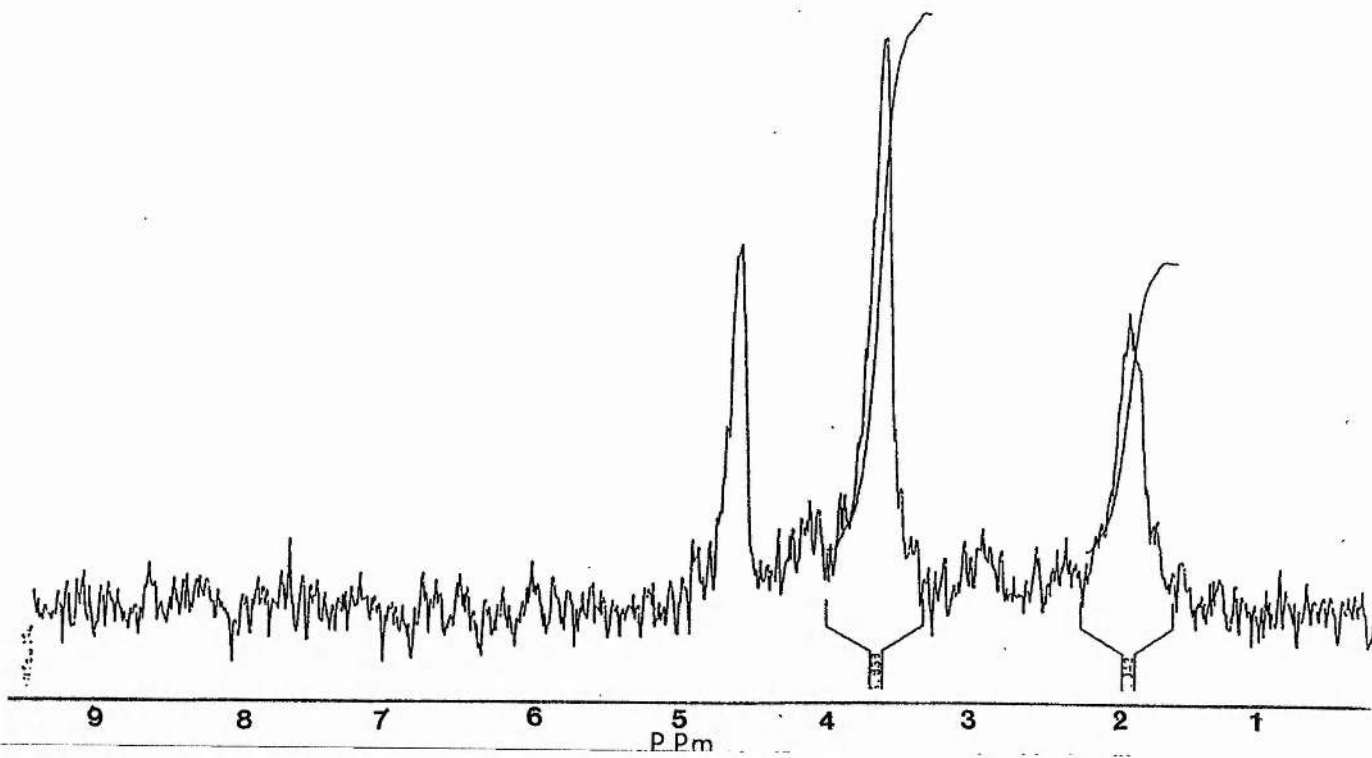


Figure 2.13 ^{1}H nmr spectrum of S-methylcysteine.
(reference sample)

