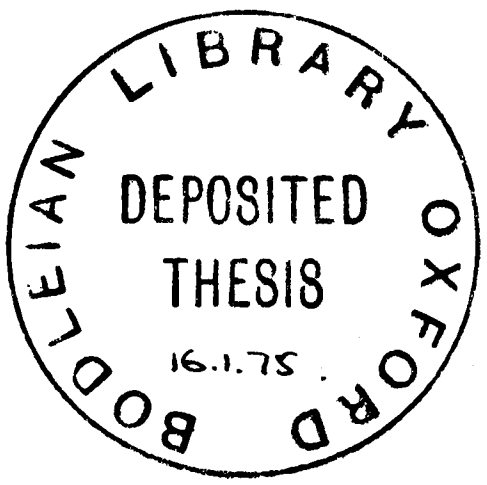
NUCLEAR MAGNETIC RESONANCE STUDIES OF ALAMETHICIN



D. Phil. Thesis

Oxford, 1974

D. R. Martin

Merton College

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to my supervisor Professor R. J. P. Williams F. R. S. for his continual help and encouragement with this project.

I would also like to thank the large number of colleagues with whom I have had many interesting and valuable discussions, in particular Dr. C. M. Dobson, Dr. B. A. Levine, Dr. I. D. Campbell and Dr. G. T. Young.

Finally I would like to thank those who performed some of the experimental work reported in this thesis and who are acknowledged at the appropriate place in the text.

INDEX

Abstract

Abbreviations

CHAPTER ONE Introduction 1

CHAPTER TWO Theoretical

Part I General theory 8

Chemical shift 8

Relaxation 12

Fourier Transform spectroscopy . . 14

Part II Paramagnetic effects 21

Chemical shift effects 21

Relaxation effects 24

Summary of paramagnetic effects . . 25

CHAPTER THREE Chemical nature and sequence of alamethicin

Part I Chemical composition and preparation of alamethicin 30

Part II Sequence of alamethicin F30 35

Previous sequence and composition work 35

The aromatic moeity in alamethicin, initial evidence . . 42

IDENTIFICATION OF THE AROMATIC MOEITY 47

Experiments on the complete hydrolysate of F30 48

Experiments on intact F30 56

Further hydrolysate experiments 59

Identification of PHXH 72

Location of phenylalaninol in the sequence 75

THE N-TERMINAL BLOCKING GROUP . 88
Partial hydrolysis experiments . . 91
Isolation of XH 94
Identification of XH and X . . . 95
Experiments on very pure F30 . . . 102
DISCUSSION OF THE FINAL SEQUENCE 102
SUMMARY (of the whole of Part II) . . 107
Part III The Sequence of F50 . . . 111

CHAPTER FOUR

Materials and Method

Instrumentation 122
Materials 124
METHOD 125
Solvent 125
Control of pH 127
Water/D_2O content 131
The use of lanthanides to determine conformation 132
Shift data 135
Axial symmetry 138
Non-axial symmetry . . . 139
Relaxation data 141
SUMMARY 143

CHAPTER FIVE

Preliminary experiments and lanthanide shift experiments

ASSIGNMENT 146
LANTHANIDE SHIFT EXPERIMENTS 156
Effect of pH 156
Effect of D_2O content . . . 162
Conditions for the lanthanide method 167

DETERMINATION OF THE CONFORMATION
BY SHIFT EXPERIMENTS 172
Lanthanum results 173
Paramagnetic lanthanide experiments 175
Intermediate pH experiments 176
Low pH experiments 185
Interpretation of results 186
Conclusions concerning the conformation 190
SUMMARY 193

CHAPTER SIX — Determination of the conformation by relaxation studies 195

Part I Determination of the conformation of F30 200
The high pH complex $M(A^-)$ 200
The low pH complex M(HA) 207

Part II Determination of the conformation of F50 214

APPENDIX ONE — Theoretical
This is separately indexed on page . . 216

APPENDIX TWO — Non-axial symmetry 234

APPENDIX THREE — Experimental
This is separately indexed on page . . 242

The references are given at the end of each chapter (or appendix)

ABSTRACT

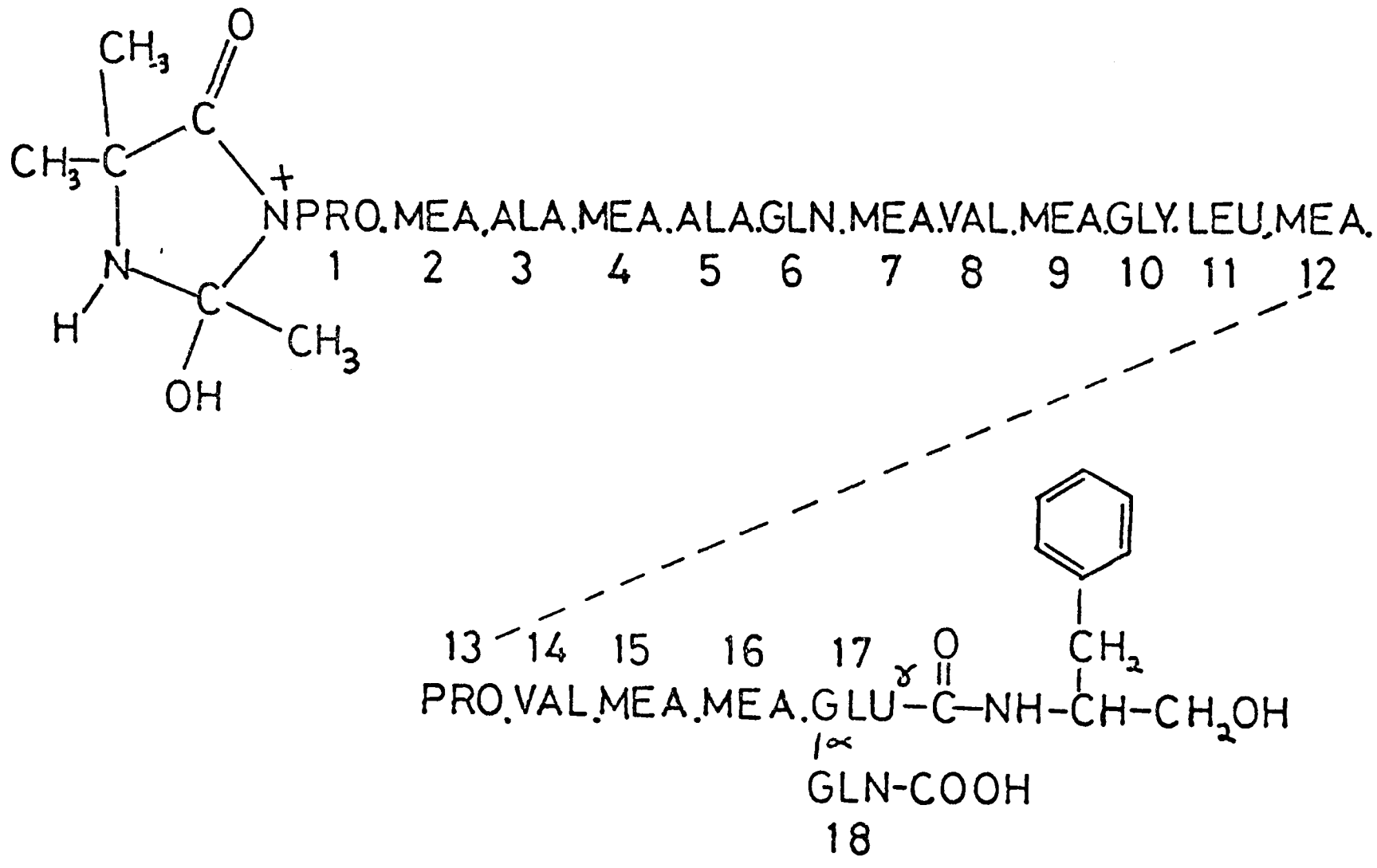
NMR STUDIES OF ALAMETHICIN

Alamethicin is a polypeptide with many interesting biological properties, in particular it shows voltage dependent conductance in lipid bilayers and also under the correct conditions can mimic nerve action potentials. It was chosen for study because of these properties and also because it is of suitable size (MW 2,000), it had apparently been fully characterised and sequenced and it binds metal ions and hence its conformation could be studied using the lanthanide ions.

It was shown by workers at Porton Microbiological Research Establishment that 'alamethicin' is a mixture of several similar chromatographically separable components the major ones being 'F30' ca 85% and 'F50' 12%. All previous literature work has been done on the unseparated mixture of these various components. Most of our work was performed on F30 which has an ionisable carboxyl group (GLN 18). Porton supplied the alamethicin F30 and F50 for our studies. A very pure sample of F30 was prepared in Oxford by Dr. J. D. Priddle.

The original aims of the project had to be subs tantially modified when it was found that the well-established and widely-quoted sequence of alamethicin proposed by J. W. Payne et al, and confirmed by A. A. Kiryushkin et al, was in fact incorrect. This discovery was made from the preli minary Nmr spectra of F30 (and F50). These spectra showed two unexpe cted sets of peaks which could not come from any groups in the published sequence. Nmr spectra of a sample of the 'Upjohn' alamethicin used in almost all previous published studies of alamethicin showed these same

F30 SEQUENCE



The final sequence of alamethicin F30 showing the two blocking groups in detail and also the free carboxyl group of GLN 18. Other side-chains are not shown.

extra peaks were present and hence the problem was not unique to our alamethicin samples.

The nature of both of these extra moieties was determined by detailed Nmr studies of their properties in the intact F30, in partial hydrolysates and in complete hydrolysates of the F30. Both of the extra moieties were successfully isolated after they had been hydrolysed off the alamethicin, and hence their identity could be determined beyond all doubt. It was also shown that these extra moieties were covalently bound into the alamethicin and the nature of the linkages involved was established as a result of the experiments above.

The only weakness in the published sequence is the supposed GLU 17 γ carboxyl to PRO 1 peptide linkage which cyclised the alamethicin 'ring'. The presence of this link was never directly demonstrated. It was shown from our studies that one of the extra moieties, phenylalaninol was attached to the GLU 17 γ carboxyl group by an amide linkage, and the hydroxyl group of the phenlalaninol was free and hence the 'ring' was not closed and so alamethicin is linear. The other extra moiety, was known from the lanthanide studies to be a very long way from the phenylalaninol and this other moiety was shown to be attached to the PRO 1 hence blocking the N-terminus.

This finding that alamethicin F30 is not cyclic but is a linear peptide with the C- and N- termini blocked by groups which are undetected by conventional procedures is not in fact in disagreement with any of the previous sequence/composition studies of alamethicin. This finding is obviously of importance in trying to understand the interesting conduction properties shown by alamethicin.

A similar set of experiments on F50 showed that it too is linear and has the C- and N- termini blocked in an identical fashion to F30. The rest of the sequence of F50 is virtually identical to that of F30.

The conformational studies of alamethicin were performed in deuterated methanol as here, in contrast to aqueous solvents, alamethicin is monomeric and does not aggregate. The pH in methanol was measured using a directly calibrated indicator system. Control of pH is important as the lanthanide ions bind strongly to both ionised and unionised F30.

The method of determining the conformation involves binding a lanthanide ion to a single site in the molecule and then observing the two principal effects which the paramagnetic ions may produce on the Nmr spectrum of the molecule, namely changes in the resonance energy of the nuclei (i. e. shifts of the lines) and relaxation of the nuclei (broadening of the lines). It was shown that the required conditions of fast exchange, 1:1 complex and no contact effects were present and hence the magnitude of these effects depends on various geometric factors relating the bound metal ion to the observed nucleus.

The magnitude of the shift effect gives distance and angular information, however the simplifying case of axial symmetry which has been found in all previous studies by our group using lanthanide ions was not present in this case. It was shown that the case of non-axial symmetry is fundamentally insoluble and so it was only possible to obtain qualitative information about the conformation from these studies.

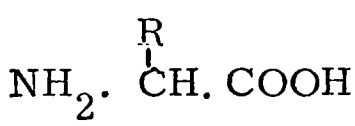
The magnitude of the relaxation effect depends only on distance. Analysis of the relaxation data enabled more detailed conformational information to be obtained, although for various reasons no attempt was made to obtain absolute distances. The relaxation results agreed with the shift results and this is an important check on the correctness of the conformation as the geometric dependence of the shift and relaxation effects is very different.

These studies showed that the conformation was pH dependent, i.e. depended on whether the GLN 18 carboxyl group was ionised or not, and that the binding was strong to both ionised and unionised forms.

Studies on the minor component F50 were very much less detailed but showed that the conformation of the F50-lanthanide complex was very similar to that of the unionised F30-lanthanide complex as might be expected.

ABBREVIATIONS

The abbreviations used in this thesis are given below in alphabetical order. They are mostly standard abbreviations for ∝ amino acids which usually have the structure:-



The nature of the side-chain R is given below. Technical abbreviations are given before the theoretical chapter (two) though a few common ones are given here as well.

ALA ... Alanine R = CH_3

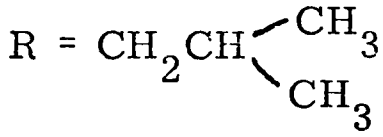
GLN ... Glutamine R = $CH_2.CH_2.CONH_2$

GLU ... Glutamic acid R = $CH_2.CH_2COOH$

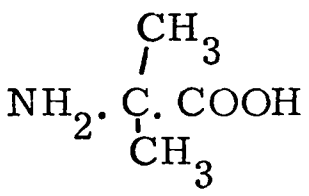
GLY ... Glycine R = H

H_o ... The main applied magnetic field in a Nmr experiment

H_1 ... The radio-frequency magnetic field in an Nmr experiment

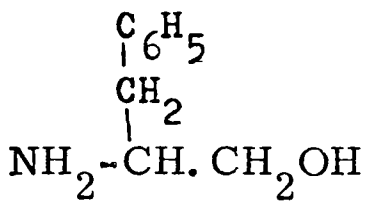
LEU ... Leucine R = 

MEA ... Methyl alanine, strictly C-methyl alanine or 2-methyl alanine or alternatively ∝ amino-isobutyric acid, an unusual ∝ amino acid with the following structure:-



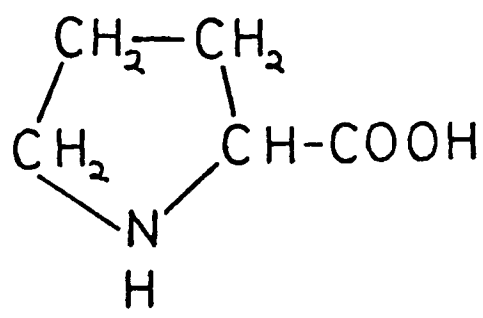
PHE ... Phenyl alanine R = $CH_2.C_6H_5$

PHO ... Phenylalaninol, a none-standard abbreviation for the following:-



PPM ... This is the chemical shift, or change in chemical shift in parts per million. When used as an absolute chemical shift in this thesis it is invariably measured downfield (as positive) from the standard of tetra-methyl-silane whose chemical shift is 0 PPM.

PRO ... Proline, an α amino acid whose structure is:-

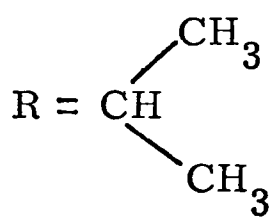


R.E. ... Resolution enhancement, a technique for changing line shapes and improving the resolution of a spectrum (see chapter 2).

TMS ... Tetra-methyl-silane, which is invariably taken as the standard for measurement of chemical shifts, see PPM above.

TSS ... A water-soluble derivative of TMS which was used as the shift standard for experiments in D_2O, and which has the same shift as that of TMS itself.

VAL ... Valine



CHAPTER ONE

INTRODUCTION

Many peptides of molecular weight about 2000 are important in biological control and it is of considerable importance to obtain conformational knowledge about them. Alamethicin was chosen for this study because it is a molecule with many interesting biological properties, of suitable molecular weight (ca. 2,000), apparently it had been fully characterised and sequenced and it can bind metal ions. In fact alamethicin shows many interesting metal ion transport properties which have been likened to the transport properties of nerve membranes.

A straight forward attack on the conformation of the peptide was prevented by the discovery early on in the work that the composition and sequence of alamethicin, reported by various groups of workers, was incomplete. The original aims of the project had then to be considerably modified.

The work discussed in this thesis may be broadly divided into two, the elucidation of the chemical nature of alamethicin, and the study of the monomeric solution conformation of alamethicin using paramagnetic probes.

Properties of alamethicin

Alamethicin is an ionophorous antibiotic produced by Trichoderma Viride. It is a highly hydrophobic polypeptide with two non-amino acid moeities which were not detected in the published works

on the composition and sequence of alamethicin. The crude alamethicin contains two major and several minor components, which are distinguished in this thesis by their R_f values in the chromatographic system for their separation. (See page 30) The two major components are the 'F30' and 'F50', which occur to the extent of roughly 85% and 12% respectively. The component on which most of the experiments were done is the 'F30' which contains a single ionisable group, a carboxyl. F50 contains no ionisable groups. All the previous work reported in the literature was performed on the unseparated mixture of these (similar) components.

Alamethicin aggregates strongly in aqueous solution, and micellisation takes place in aqueous solvents at very low concentrations, these properties also being influenced by pH, and ionic strength. However alamethicin is monomeric (and highly soluble) in ethanol or methanol. The single pK of the F30 is at about 5.5-5.8 in aqueous solvents.

The main property of biological interest is alamethicins conductance properties in lipid bilayers. Many detailed studies of conductance by alamethicin have been made [2, 3, 4, 5]. They show that alamethicin is one of the very few compounds to demonstrate a voltage dependent conductance. This conductance varies with the sixth power of the alamethicin concentration, the sixth power of the concentration of salt and the sixth power of the voltage. Under the correct conditions of salt, concentration of alamethicin, and presence of substances such as protamine, alamethicin will give bistable voltage states and single or rhythmic action potentials, properties of interest in the understanding of nerve impulse action.

There has been much discussion about the significance of the kinetics of alamethicin conduction, in particular with

respect to conduction by a 'pore' mechanism (rather than the discrete carrier mechanism shown by many other ionophores), and possible cooperative interactions between the molecules[5]. However as has been pointed out by Haydon[6] the complexity of theoretical treatments of conductance is very considerable and it is worth remembering that these conductance studies have all been performed on impure alamethicins. Also our findings on the sequence of alamethicin have invalidated all detailed discussions of possible 'pores'.

The subject of ion transport across thin lipid membranes by ionophorous molecules in general has been reviewed by Haydon et al[6].

Determination of conformation by Nmr spectroscopy

Nmr spectroscopy is becoming an increasingly powerful tool for the study of conformation of biologically interesting molecules[7-9]. The advent of Fourier Transform spectroscopy has enabled much lower concentrations, (e. g. a few mM protons), to be studied; and super-conducting magnets with their very large fields have made it possible to examine larger molecular weights. The most important advantage of nmr is that it gives a very large number of probe points in a molecule, and so there is a considerable amount of structural data available.

The three principal quantities which may be measured in an Nmr experiment are the resonance energy (i. e. the chemical shift), the relaxation times of the nuclei, and in molecules of not too large molecular weight, coupling constants. The information which may be obtained from studies of these in a diamagnetic molecule is rather limited, although conformations have been deduced for small molecules from such measurements in some cases (usually using NH-αCH

coupling constants and the temperature dependence of NH proton chemical shifts).

However the introduction of a paramagnetic probe to a single site in the molecule, can cause considerable changes in the chemical shifts and relaxation times of the nuclei which, under the right conditions, yields geometric information about the position of the nuclei observed relative to the bound probe. Hence geometric information about all the individual nuclei observed can be obtained.

The method used involves ions of the lanthanide series and is discussed in detail in chapter 4. The lanthanides possess several advantages over other paramagnetic probes, one of which is that both distance and angular information is available under the right conditions, thus providing an important crosscheck. The method has already been applied by us successfully to small molecules such as the mononucleotides[15-17] and it was our desire to extend the method to larger molecules.

The binding of lanthanide ions is obviously liable to perturb the conformation, but in the case of alamethicin the metal bound conformation is of more interest. (All paramagnetic probes give the conformation of the bound molecule only).

Previous conformational/Nmr studies of alamethicin

Conformational studies of alamethicin have been published using optical rotary dispersion and circular dichroism measurements[10], but the interpretation of these results is invalidated by our discovery of an aromatic moiety in the molecule. A theoretical calculation has also been performed to evaluate the conformation[12], but again since this used the incorrect sequence it is of no value.

There has been no previous high-field, Fourier transform. Nmr study of alamethicin. Chapman mentioned the 'sharpness' or otherwise of alamethicin spectra under various conditions[13], and he also published a 60 MHz spectrum which although of poor quality clearly shows the aromatic moiety, but which is interpreted by Chapman as "amide" protons[14]. These spectra were run as part of his studies of the interaction of alamethicin with phospholipids, and not to study the conformation of alamethicin itself.

In this thesis the results are described of a study of alamethicin using a 270 MHz Fourier Transform Nmr. spectrometer, from which we have been able to deduce the chemical nature of alamethicin and determine the solution conformation of both of the major components.

REFERENCES

1. B. C. Pressman — Fed. Proc. 27, 1283 (1968)
2. P. Mueller, D. O. Rudin, J. Theoret. Biol. 18, 222 (1968)
3. P. Mueller, D. O. Rudin, — Nature, 217, 713 (1968)
4. R. J. Cherry, D. Chapman, J. Membrane Biol. 7, 325 (1972)
5. L. G. M. Gordon, D. A. Haydon — Biochem. Biophys. Acta 255, 1014 (1972)
6. D. A. Haydon, S. B. Hladky — Quart. Rev. Biophys. 5, 187 (1972)
7. E. A. Trull — Ann. Rev. Phys. Chem. 21, 317 (1970)
8. O. Jardetsky — Ann. Rev. Biochem. 40, 605 (1971)
9. R. A. Dwek — N. m. r. in Biochemistry Clarendon Press Oxford 1973
10. A. I. McMullen, D. I. Marlborough, P. M. Bayley Febs. Letters 16 no. 4 278 (1971)
11. A. I. McMullen, J. A. Stirrup — Biochem. Biophys. Acta 241, 807 (1971)
12. A. W. Burgess, S. J. Leach — Biopolymers 12, 2691 (1973)
13. D. Chapman et al — Nature Lond. 224, 692 (1969)
14. D. Chapman, H. Hauser, E. G. Finer, J. Molec. Biol. 53, 519 (1970)
15. A. V. Xavier, R. J. P. Williams, C. D. Barry, Nature 232, 236 (1971)
16. C. M. Dobson, R. J. P. Williams et al in Press
17. C. D. Barry, D. R. Martin, R. J. P. Williams, A. V. Xavier J. Mol. Biol. 84, 491 (1974)

CHAPTER TWO

THEORETICAL

Abbreviations

k	The Boltzmann constant
h	Planck's constant
$\hbar$	$h/2\pi$
τ	Frequency in Hz (Hertz)
ω	Frequency in radians per second
g	The 'g' value for the electron
g_L	The Lande 'g' factor
g_N	The nuclear 'g' factor
β_N	The nuclear Bohr magneton;
γ	The magnetogyric ratio for the nucleus
H_o	The main static applied magnetic field in an nmr. experiment
H_1	The radio-frequency field
M_z	The (macroscopic) magnetisation along the z axis
M_o	The equilibrium value of M_z, in the absence of radio-frequency field
T_1	The spin lattice relaxation time
T_2	The spin-spin relaxation time
χ_z	The value of the susceptibility in the z direction
T	Temperature ^oK
$\delta_{Obs.}$	Observed induced shift
A_N	The hyperfine coupling constant

This chapter is divided into two parts. Part I will give a brief and elementary discussion of general Nmr. theory. Part II will give a brief discussion of paramagnetic Nmr. theory. Detailed discussion of most of the topics covered in both of these parts will be found in Appendix 1., and reference should be made to the separate index for Appendix 1 on page 216.

PART I GENERAL THEORY

The most important parameters which may be obtained in an Nmr. experiment are the chemical shift and the relaxation times.