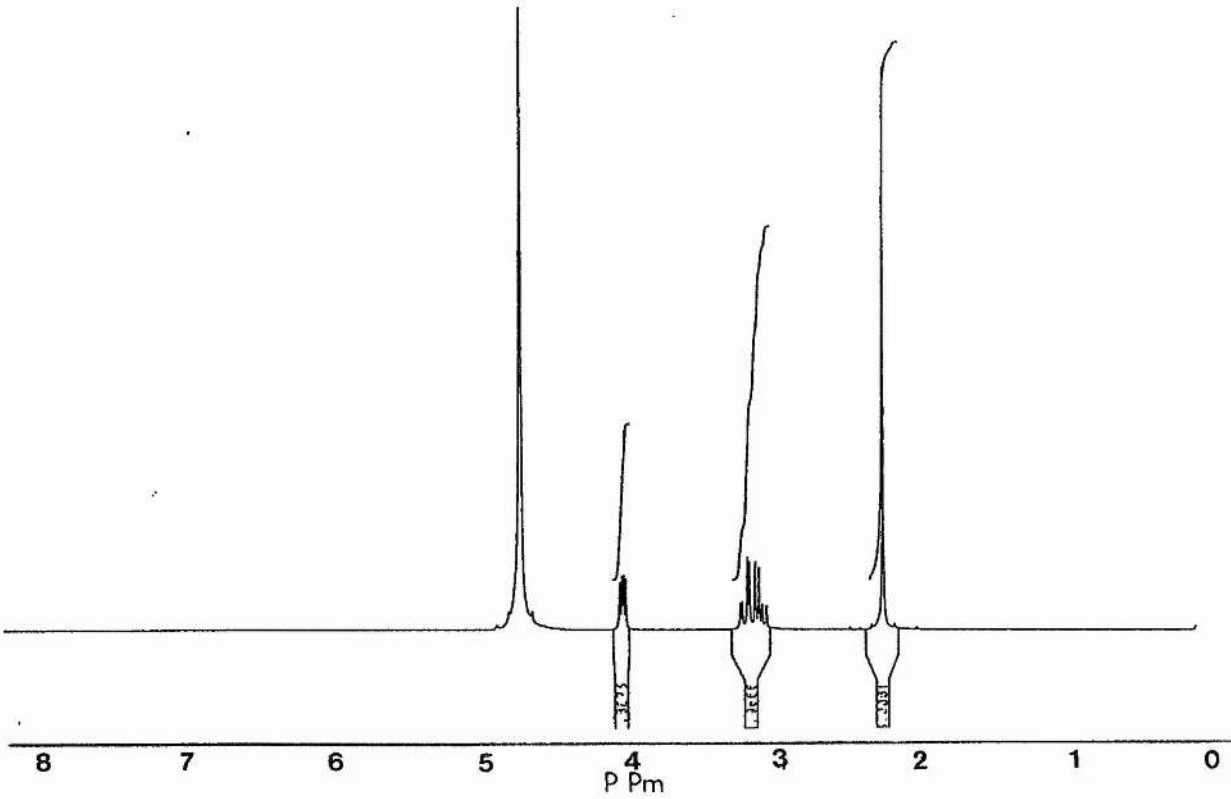


Figure 2.14 ^{1}H nmr spectrum of [2, 3-^{2}H]-S-methylcysteine.
(pyridoxal phosphate used as coenzyme)

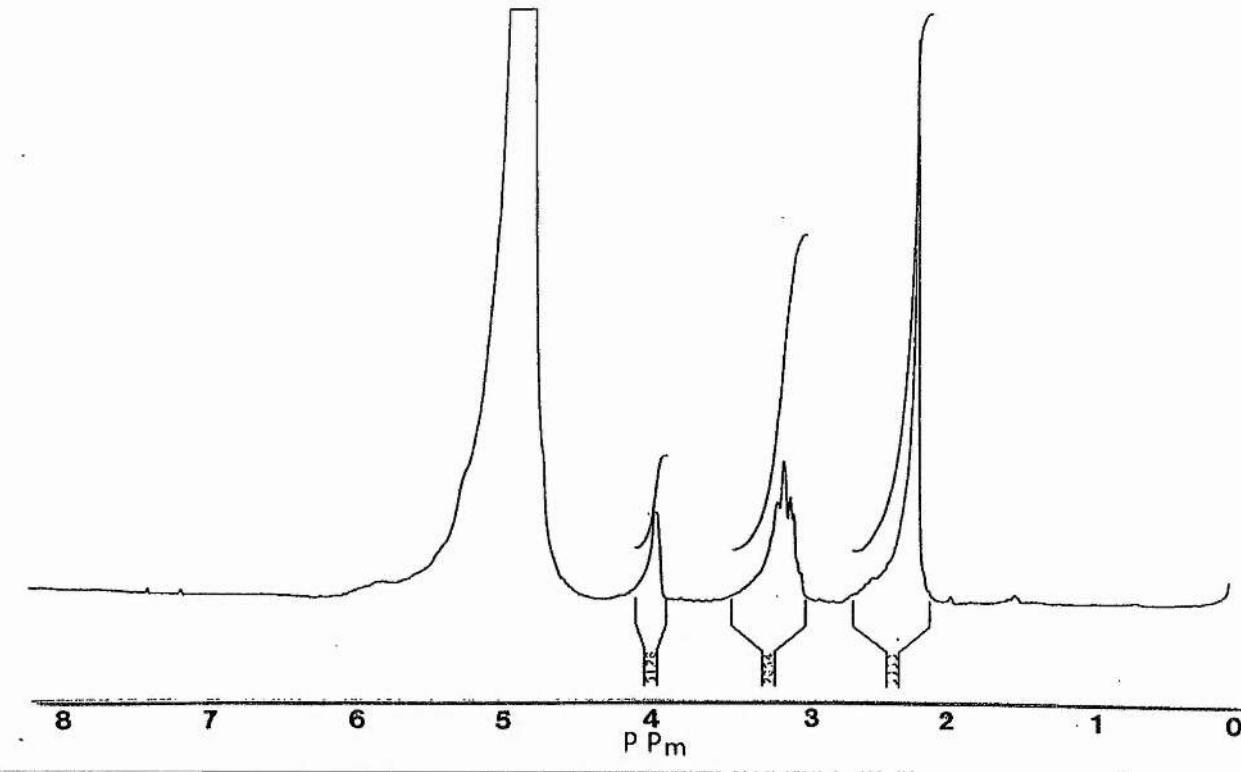


Figure 2.15 ^{2}H nmr spectrum of [2, 3-^{2}H]-S-methylcysteine.
(pyridoxal phosphate used as coenzyme)

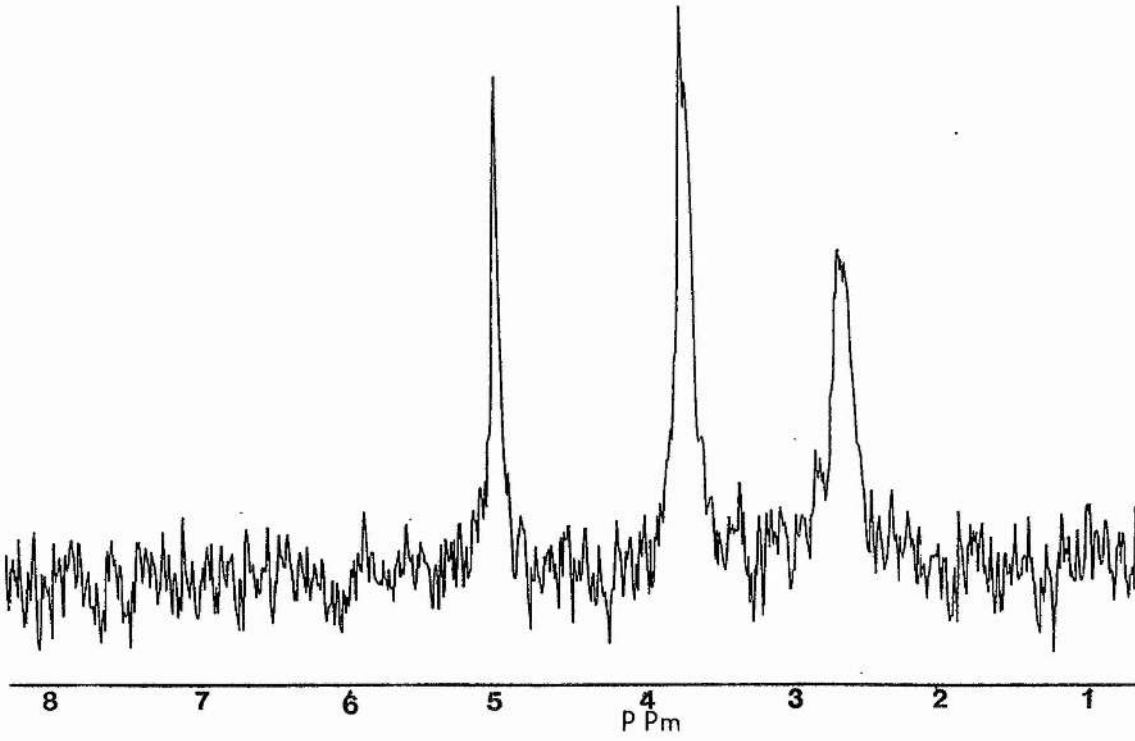


Figure 2.16 ^{1}H nmr spectrum of [2-^{2}H]-glycine.
(pyridoxal phosphate used as coenzyme)

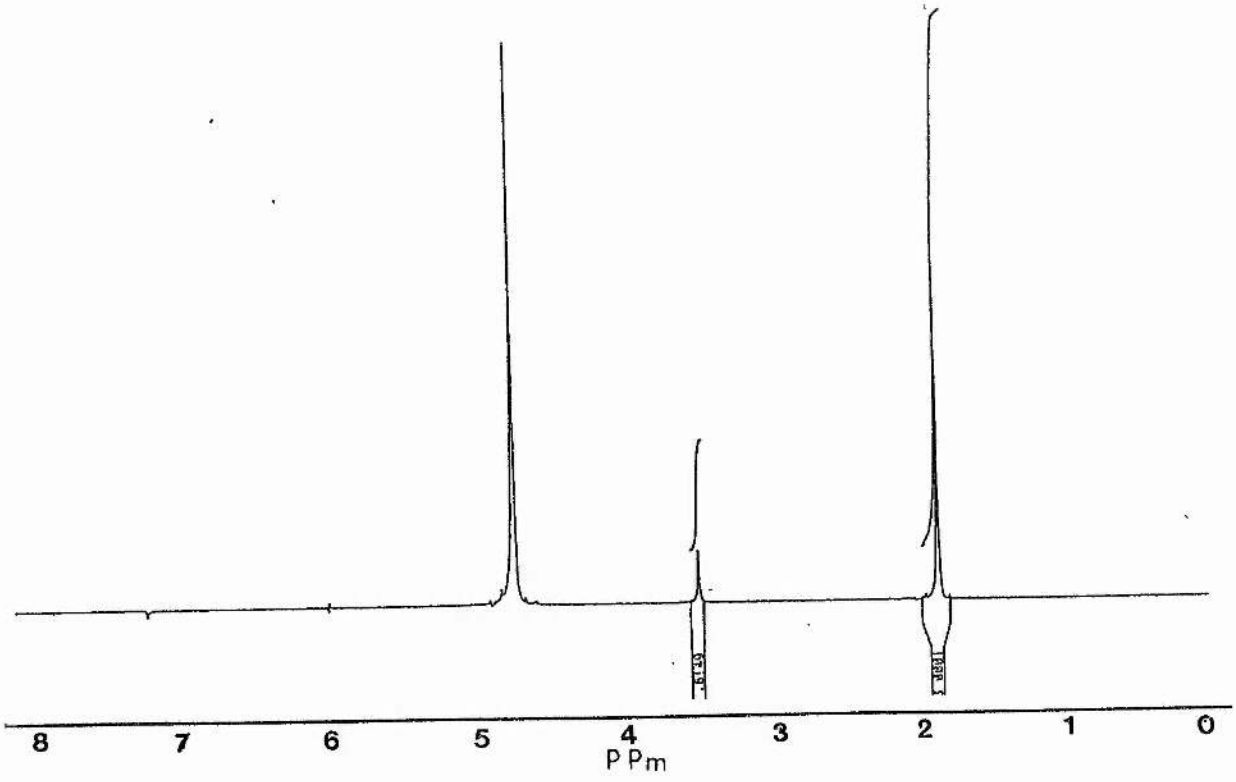


Figure 2.17 ^{2}H nmr spectrum of [2-^{2}H]-glycine.
(pyridoxal phosphate used as coenzyme)