Chemical shift

This is the energy of transition of the nucleus between the various possible orientations of the nuclear magnetic dipole in a magnetic field (two such orientations for spin $\frac{1}{2}$ nuclei such as ^{1}H, ^{13}C, ^{19}F). The energy of such a transition is given by:-

$$h\nu_0 = g_N \beta_N H_{eff} \qquad 2.1.1$$

where $H_{eff} = H_0(1 - \sigma)$

H_0 is the main applied magnetic field and H_{eff} is the effective field felt by an individual nucleus and differs from H_0 in the case of protons by a few parts per million (PPM), due to the shielding of the nucleus by surrounding electrons. This shielding, represented by σ in equ. 2.1.1, is principally determined by the chemical group of which the nucleus is a part, though secondary diamagnetic interactions also change it.

For protons in diamagnetic systems, almost all transitions fall within a range from 0 - 10 ppm downfield of the Tetra-methyl silane reference. Typical shifts are given in Fig. 2.1.1.

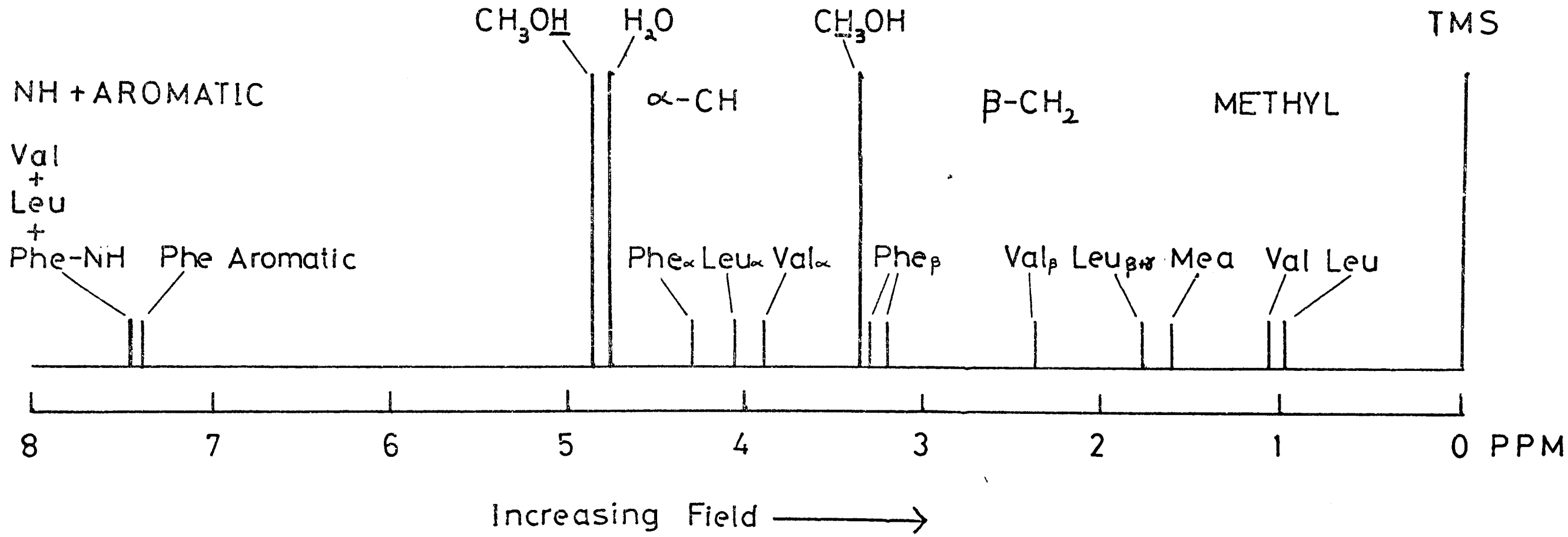


Fig. 2.1.1.

Typical chemical shifts of some common amino-acids, showing the general regions of the Nmr spectrum where the various types of proton peaks occur. Some common solvents are included.

Other nuclei can give much larger chemical shifts e. g. 200 PPM for ^{13}C.

Typical frequencies for different nuclei for a value of the main field H_o of 23, 500 guass (2. 35 Tesla) are:-

^{1}H	100 MHz
^{19}F	94. 1 MHz
^{31}P	38. 1 MHz

Thus the energies of transition are in the radio-frequency region of the spectrum. The transitions are induced by applying an oscillating radio-frequency signal along the x axis, the main field H_o is always assumed along the z axis.

The 'fine structure' of an nmr. spectrum arises from spin-spin coupling. This is an interaction between the spins of neighbouring nuclei which is transmitted through the chemical bonds and thus falls rapidly with the number of bonds. Proton-proton couplings are rarely larger than 10 Hz and this energy of interaction is independent of the strength of the main magnetic field H_o. In the simple case, (where the difference in chemical shift of the nuclei involved is much larger than the coupling constants), couplings to N equivalent neighbouring nuclei will split a single line into N + 1 lines.

The interaction is removed if one of the nuclei is caused to undergo very rapid transitions, this may happen in two ways:-

1. In a spin 'decoupling' experiment where the sample is irradiated with radiation corresponding to the resonance energy of one of the nuclei concerned whilst the spectrum is being observed. This is of course a very useful assignment tool.

2. Rapid relaxation of the nucleus, e. g. by addition of certain paramagnetic materials will remove the coupling.

Use of spin decoupling in assignment

This technique consists of irradiating a very narrow band of the spectrum whilst observing the rest of the spectrum normally. The effect of the irradiation is to cause very rapid transitions of the nucleus or nuclei with resonant frequencies at the irradiation frequency. These very rapid transitions remove all the spin-spin interactions in which the irradiated nucleus may be involved, and will thus cause simplification of the fine structure of any peaks from nuclei to which the irradiated nucleus is normally coupled. If the irradiated nucleus is the only nucleus coupled to a particular observed nucleus then the peak from that observed nucleus will be completely decoupled to a singlet.

It is thus possible in theory to find all the protons of a particular molecule, if they are all coupled to at least one other proton in the molecule, by successive decoupling experiments.

The 'width' of the irradiation band depends on the power applied at the irradiation frequency, but it is quite possible to reduce the width of effective decoupling to only several hundredths of a PPM.

Details of spectrometers and methods of obtaining 'high resolution' are discussed in standard texts.[1-5]

Relaxation

When no radio-frequency field H_1 is being applied and there is only the main applied static field H_o, then M_z has an equilibrium value M_o which is dependent on the Boltzmann distribution between the various allowed orientations of the magnetic dipole in the field, and M_x and M_y are effectively zero (i. e. the nuclei are 'precessing' about H_o which is always assumed along the z axis).

When this equilibrium is disturbed the magnetisation 'relaxes' back to equilibrium by (approximately) first order processes and the time constants for these are the two relaxation times T_1, the spin-lattice, or longtitudinal relaxation time, which describes the relaxation of M_z,; and T_2, the spin-spin or transverse relaxation time which describes the relaxation of M_x and M_y. The equations have been derived by Bloch[6]:-

$$\frac{dM_x}{dt} = \omega_o M_y - \frac{M_x}{T}$$

$$\frac{dM_z}{dt} = -\frac{(M_z - M_o)}{T_1} \qquad 2.1.2$$

In solution T_1 and T_2 are often equal but T_1 may be longer than T_2 because different processes cause the relaxations. These processes may be simply summarised as follows:-

For T_1

This is determined by the component in the xy plane of the randomly fluctuating magnetic field which is at the Larmor (resonance) frequency as this will cause transitions in an analaguous manner to externally applied fields. Such transitions obviously change M_z and cause it to relax to M_o in a first order manner. These randomly fluctuating magnetic fields are produced

by tumbling of the molecules (which will contain magnetic nuclei) and various other processes such as electron relaxation of paramagnetic species.

For T_2

Any process which contributes to T_1 will contribute to T_2 because after a transition the components m_x and m_y of the individual nucleus concerned are randomised and thus the macroscopic components M_x and M_y tend to their equilibrium value of zero.

Additional contributions to T_2 are as follows. Any field along the z axis, whatever its frequency even zero, will change the resonance energy and thus change the 'precession' rate of the nucleus (which is equal to the frequency of the electromagnetic radiation of correct energy to cause a transition) and will thus relax M_x and M_y. In solids the static field from nearby nuclei is responsible for the very efficient transverse relaxation i.e. short T_2 and very broad lines which are observed. Magnetic field inhomogeneity will also cause relaxation and thus broaden the lines. There is a further mechanism which contributes to T_2, this is the spin-spin mechanism whereby one nucleus flips 'down' and a neighbouring nucleus absorbs the radiation and thereby flips 'up'. This process leaves M_z unchanged, but m_x and m_y for the nuclei involved become randomised and thus relaxation occurs.

In a 'continuous wave' Nmr. experiment the line-width is proportional to T_2^{-1} and does not depend on T_1 unless too much radio-frequency power is used (saturation). In the Fourier Transform mode the line-width is always proportional to T_2^{-1}. In both modes of operation the total intensity of a signal, i.e. its area, under normal operating conditions, is dependent only on the number of protons giving rise to the signal(and the concentration).

Measurement of the spectrum will now be considered and an important new technique developed in our laboratory for changing the line shape and hence improving the resolution of the spectrum. This technique is known as Resolution Enhancement. All our measurements were made using a Fourier transform spectrometer rather than the older continuous wave spectrometer. The two modes of operation are fundamentally different, and only the Fourier transform mode will be considered in any detail here.

Fourier Transform Spectroscopy[7, 8]

The principal advantage of this technique compared to the older continuous wave mode is the much better signal to noise which may be obtained. An improvement in signal to noise of a factor of ten for a given time is easily obtained, this corresponds to a saving in time (for given signal to noise) of a factor of a hundred. This has extended greatly the range of concentrations which it is feasible to study, down to a few mM with protons. It also makes a study of nuclei such as ^{13}C, in natural abundance feasible.

The essential reason for this improvement is that in Fourier Transform spectroscopy information is obtained from the whole spectrum all the time whereas in the continuous wave mode only a very small part of the spectrum is being inspected at any one time.

In order to understand the resolution enhancement technique for changing the line shape mentioned above, it will be necessary to consider Fourier Transform in a little more detail.

In the Fourier Transform experiment a very powerful pulse of radio-frequency radiation is applied to the sample for a very short time. The effect of this pulse is to turn the net magnetisation of all the nuclei which normally point along the (applied field) z direction, into the xy plane. (Leaving $M_z = 0$). When the pulse is removed the magnetisation starts to relax from this non-equilibrium state according to the Bloch equations (page 12) The detector measures the magnetisation in the xy plane and thus 'sees' an initially large magnetisation decay in a first order process. (i. e. exponentially) to zero in a time governed by the spin-spin relaxation time T_2. This free induction decay is fed via an analogue to digital converter directly into the memory of an on-line computer. The whole process is repeated many times to average out the noise. The equation for the behaviour of the magnetisation with time, as seen by the detector, $M_{det.}(t)$ is the sum of exponentially damped sine waves as:

$$M_{det.}(t) = \sum^{i} \mathrm{Sin}(\omega_i - \omega_{r.f.})t \; . \; \exp(-t/T_{2i}) \qquad 2.1.3.$$

ω_i = frequency of the i^{th} nucleus

$\omega_{r.f.}$ = frequency of the applied RF pulse

T_{2i} = T_2 of i^{th} nucleus.

Thus this free induction decay 'contains' the same information as a normal spectrum, i. e. the resonant frequencies ω_i and the line widths ($\propto T_2^{-1}$) of all the nuclei in the sample.

The data thus obtained may be converted into the conventional spectrum by the process of Fourier Transformation, which is performed by the computer. However it is an important advantage of Fourier Transform spectroscopy that because the data is in digital form in a computer, various mathematical tricks may be performed on it using standard operations which are programmed into the computer. One important 'trick' will now be considered.

Resolution Enhancement

The advent of very large magnetic fields has helped to alleviate one of the main problems of Nmr. namely, overlapping resonances, but has not really removed the cause of the problem, which is the Lorentzian shape of the resonance lines. Lorentzian lines have relatively large, long wings and thus worsen the overlap problem.

The idea of narrowing lines without changing their shape has been known for some time, but in practice it has many problems and the procedures used[20, 21] still do not get rid of the large wings. However it is possible to change the basic shape of the lines and so cut out these long wings, to a large extent, by mathematical manipulation of the free induction decay (or the lines themselves).

The method consists, in essence, of subtracting two lines of Lorentzian shape but with different linewidths. This is usually achieved by manipulation of the free induction decay rather than the peaks themselves (though the two processes are of course entirely equivalent). The detailed mathematics is given in the appendix, but in brief the free induction decay is multiplied by a function of the form:-

$$\exp(-t/\tau_1) - K \exp(-t/\tau_2)$$

Typical values might be

$\tau_1 = 0.194$ sec. $\tau_2 = 0.272$ sec. $K = 1.02$

The constants are adjusted empirically to give the desired result.

The results of this operation on a simulated peak are shown in Fig. 2. 1. 2., where the free induction decays corresponding

RESOLUTION ENHANCEMENT

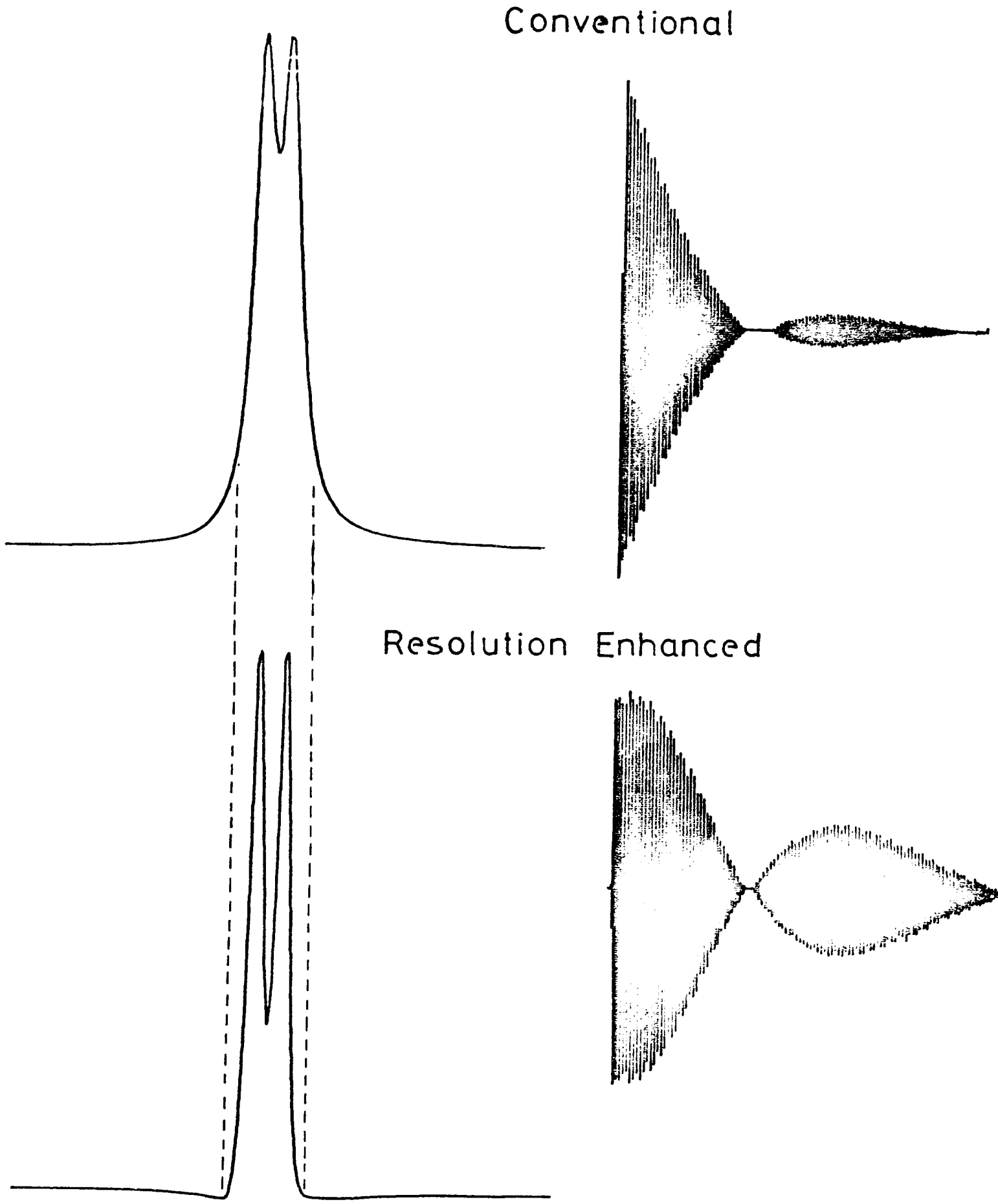


Fig 2.1.2

The effect of resolution enhancement on simulated peaks. Note how the long "wings" of the Lorentzian peak (top) are removed. The corresponding free induction decays are given on the right.

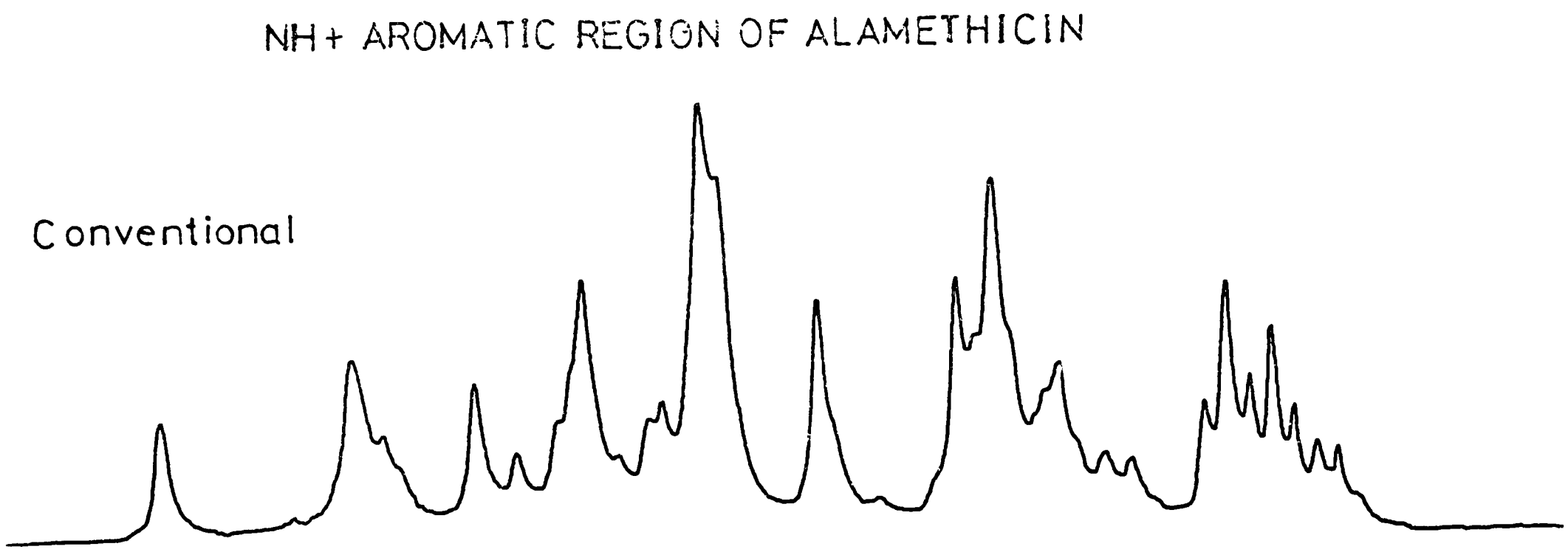


Fig. 2.1.3

The effect of resolution enhancement on a typical spectrum in methanol at 298°K .

to the peaks are also shown. The result on part of an actual spectrum of alamethicin is shown in Fig. 2.1.3. As can be seen from this figure, peaks which appear as barely resolved shoulders in a conventional Fourier Transform spectrum are clearly resolved in the Resolution Enhanced Spectrum. It should be pointed out that relative areas are not preserved by this technique unless the peaks have the same line-width (see appendix 1).

Considerable use was made of this technique and most of the spectra shown in this thesis are resolution enhanced. The abbreviation R.E. will be adopted for such spectra.

Exchangeable protons

The phenomenon of exchange of protons with solvent nuclei is a limitation on the types of protons which are observable by n.m.r. There are two distinct possible consequences of this exchange:-

1. Most nmr spectra are run in fully deuterated solvents and if the solvent deuterons are exchangeable then the exchangeable protons in the molecule under observation will be steadily replaced by deuterons (of which there are a vast excess) and will hence vanish from the spectrum irreversibly.
2. If the spectrum is run in a normal 'protonated' solvent, or alternatively if the deuterated solvent contains no exchangeable deuterons (e.g. fully deuterated dimethyl sulphoxide), there will still be problems if there are hydrogen nuclei with which those of the observed molecule can exchange, (e.g. traces of water in dimethyl sulphoxide). In this case the exchange will cause a broadening of the signal from the exchanging nucleus, and if exchange is sufficiently rapid the broadening will be so great that the signal will be quite unobservable and also the signal will merge with that from the (solvent) protons with which it is exchanging. This process is reversible in that if the

conditions are changed such that exchange becomes slow again, for example by changing the pH of the sample, the signal from the proton will reappear in the spectrum.

Protons in peptides may be roughly classified as follows as regards their exchange rates:-

1. Any proton bound directly to a carbon is quite unexchangeable.
2. Peptide NH protons are exchangeable, but it is usually possible to observe them in deuterated solvents at the correct pH for reasonably long periods of time, and they are usually observable indefinitely in 'protonated' solvents at the correct pH. The exchange rate is, however, very dependent on the solvent as well, and for example in deuterated dimethyl sulphoxide NH protons are more or less indefinitely stable.
3. Hydroxyl protons exchange extremely rapidly and are normally quite unobservable except in solvents such as dimethyl sulphoxide (chloroform etc.) and then extremely careful drying, to remove water, is required. So that in practice hydroxyl protons are very difficult to observe.

PART II. PARAMAGNETIC EFFECTS

The theory in this section will not be needed until the material in and beyond chapter four is considered, and it may therefore be useful to return to this section before reading those chapters.

The paramagnetic effects[10, 11, 12] are caused by the large fluctuating magnetic fields produced by the very large magnetic moment of the unpaired electron. The precise magnitude of the effects however depends on the relative time-scales of various events such as molecular tumbling, rate of electron relaxation, rate of exchange (on and off) of the paramagnetic species binding to the molecule under observation, relaxation rates and chemical shifts of the nuclei involved.

The two principal effects of a paramagnetic centre which are of interest are:-

1. Changes in resonance energy (i. e. chemical shift) of nearby nuclei by two distinct mechanisms, Fermi contact and Pseudo-contact.
2. Changes in relaxation times (both T_1 and T_2).

These effects will now be discussed in a little more detail:-

1. Changes in chemical shift

(a) Fermi contact[13]

This is a direct interaction between the nucleus and unpaired electron density at the nucleus, and is thus transmitted through the bonds. The shift for lanthanides other than Europium is given by:-[14]

$$\delta_{f.b.} = \frac{A_N}{\hbar} \frac{J(J+1)g_L(g_L-1)}{3kT} \qquad 2.2.1$$

It will be shown that this mechanism is negligible for most of the protons in our system.

(b) Pseudo contact

This is a through space dipolar interaction between the unpaired electron(s) and the nucleus. It involves electron spin-orbit; electron orbit-nuclear spin; and electron spin-nuclear spin; dipolar couplings. It is an isotropic interaction, i.e. to a first approximation it is independent of the orientation of the molecule with respect to H_o.

The theory of the pseudo-contact shift has been evaluated for ions with anisotropic g values by McConnell and Robertson[15]. J. P. Jesson[16] showed that McConnells approach was valid for a number of Kramers doublets of different energy and derived various values for the shift dependent on the relative values of the molecular tumbling, the electron relaxation rate, and the Zeeman anisotropy energy. This however involves approximations as has been pointed out[17]. There are also various assumptions[18] in their derivation.

Bleaney has argued[19] that the above treatments, which perform the calculations in terms of anisotropic 'g' values, are invalid for the case of lanthanide ions, where the lifetime of an ion in any one state is exceedingly small compared with the Nmr lifetime. Bleaney calculates the pseudo-contact shift in terms of anisotropic susceptibility. The important point however is that both treatments give the same geometric dependences for the shifts though the values of the 'constants' in the expressions vary.