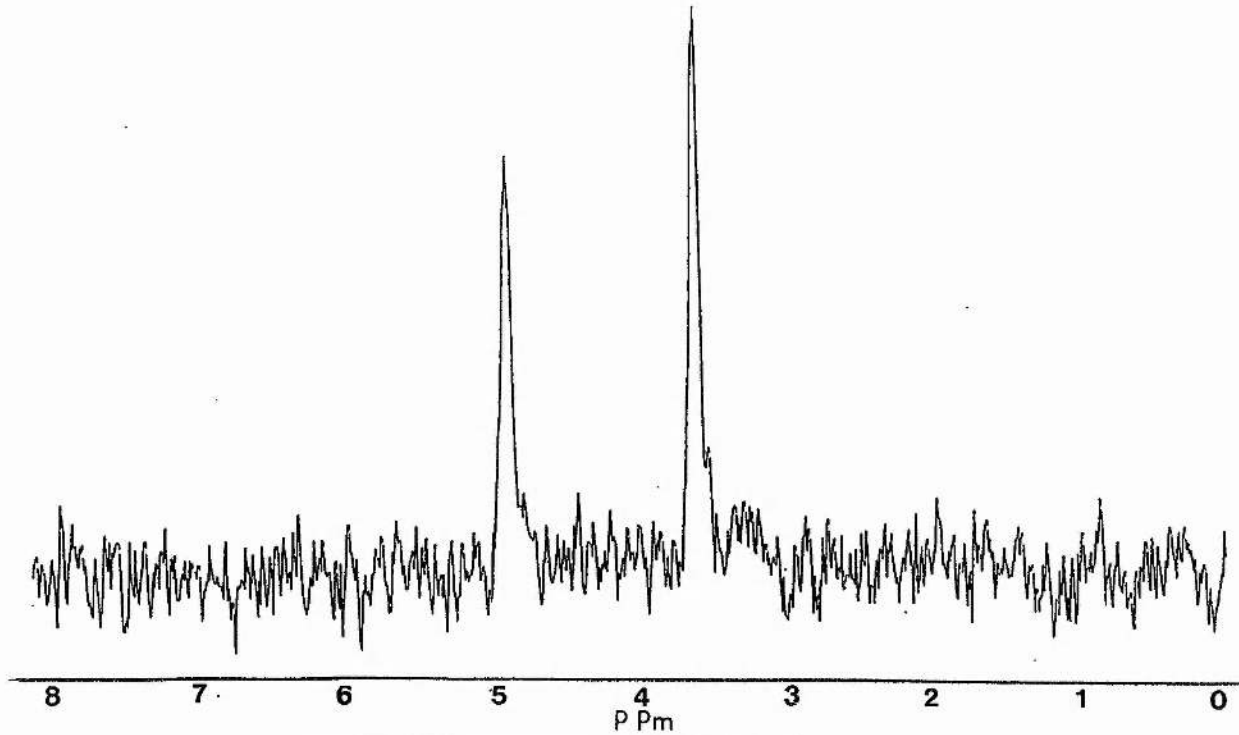


Figure 2.18 ^{1}H nmr spectrum of [2, 3-^{2}H]-alanine.
(pyridoxal phosphate used as coenzyme)

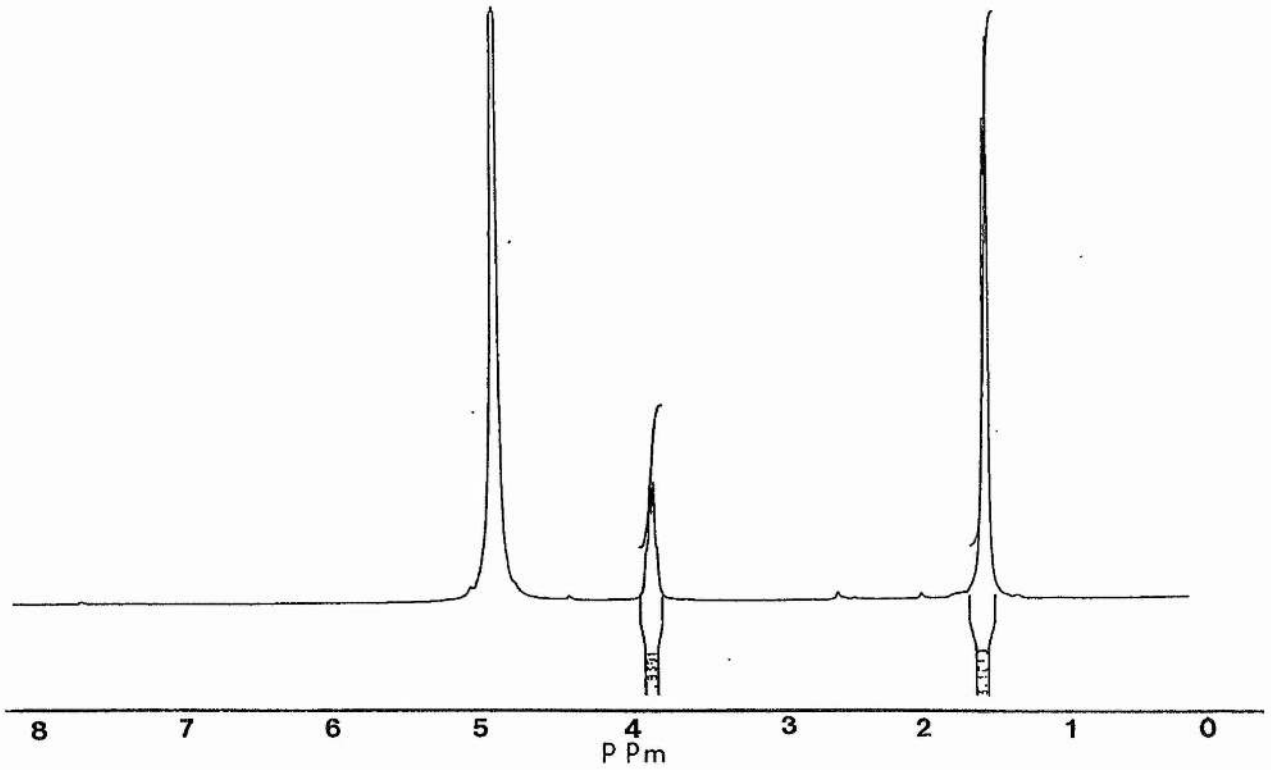


Figure 2.19 ^{2}H nmr spectrum of [2, 3-^{2}H]-alanine.
(pyridoxal phosphate used as coenzyme)

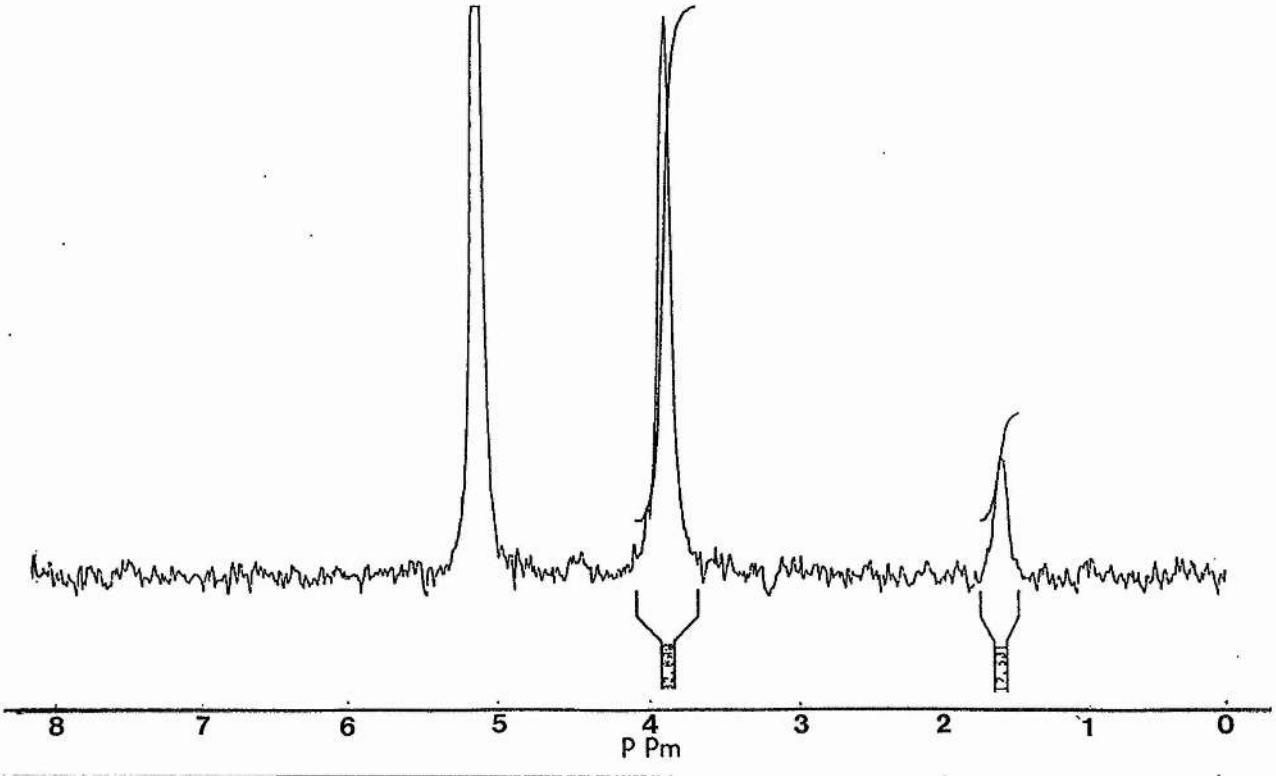


Figure 2.20 ^{1}H nmr spectrum of phenylalanine.
(reference sample)