In the simplifying case of axial symmetry when $\chi_x = \chi_y$ the equations are of the form,

$$\delta_{f.b.} = K \frac{3\cos^2\theta - 1}{r^3} \qquad 2.2.2$$

K is a constant for any particular lanthanide at constant temperature

θ is the angle between the principal symmetry axis and the vector joining the paramagnetic centre and the nucleus in question.

r is the distance from the paramagnetic centre to the nucleus in question.

$\delta_{f.b.}$ is the change in chemical shift caused by the paramagnetic species.

This equation gives the induced paramagnetic shift, for which the abbreviation shift will be commonly used throughout this thesis.

Thus if the ratios are taken of the (induced paramagnetic) shifts of different nuclei in the same molecule for a particular lanthanide the unknown 'constant' is eliminated and only the geometric factors are left. Thus the conformation may be elucidated (this is discussed in more detail on page 13).

However the general form of the equation is as follows:[15-19]

$$\delta_{f.b} = A \frac{3\cos^2\theta - 1}{r^3} + B \frac{\sin^2\theta . \cos 2\phi}{r^3} \qquad 2.2.3$$

A, B are constant for any one lanthanide at constant temperature

ϕ angle between the subsidiary axis and the paramagnetic centre–nucleus vector.

The ratio method does not now eliminate the unwanted constants and the whole situation is much more complex (this is discussed in more detail on page 139).

Effect of 'exchange' on the shift

The above treatment gives the shift for the fully bound complex of paramagnetic species and observed molecule from the diamagnetic

position. In practice for the systems we will consider there are appreciable concentrations of unbound molecules, and the paramagnetic species will be exchanging on and off the molecule observed. It was demonstrated experimentally that there was fast exchange, when the exchange rate is much greater than the fully bound chemical shift. In this situation the observed resonance position is a weighted average of the positions in the bound and unbound species. Thus the shift from the unbound position on binding a paramagnetic species M to a ligand L is simply:-

$$\delta_{obs.} = \frac{[ML]}{[L_t]} \times \delta_{f.b.} \qquad 2.2.4$$

where $[L_t]$ is the total ligand concentration

$[ML]$ is the concentration of complex

$\delta_{f.b.}$ is the fully bound shift (from the unbound position).

The fraction bound $[ML]/[L_t]$ factor is obviously constant for the different nuclei of a particular molecule, thus the geometric dependence of the shifts given in equations 2.2.2 or 2.2.3 is preserved.

2. Paramagnetic relaxation effects

The equations for the relaxation times T_{iM} in the fully bound complex may be written,

$$(T_{1M})^{-1} = Ar^{-6} + [B]$$

$$(T_{2M})^{-1} = Ar^{-6} + [B] \qquad 2.2.5$$

It can be shown for our systems that the contact term B in brackets is negligible. (Like the contact shift term is is a through bond effect). This simply leaves the term in r^{-6} which is a dipolar through space

interaction, and relative distances can be obtained from the values of T_{1M} and T_{2M} without needing the value of the constant.

Effect of exchange

The same comments are relevant here as for the case of shifts. The situation can be very complex (see appendix 1), but in the case of fast exchange, the observed values of T_1 and T_2 are simply weighted averages of the bound and unbound values so that we may simply write:-

$$\frac{f}{T_{1M}} = \frac{1}{T_{1obs}} - \frac{1}{T_{1A^o}} - \frac{1}{T_{1A''}} = \frac{f\ Const.}{r^6} \qquad 2.2.6.$$

f = fraction of ligand bound

$T_{1A}{}^o$ is the relaxation time at the unbound site in the absence of added paramagnetic ion.

$T_{1A''}$ is a term to account for the bulk susceptibility effect of the added paramagnetic ion.

Identical equations apply for T_2.

All the quantities on the right hand side of the equation can be measured ($T_{1A''}$ is estimated by use of a suitable blank) and so $T_{1M}{}^{-1}$ may be obtained and thus the relative distances of the various nuclei from the bound paramagnetic species.

Summary of paramagnetic theory with application to conformational studies

If a paramagnetic species, such as a lanthanide ion, is bound to a single site in a molecule, and if through-bond (i.e. contact) effects are negligible, then geometric information can be obtained from the through space, (dipolar) effects. The situation is greatly simplified if exchange between the bound and unbound species is rapid.

The changes in chemical shifts are given by an equation of the form:- (fast exchange, non-axial symmetry)

$$\delta_{obs.} = f\left[A\,\frac{3\cos^2\theta - 1}{r^3} + B\,\frac{\sin^2\theta\,\cos 2\phi}{r^3}\right] \qquad 2.2.7$$

f = fraction of ligand bound = $\frac{[\text{complex}]}{[\text{total ligand}]}$

so that the resonances can be 'titrated' by adding more and more paramagnetic species. This experiment will yield distance r and angular (θ , ϕ) information. The most useful situation is when there is axial symmetry, in this case the second term of equation 2.2.7 disappears and the shift now depends only on one angle, θ, so that by taking ratios of shifts for any one lanthanide the constant in the expression can be eliminated.

The changes in relaxation times, are given by:-

$$\frac{1}{T_{1obs}} - \frac{1}{T_{1A'}} - \frac{1}{T_{1A''}} = \frac{f\,Const.}{r^6} \qquad 2.2.8$$

(for the case of fast exchange)

Again by adding progressively more of the paramagnetic species a steadily increasing effect is observed. This experiment yields distance information, (though note the distance dependence is different from that in the shift case). In order to obtain relative distances it is not necessary to evaluate the constant.

It is a singular advantage of the lanthanide ions that they are a virtually isomorphous set of ions which show both the shift and relaxation effects above to differing extents, and so it is possible to choose the ions which are most suited to the study of the particular system, and also by use of several of these ions it is possible to test whether there is the simplifying case (for the shifts) of axial symmetry.

It is therefore the case that a detailed analysis of the shift and relaxation data will lead directly to the conformation of a molecule.

A detailed discussion of the lanthanide method is given in chapter 4.

REFERENCES

1. J.A. Pople, W.G. Schneider, H.J. Bernstein 'High Resolution Nmr.' McGraw -Hil (1959)
2. A. Carrington, A. McLachlan, 'Introduction to magnetic resonance' Harper and Row (1967)
3. A. Abragam 'The Principles of nuclear magnetism Clarendon Press Oxford (1961)
4. J.D. Roberts 'Nuclear magnetic resonance'
5. J.W. Emsley, J. Feeney, L.H. Sutcliff, 'High resolution N.m.r. spectroscopy' Pergamon Press (1965)
6. F. Bloch Phys. Rev, 70, 460 (1946)
7. R.R. Ernst Rev. Sci. Instr. 37 no. 1, 93 (1966)
8. T.C. Farrar, E.D. Becker 'Pulse and Fourier Transform Nmr; introduction to theory and methods' Academic Press (1971)
9. I.D. Cambell, C.M. Dobson, R.J.P. Williams and A.V. Xavier J. Mag. Res. 11, 172 (1973)
10. D.R. Eaton, W.D. Phillips, Adv. Mag. Res. 1, 103 (1965)
11. G.A. Webb Ann. Rep. Nmr. 3, 211 (1970)
12. H. Van Willegan Prog. in Nmr. 2, 111 (1967)
13. E. Fermi Zeit Physik 60, 320 (1930)
14. J. Reuben J.C.P. 51, 4909 (1969)
15. H.M. McConnell, J.C.P. 29, 1361, (1958)
16. J.P. Jesson J.C.P. 47, 579 (1967)
17. W. DeW. Horrocks In. Chem. 9, 690 (1970)
18. R.J. Kurland, B.R. McGarvey, J. Mag. Res. 2, 286 (1970)
19. B. Bleaney J. Mag. Res. 8, 91 (1972)

20. R. R. Ernst, R. Freeman, B. O. Gestbloom & T. R. Lusebrink
Mol. Phys. 13, 283 (1967)

21. R. R. Ernst
'Advances in Magnetic Resonance'
J. S. Waugh Ed. pp 1-132
Academic Press New York

CHAPTER THREE

CHEMICAL NATURE AND SEQUENCE OF ALAMETHICIN

The chapter will be divided as follows:-

1. Part I, will deal with the chemical composition of alamethicin, its preparation and the chromatographic separation of the similar components which constitute what is normally termed 'alamethicin'. The preparation of a very pure sample of the major component will also be discussed.
2. Part II, will deal with the problem of the sequence of the major component the so-called F30.
3. Part III, will deal with the problem of the sequence of the second most common component, F50.

PART I

Chemical composition of alamethicin

The material usually referred to as 'alamethicin' in fact consists of a mixture of similar components which are designated in this thesis by their approximate R_f values on the chromatographic system used for their separation. Thus components F20, F30, F40, F50, F60, F70 have all now been isolated by workers at Porton Microbiological Research Establishment who first reported chromatographic separation of alamethicin[5]. Only two components occur to any substantial extent, F30 (ca. 85%) and F50 (ca. 12%). These two differ, inter alia, in the possession of a carboxyl group so that the differences are not trivial. These various components are not to be confused with the microheterogeneity found in the sequence determinations[3, 4].

All the previous work in the literature has been performed on the unseparated mixture of the various components comprising alamethicin. Thus physical studies of alamethicin[6, 7], conductance studies[8-11], conformational studies[12] and the sequence[4] were all performed on samples of alamethicin supplied by the Upjohn Company. Dr. J. W. Payne kindly supplied us with some of the Upjohn material which was unused in the sequence determination, and the Nmr spectrum of this material has been run and confirms that, as expected from the preparation procedure, the Upjohn alamethicin is indeed a mixture of the various components. The determination of the sequence by mass spectrometric methods[3] by the Russian workers was also performed on a mixed sample.

Preparation of alamethicin

Alamethicin is produced by the mould Trichoderma Viride, and previously various methods have been used for obtaining the alamethicin from the culture medium. The preparation originally described by Meyer and Reusser of the Upjohn Company[1], involves the use of activated charcoal and various solvent extractions, followed by crystallisation. This is similar to the method used by the Russian workers[2, 3]. Neither of these methods separate the several similar components discussed above which constitute alamethicin.

The preparation procedure used at Porton by Dr. J. Melling to prepare the separate components of the alamethicin supplied to us for study will now be discussed. The Trichoderm Viride was grown in a peptone salts and starch medium, the culture liquid was filtered and concentrated, by a factor of ten and then extracted with butanol. This was evaporated under reduced pressure, dissolved in ethanol and put down a DEAE cellulose column. Then the resultant was put down another DEAE cellulose column in aqueous phosphate buffer, the liquid was then washed with ether, extracted with alcohol

and put down a final column which achieved the chromatographic separation of the various components. This column was Kieselgel G (manufactured by Merck) and the solvent system was chloroform 70%, methanol 24%, water 4%. The major components which separate out are the so called F30, approx. 85%, and F50 12%, and these are the two components which have been supplied to us for study. There are also very small amounts of F20, 40, 60, 70, obtained in the above procedure.

Purity of the Porton alamethicin

The above procedure was believed by the workers at Porton to produce very pure alamethicin, and it was the original intention of the project to be able to use the alamethicin without further purification. The initial batches of both F30 and F50 supplied to us were however found by Nmr and other procedures to contain small amounts (a few per cent by weight) of various impurities which absorbed very strongly in the ultra-violet region at about 300 nm. It was also found for example, that one batch of F30 gave a positive ninydrin test which corresponded to approximately 10-15% per mole of a primary amine impurity (F30 itself has no free amine groups). None of these impurities was however of a type, (orpresent in sufficient amount) to interfere with our Nmr studies.

Unfortunately later batches prepared by an apparently identical procedure were more heavily contaminated, and some batches also contained inorganic salt. In particular one large batch supplied to us was found to contain 23% of potassium, and 4% of sodium by weight, probably as the phosphate salts making a total of some 50% by weight of salt! It was therefore necessary

for us to attempt purification of some of the alamethicin ourselves to avoid undue delay to the project, as the problem of contamination by salt was not overcome by the workers at Porton for a considerable time (over six months in fact, after copying a procedure devised by Dr. J. D. Priddle of the Department of Molecular Biophysics at Oxford).

A very pure sample of the major component F30 was prepared for us by Dr. J. D. Priddle of the Department of Molecular Biophysics at Oxford. Cruder samples which were reasonably free from salt contamination were prepared by us by repeated extraction of the salt-contaminated alamethicin with water at low pH, in which alamethicin is virtually insoluble and not present in the ionised form hence binding metal ions more weakly.

Preparation of a very pure alamethicin F30

This preparation was performed by Dr. J. D. Priddle. The starting material was the batch of F30 from Porton which contained a little over 50% by weight of salt. This was dissolved in the minimum quantity of 10% ethanol, 90% water and put down a G 25 sephadex column, no buffer was used. The following materials were eluted and are given in the order in which they came off the column:-

1. A high molecular weight material absorbing at 250-260 nm.
2. Moderately pure F30
3. Numerous peaks from low molecular weight materials absorbing at around 300 nm.

The very pure F30 was obtained by taking a very narrow cut from the centre of the F30 peak and putting this down

the column again to achieve a better separation from the higher molecular weight material.

The ultra violet spectrum of this very pure F30 showed only the symmetrical peptide absorption and the very weak absorption ($\varepsilon \sim 200$) at about 260 nm from the aromatic moiety in the molecule (see Part II).

It was decided to repeat this successful purification on a G50 column, to obviate the need to do the separation twice, because G50 would give a better separation of the high molecular weight materials from the F30, as the F30 is only very slightly retarted on G25. This purification was performed by Dr. J. Melling at Porton using a different starting sample of F30, and a good separation of materials was obtained into the same categories as given above. The pure F30 peak was not however homogeneous, and this may be the result of the microheterogeneity found by J. W. Payne and co-workers[4] and Kiryushkin et al[3].

The Nmr spectra of the very pure material have been run under a wide variety of conditions and show that the non-salt impurities in the usual samples of F30 are only a few per cent by weight and unimportant. These experiments are discussed in a little more detail in part II.

PART II

SEQUENCE OF ALAMETHICIN F30

The sequence of the major component of alamethicin, F30, will now be discussed. Before the results of the present Nmr studies were known, the composition and sequence of alamethicin seemed to have been established beyond all reasonable doubt by the work of various groups (although the work was in fact performed on the mixed alamethicins). Hence the sequence proposed by Payne et al[4] and confirmed by Kiryushkin et al[3] is widely quoted in papers and reviews on alamethicin. The results of our Nmr studies, discussed in detail in this section, show that the sequence is in fact incorrect and that F30 contains various non-amino acid moieties. Before discussing these results a fairly detailed summary of the previous work, particularly that of Payne et al[4] and Kiryshkin et al[3] will be given.

Previous sequence and composition work

Meyer and Reusser in their original paper[1] on alamethicin concluded that alamethicin was a cyclic polypeptide with composition:- $GLN_2\ PRO_2\ GLU_1\ ALA_2\ VAL_2\ LEU_1\ MEA_8$, where MEA is C-methyl alanine or α-amino isobutyric acid. They showed that all the active amino acids were in the L-configuration

J. W. Payne, R. Jakes and B. S. Hartley have performed[4] a complete sequence of alamethicin by conventional chemical techniques and A. A. Kiryushkin et al independently determined the sequence by mass spectrometric techniques[3].

Both groups of workers obtained a composition for alamethicin which differed in having seven and not eight residues of methyl alanine (as reported by Meyer et al[1] see above). However both groups of workers proposed that alamethicin was microheterogenuous and that in some molecules ALA 3 or 5 was replaced by an extra methyl alanine. Both groups of workers found that the supposed cyclising link of alamethicin was very readily cleaved to give a linear polypeptide (which we will refer to as peptide 1), and they unequivocally established the sequence of the peptide 1 as:-

PRO. MEA. ALA. MEA. ALA. GLN. MEA. VAL. MEA. GLY. LEU. MEA.
1 2 3 4 5 6 7 8 9 10 11 12

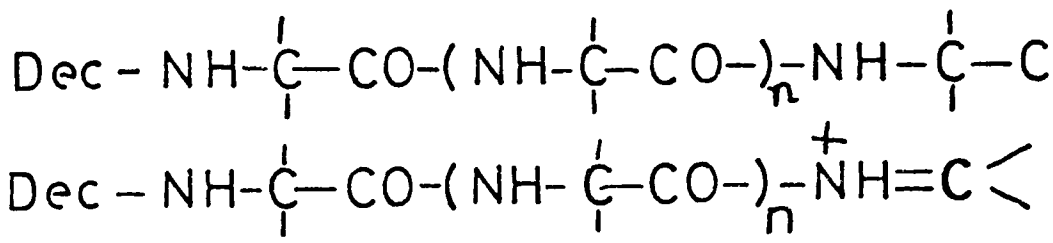
PRO. VAL. MEA. MEA. GLU. GLN. (PRO 1 is the N-terminus).
13 14 15 16 17 18

J. W. Payne et al reported the complete resistance of both the intact alamethicin and peptide 1, above to enzymic digestion by a wide range of enzymes. They used the dansyl-Edman procedure for sequential analysis. The methyl alanine residues made the analysis more difficult than usual, because of poor yields of DNS-methyl alanine and occasional incomplete cleavage of N-terminal methyl alanine.

They isolated a total of twentyfive peptides from various hydrolysis procedures, and many of these were subjected to sequential Edman degradation. If one considers only the sequential degradations then there are gaps in the above linear sequence at 9-10 and 12-13, but both of these gaps are covered by the quantitative amino-acid analysis of the various peptides.

The method used by Kiryushkin et al[3] was to do a partial hydrolysis of the alamethicin, and then separate the peptides on an ion exchange column, with detection by

ninhydrin-complex absorption at 570 nm. The amino terminus of the peptide was then acylated with the n-decanoyl group, and the carboxyl terminus converted to the methyl ester. If such peptides are put into a mass spectrometer they fragment in a relatively simple way, the main ions formed being:



The amino-acids fragment successively from the carboxyl terminus giving the sequence of the entire peptide from one spectrum. Hence by studying a number of peptides from the partially hydrolysed alamethicin the sequence of the whole of alamethicin was built up. The sequence of peptide 1 was well overlapped by the peptides isolated. Also Kiryushkin et al actually isolated peptides with the ALA at position 5 replaced by MEA (see comments on microheterogeneity above).

Thus the result of these two studies is to establish beyond all possible doubt the sequence of peptide 1 given above.

The cyclic sequence for the intact alamethicin, which consists of linking the γ carboxyl group of GLU 17 to PRO 1, was proposed by Payne et al[4] and was confirmed by Kiryushkin et al[3] and is shown in Fig. 3.1. It was arrived at by the following additional arguments:-

1. Mild hydrolysis of alamethicin rapidly yields peptide 1:- PRO^{1}. MEA. ALA. MEA. ALA. GLN. MEA. VAL. MEA. GLY. LEU MEA. PRO. VAL. MEA. MEA. GLU^{17} GLN^{18} and this peptide

ALAMETHICIN SEQUENCE,(INCOMPLETE)

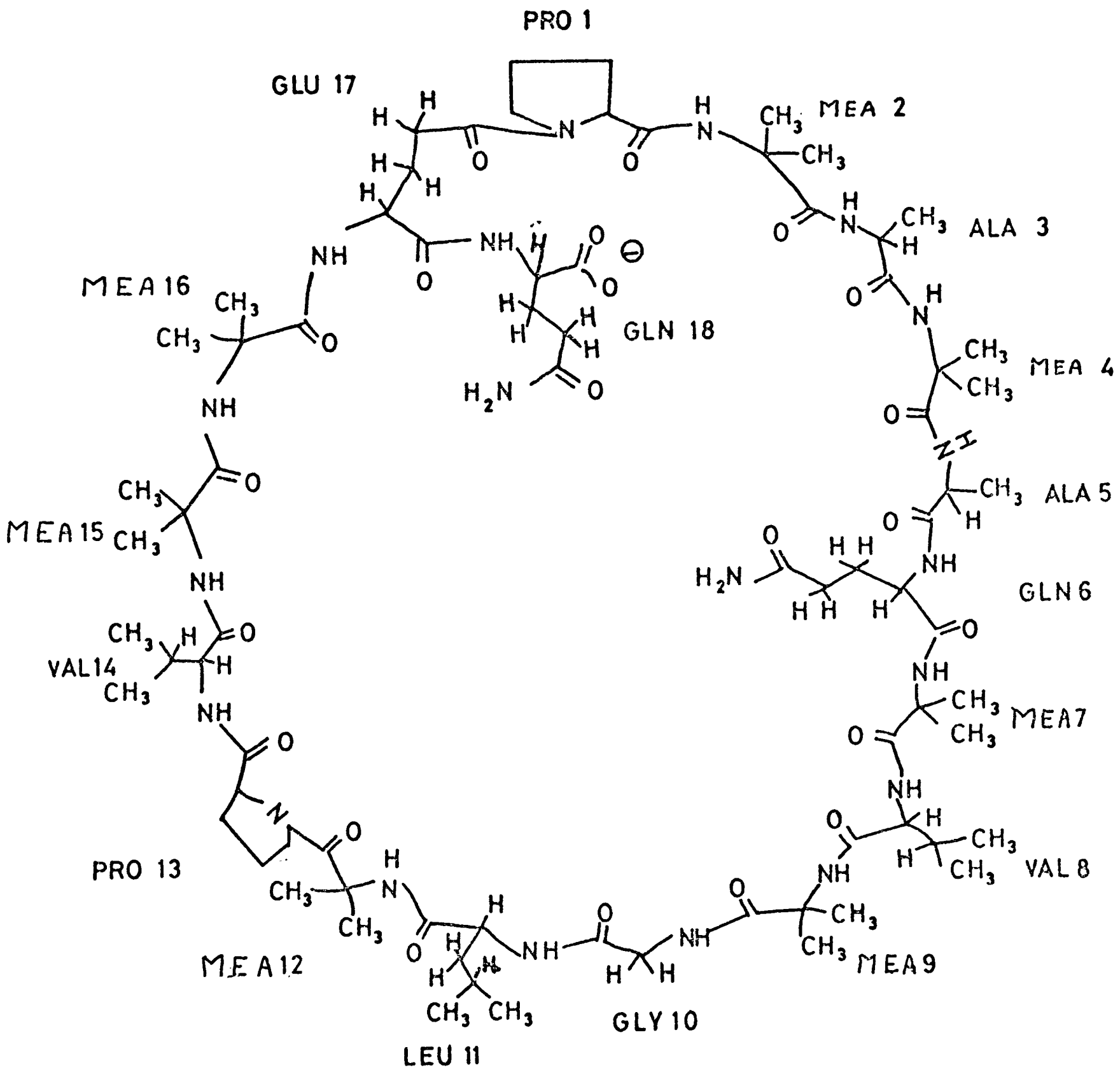


Fig 3.1

The cyclic alamethicin sequence proposed by J.W. Payne et al and confirmed by Kiryushkin et al.

contains all the amino acids found by Payne et al and Kiryushkin et al in alamethicin.

2. There is no free amino group, as shown by titration, ninhydrin test, dansylation[13] and dinitrophenyl[14] methods. There is only one free carboxyl group, and Payne et al[4] and Kiryushkin et al[3] came to the conclusion, by different methods, that the free carboxyl group was α COOH of GLN 18 and not the γ group of the GLU 17.
3. All the peptide bonds in peptide 1 are α linkages since the residues can be removed by Edman degradation. This was confirmed by the mass spectrometric studies and both groups of workers also independently showed that the two GLN in the sequence were normal and not iso-.

Hence it seemed reasonable to link the γ GLU 17 carboxyl group to PRO 1 thus cyclising the alamethicin as shown in Fig. 3.1. The lability which this γ -link can be expected to possess can then be invoked to explain the complete failure of both groups to isolate any peptides which crossed the supposed cyclising link.

This failure to provide direct evidence of the cyclising link was the crucial weakness in the sequence determinations.

There is however one further finding to consider which is not reported in the literature, but was communicated to us via Dr. A. I. McMullen. This concerns some mass spectrometric studies undertaken by W. R. Gray, which appeared to give direct evidence of a direct GLU-PRO linkage. W. R. Gray reduced alamethicin with sodium in liquid ammonia, a technique which when applied under the correct conditions[15] to pure peptides will

selectively cleave the peptide at the N-terminal side of PRO residues converting the preceding amino acid to the aldehyde or alcohol. W. R. Gray succeeded in identifying by mass spectrometry the peptide:-

PRO. VAL. MEA MEAGLU*GLN
13 14 15 16 17 18

where GLU* is the semi-aldehyde of glutamic acid. W. R. Gray put this forward as direct evidence to indicate that the GLU is bonded to the PRO as postulated by Payne et al[4].

To summarise, the work of Payne et al and Kiryushkin et al unequivocally established the sequence of peptide 1 and also apparently provided strong circumstantial evidence for the cyclising of alamethicin by linking the γ carboxyl group of GLU 17 to PRO 1. This seemed to be directly confirmed by the unpublished work of W. R. Gray.

However the Nmr work presented in chapter conclusively shows that the published sequence is incorrect. A considerable amount of experimental work was involved and it will be considered in the following order:-

1. The elucidation of the nature of an extra aromatic moiety in alamethicin. The unexpected peaks from the aromatic group of this moiety were immediately evident in the very first spectra run, (alamethicin supposedly contained no aromatic groups at all see Fig. 3. 1). The proof that the peaks were not from an impurity was followed by the isolation of the aromatic moiety from a complete hydrolysate of the alamethicin, and its identification. Finally there was the difficult problem of placing this moiety in the sequence keeping in mind the considerable number of constraints represented by the previous work on alamethicin.

2. The second extra moeity in alamethicin which gave anomalous peaks in the Nmr spectrum of alamethicin, this time in the methyl group region. This moeity was initially considered to be an impurity, but it was subsequently shown to be from a group covalently linked to the rest of the alamethicin molecule. The isolation of the hydrolysis product of this group from a partial hydrolysate of alamethicin, along with the placement of the intact group in the sequence cleared up the rather perplexing problems left by the extra aromatic moeity in 1. above.
3. At the end of this section there will be a summary of all the evidence and important experiments involved in the determination of the sequence.

1. THE AROMATIC MOEITY IN ALAMETHICIN

Initial evidence for the aromatic moiety

This was obtained when running the initial spectra with the alamethicin dissolved in fully deuterated methanol CD_3OD (see chapter 4 for discussion of choice of solvent). A typical spectrum is shown in Fig. 3.2. and it can be seen that there is an anomalous peak in the aromatic + NH region (7-9 PPM). This anomalous peak is at 7.3 PPM, and it cannot be from an NH proton of any type, for the following reasons:-

1. It is relatively sharp and exhibits a high degree of spin-spin coupling.
2. In contrast to the NH protons which exchange extremely rapidly at room temperature and high pH with the deuterons of the solvent, hence disappearing from the spectrum, this peak is quite unaffected even after 24 hrs at $80^{o}C$ in D_2O at high pH (see Fig. 3.2 insert).
3. In a ^{13}C Nmr spectrum aromatic peaks appear in a distinct region separated from peaks from other types of carbon. A ^{13}C spectrum of alamethicin clearly shows four peaks in the aromatic region, probably corresponding to six 'aromatic' carbon atoms.

There were now two possibilites,

(a) The aromatic moeity was an impurity which was strongly adsorbed to the alamethicin, the line-width quite definitely showing that it could not be a small molecule present free in solution, and the molar proportions 1:1 showing that it cannot be a large molecule unless the alamethicin is extremely impure which is very unlikely after all the separation procedures it has been through.

(b) It is covalently bound to the alamethicin, and hence the sequence is incorrect.

270 MHz SPECTRUM OF ALAMETHICIN (F30) IN CD_3OD

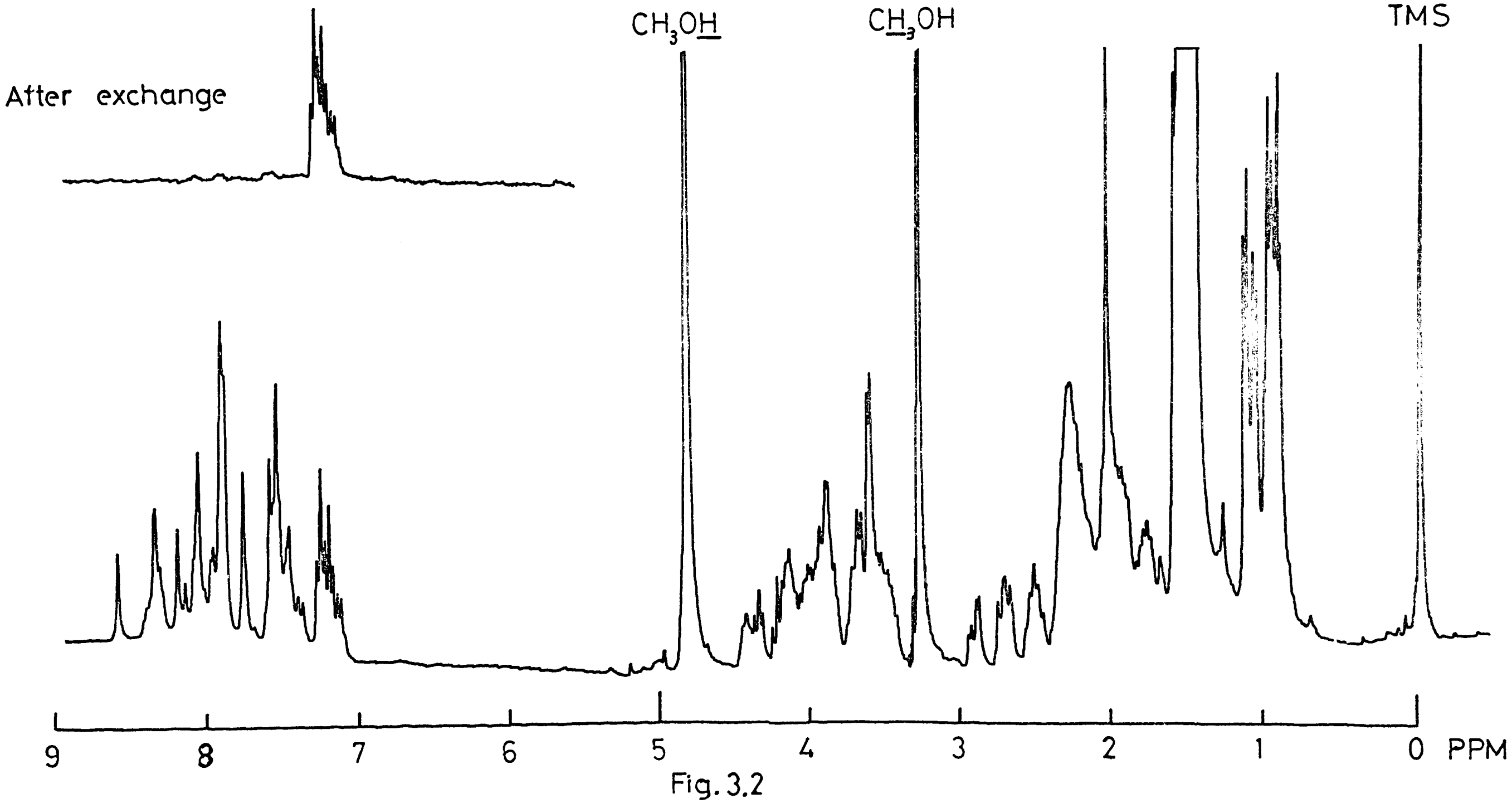


Fig. 3.2

The evidence was strongly against (a) even at this stage for the following reasons:-

1. In contrast to various true impurities, the amounts of which varied wildly from batch to batch, the intensity of this aromatic peak was constant for all the batches i.e. the aromatic group was always present in the same molar proportions, in fact the molar proportion was 1:1 with the alamethicin.
2. The peaks from this aromatic moiety were present in identical form in both F30 and F50, and it is unlikely that an impurity is going to appear in molar proportions in two chromatographically separable materials.

The conclusive evidence that this aromatic group was not an impurity but was indeed covalently bound into the alamethicin accumulated as the experiments progressed but it is convenient to summarise the evidence here:-

1. The peaks from the aromatic group of the aromatic moiety, and also other peaks in different regions of the spectrum which were subsequently found to come from non-aromatic protons in the aromatic moeity, are all present in identical form and unchanged intensity in the spectra of the very pure sample of F30 prepared as described at the beginning of this chapter.
2. It was shown that the free amine group present in the aromatic moeity after it had been hydrolysedoff the alamethicin, was converted into an amide linkage, -CO-NH-, in the intact alamethicin hence directly establishing the presence of at least one covalent bond.
3. The hydrolysis of the aromatic moiety off the alamethicin was directly observed by Nmr as it was occurring, under mild conditions, with a time-scale which depended on the concentration of acid but which was of the order of hours (2 hrs, 3.5 Molar acid, 37^{o} C in 75% methanol).

These experiments are all discussed in more detail later in this section.

It was necessary to start by making a few simple checks. Firstly there is the possibility that the alamethicin supplied to us was different from the alamethicin produced by the Upjohn Company, on which all previous work, except that of the Russian workers had been performed. Dr. J.W. Payne who was one of those who performed the chemical sequence work on alamethicin[4], kindly supplied us with some of the Upjohn alamethicin which was unused in the sequence determination. The Nmr spectrum of this material showed identical aromatic peaks, with the same area as those of our samples of alamethicin. Further a previously published spectrum of Upjohn alamethicin[16], although run on a much inferior spectrometer and therefore of poor quality, does clearly show a broad peak in the aromatic region which does not exchange. The authors of this paper try to explain this peak by assigning it to unexchangeable amide protons, but this is hardly possible in a molecule such as alamethicin. Hence it is evident that all previous samples of alamethicin have contained this aromatic moiety.

It was alsc decided to perform a conventional amino acid analysis on the F30. This was performed for us by Dr. G. Gonzales of the Department of Molecular Biophysis at Oxford. The result was the same as had been obtained by all previous workers.

There are now two problems to be solved, firstly the identification of this extra unit, and secondly how it is to be joined onto the rest of the alamethicin. Although chemical techniques were used to some extent in this study almost all the work

was Nmr work and Nmr is a good technique for this type of problem for the following reasons:-

1. If there were any suggestion that some reorganisation of the PRO 1 to GLN 18 sequence were necessary obviously Nmr would be a quite unsuitable technique to use. However the PRO 1 to GLN 18 sequence can be taken as immutable, and so the principal problem is one of identification and then attaching the extra unit into the molecule. A study of the various protons present in the aromatic moiety hydrolysed off the alamethicin but not in the intact aromatic moeity will show how the unit is bound in.
2. Nmr does possess the singular advantage over almost all other physical and chemical techniques of having virtually no 'blind spots'. Those which do occur in diamagnetic systems, namely exchange of protons, and protons obscured by the solvent peak, are well understood and easily overcome. This means that almost every single proton in the molecule can be seen in the Nmr spectrum under the right conditions, and furthermore the chemical shift of the protons and their spin-spin couplings provide a tremendous amount of information concerning the atoms attached to the carbons bearing the protons and this must eventually lead to a solution of the problem. For a molecule the size of alamethicin (molecular weight ca. 2000), and with present very high field spectrometers and the various techniques available it is difficult for any unexpected protons to remain hidden in the spectrum for very long and it is certainly not possible for a whole unit possessing several protons in different regions of the spectrum, as it almost certainly must, to go undetected for very long. It will indeed be seen in the discussion of the experimental work that extra, quite unexpected protons were discovered at various times.

IDENTIFICATION OF THE AROMATIC MOIETY

The abbreviation PHX will be used to denote the whole of the aromatic moiety as it is present in the intact alamethicin F30. The abbreviation PHXH will be used to denote the aromatic moiety which is present in solution after a complete hydrolysis of the alamethicin F30.

Preliminary evidence

It is important to know the accurate area of the aromatic proton peak. However it is extremely difficult to integrate a spectrum so accurately as to be able to say categorically that a single peak is from five rather than four protons. So the peak was split up into three distinct peaks by addition of the europium ion, which induces shifts of the peaks, these three peaks were of area 2:2:1 and so it can be definitely stated that there are five aromatic protons per molecule. It was also possible to perform decoupling experiments on these three shifted peaks and so these peaks could be assigned to the ortho, meta and para proton(s) of a phenyl ring.

The chemical shift of the proton peak and its area, and also the ^{13}C spectrum clearly show that there is a phenyl ring attached to a non-electron withdrawing carbon. A close inspection of the F30 spectrum also shows a pair of quartets in the CH_2 region (each of single proton area), which are most unlikely to have come from any of the amino acids in the published alamethicin sequence, and which are in the correct position and have the correct coupling for the peaks from the β-CH_2 of a phenylalanine, or a

molecule very similar to PHE. It seemed most likely at this stage that the extra moeity was an amino acid and PHE was therefore tentatively considered the most likely residue to account for the extra peaks. Since it was inconceivable that a simple PHE could have been missed in all the previous composition and sequence studies the full explanation was obviously not as simple as that.

Experiments on the complete hydrolysate of F30

These will be particularly useful for solving the problem of the identification of the PHXH because it is easy to find out precisely where the peaks from the known amino acids of alamethicin should be, under a given set of conditions, and it will hence be at once obvious which peaks in the spectrum are extra or misplaced. It is not possible to know precisely where all the peaks of the amino acids should be in the intact F30 because, of course, they are shifted by peptide bond formation and the secondary structure of the F30.

In order to determine what the Nmr spectrum of a complete hydrolysate of alamethicin should look like the spectra of constituent amino acids of alamethicin were run separately under identical conditions (i.e. dissolved in D_2O at pH = 1.7*, temperature 25^{o}C and at 270 MHz), and then run together in the correct molar proportions, so that an assigned spectrum of the theoretical hydrolysate was obtained. One mole of phenyl alanine (which is NOT of course in the published sequence) was also included in this synthetic hydrolysate.

*(All D_2O 'pH' values in this thesis were measured by use of an ordinary glass electrode. It can be shown that the true pD is given by 'pH' + 0-4 to a reasonable approximation[20]. However the precise value of the pD's is unimportant and the meter reading i.e. 'pH' will simply be used)

Alamethicin was hydrolysed at first for 20 hrs at 45°C in 12 M HCl, this produced substantial but not complete hydrolysis. Complete hydrolysis was achieved after about 100 hrs at 60°C again in 12 M HCl. After hydrolysis the hydrolysate was freeze-dried and dissolved in D_2O and the pH adjusted to 1.7. The spectrum of this hydrolysate and that of the synthetic hydrolysate are compared in Fig. 3.3 A detailed comparison of chemical shifts in these spectra is given in table 3.1.

As well as measuring the chemical shift positions it is possible to measure the areas of the various peaks and this area is proportional to the number of protons giving rise to the signal (and their concentration). It is very difficult in practice to get an accuracy of greater than 10-20% for a single determination. The relative areas for two different hydrolysates are given below:- (VAL + LEU methyl group areas = 18 protons are taken as standard).

Table 3.2 Hydrolysate peak areas

		AREA		
PEAK	sample	1	2	Theoretical
??? Aromatic		4.4	4.4	?
1LEU +3 GLU + 2 ALA	α CH	5.0	4.9	6.0
2 VAL	α CH	1.0	1.6	2.0
1 GLY,	α CH_2	2.9	2.3	2.0
??? doublet at 3.64 PPM		1.8	1.5	??
2 PRO	δ CH_2	3.4	3.6	4.0
??? pair quartets at 3.0 PPM		2.8	2.8	??
3 GLU	β CH_2	6.2	5.7	6.0
7 MEA + 2 ALA	methyl	54.3	62.1	48.0
2 VAL + 1 LEU standard	methyl	-	18.0	-

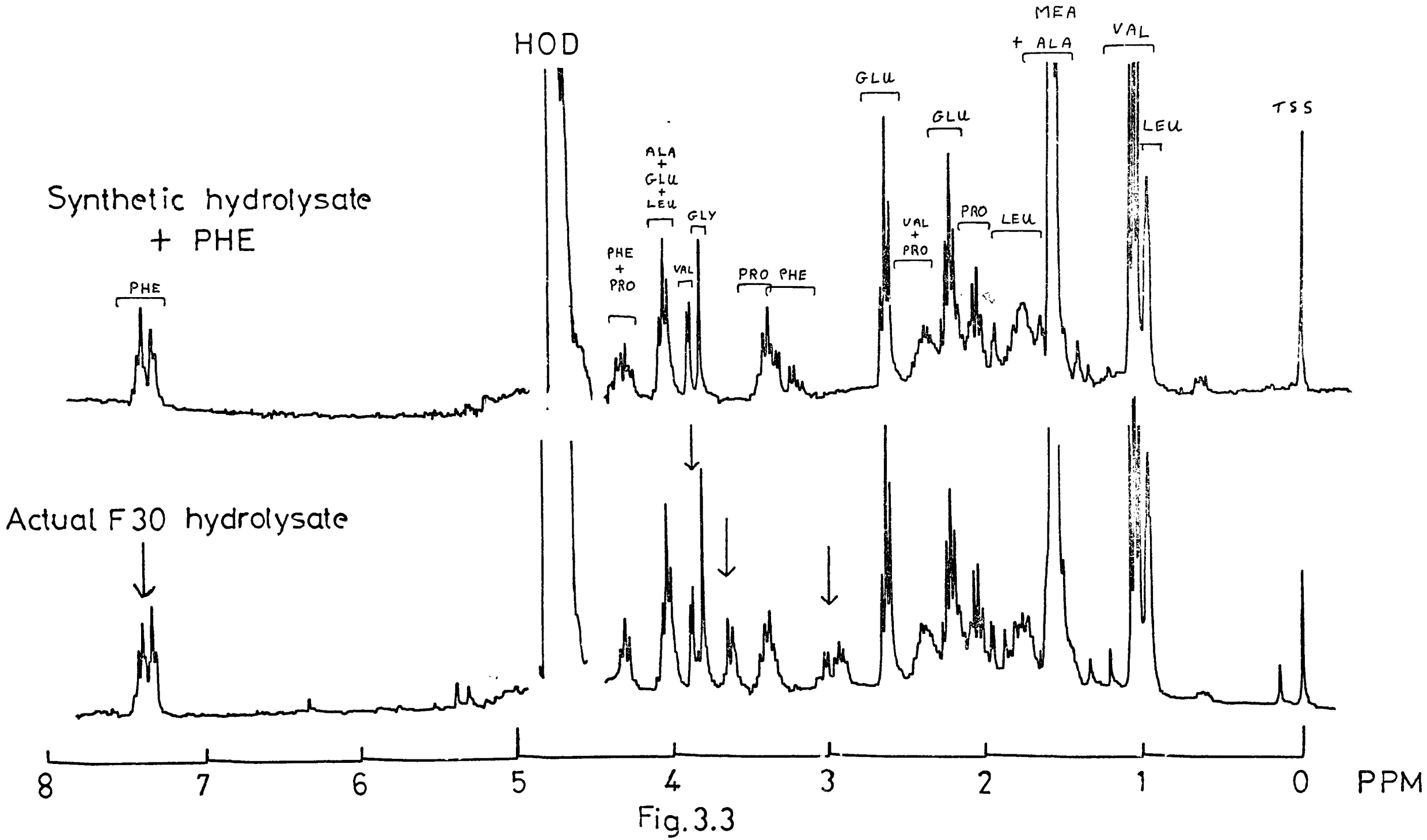


Fig. 3.3

Comparison of synthetic and actual hydrolysate spectra. The extra peaks are arrowed.

Table 3.1

Assignment		Chemical shift PPM 1.	Chemical shift PPM 2.	Assignment	Chemical shift PPM 3.	Chemical shift PPM 4.	Chemical shift PPM 5.
Aromatic		7.398*	-	Aromatic	7.408*	7.378*	7.397*
		7.340*	-		7.345*	7.356*	7.332*
PRO	α	4.311T	4.331T				
				α CH	4.275T	4.548T	4.153T
GLU+LEU+ALA		4.090T	4.060T				
VAL	α	3.927D	3.907D				
GLY	α	3.848	3.832				
???		3.64D*	-				
PRO	δ	3.41D*	3.39 D*				
???		3.05 Q	-	β CH_2	3.35 Q	3.17 Q	3.318D
???		2.94 Q	-		3.20 Q	3.00 Q	3.297D
GLU	β	2.630T	2.634T	Methyl	-	-	2.734
PRO + VAL	β	2.387*	2.381*				
GLU	β	2.217T	2.227T				
PRO	γ	2.062*	2.064*				
LEU	$\beta+\gamma$	1.780*	1.766*				
MEA+ ALA		1.594	1.593				
VAL		1.048T	1.056T				
LEU		0.964	0.968				

D,T,Q,* denote doublet,triplet,quartet and complex fine structure.

1 . The shifts of an actual F30 hydrolysate.

2. The shifts of the synthetic hydrolysate without the extra PHE

3. The shifts of PHE itself run under corresponding conditions.

4. The shifts of β phenyl lactic acid.

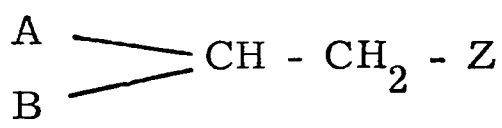
5. The shifts of N-methyl phenylalanine.

Discussion

The chemical shifts given in table 3.1 show, that the with exception of the extra peaks underlined, the chemical shifts of the peaks in the hydrolysate agree extremely well with the free amino acid values given for the synthetic hydrolysate spectrum based on the amino acid composition of alamethicin. The same is true of the areas in table 3.2 within experimental error.

There are however the extra peaks, indicated by the four arrows in the bottom spectrum of fig. 3.3., and underlined in tables 3.1 and 3.2. These will now be considered,

1. The extra peak at 3.64 PPM. This peak will be returned to later.
2. The extra peak between the VAL α CH and GLY CH_2. This was interpreted at this time to be due to an incompletely hydrolysed VAL peptide as the conventional amino acid analysis (performed in Oxford) indicated that the VAL was slow to be liberated. Also the total areas of the VAL + GLY are apparently correct.
3. This leaves the aromatic peak and the pair of quartets at around 3 PPM. It is worth noting a few points about these peaks:-
   (a) the obvious similarity of the fine structure and chemical shift position of the aromatic peak in the actual hydrolysate and the PHE added to the synthetic hydrolysate, (though there are some differences).
   (b) The pair of quartets at 3 PPM in the actual hydrolysate is very similar in coupling to the β-CH_2 peaks of the PHE in the synthetic hydrolysate but has a different chemical shift. The pair of quartets can be said from the coupling pattern to come from the CH_2 group of a unit of the type:-



Where the A, B groups are NOT protons and Z cannot be an atom bearing any protons.

(c) There is no peak in the actual hydrolysate at the position which would be occupied by a free PHE α CH.

There are two possible explanations of the above observations:-

1. The peak at 3.64 PPM is not part of the PHXH but from another unknown moeity. The remaining extra peaks in the spectrum which are very like those of a PHE, but are displaced from their 'expected' positions could be from a molecule such as N-methyl PHE, , or β-Phenyl lactic acid (the hydroxy analogue of PHE). These two attractive possibilities, which would have been undetected in the sequence work, were eliminated by running their spectra under our standard conditions (D_2O pH = 1.7, 25°C) see table 3.1.
2. All the unknown extra peaks come from the PHXH moeity. A definite hypothesis was formed as to what precisely PHXH might be, and it had many very attractive features and one serious weakness, however since it eventually proved incorrect it will not be discussed.

A further experiment on the hydrolysate was then performed to see whether all the extra peaks in the spectrum were from the PHXH, and also as a test of the hypothesis mentioned in 2. which predicted that PHXH would possess no free carboxyl groups.

Europium chloride experiment

This consisted of adding europium chloride to the hydrolysate sample at pH 4.0. The Europium ion

will bind to any free carboxylate groups at this pH and the resonances of the molecule to which it binds will all be shifted. Obviously all free amino-acid resonances should be affected. The result of this experiment was that, as expected, all the free amino-acid peaks showed considerable shifts upfield, up to 1 PPM or so, however the following peaks were unaffected, staying constant in position to within 0.01 PPM:-

1. The aromatic peaks at around 7.3 PPM.
2. The pair of ' β-CH_2 ' quartets at around 3.0 PPM.
3. The other extra peak at 3.64 PPM.

However due to the complexity of the spectra which was increased by addition of the europium ion it was by no means possible to observe all the peaks in the whole spectrum for the whole experiment.