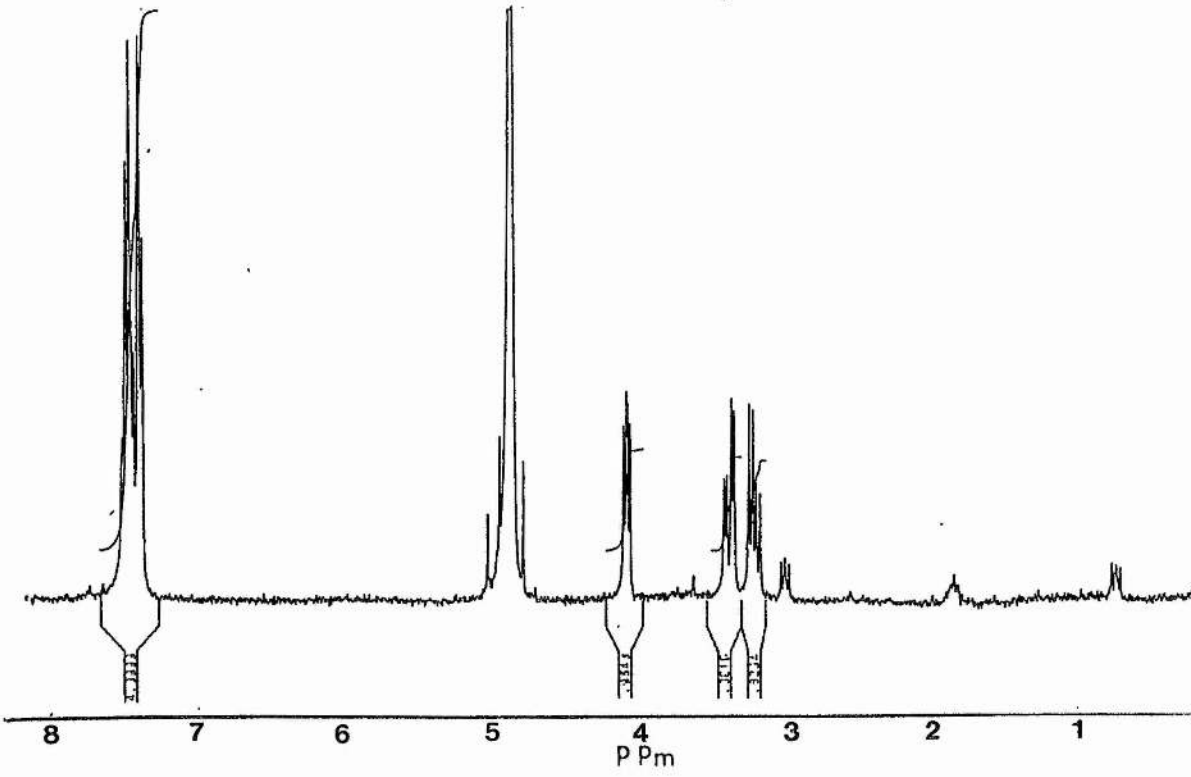


Figure 2.21 ^{1}H nmr spectrum of [2-^{2}H]-phenylalanine.
(pyridoxal phosphate used as coenzyme)

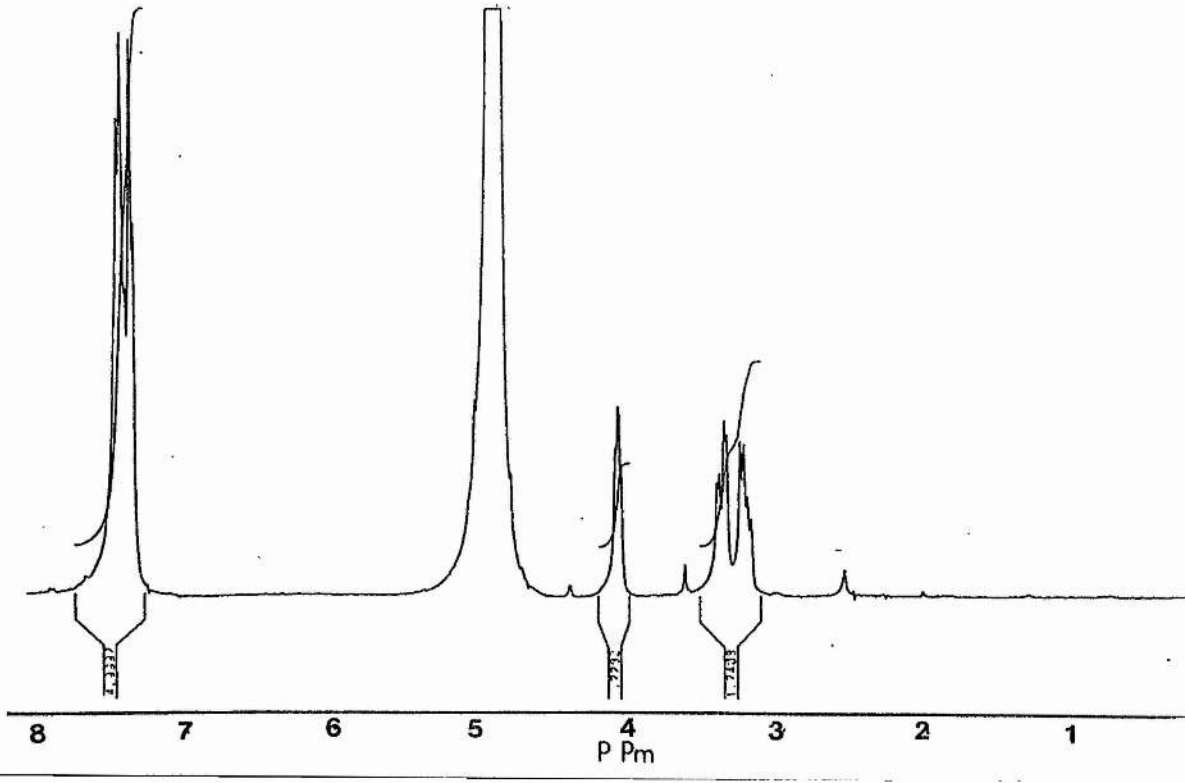
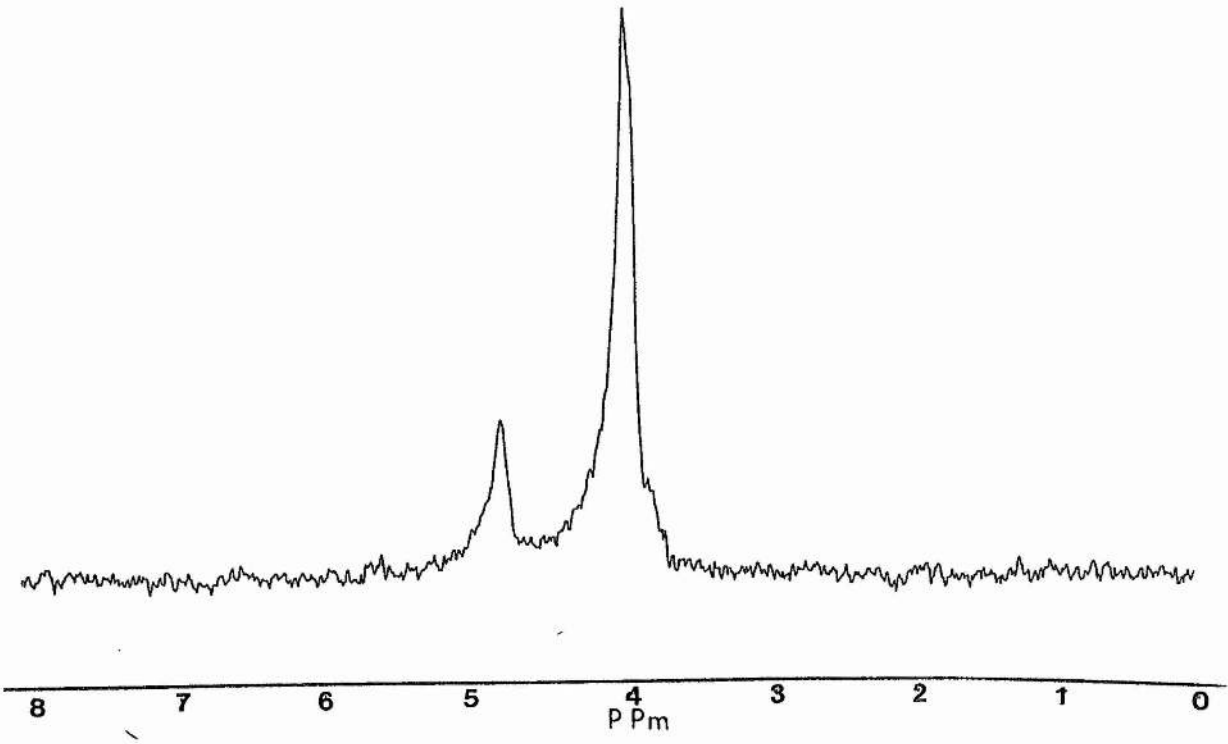