Nevertheless this is an important experiment because it shows that the extra peaks at 7.3 PPM and at 3.6 PPM and the ' β ' CH_2 at 3.0 PPM are all indeed part of the same PHXH moeity, and it also conclusively demonstrates that the PHXH moeity does not contain a free carboxyl group.

To sum up, at this stage experiments on the hydrolysate showed that the extra PHXH moeity contained:-

1. Five aromatic protons very similar to those of a PHE.
2. Two ' β-CH_2 ' protons very similar to those of a PHE but with a different chemical shift showing that the PHE is either not free (i.e. incompletely hydrolysed) or is not a PHE at all but a very similar molecule. These protons were shown to be part of the PHXH by the europium experiment.

3. The ' α-CH' proton. This had not actually been found in the spectrum but the coupling pattern of the β-CH_2 peaks showed that the β CH_2 were coupled to another single proton.
4. The complex doublet at 3.64 PPM, which the europium experiment shows to be part of the PHXH.

The rest of the hydrolysate spectrum was apparently as expected, the extra peak between the GLY and VAL α CH being easily accounted for as from an incompletely hydrolysed VAL peptide.

Attempted separation of PHXH

At this stage it was decided that it was worth trying to separate the PHXH from the hydrolysate.

Dr. J. Priddle performed this attempted separation for us by passing the hydrolysate down a G10 column and monitoring the absorption at 260 nm to find the aromatic moeity. However, only a very small amount of aromatic emerged from the column and its nmr. spectrum was completely unlike that of the PHXH. in the hydrolysate. This aromatic was presumably the hydrolysis product of the impurity absorbing at 250 - 260 nm which was subsequently found to be present when the very pure F30 was prepared (see page 33)

A further attempt to separate the PHXH was then made. This time the hydrolysate was passed down the G10 column and a large number of fractions were collected and each of these was dried down and then run in the Nmr. This attempt was again unsuccessful and no trace of the peaks corresponding to the PHXH could be found. The reasons for this only became apparent later.

Experiments on intact F30

At the same time as the experiments on the hydrolysate were proceeding some important experiments were being done on the intact F30. These experiments were intended to find the other protons of the PHX by decoupling experiments, and most i mportantly to try and find the NH which ought to be present in the supposed peptide link joining this residue into the molecule. As the NH protons exchange much less rapidly in dimethyl sulphoxide than in methanol these experiments were initially performed in deuterated dimethyl sulphoxide.

The β-CH_2 protons of the PHX are clearly identifiable in the Nmr spectrum of the intact F30 as a pair of quartets at about 3.0 PPM these quartets cannot come from any of the amino acids in alamethicin.

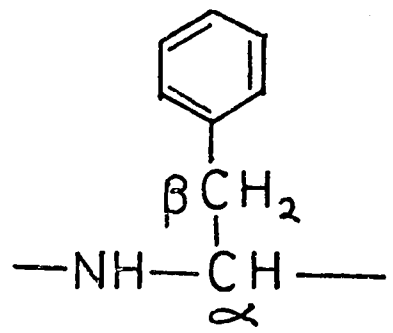
Attempts to find the PHX α CH peak by irradiation of the PHX β-CH_2 peaks failed, one reason being the difficulty of effectively irradiating both β CH_2 peaks at once. However the reverse experiment was successful, namely searching through the α CH region to find a peak which upon irradiation decoupled the two peaks from the β CH_2 of the PHX. Irradiation of this PHX α CH peak simultaneously caused the decoupling of a doublet peak in the NH region. Before this latter peak could be definitely assigned as being from the PHX NH proton it was necessary to demonstrate that the irradiation was not hitting another α CH peak as well. The PHX α CH being coupled, of course, to the PHX β CH_2, and the other α CH being coupled to the doublet in the NH region. This was done in two ways:-

1. Firstly the power of the irradiation was reduced so that the irradiation was made highly specific, and then the irradiation position was changed by very small amounts so that the irradiations were performed at very closely spaced intervals covering the

region of interest. It is then possible to follow the decoupling of the peaks very precisely, because partial decoupling effects are observed as well. It was shown that the NH and β CH_2 were decoupled simultaneously to within a few hundredths of a PPM.

2. The irradiations were successfuly repeated at 310^{o}, 330^{o} and 350^{o} K and also in the presence of the lanthanide ions lanthanum, and europium. These various conditions would be expected to move any overlapping peaks by different amounts. The fact that the decouplings were simultaneous under all these various conditions is very strong evidence that the observed effect is only due to one α CH peak, that of the PHX moeity.

This experiment (along with the other evidence) meant that in the intact material the presence of the following unit had been established:-



and the following further points may be made about the groups attached to the NH and the CH:-

1. The group attached to the NH must almost certainly be a carbonyl, or alternatively it must have no protons on it and have the same electron withdrawing power as a carbonyl.
2. The group attached to the α CH must have a strong electron withdrawing power very like that of a carbonyl group to account for its observed chemical shift.

The above experiment also provided another very important result, it showed that there was possibly another peak being affected by the irradiation of the PHX αCH peak, however this other peak was near the solvent peak in DMSO and so it was not easy to be sure of the effect, for this reason these decoupling experiments were repeated in methanol to ascertain whether the effect was genuine.

The results of the decoupling experiments in methanol were first to confirm the DMSO results, in that irradiation of the PHX α CH produced decoupling of the PHX β CH_2 quartets and also of a doublet in the NH region, which must hence be the PHX NH peak, and secondly to show that a nother peak was indeed being decoupled by the irradiation of the PHX αCH. This peak was a clear doublet at 3.64 PPM which on irradiation of the PHX α CH decoupled to a single peak.

It was again necessary to check very carefully that the effect of the irradiation was not due to hi tting more than one αCH. This was done as follows:-

1. By reduction of the irradiation power and careful sweeping of the irradiation through the α CH position, as for the DMSO irradiations. It was clearly shown that the NH, β-CH_2 and the 3.64 doublet were all decoupled simultaneously.
2. After the addition of lanthanum which shifted the α CH by 0.2 PPM whilst leaving almost all other peaks unaffected, the entire set of deccupling experiments was repeated successfuly (though the NH could not be seen in this experiment).
3. By detailed examination and integration of that part of the spectrum containing the irradiated PHX α CH it was shown that there were no other α CH protons there which could be coupled to the 3.64 PPM doublet.

The somewhat complex details of these experiments are given in appendix 3.

These experiments all conclusively showed that the unexpected extra peak at 3.64 PPM was indeed coupled to the PHXαCH. This is very important because it means that there must be one (or two) protons on the carbon next to the αCH and this is therefore direct proof that PHX cannot be an α amino acid. The full explanation of these experiments did not become evident until the PHXH was isolated from the hydrolysate.

Further hydrolysate experiments

The above decoupling result which shows that the PHX αCH as well as being coupled to the β CH_2, as we always knew it was, and the NH, as was expected, is also coupled to some other proton(s) makes it important to try and find the PHXH αCH and all the peaks to which it is coupled in the hydrolysate. (Although there is always the possibility that the hydrolysis changes the PHX in the intact material to a different 'PHXH' in the hydrolysate).

For this purpose another hydrolysate was prepared, again by hydrolysis with 12 M acid at 60^{o}C for 100 hrs. The sample of alamethicin used for this was the sample supplied from Porton with 50% by weight of salt, as it was not thought necessary to use up valuable salt-free F30 for this experiment. However the spectrum of this new hydrolysate produced a surprise. The appreciable ionic strength produced by the salt moved the peaks slightly from their expected positions and in particular moved VAL α CH and the GLY CH_2 peak so as to reveal the peak that was previously between the VAL α CH and GLY CH_2 peaks. This peak was interpreted in all previous hydrolysates to be from an incompletely

hydrolysed VAL peptide. However the movement of the GLY revealed not a doublet, as would come from valine, but a triplet of area approximately one proton. This is most important as it means there is now another 'extra' peak in the spectrum to be explained.

The extra peaks now in the spectrum and their positions and areas are given in table 3.3.

Table 3.3

	'Aromatic'			' β CH_2'	
Area.	5 protons	1 proton	2 protons	2 protons	
Δ PPM.	7.405 7.356	3.816	3,660, 3.631	3.04	2.92
structure.	complex	Triplet	complex doublet	quartet	quartet

The extra peaks, except for the aromatic, are shown in Fig. 3.4., which also gives the corresponding part of the spectrum of the synthetic hydrolysate of F30 with a mole of PHE added. The shift of the GLY due to the high ionic strength in the actual hydrolysate is clearly visible. (The pH of the sample was accurately measured by using a thin glass electrode which could be inserted into the sample actually in the nmr tube. The pH of these samples is again 1.70).

Decoupling experiments

A comprehensive set of double irradiation experiments was then performed on the hydrolysate. First the α CH and β, γ protons of the peaks from the free amino acids were all irradiated just to check that the expected decoupling effects were observed and they were. Then a set of irradiations was performed to try and obtain some more information about the extra peaks.

PART OF COMPLETE HYDROLYSATE SPECTRA

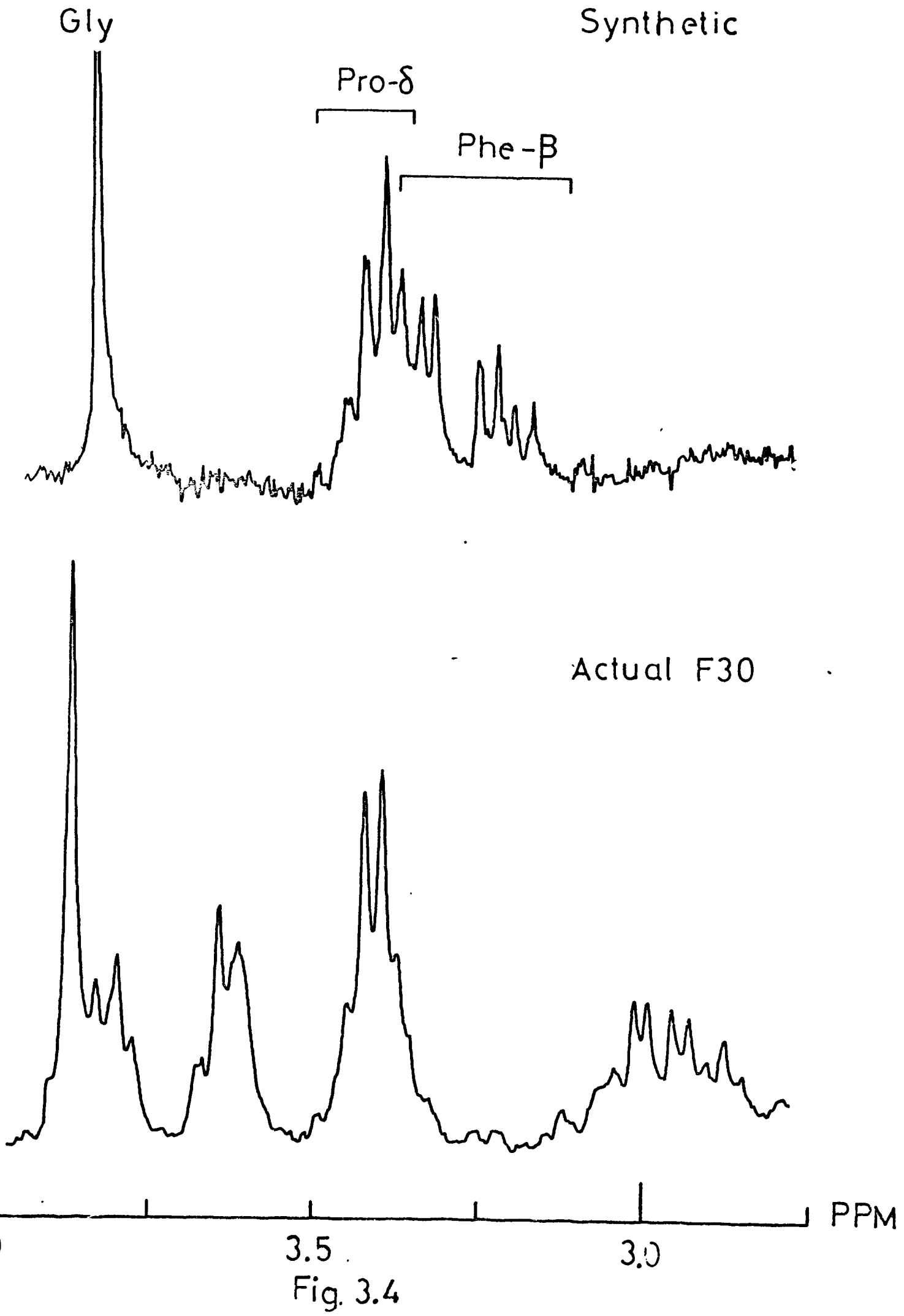


Fig. 3.4

Spectra in D_2O, pH=1·8, showing all the 'extra' peaks except the peaks from the aromatic protons.

Irradiation of the extra two-proton peak at 3.64 PPM (see table 3.3) decoupled the β CH_2 at 3 PPM. Hence one of the two protons under this complex doublet is the PHXH α CH The irradiation of the peak at 3.64 PPM also showed signs of affecting the triplet at 3.816 PPM, but the proximity of these peaks (see Fig. 3.4) and the small nature of the effect meant that the result was not conclusive, so the experiments were repeated at higher temperature.

The above experiments were all conducted at the 'usual' temperature of 25°C. On raising the temperature to 67°C, the 3.816 Triplet moves a little further from the GLY, and the 3.660, and 3.631 peaks appear to swap places with each other and the β CH_2 peaks at 3 PPM collapse from a pair of quartets to a single triplet. The following irradiation experiments were then performed:-

1. The β CH_2 peak was irradiated and produced partial decoupling of the PHXH α CH peak.
2. The doublet at 3.64 PPM was irradiated which decoupled the PHXH β CH_2 peaks partly, and also produced a definite change in the 3.816 triplet. Irradiation of the 3.816 triplet also seemed to produce changes in the 3.64 doublet, but again the proximity of the peaks made it difficult to be absolutely categorical.

These irradiation experiments confirm those at 25°C and indicate rather more strongly than the experiments at 25°C that irradiation of the 3.64 peak also affects the 3.816 peak and vice versa.

The peaks were found to shift by small amounts when the hydrolysate was dissolved in dimethyl sulphoxide, DMSO. In this solvent the extra peaks change as follows, the 3.816 'triplet'

changes to a simple doublet of large splitting (1 proton) the 3.64 complex doublet splits giving a triplet (1 proton) and the PHXH α CH peak was shifted to overlap with the genuine PRO δ CH_2 peak, with the β CH_2 peaks a pair of quartets as usual. Double irradiations were also performed on this sample and confirmed the results above, but gave no new information.

These double irradiation experiments show that the complex doublet at 3.64 PPM contains the PHXH α CH proton. The experiments also indicate that there is some coupling between the 3.816 triplet and one of the protons in the 3.64 doublet.

These experiments provided useful information but in order to investigate decoupling properties in more detail and identify the PHXH it was desirable to separate the PHXH from the hydrolysate. However two previous separation attempts failed and it was decided to try and obtain some information about the properties of PHXH which might be useful in designing a separation experiment and so a pH titration was performed.

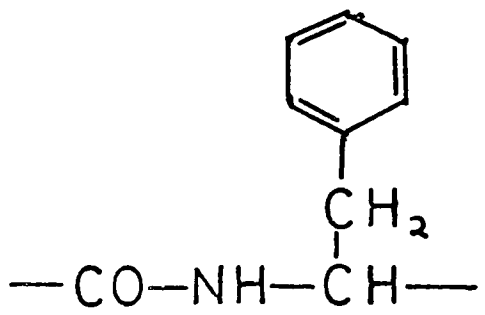
Hydrolysate pH titration

The europium experiment showed that there is no carboxyl group, and a primary amine group would not be expected (as this would be ninhydrin positive), nevertheless the experiment was considered worth doing. The measurement of pH was again performed with the very narrow glass electrode which was capable of measuring the pH of the sample actually in the nmr tube.

The nmr does not see the ionised and unionised species separately, of course, but the shifts of the peaks will be averages of the shifts of the ionised and unionised species

weighted by the concentrations of these species. Thus a graph of chemical shift against pH gives a typical sigmoidal pk curve.

The pH was gradually increased from the 1.7 starting value. The free amino acid peaks especially the α CH peaks all clearly showed the carboxylate pK at around 2. The extra 3.660, 3.631 peaks were visible until pH 2.92, by which time they had not shifted at all, similarly the peaks from the PHXH β CH_2 and aromatic protons which were visible over the entire pH range did not show any carboxylate pK. The PHXH β CH_2 peaks did however show a clear pK at a nominal pH of between 9 and 9.5. The aromatic peak gave an identical pK curve. The shift of the PHXH α CH peak could not be observed directly but it was shown to have shifted at high pH by finding its position by double irradiation. Thus at pH 9.6 it had shifted from its starting position of 3.64 PPM to 3.27 PPM (irradiation at this position decoupling the pair of PHXH β CH_2 quartets). The pH was lowered again to make sure that the pK was reversible and was not due to some decomposition. The total pH range in the titration was 1.74 to 11.18. This experiment thus confirms the absence of any carboxylate pK in the PHXH and shows that the PHXH moiety has a pK at 9 - 9.5. In view of the finding of an NH peak in the decoupling experiments on the intact material, the most likely group to give such a pK was thought to be a primary amine group. (Though this raises the problem of why the PHXH being now ninhydrin positive did not appear as an anomalous peak in the amino acid analysis). This experiment is very important because there is no amino pK in alamethicin itself, and so this finding, along with the finding of a single NH proton by the decoupling experiments discussed earlier provides direct evidence that the PHX moeity is covalently bound into the alamethicin in the form of the following unit:-



and that on hydrolysisthis yields as would be expected, a free amino group.

A final experiment on this hydrolysate was to run a spectrum with a very large sweep width just to make sure that no peaks could be missed, in particular it was just possible that at aldehyde proton which occurs at very low field might otherwise be missed. (The PHX moiety could conceivably possess an aldehyde group resulting from decarboxylation of an α hydroxy acid). However the wide sweep showed no extra peaks.

Isolation of the PHXH

At this stage all the simple experiments which could be performed on the hydrolysate mixture had been performed. It was now necessary to try and solve the problem of separation again. The failure of the attempted separation of the hydrolysate mixture on a G 10 column (which was repeated with another sample), made it necessary to consider other ways of separation. The fact that the PHXH moiety only has a single pK in contrast to the two pK of the amino acids meant that a simple solvent extraction could be tried. The PHXH, as it contains a phenyl ring,is obviously fairly hydrophobic and might well extract into chloroform from water when not charged i.e. at high pH, whereas the amino acids, which will of course be negatively charged at high pH, are most unlikely to favour chloroform over water.

It was therefore decided to attempt a solvent extraction using deuterated water and deuterated chloroform, so that the various stages of the extraction procedure could be simply and rapidly followed by measuring Nmr spectra directly on the samples.

The hydrolysate dissolved in D_2O was first extracted with chloroform at low pH to remove the small amounts of hydrophobic impurities which were known to be present in the hydrolysate. Nmr spectra confirmed that a few small peaks were removed but that the PHXH stayed almost completely in the D_2O phase. The hydrolysate pH was then changed to about 11.5 and the D_2O phase again extracted with $CDCl_3$, this time nmr spectra showed that the PHXH had disappeared almost completely from the D_2O phase, whereas the amino acids were almost unaffected. Unfortunately, by no means all of the PHXH which disappeared from the D_2O phase reappeared in the $CDCl_3$ phase, quite a lot of the PHXH was obviously present at the rather messy interface between the two phases, nevertheless there was ample PHXH in the $CDCl_3$ phase to obtain reasonable nmr spectra. Some of the hydrophobic amino acids were also extracted into the $CDCl_3$ in small amounts, namely leucine and valine and particularly methyl alanine, (of which there is of course a large amount in the hydrolysate). However the chemical shift of these peaks is known and they did not interfere with the spectra of the PHXH.

Following the successful extraction into the chloroform and running of spectra the PHXH was re-extracted back into (clean) D_2O at low pH so that the spectra could be directly compared with the usual hydrolysate spectra. The following peaks were obtained in the D_2O spectrum, pH1.8 and are identical with the extra peaks in the complete hydrolysate spectrum given in table 3.3.

Table 3.4

Aromatic	?	PHXH α CH+?	' β CH_2'
5 protons	1 proton	2 protons	2 protons
7.403, 7.343	3,810	3.652, 3.625	3.03, 2.92 PPM
complex doublet	triplet	complex doublet	pair of quartets

A pH titration was then performed on this extracted sample in the hope of splitting apart the 2-proton complex doublet peak at 3.63 PPM into the two individual one-proton peaks of which it was made up, and also to confirm that the 3.816 PPM triplet and the peaks comprising the 3.63 PPM doublet did show a pK at $\sim$ 9-9.5 as the PHXH β CH_2 , α CH and aromatic peaks had done. (These other peaks were obscured for much of the pH titration on the complete hydrolysate).

Raising the pH to 4.1 had no effect on any of the peaks (except those due to small amounts of the amino acids) as expected. The pH was then increased to 10.8. This had a dramatic effect on the spectrum, not only were all the peaks in the α CH and β CH_2 region now separated from each other into single proton peaks but the fine structure changed, giving four quartets and an unresolved broad peak. The spectrum of the PHXH at this pH is shown in Fig. 3.5. The top spectrum shows the complete spectrum, and the middle spectrum is an enlargement of the part indicated, showing the four quartets and the unresolved broad peak. Irradiation of the PHXH α CH, indicated by the arrow decoupled all four quartets <u>simultaneously</u>. (Leaving only the internal coupling between the protons of each CH_2 unit).

This decoupling experiment unequivocally establishes the presence of the following unit for the PHXH in the hydrolysate:-

EXTRACTED AROMATIC, D_2O pH=11

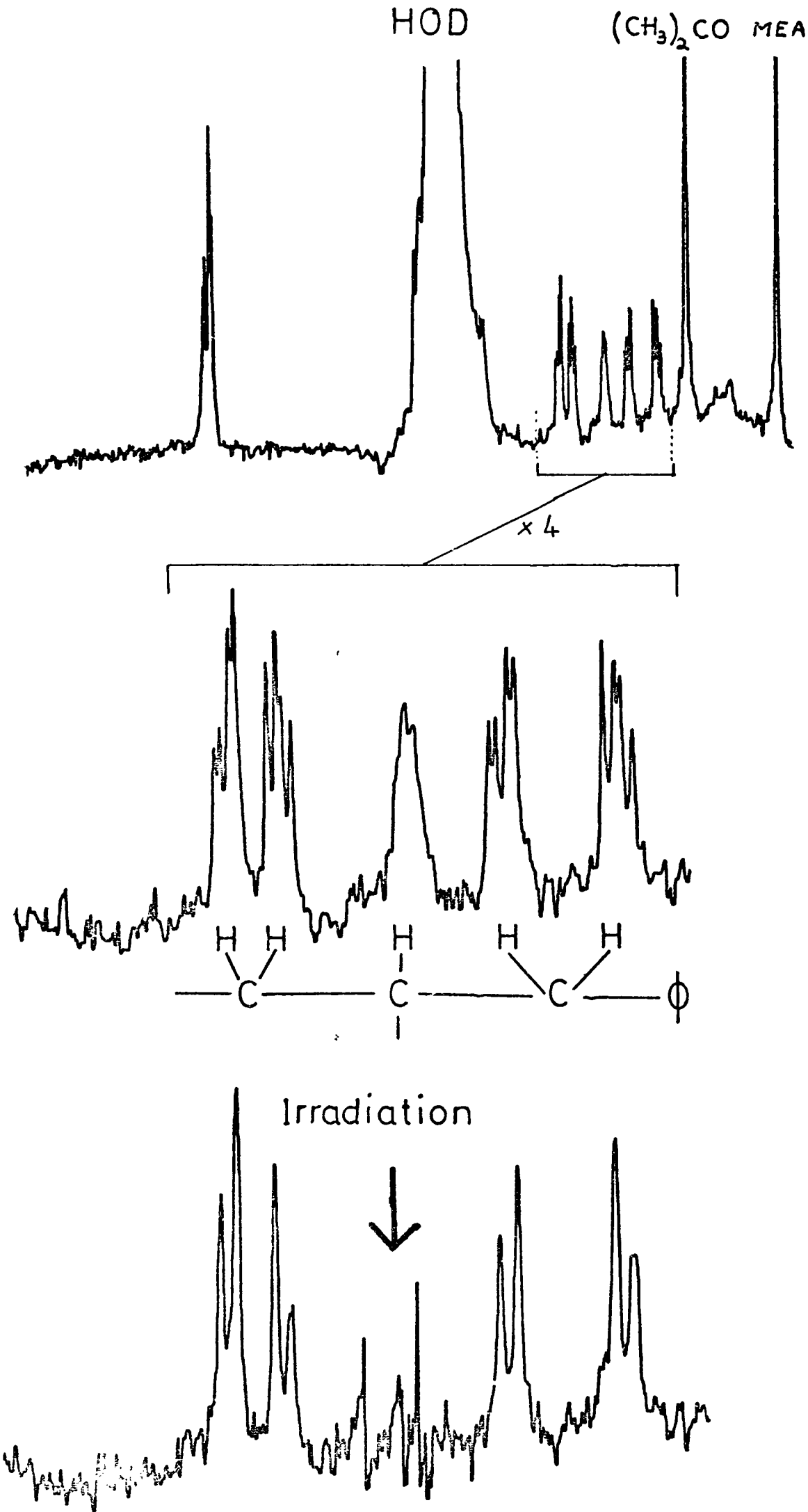
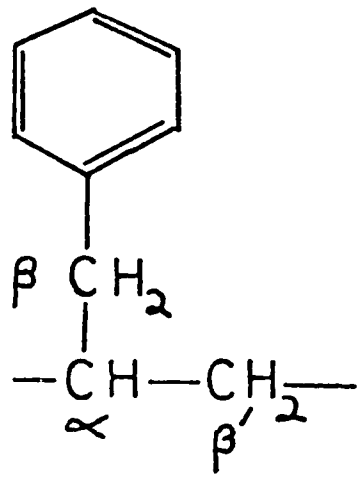


Fig. 3.5

Spectra of the extracted aromatic PHXH, details in the text.



As indicated in Fig. 3.5 the upfield pair of quartets is the PHXH β CH_2 next to the aromatic ring which have always been present in the spectrum as quartets. The lower pair of quartets come from the so called β' protons and correlate with the low pH peaks as shown in the table below:-

Table 3.5

Aromatic	triplet	complex	doublet	pair of quartets	
7.403, 7.343	3.810	3.652	3.625	3.03, 2.92	pH 1.8
7.38, 7.307, 7.359	3.58	3.43	3.09	2.83 2.52	pH 10.8
Aromatic	quartet	quartet	broad	pair of quartets	

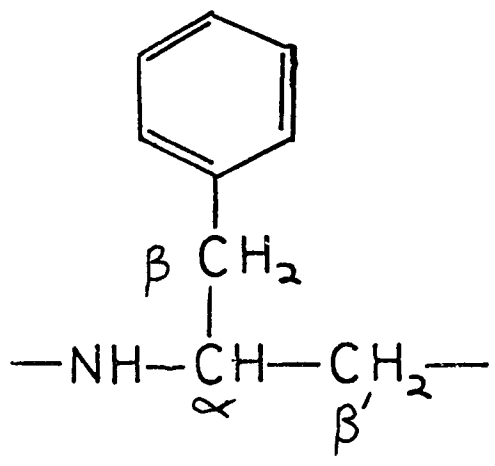
The reason that the other peaks do not appear as quartets at lower pH is that the two inner components may merge together and become unresolvable so making the peak appear a triplet. Also if either the coupling constants to the α CH are very small because of the geometry of the various bonds or alternatively the two CH_2 protons are equivalent then the quartet will simply become a doublet. It is interesting to compare the 'extra' peaks in the spectrum on page 61 Fig. 3.4 with the peaks in Fig. 3.5. Several other important points should be noted:-

1. The spectrum of the PHXH run in $CDCl_3$ and D_2O, with their different solvent peak positions definitely showed that the ONLY non-exchanging protons in the PHXH are those given in table 3.5.

A few other peaks were present in the spectrum but these were either clearly identified amino-acid peaks or peaks occurring in small non-integral amounts which did not re-extract with the PHXH peaks.

2. The group attached to the so called β'-CH_2 above must be strongly electron withdrawing, to account for the chemical shift of these protons.

3. The finding of the above unit explains the previously puzzling decoupli ng experiment performed on the intact F30. This experiment was discussed in detail on page 58 and is shown in Fig. 3.5A which shows that irradiation of the PHX α CH peak decouples the doublet NH peak, the pair of upfield quartets from the β- CH_2 and the large doublet at 3.64 PPM. It was the decoupling of this latter peak which was so puzzling at the time (page 58), but now it is obvious that this single doublet arises from the two equivalent β'-CH_2 protons, the coupling being that due to the α CH. (Because the β'- CH_2 are equivalent the coupling of each of the β'- CH_2 to each other is not seen). So that when the PHX α CH peak is irradiated the β'-CH_2 peak decouples to give a singlet. This decoupling experiment conclusively establishes the presence of the following unit in the intact F30:-



F30 PHX ∝CH IRRADIATION

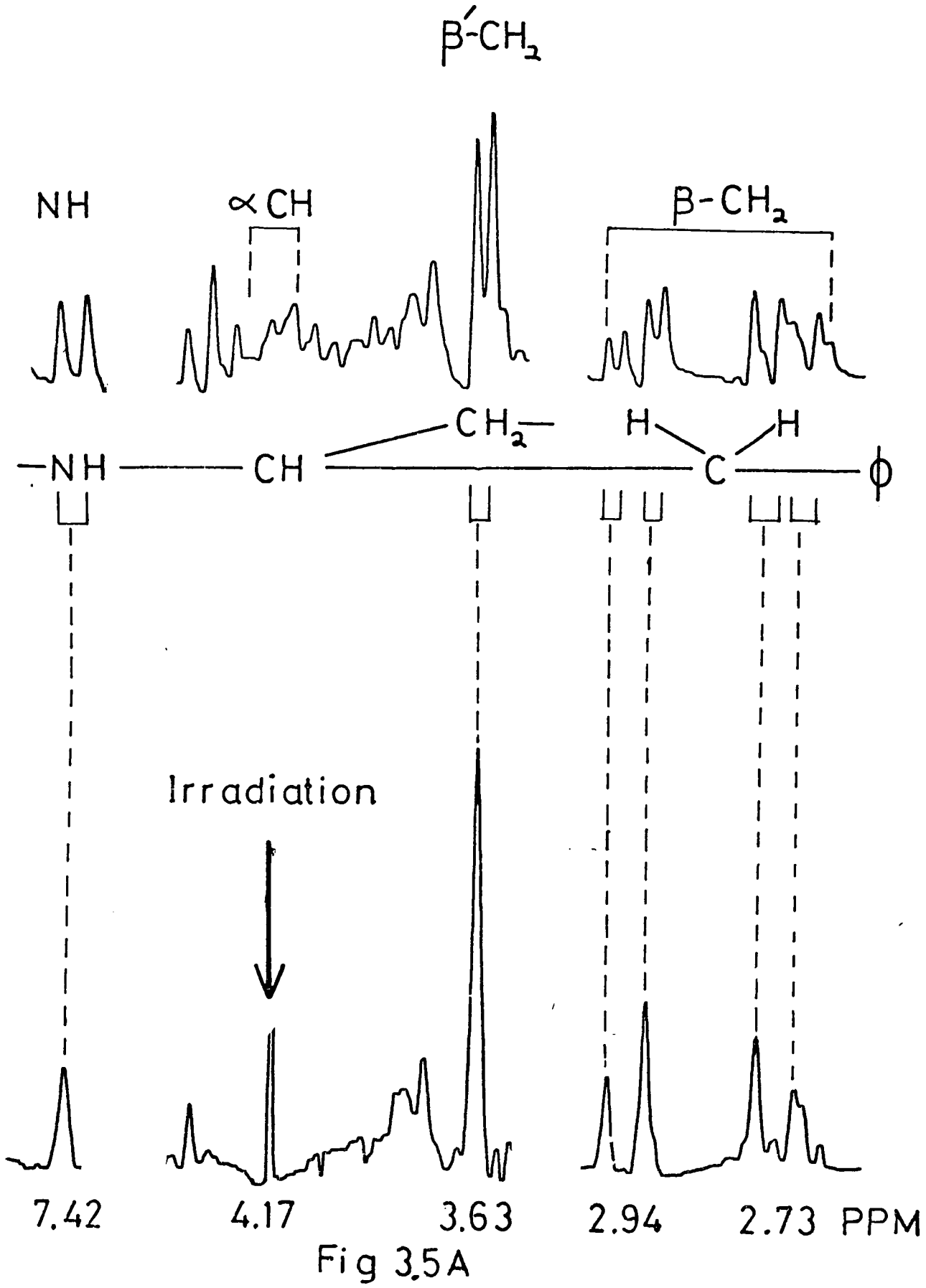


Fig 3.5A

R.E. spectra in methanol showing the irradiation of the PHX ∝CH proton in intact F30, and the decoupling of the NH, β-CH_2, and β'-CH_2 protons.

Identification of the PHXH

There is now sufficient information about the PHXH present in the hydrolysate to enable its identification.

Firstly it is known from the decoupling experiment on the intact material that there is an NH attached to the α CH in the intact material as shown above. This N atom is obviously still going to be present in the hydrolysed PHXH. It should also be remembered that the hydrolysed PHXH gives a pK at about 9, and that no further groups with non-exchangeable protons can be put into the PHXH. The only group that will satisfy these restrictions is a simple primary amine group attached to the α CH.

It is now necessary to attach a group to the β' CH_2, and it must satisfy the following conditions:-

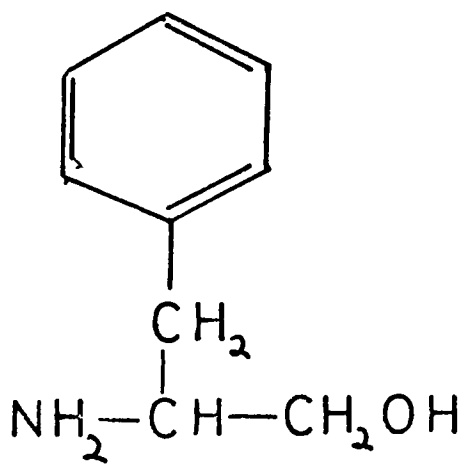
1. It must be strongly electron-withdrawing in order to account for the observed chemical shift of the β' CH_2 protons. This rules out attachment of a carbon atom even with oxygen on it, and also an uncharged nitrogen.
2. There must be no pK in the range 2 - 11.5, (unless it happens to be almost identical with the NH_2 pK at 9 - 9.5).
3. There can be no further non-exchangeable protons.

The most likely group to satisfy these requirements is the hydroxyl group, and this would be expected to give chemical shifts for the β' CH_2 close to those which are actually observed.

This means that PHXH is phenylalaninol, i.e. the alcohol obtained by reduction of phenyl-alanine. Alternatively it may be regarded as a derivative of ethanolamine. Ethanolamine

itself has of course been previously found in peptide antibiotics [17-19].

Thus the structure of the PHXH is:-



PHENYLALANINOL

Although there seems no other possibility for PHXH it was decided to obtain absolute confi rmation by obtaining a commercial sample of phenylalaninol (from Fluorochem. Ltd) and running the Nmr spectrum of this to compare the the spectrum of PHXH. This was done and the results are given in table 3.6

Table 3.6

Peak	chemical shift 'PHXH'			commercial phenylalaninol		
Aromatic	7.38	7.359	7.307	7.383	7.356	7.302
β'- CH_2		3.58	quartet		3.574	quartet
		3.43	quartet		3.429	quartet
α-CH		3.09	broad,complex		3.072	broad,complex
β- CH_2		2.83	quartet		2.819	quartet
		2.52	quartet		2.518	quartet

Both spectra were run at pH 11.2 in D_2O. The fine structure of the peaks was also identical. This experiment unequivocally identifies the unknown PHXH as phenylalaninol.

Isolation of pure phenylalaninol from the hydrolysate

This was achieved by Dr. J. D. Priddle using a G10 sephadex column. A sample of the very pure F30 was hydrolysed in the usual manner and the Nmr spectrum of this material was run just prior to it being put on the column. The hydrolysate was put down the column in 0.1 M ammonia solution. The absorption of the material coming off the column was monitored at 256 nm, and the appropriate fraction collected. This fraction was run in the Nmr and although it was pure, and gave the same spectrum as the impure material minus impurities, only about five percent of the material put on the column emerged in this sample. Even this fraction did not in fact appear until after three void volumes had been collected, showing that the phenylalaninol was adsorbed on the sephadex, and not just included. The remaining material seems to have been very strongly adsorbed, though it is rather puzzling that a peak of some of the phenylalaninol should come off the column in those circumstances.

This strong absorption by the column and poor yield of the process probably accounts for the previous failures to achieve a separation on G10. Further the impure F30 used to prepare the hydrolysates in the previous attempts contained impurities absorbing in the same region as the phenylalaninol and thus further confusing the issue.

LOCATION OF THE PHENYLALANINOL IN THE SEQUENCE

Phenylalaninol will be denoted by the abbreviation PHO.

The only side chains in the molecule are the GLN 6 and GLN 18 amide groups and the GLN carboxyl group. The latter was clearly established as free in the chemical work of Payne et al and Kiryushkin et al. Both groups also found the expected two amide groups using conventional destructive estimation procedures. It must also be remembered that their work makes the PRO 1 to GLN 18 sequence almost immutable and hence the most likely method of attaching this aromatic moiety is to break open the supposed GLU 17 to PRO 1 linkage, the presence of which was never directly demonstrated. W. R. Grays mass spectrometric work which apparently demonstrated directly the GLU 17 to PRO 1 linkage (page 39) can now be discounted because there is no reason to suppose that the reduction which he used, which is specific for PRO bonds in pure peptides, will retain that specificity in the presence of non-amino acid moieties and non-peptide linkages.

There was one further piece of experimental evidence to bear in mind, the lanthanide work which is described in later chapters, showed that the PHO was very close to the metal binding site which is itself close to the GLN 18 residue. Hence this supported the placement of the PHO near to GLU 17.

The difficulty of putting the PHO into the GLU 17 to PRO 1 linkage is evident and it was therefore decided to perform experiments which would show that there was no possibility of attachment of the PHO to any of the side chains in alamethicin. It is important to confirm that the two amide side-chains of the GLN 6

and 18 are indeed intact, as the chemical evidences for their existence though strong cannot be regarded as conclusive because any group which hydrolysed in acid under relatively mild conditions to give an amide group would give the results obtained. It was also decided that a simple and useful experiment was to try and estimate as accurately as possible the number of NH protons in the molecule.

Some of the experiments to establish the presence of the GLN amides were done in DMSO, where the exchange of the NH and amide protons is slow. A temperature dependence study of the NH region revealed the four individually visible GLN amide peaks which are characterised by their chemical shifts and rapid exchange leading to broadening at high temperatures. These spectra are shown in Fig. 3.6, hence the presence of the GLN 6 and 18 amide groups is confirmed.

Experiments were also performed in protonated methanol, where the exchange of the NH protons is sufficiently slow, at low pH to render them observable. The spectra showed four extra peaks not observed in deuterated methanol, and these are from the four rapidly exchanging amide protons. The shifts of these peaks were similar to the shifts in DMSO. It was found that lowering the pH slightly caused sufficiently rapid exchange of these amide protons that they became unobservable. It was then possible to perform difference spectra between the samples with and without these four protons and this very clearly revealed the four protons, the rest of the NH being relatively unaffected by the pH change and hence subtracting to zero in the difference spectra. (The technique of difference spectra simply consists of subtracting one spectrum from the other in the computer). These experiments were performed on the very pure F30, and again confirm the presence of the GLN 6 and 18 amide groups.

GLN $-CONH_2$ TEMPERATURE DEPENDENCE

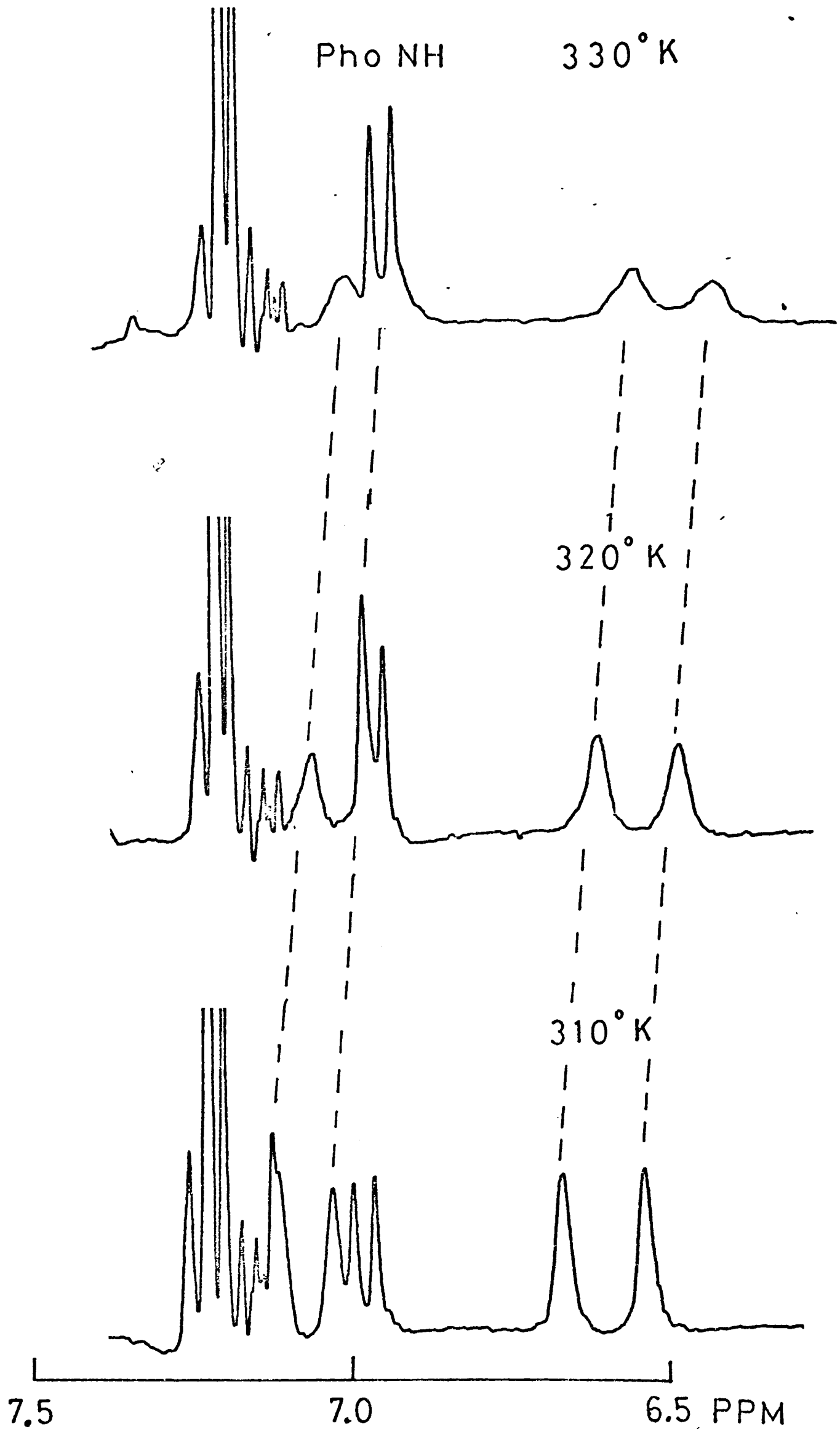


Fig. 3.6

R.E. spectrum of F30 inDMSO clearly showing the four individually visible amide protons.

To determine the total number of NH present, spectra were run in DMSO at two different temperatures and taking care to use a long delay between the radio-frequency pulses so as to avoid any possibility of saturation. The results were as follows:-

NH protons	Aromatic + PHO + GLN amide			
16	5	1	4	Theoretical
16.2	- -	10.8	- -	298°K
18.1	- -	10.2	- -	350°K

This experiment was again performed on the very pure sample of F30. The results indicate close agreement with the expected values, though it is possible that there is one or even two NH protons over and above those expected from the Payne sequence with a PHO added. In particular in DMSO there is present, as is present also in methanol one very low field NH peak which exchanges extremely rapidly and which may be an 'extra' NH.

These experiments demonstrate that the GLN amide groups are intact and also show that there are a maximum of only one or two NH protons which could be present over and above those expected from the published sequence with a PHO added.

It is evident that the PHO must indeed be placed somehow between the GLU 17 and the PRO 1. There is however one important possibility which has not been considered so far, namely that alamethicin is not cyclic at all but resembles the gramicidins in possessing blocked C- and N- termini. Gramicidin A, B, and C were originally thought to be cyclic but it was eventually found that they had the C- terminus blocked by ethanolamine and the N- terminus blocked by the formyl group[17-19] as:-

H. CO. N— Normal Polypeptide —CO. $NHCH_2CH_2OH$

Phenylalaninol is of course a derivative of ethanolamine, and could block the GLU 17 γ carboxyl group. It would then be necessary to find a group blocking the PRO 1 N- terminus.

Partial hydrolysis experiments

It was decided to attempt to find out more about the attachment of the PHO to the alamethicin by performing partial hydrolysis experiments. Hence for example if alamethicin is cyclic then the phenylalaninol must be bound to both GLU 17 and PRO 1 in some way, and it should be possible to find a peptide in the hydrolysate in which the PHO is still attached by the linkage involving the -OH group to the alamethicin, but with the amine group free. It would be easy to see if such a peptide existed even in a mixture, as the amine group at low pH would be positively charged and would bind the chromicyanide ion and this would be seen in the Nmr as the paramagnetic properties of the chromicyanide ion cause rapid broadening of peaks from protons near the bound chromicyanide ion. It can be fairly safely assumed from the previous chemical work, and anyway will be evident from the Nmr, that there should be little cleavage of the peptide bonds around the PHO in the alamethicin molecule under mild conditions.

It is obviously much better to monitor the hydrolysis as it is actually occurring if this is possible. It is a further advantage of Fourier transform Nmr as compared with continuous wave techniques, that because of the different mode of operation it is possible to run perfectly good Nmr spectra of samples made up in very strong acid. (The very high conductivity of such samples leads to very poor signal to noise in the continuous wave mode of operation). So in these partial hydrolysis experiments the hydrolysis was observed by Nmr as it was actually occurring in the sample placed in the spectrometer.

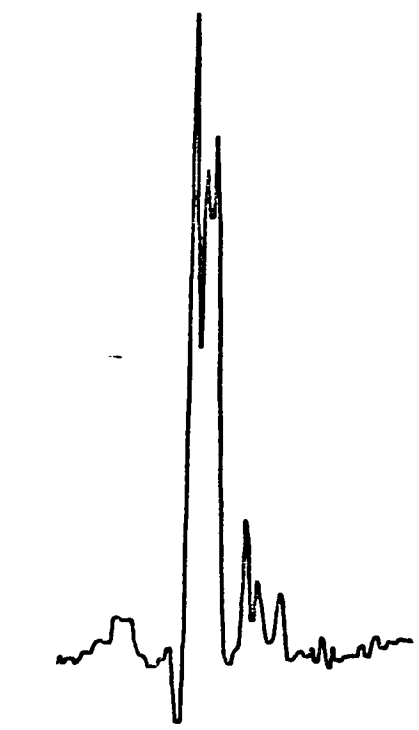
Because of the need to ensure that the hydrolysis was reasonably selective and took place at a slow rate, i.e. over the space of a few hours, and because alamethicin is insoluble in dilute acid, it was decided to perform the hydrolysis in 75% methanol where the alamethicin would be soluble and monomeric and give an nmr spectrum not very different from the usual spectra obtained in pure methanol. Hence a sample was made up with 75% methanol, and 25% D_2O with a concentration of DCl in the sample of ca. 1.5 Molar, this sample was placed in the nmr spectrometer with the temperature at $37^{o}C$. The peaks were correlated with the peaks of the methanol spectrum at normal pH by means of a titration, so it was possible to assign the peaks in the 1.5 Molar DCl spectrum and to say that no decomposition had occurred after the running of the first spectrum (ca. 5 minutes after making up the solution). This sample was then simply rerun at periodic intervals to observe the changes taking place.