Figure 2.22 ^{2}H nmr spectrum of [2-^{2}H]-phenylalanine.
(pyridoxal phosphate used as coenzyme)



2.4 Discussion.

The catalytic deuteration of several amino acids and amino acid derivatives using whole cells of Pseudomonas putida has been shown to be feasible. However, attempts to utilize crude and semi-purified extracts of the enzyme methionine-γ-lyase as catalysts for deuteration of amino acids were unsuccessful.

There are several distinct differences between the results observed for the deuteration of amino acids and their derivatives using whole cells of Pseudomonas putida (ATCC 2032) and those reported by Esaki et. al. [5] for methionine-γ-lyase. In the latter case, the α- and ß-positions were deuterated only for L-methionine, L-S-alkylcysteines and linear L-α-amino acids while labelling of the α-position occured with glycine, L-phenylalanine and L-tryptophan. It was shown by the research described herein that the labelling of L-leucine was possible but other branched-chain amino acids did not label. The deuteration of L-tryptophan using whole cells was not possible. Replacing the methyl group of methionine with an ethyl group did not affect the ability of the α- and ß-protons of the amino acid to undergo exchange labelling with deuterium.

It was found that the D- and DL-amino acids did not undergo deuteration with Pseudomonas putida. Attempts to deuterate N-substituted amino acids, simple dipeptides, ω-amino acids dicarboxylic amino acids and hydroxy amino acids

were also unsuccessful. These observations were in agreement with results obtained by Esaki et. al.[5] using methionine-γ-lyase.

There are several possible explanations for the differences between the data obtained by Esaki et. al. [5] and those reported here. Firstly, Esaki et. al.[5] used a different strain of Pseudomonas putida than that used for this work. A more likely reason is the fact that the whole cells will contain a mixture of enzymes and other cellular compounds which may affect the deuteration of amino acids. It is conceivable that, in addition to methionine-γ-lyase, several other transaminases might exist in Pseudomonas putida and these could even promote similar deuteration reactions. Indeed the latter case would seem quite plausible when one considers that Pseudomonas aeruginosa and Escherichia coli have been shown to contain several transaminases which overlap in their ability to catalyse different biochemical pathways [12, 27].

The investigation of the effects of different coenzymes on the deuteration of amino acids has been carried out using pyridoxine derivatives. The deuteration of amino acids in the presence of either pyridoxal phosphate or pyridoxine led to similar labelling patterns for the amino acids (see Tables 2.6-2.9 and Tables 2.16-2.19). When pyridoxal hydrochloride was used as the coenzyme, noticeable differences in the labelling pattern of the amino acids were observed. It can be seen from Table 2.14 that in the case

of alanine and norvaline the majority of the deuterium was incorporated into the ß-positions. The other amino acids showed a lower level of deuterium incorporation for reactions using pyridoxal hydrochloride as coenzyme than those with pyridoxine and pyridoxal phosphate. The deuteration of methionine in the presence of pyridoxal hydrochloride led to a product that was labelled mainly in the α-position. An additional chemical shift at δ 2.0 ppm was observed in the ^2H nmr spectra of labelled methionine and can be assigned as the ß-methylene group of the corresponding α-keto acid.

The above observations for experiments in which pyridoxal hydrochloride was present as a coenzyme implied that both transamination and hydrogen isotope exchange reactions were competitively occuring. Indeed both reactions depend upon a common Schiff's base as an intermediate and so it is possible that either reaction pathway can be followed. The structure of the coenzyme possibly dictates which reaction predominates. Esaki et. al [5] also reported the formation of an α-keto acid during the deuteration of methionine by methionine-γ-lyase and pyridoxal phosphate.

Successful immobilization of Pseudomonas putida cells was achieved using a biofix C2 ceramic support. The bacteria cells were loaded directly onto the column of biofix C2, which had been pre-washed with phosphate buffer solution. Although the method of immobilization had not been optimized for these studies, it can be seen from plate

counting results in section 2.3.3 that about 5% of the cells were immobilized.