It was found that the PHO started to hydrolyse off the alamethicin, this was shown by the appearance next to the aromatic peak of the PHO of a sharp peak which increased in intensity with time, in a manner corresponding to the decrease in intensity of the aromatic peak from the PHO in the intact F30. The other peaks which belonged to the PHO residue also started to disappear, and sharper peaks appeared close to these, although it was not so easy to observe the regions of the spectrum where these peaks occured. The hydrolysis under these conditions (1.5 Molar acid $37^{o}C$) was very slow and so the experiment was repeated using an acid concentration of 3.5 Molar but under otherwise identical conditions and these results will now be considered in a little more detail.

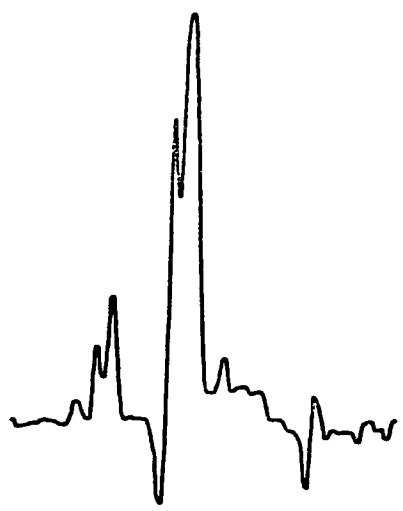
The hydrolysis of the PHO as seen in the changes in the aromatic peak is shown in Fig. 3.7 for the 3.5 Molar acid experiment. The half-life of the PHO under these conditions was about ninety minutes.

IN-PROBE F30 HYDROLYSIS

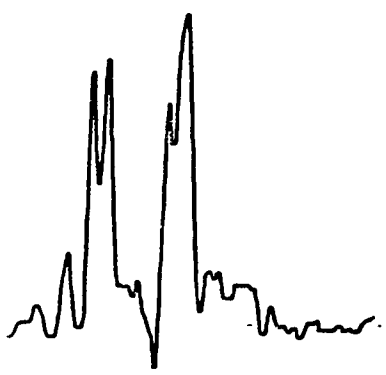
Aromatic peak



10 MIN.



30 MIN.



70 MIN.

Fig.3.7

R.E. spectra of the aromatic peak of the PHO showing the hydrolysis of the PHO off F30, in 75% methanol 25% water 3·5 Molar DCl at 310°K.

The hydrolysis was quenched after 90 minutes and the sample was lyophilised and redissolved in pure deuterated methanol under the usual conditions. This enabled direct comparison of the spectra of the hydrolysed material with those of the intact material, and also further experiments were performed in the form of pH titrations and experiments with paramagnetic ions to try and find the ionisable groups released. The results of these experiments may be summarised as follows:-

1. As expected the peaks associated with the PHO residue were all changed substantially in the spectrum of this partial hydrolysate though about 50% of the material was unhydrolysed and hence gave rise to the usual peaks. There was also one other important change which is discussed in the section on the other extra moiety in alamethicin. The remainder of the spectrum was largely unchanged.
2. The pH experiments and the experiments with paramagnetic ions showed that other cleavages of the molecule, apart from that of the PHO residue, had taken place. This is not unexpected from the previous chemical work on the hydrolysis of alamethicin which showed that at 37^{o}C in 12 Molar acid after only 30 minutes there was cleavage between MEA 12 and PRO 13.
3. Peaks from the PHO completely hydrolysed off the alamethicin were present and these showed the expected amine pK value (of 9) and also, as expected due to the positive NH_3^+ group present at low pH, bound the paramagnetic $Cr(CN)_6^{3-}$ ion which resulted in considerable broadening of the peaks.
4. There were other peaks which were not from the PHO in the intact F30 and not from the PHO completely hydrolysed off the F30 and did not bind the paramagnetic $Cr(CN)_6^{3-}$ ion. These peaks must be from the PHO which is part of some peptide changed by the hydrolysis. No peaks were found which corresponded to the PHO still attached to the alamethicin but with the NH_2 group of the PHO free. This is important and suggests that the PHO is bound to the rest of the alamethicin only by its NH_2 group in the

-CO-NH- type linkage which previous experiments have shown to be present, and that the -OH group is unbound.

The above experiments are important in confirming the general ideas about the PHO residue, and show that it hydrolyses off comparatively rapidly (as it must to explain why it was missed in the previous chemical work). They do not provide conclusive evidence concerning whether or not the PHO residue does close the ring by linking to both GLU 17 and PRO 1, but suggest that this may not be the case. There is one unequivocal experiment which would show whether or not alamethicin is cyclic, and that would be to look for the -OH proton of the PHO. Since it is known that the PHO is bound to the rest of the alamethicin by its NH_2 group, if the -OH proton were missing this would show that the PHO must also be linked by its -OH group and the molecule could be presumed cyclic. The reason why this experiment was not attempted earlier is that finding -OH protons is very difficult, and requires the preparation of extremely dry DMSO, and further it would always be difficult to make a negative result really convincing. However since the partial hydrolysis experiments did not provide the hoped-for information it was decided to attempt this experiment.

Dry DMSO experiments

The deuterated DMSO for this experiment was taken from the sealed ampule in which it is supplied and dried with molecular sieve (size 3A). This was found to remove most of the water from the DMSO. There is however a further difficulty, namely that the lyophilised alamethicin does absorb traces of water. For this reason it was decided that a method would have to be devised for drying the sample itself once it had been made up in the Nmr tube. This was achieve by making a constriction in the nmr tube fairly near

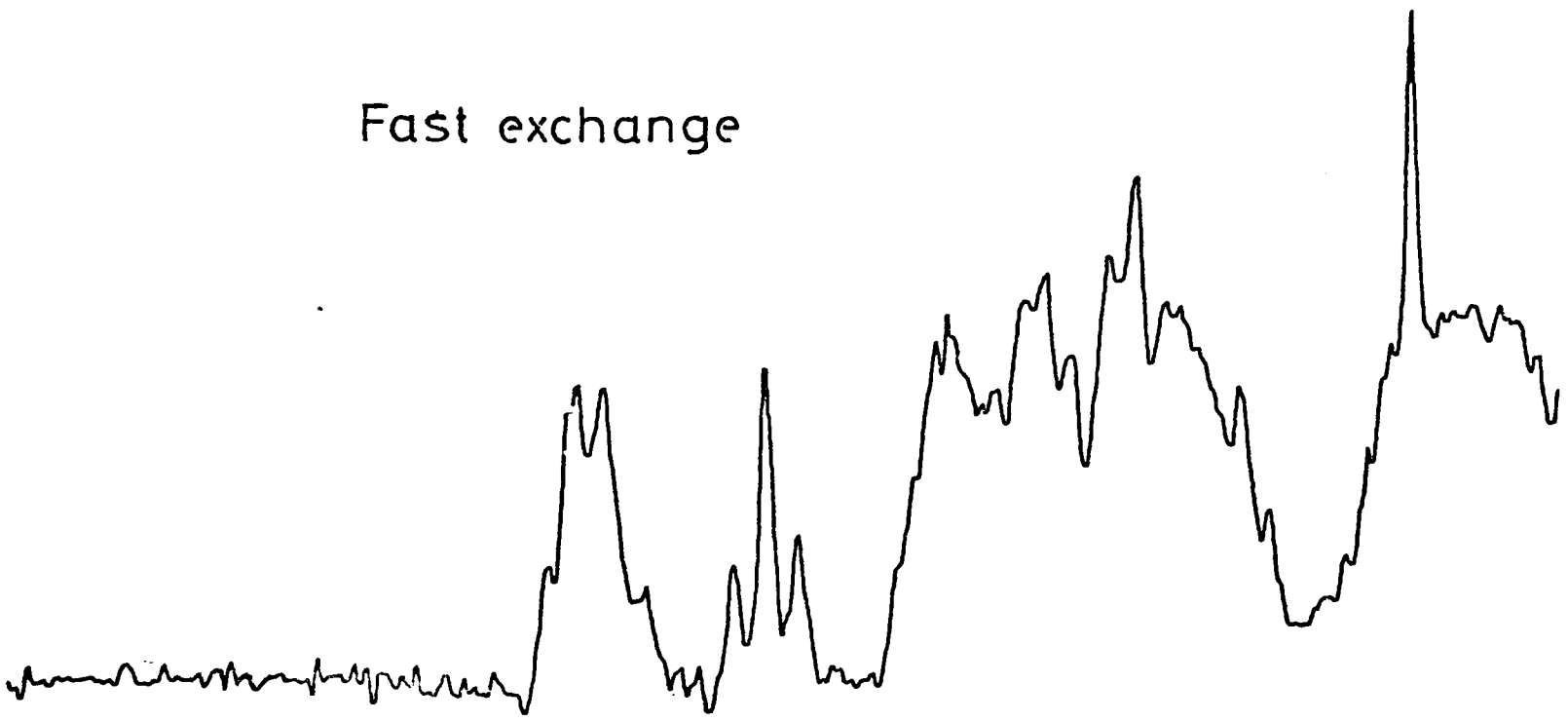
the top so that molecular sieve could be placed in this top part of the tube and the tube capped in the usual manner. Inversion of the tube brings the sample into contact with the molecular sieve to dry it thoroughl y just before the sample is run. The tube is then re-inverted and run in the usual manner. (The molecular sieve cannot of course be in the sample itself whilst the spectrum is accumulated as it would ruin the field homogeneity).

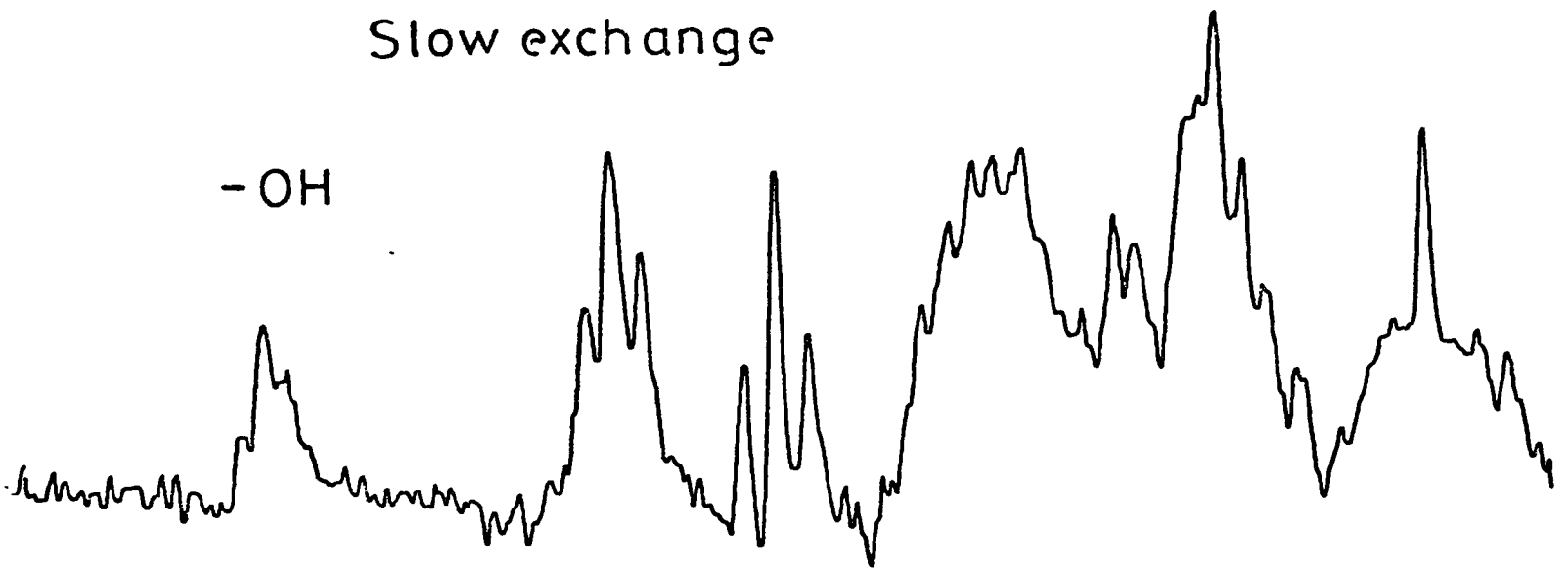
It is interesting to note that under these very dry conditions the proton of the free carboxyl group (GLN 18) can be observed as a broad peak at very low field ca 10 PPM; the position of the peak is very dependent on the amount of the remaining traces of water in the sample.

Even with all the above precautions it was not possible to observe the hydroxyl proton in F30 at first (though it was seen in F50), however it was found that by changing the pH of the DMSO (which was not completely dry) the exchange rate of the hydroxyl proton was slowed and hence the proton became visible and also decoupling experiments could be performed on it.

This extremely important set of experiments is shown in Fig. 3.8. The top spectrum shows the region of interest, where the exchange rate of the hydroxyl proton is so rapid that is is very broad and hence unobservable. When the pH is lowered the hydroxyl proton peak gradually appears and becomes sharper, though it was never possible to prevent some exchange of the proton, and so the peak was always fairly broad (hence its relative small intensity in the resolution enhanced spectra). The proton was successfully decoupled from the β'-CH_2 of the PHO to which it is coupled and this experiment is shown in the bottom spectrum of Fig. 3.8

F 30 PHO HYDROXYL PROTON





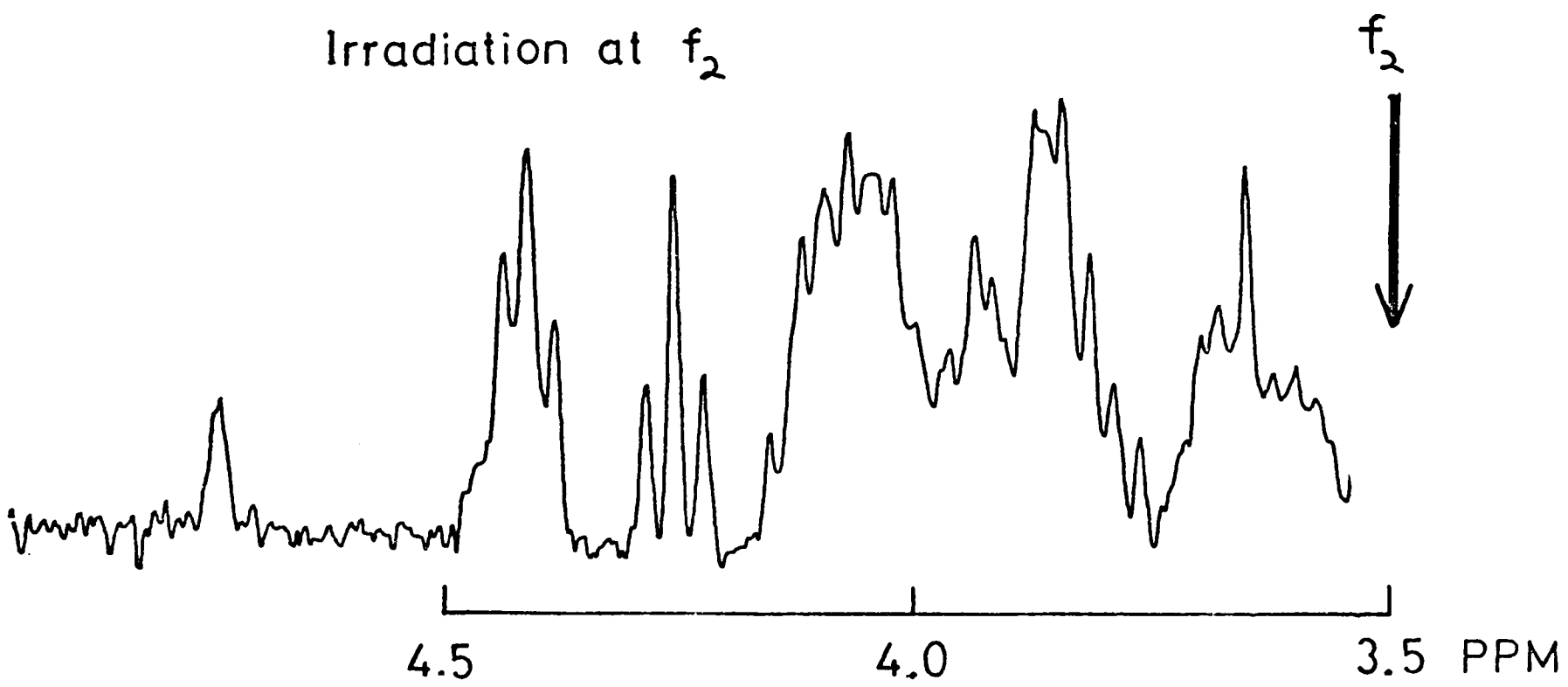


Fig 3.8

R.E. spectra in very dry DMSO at 298°K. Details in text.

The quality of the spectra is not very good because it was not possible to entirely prevent exchange of the -OH with the last traces of water in the DMSO. However the experiments were successfully repeated on two different F30 samples with identical results.

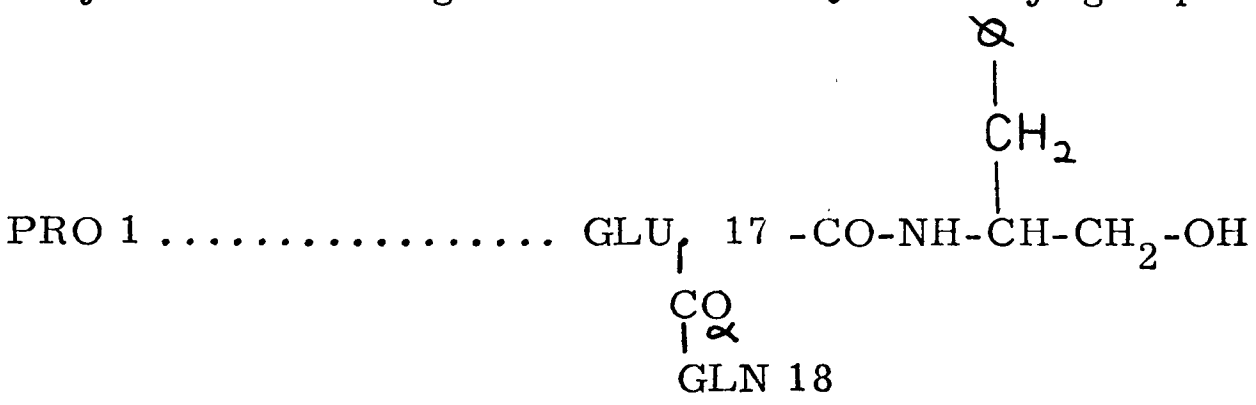
The chemical shift, extremely rapid exchange and area of the peak (in non resolution enhanced spectra) unequivocally identify this as a single hydroxyl proton. The triplet fine structure of this peak shows that it is coupled to two other protons, and the decoupling experiment irradiating the two equivalent $\beta'CH_2$ PHO protons establishes these as the two protons to which it is in fact coupled. Thus this peak is clearly identified as from the PHO hydroxyl proton.

(The extremely remote possibilily of some ethanol contaminant surviving the lyophilisation was directly ruled out by running ethanol in DMSO under these conditions, where it gives a quartet hydroxyl peak at 4. 347 PPM, the observed F30 triplet hydroxyl peak being at 4. 744 PPM)

To sum up the evidence regarding the attachment of the PHO moeity:-

1. It is bound via the NH_2 group only to the rest of the alamethicin, and the linkage involving the NH_2 is almost certainly a -CO-NH- linkage.
2. The work with the lanthanide ions (chapters 5 and 6) showed that the PHO was very close to the lanthanide binding site near the GLN 18 residue.
3. The -OH group is free and hence the ring cannot possibly be closed and so alamethicin must be a linear molecule.
4. The previous chemical and mass spectrometic sequence work unequivocally rules out interference with the PRO 1 to GLN 18 linear sequence. The presence of the two intact GLN amide

groups was confirmed by Nmr experiments. The free carboxyl group was clearly shown by the chemical work to be that of GLN 18 (not GLU 17). The only place to attach the PHO residue then is by an amide linkage to the GLU 17 γ carboxyl group as:-



This leaves one very important question, what is blocking the N-terminus of the PRO 1? The answer to this is provided by the other extra moeity in alamethicin which will now be discussed.

THE N-TERMINAL BLOCKING GROUP IN ALAMETHICIN

The experimental work involved here is not as extensive as that for the PHO moeity but there is still quite a lot to consider. The experiments will be considered in chronological order, though it must be remembered that many of them were being done in parallel with the experiments on the PHO moeity and that most of them were done before it was established that alamethicin is in fact linear. The unknown moeity in this section will be denoted for convenience by the symbol 'X', for the intact F30 and XH for the X present in solution after partial hydrolysis of F30.

Preliminary evidence for another extra moeity

The first spectra of alamethicin not only showed the unexpected peaks of the aromatic but also another unexpected peak. This can be seen in Fig. 3.2 page 43 , as the single sharp peak in the methyl region at about 2.0 PPM. There is certainly nothing in the sequence of alamethicin that could give this peak. Another peak at 1.46 PPM was found to be part of the X as well. These peaks were regarded for some time as probably being from impurities for the following reasons:-

1. The alamethicin supplied to us often gave fairly small singlet peaks in the methyl region where the peaks from the X moeity were. It was difficult to accurately determine the area of either the 2.0 PPM or 1.46 PPM peak and hence it was difficult to be sure whether these peaks were present in different batches in different amounts, which would have clearly showed that they were impurities.
2. Before it was discovered that alamethicin was linear there was no where to bind any such extra moeity to the alamethicin. This is because the lanthanide experiments clearly showed that X was a very long way from the lanthanide binding site near GLN 18 and

hence from the only weak point in the cyclic Payne sequence where an extra group might conceivably be inserted.

3. The discovery fairly early on in the work that this group bound the $Cr(CN)_6^{3-}$ ion very strongly and must therefore almost certainly be very near a positive charge seemed to show that X must be an impurity as it seemed impossible that there could be such a positive centre in alamethicin.

4. This group apparently does not survive the complete hydrolysis procedures used to study PHO moeity.

Despite the apparently baffling problems presented by points 2. and 3. above there was one point which was difficult to explain if one said that these peaks were from impurities namely the fact that these peaks were (in contrast to the true impurities) present in all the batches of F30 and further were present in very similar form in the two major components of alamethicin F30 and F50.

Evidence gradually accumulated which made it more and more difficult to say that these peaks were from groups which were not covalently bound to the alamethicin and finally conclusive evidence was obtained which showed that these extra peaks came from another extra moeity which was covalently bound to the alamethicin, this evidence was as follows;-

1. The very pure F30 prepared as described at the beginning of this chapter contained the peaks at 2.0 PPM and 1.46 PPM in identical form to that of the normal samples.

2. The in-probe hydrolysis experiments which were described in part in the previous section clearly showed the peaks at 2.0, 1.46 were associated together in a moeity which rapidly hydrolysed off the alamethicin. These experiments will be described in detail later.

The experiments which provided progressively more information about this extra 'X' moeity and eventually lead to the

determination of its identity will now be discussed.

Experiments with the chromicyanide ion

It is worth discussing the chromicyanide experiment mentioned in 3. above in more detail as this experiment revealed the less obvious peak at 1.46 PPM in the spectrum which was associated in some way with the peak at 2.0 PPM. This experiment simply consisted of adding potassium chromicyanide dissolved in methanol to a sample of the intact F30 in methanol. The chromicyanide ion is a large negative ion which binds weakly to positively charged centres. The paramagnetic properties of the ion are such that it causes broadening of peaks from nuclei near the bound ion and this effect falls off as the inverse sixth power of the distance.