In general, as can be seen from the results given in sections 2.3.4, 2.3.5 and 2.3.6, the labelling of amino acids using immobilized cells resulted in a higher incorporation of deuterium than the corresponding experiment with free cells. Indeed in the case of L-methionine, which was labelled in the presence of pyridoxal phosphate as coenzyme, an increase in deuterium incorporation of over 50% was observed at the α-position. However, it should be pointed out that the number of bacteria cells used in deuteration studies using immobilized cells was approximately tenfold that for experiments using free cells.

A study of the reusability of the immobilized Pseudomonas putida cells has been carried out. It was found that regeneration of the biocatalyst was necessary to restore the catalytic activity of the Pseudomonas putida. The regeneration of the biocatalyst was achieved by leaving the immobilized Pseudomonas putida cells overnight in a 10% peptone solution. As can be seen from Table 2.10, the degree of deuterium incorporation obtained using the regenerated biocatalyst was greater than that for biocatalyst which had been directly reused but less than with freshly prepared catalyst. Although the regeneration method employed may not have restored the catalyst to its full biological activity, the results obtained indicated that it was possible to regenerate the immobilized

Pseudomonas putida cells. The reproducibility of the deuteration method was investigated by using three batches of Pseudomonas putida to label methionine. It can be seen from Table 2.11 that no significant difference in the level of ^{2}H incorporated into methionine was observed for the different batches of Pseudomonas putida.

The use of semi- and non-aqueous solvent media for deuteration experiments has been investigated. The deuteration of amino acids was found to be possible in 10% DMSO, 10% DMF and 40% glycerol (see Section 2.3.8). Attempts to use aqueous ethanol and chloroform as solvent media were unsuccessful. There was no overall pattern to the degree of ^{2}H incorporated into the amino acids in semi-aqueous solvents and thus it is not possible to determine the best system to employ. In addition, the deuteration of glycine esters in DMSO and DMF was also found possible. The loss of the ester groups from the amino acid derivatives did not occur in these solvent systems. The retention of the ester group implied that the carboxyl group of the amino acid may not play a part in the reaction.

Indeed there is some supportive evidence for the deuteration of amino acids by Pseudomonas putida in semi- and non-aqueous media. For instance, the interconversion of glycine and glyoxylate by serine:glyoxylate transaminase is induced eightfold in the presence of methanol and methylamine[12]. Transamination has also been reported to occur in micellar phase[26]. Thus, it is conceivable that a

transaminase would be capable of promoting deuterium labelling, since both transamination and hydrogen isotope exchange reaction pathways involve a common Schiff's base.

The ability to deuterate amino acids and and amino acid esters in semi- and non-aqueous solvent systems may be dictated by the physical properties and toxicity of the solvents. Those solvents with high dielectric constants, such as water (80.2), DMSO (46.7), DMF (36.7) and glycerol (42.5), were found to be suitable media in which to carry out deuteration experiments. In solvents with low dielectric constants, such as chloroform (4.8), THF (7.6) and ethanol (24.6), no deuteration of the amino acids occurred. In solvents with high dielectric constants the α-carbanion (iminium ion), formed by α-hydrogen abstraction from the coenzyme-substrate Schiff's base, may be less stable than the resulting deuterated amino acid. In the case of solvents such as DMSO and DMF, which have higher dipole moments than water, the deuteration of amino acids may occur due to the lowering of the activation energy. In these cases the nature of the solvents may destabilize the iminium ion relative to the products.

The effects of reducing the amount of water in the reaction media upon the behaviour of the Pseudomonas putida cells and the transaminase must be considered. One aspect of increasing the proportion of organic solvent in the system will alter the osmotic conditions and this may lead to dehydration of the bacteria cells and denaturing of the

enzyme. Also since D_2O was used as the labelling reagent, then it was desirable that the organic component of the solvent system had a high degree of aqueous miscibility. This was important because it was hoped to promote an intracellular reaction rather than a free enzyme process. In the case of chloroform, which has a poor miscibility with water, the attempts to deuterate amino acids with whole cells may have been unsuccessful due to the fact that this criteria was not met.

The kinetics of the deuteration of amino acids have been studied by ^1H nmr spectroscopy. However, the first order rate constants of deuteration determined using this method need to be carefully interpreted. There are several processes which make up the overall biochemical reaction. Some of these are: (a) the absorption of amino acid into the cell and its subsequent excretion; (b) the formation of the Schiff's bases between the pyridoxine derivative and the enzyme and/ or the amino acid; (c) the isotope exchange of deuterium with the protonated enzyme Schiff's base and subsequent labellimg of the amino acid. The first order rate constants for the deuteration of glycine methyl ester and glycine ethyl ester in d_6-DMSO were found to be $8.68 \times 10^{-5} s^{-1}$ and $7.73 \times 10^{-5} s^{-1}$ respectively.

In summary, it was found possible to deuterate amino acids using whole cells of Pseudomonas putida. The deuteration was found to be dependant upon the pyridoxine derivative used as the coenzyme. In the case of experiments

employing pyridoxal hydrochloride as the coenzyme it would appear that both transamination and hydrogen isotope exchange reactions occur.

It was found that Pseudomonas putida cells could be immobilized using a biofix support. The recovery and regeneration of immobilized bacteria cells has been successfully carried out. The deuteration of amino acids using immobilized bacteria was more efficient than experiments using free cells of Pseudomonas putida.

The deuteration of amino acids in semi-aqueous and non-aqueous solvents has been achieved using immobilized Pseudomonas putida cells. The deuterium labelling of glycine esters in DMSO and DMF have been achieved. The glycine esters did not undergo loss of the ester groups during the hydrogen isotope exchange reactions. ^{1}H Nmr spectroscopy has been used to follow the deuteration of glycine esters in DMSO.

2.5 References.

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