Addition of this material to the F30 had a large effect, it broadening the peak at 2.0 PPM to such an extent that it was completely removed from the spectrum and there was also a considerable effect on another peak at 1.46 PPM, a singlet peak on the upfield side of the enormous methyl alanine methyl peak. There was one other peak in the spectrum which showed a moderate broadening, an unassigned triplet in the α CH region at 4.249 PPM. The rest of the spectrum was unaffected, though it should be remembered that most of the NH peaks had exchanged from the spectrum under the conditions of the experiment. This experiment showed that there was another singlet peak at 1 .46 PPM which was associated with the peak at 2.0 PPM. This experiment was also used to determine the area of the peak at 2.0 PPM in the intact alamethicin. This was determined from difference spectra as it is not easy to determine the areas directly. It will be remembered that binding of the chromicyanide ion completely removes the 2.0 PPM peak from the spectrum whilst leaving the other

peaks in the same region unaffected. If the spectra with and without the chromicyanide ion are simply subtracted from each other in the computer all the unaffected peaks will subtract to zero and only the peak affected by the chromicyanide ion i.e. the 2.0 PPM peak will appear, and its area may be determined. This experiment shows that the 2.0 PPM peak is almost certainly 3 protons. (The area of the 1.46 PPM peak cannot be found from this experiment because it is not completely broadened by the chromicyanide). It is also clear from spectra of the intact F30 which have been subjected to a convolution technique, which sharpens the lines and preserves their area relationships (but unfortunately introduces considerable noise) that the peak at 1.46 PPM is much more than three protons and is almost certainly 6 protons arising from two methyl groups.

Hence these experiments show that X probably contains one methyl group giving the peak at 2.0 PPM and two further methyl groups giving the peak at 1.46 PPM, and that all of these methyl groups, but particularly the one giving the peak at 2.0 PPM, are very close to a centre of positive charge, where the chromicyanide ion binds.

Partial hydrolysis experiments

These are the experiments conducted in 3.5 Molar DCl at 37°C in a solvent of 75% methanol. In the previous section the rapid hydrolysis of the PHO moeity off F30 was discussed. However hydrolysis of the X moeity could be clearly seen from the changes in the peaks at 2.0 and 1.46 PPM. As the experiment proceeded small very sharp peaks appeared close to both the 2.0 PPM and 1.46 PPM peaks and gradually increased in intensity whilst the relatively broad peaks at 2.0, 1.46 PPM from the intact group decreased in intensity in a corresponding manner. The significance of the line-widths of these peaks is simply that a molecule of molecular weight of 2000 gives broader lines than a molecule of

molecular weight say 200, because of the much more rapid tumbling of the latter in solution. Hence the sharpness of the peaks from the hydrolysed material show that they come from a small group which has come off the alamethicin. The half life of the peaks of the X in intact F30 under the above conditions was of the order of two hours, so that this corresponds to a rapid hydrolysis compared with that of the peptide bonds.

After the above hydrolysis had proceeded for about two hours it was quenched, and after lyophilisation the sample was redissolved in methanol at normal pH. The pH titrations and experiments with paramagnetic ions described in the section on PHO were performed on the sample and quite a lot of information was obtained about the XH peaks:-

1. Both peaks appeared in the new sample without loss of intensity, this shows that the molecule from which they arise is not volatile enough to be lost in the lyophilisation, and this is important because it should be remembered that peaks corresponding to those observed after the above mild hydrolysis do not appear after prolonged hydrolysis, although the 2.0 PPM peak in particular would be very easily seen. Hence this mild hydrolysis must not break down the X moeity fully.
2. The XH peak coming from the intact X peak at 2.0 PPM showed a substantial shift between the original sample, in 3.5 Molar DCl 75% methanol and the new sample in pH 1 methanol. Hence the hydrolysed molecule would appear to possess a pK at very low pH (and in fact the intact molecule appears to as well). The values of the chemical shifts were as follows:-

Intact alamethicin		hydrolysed	
CD_3OD pH 1	CD_3OD/3.5 M DCl	CD_3OD/DCl	CD_3OD pH 1
1.46	1.51	1.49	1.43
2.06	2.18	2.20	1.91

3. The pH titration showed that the XH apparently possessed no pK (above pH = 1) though it must be remembered that the hydrolysis was really a methanolysis and a carboxyl group might well esterify under these conditions.
4. In striking contrast to the intact X the XH does not bind chromicyanide at all, and so the positive centre must have been destroyed. The 4.249 Triplet peak affected by chromicyanide in the intact alamethicin, is still affected after the X group has been hydrolysed off and therefore is near some new positive centre created by the hydrolysis. (In fact the discovery that alamethicin is linear along with the gadolinium relaxation properties show that this 4.249 PPM triplet must be the α CH peak of PRO 1, and this is direct evidence that X is indeed bound to PRO 1 .)

The above information was not particularly helpful at the time in solving the problem of the X moeity, though it proved helpful later. At the time these experiments were performed it was still not realised that alamethicin was linear, and there was always therefore one particularly puzzling feature of the properties of X. It was known that X was one of the groups furthest away from the lanthanide binding site near GLN 18, and hence it was not possible to bind the X moeity in some way in the only weak place in the sequence namely the supposed GLU 17-PRO 1 cyclising link, and it therefore appeared necessary to bind the X moeity in some way to some other part of the alamethicin molecule, although the chemical, mass spectrometric work showed that it was impossible to interfere with any part

of the sequence of alamethicin except the GLU 17 to PRO 1 part. This paradox was immediately resolved by the discovery that alamethicin was linear, as it was then evident that the X moeity must be the N-terminal blocking group on the PRO 1, which since the alamethicin is now linear will indeed be a very long way from the lanthanide binding site near GLN 18.

Although the probl em of where to bind X had been resolved by the discovery of the linearity of alamethicin there was still no real clue as to what X was. It is surp risingly difficult to think of (sen sible) molecules whose Nmr spectrum consists of two singlet resonances which both come from methyl groups with very little, if any, scope for introducing any other non-exchangeable protons into the molecule, and in addition it was necessary to explain the lability of the linkage to PRO 1 and if possible to introduce a positive charge into the system! It was evident that it was necessary to attempt to separate XH after a hydrolysis and then identify it. It is of course difficult to design good separation experiments when so little is known of the chemistry of the group. It was decided to attempt a very simple method of separation and if this failed to then use column procedures to try and separate the XH.

Isolation of XH

This separation attempt was combined with an experiment which it was necessary to perform anyway, namely a hydrolysis in pure D_2O, rather than in 75% methanol, so that there was no possibility of esterifying any carboxyl groups released. For this experiment the alamethicin was hydrolysed in 3.5 Molar DCl in D_2O at 37^oC. Alamethicin is not very soluble under these conditions and it was hoped that the X moeity (perhaps along with the PHO) would hydrolyse off and dissolve in the D_2O and raising of the pH to about one would then precipitate the alamethicin almost completel y and probably any hydrolysed hydrophobic peptides as well.

The experiment worked very well, the alamethicin gave very broad, weak peaks in the Nmr spectrum as the small amount which dissolved was highly aggregated, the rest was precipitated. After about twenty minutes of hydrolysis two very sharp peaks started to appear in the spectrum in just the positions expected for the hydrolysed X moeity. These peaks increased in intensity with time. After two hours the reaction was quenched by adding sodium deuteroxide until the pH was about 1. The insoluble alamethicin, and other hydrophobic peptides were spun down and the liquid containing the XH removed and put in another nmr sample tube.

Hence a fairly pure sample of the XH was prepared. The Nmr spectrum of the XH was as expected simply two singlet peaks at ca. 2.0 PPM and 1.5 PPM of relative areas 1:2. A pH titration performed on the XH showed that there was a carboxylate pK but no amine pK.

Identification of XH and X

The total amount of information on the peaks of X and XH and the properties of X and XH will now be summarised as it is sufficient to provide the solution to the problem:-

1. The X moeity must be bound to the PRO 1 by a labile bond which leaves the PRO as a normal N-terminus after hydrolysis.
2. The strength of binding of the chromicyanide indicates that there is very likely to be an actual positive charge very near X, or at least a highly positive centre.
3. The XH moeity ' hydrolyses further under rigorous conditions of hydrolysis to materials which do not stand out in any way in either the Nmr spectrum of the complete hydrolysate, which was studied in great detail when determining the nature of the PRO, or in the conventional amino-acid analysis of alamethicin which has been repeated a large number of times by many different workers. (It should be remembered that lyophilisation always preceded the nmr studies of the complete hydrolysate and this will remove

volatile materials).

4. The X and XH groups possess some pK at very low pH ca. -1 or so.
5. The XH possesses a carboxylate pK which affects the 1.5 PPM peak most. (There are no other pK above pH=1).
6. The only non-exchangeable protons in XH are those of the single methyl group at 2.0 PPM and the two equivalent methyl groups at 1.5 PPM. Further there are very severe restrictions on the type of methyl groups which can give rise to these signals. The peak at 2.0 PPM can only be from a methyl group very similar to the methyl group of an acetyl group i.e. a methyl peak attached to a strongly electron-withdrawing carbon. The peak at 1.5 PPM. can only come from methyl groups attached to either a single carbon with one powerfully electron withdrawing group such as an oxygen, as in tertiary butyl alcohol, or a carbon with two less powerful electron withdrawing groups, as in methyl alanine.
7. It must not be forgotten that this material is actually synthetised by a micro-organism! (Although alamethicin is enzymically and not ribosomally synthetised).

It is extremely difficult to write down any suggestions at all which satisfy all the above properties whilst being sensible from the point of view of 7. above, however there was one particularly convincing suggestion which is shown in Fig. 3.9. This group is not as peculiar as it might appear at first sight. It can be seen that it is simply N-acetyl methyl alanine which has been formed into a type of cyclol by interaction with the PRO 1 nitrogen. Peptides or proteins with the N-terminus blocked by acetyl are of course well known. Also cyclol derivatives without the positive charge are well known. This group would be expected to be labile but not greatly so as the two linkages, to the PRO nitrogen, though

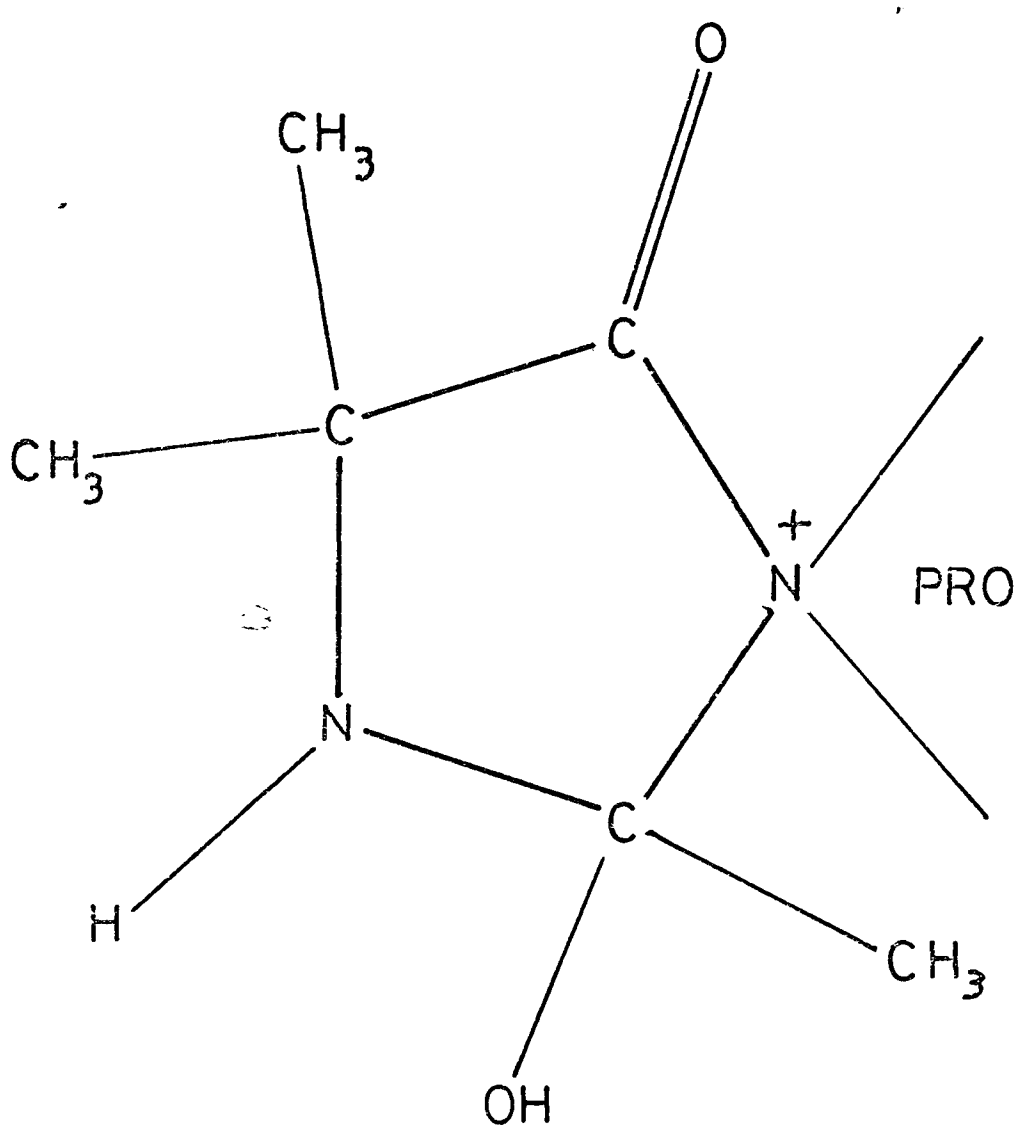


Fig. 3.9

The group blocking the N-terminus of F30 by quaternisation of the nitrogen atom of the PRO 1 residue.

unstable by themselves, will stabilise each other, and the presence of the five membered ring is a further stabilisising influence. The very strong point of this suggestion, apart from the fact that it satisfies all the chemical and Nmr properties very well, is that it uses only materials which are known to be available to the micro-organism which synthesises alamethicin.

Despite the obvious attractiveness of this suggestion it is desirable to have some direct experimental evidence and this was obtained by an unequivocal identification of the XH. On hydrolysis, the group in Fig. 3.9 would be expected to yield N-acetyl methyl alanine under the conditions of the partial hydrolysis experiments. (It is well known that the acetyl group of acetyl N-terminal blocking groups is quite slow to hydrolyse off[21].)

The identification experiment is shown in Fig. 3.10. The top spectrum shows the spectrum of the XH, and in the second spectrum some sodium acetate has been added to provide a constant intensity marker peak in the appropriate region. The third spectrum shows the crucial test, to the sample containing XH and acetate ion was added an approximately equal quantity of synthetic N-acetyl methyl alanine (to which I am indebted to Dr. M.R. Alecio) and it can be seen that the peaks of the XH simply increase in intensity. This experiment is a conclusive identification because it would be possible to separately detect peaks with chemical shifts which differed by only three thousandths of a PPM, and it is inconceivable that both of these very sharp peaks could superimpose to such a degree of precision by chance, if the hydrolysed material were not N-acetyl methyl alanine but something else, however just to make the identification quite certain the pH of the sample was changed and this spectrum is shown in the fourth (bottom) spectrum of Fig. 3.10, and as expected the peaks have moved as one hence putting the identification beyond all possible doubt.

The values of the chemical shifts were 1.999 PPM, 1.457 PPM at low pH and 1.953 PPM, 1.370 PPM at high pH.

This only identifies the hydrolysed X and not the intact X, however the suggestion shown in Fig. 3.9 is the only way in which all the properties of the intact X can be reasonably explained. It is not sufficient to simply link the N-acetyl methyl alanine to the PRO 1 by a normal peptide linkage as this fails to explain both the lability of the linkage to the PRO 1 and why chromicyanide should bind so strongly at this point. It is worth considering how the group in Fig. 3.9 explains the points listed as the properties of X a little earlier:-

1. The lability of the bond to PRO 1 to acid hydrolysis, and the pK of the carboxylate group released by the hydrolysis are evidently explained.
2. The positive centre explains the very strong binding of the chromicyanide ion to this point in the molecule.
3. The nmr properties are also explained, namely the chemical shifts, intensities and lack of splitting of the methyl group peaks. Also this group explains the anomalous NH proton which is observed both in deuterated methanol, and dimethyl sulphoxide. This proton is distinguished by two properties, firstly it is at much lower field than all the other NH (by more than 0.7 PPM in DMSO) and secondly it exchanges extremely rapidly in both solvents compared with the usual peptide NH. In a molecule such as alamethicin there is no real mechanism for shifting a peptide NH peak so far downfield, but it is now obvious that this proton is the NH of the methyl alanine of the blocking group. Reference to Fig. 3.9 shows that this NH is bound to a carbon which instead of having a carbonyl group bound to it (as it would if it were part of a peptide bond) has an oxygen and a positively charged nitrogen, both of which can be expected to be very electron withdrawing hence shifting this NH very far downfield. The rapid exchange

IDENTIFICATION OF XH

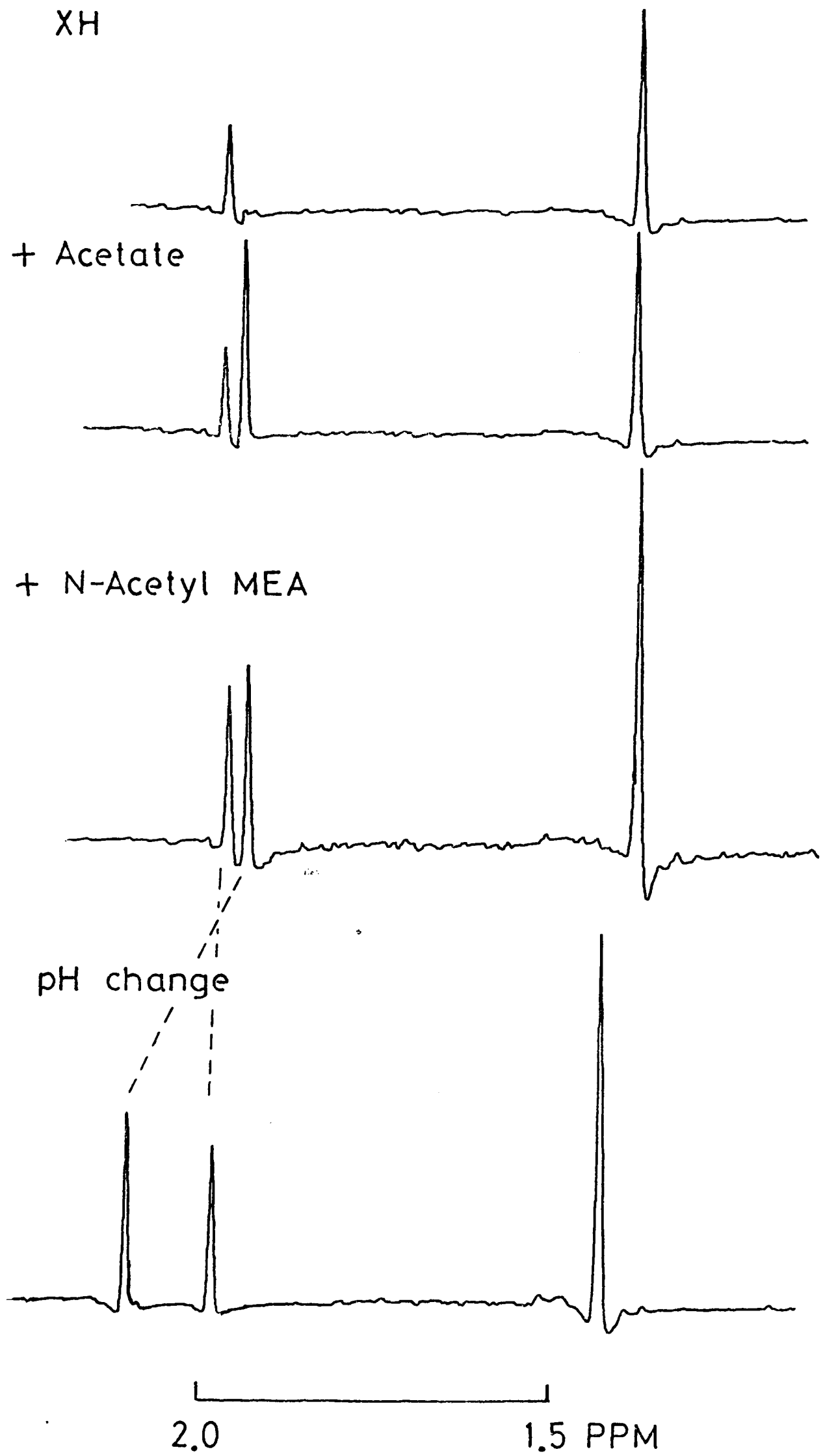


Fig 3.10

Spectra in D_2O showing the identification of XH, details in text.

is also expected for a non-peptide NH. It is interesting that there are only two other rapidly exchanging NH protons in alamethicin (under the correct conditions), one is the GLY NH which is anomalous among peptide NH in exchange properties, the other is the PHO NH which is not a proper peptide NH.

The only other proton in the blocking group is that of the -OH group. This proton was not observed even in the 'dry DMSO' experiments, but this is not surprising as the exchange rate of this proton would be expected to be abnormally rapid, even for a hydroxyl proton, as the hydroxyl group in this case is attached to such a positive carbon atom. In order to observe this -OH proton it would be necessary to use absolutely dry DMSO, and there were always traces of water present even in the 'dry DMSO' experiment discussed previously.

4. The apparent disappearance of the group on vigorous hydrolysis is also explained as the acetic acid formed will be volatile and hence removed by the lyophilisation which always preceded the running of the Nmr spectra of the complete hydrolysate. The methyl alanine released will of course be found along with the other seven methyl alanines in F30, but although integration of the methyl alanine peak in hydrolysate spectra usually gave too many protons the difference was not considered significant in comparison with the error involved.

It can thus be seen that this N-terminal blocking group explains all the various chemical and Nmr properties of X very well and seems the only possibility, bearing in mind the conclusive identification of the 'X' hydrolysed off the alamethicin as N-acetyl methyl alanine.

Experiments on very pure F30

Most of the work reported in the whole of the previous section was performed on material containing small amounts of impurities, some of which absorbed very strongly in the ultra-violet, and it is therefore worth briefly summarising the various experiments which have been performed with the very pure material which was prepared as described at the beginning of this chapter.

1. Spectra of the very pure material have been run under a wide variety of conditions, and in both CD_3OD and deuterated DMSO, where all the NH are visible and the spectra were always the same as those of the usual F30 except for a few small singlet peaks in the methyl region which were known to be impurities and which are missing in the very pure material. In particular the peaks from; the PHO and the N-terminal blocking group are absolutely unchanged.
2. The ultra violet extinction coefficient of the aromatic ring is ca. 185 at 256 nm, as would be expected for a simple phenyl ring.
3. The Nmr spectrum of a complete hydrolysate of the very pure F30 is identical to that of the usual F30 hydrolysate.
4. An amino acid analysis of the pure material revealed nothing unexpected, (the methyl alanines were not estimated).

These experiments on the very pure material hence confirm all the conclusions reached in the previous sections.

DISCUSSION

Agreement of sequence with previous work

The final sequence of alamethicin F30 is

page 109
in Fig. 3.11 and it does not in fact disagree with any of the previous composition or sequence studies of alamethicin within the error of the previous experiments. The various points raised by the proposed sequence in relation to the previous work will now be discussed in detail.

Composition studies

On complete hydrolysis of the F30 there will be, in addition to the amino acids in the cyclic Payne sequence, the products of hydrolysing the two blocking groups :-

(1). The phenylalaninol :-

Since phenylalaninol contains a primary amine group it should stain with ninhydrin (and does), and hence should appear as an anomalous peak in the amino acid analysis. In fact it was shown by using commercial phenylalaninol that phenylalaninol does not emerge from the amino acid analyser at any point between cysteic acid and arginine, and in fact does not emerge even if the analyser is run for some hours past the (very basic) arginine position. High concentrations of phenylalaninol were used in these experiments. Hence it is obvious that the phenylalaninol must stick firmly to the amino acid analyser column.

(2). The N-terminal blocking group :-

On vigorous hydrolysis the group will obviously give acetic acid and MEA, the former will be undetected chemically. The MEA will appear along with the other seven MEA residues from the rest of the alamethicin.

In the determination of the sequence by Payne et al some considerable care was taken to try and obtain an accurate figure for the number of MEA residues. This is not at all easy as MEA stains so weakly with ninhydrin, and there is the further confusing factor that the MEA is involved with the ALA in microheterogeneity. If this is taken into account

then one can calculate from the figures in Paynes paper[4] that there are 7.14 ± 0.15 MEA residues (compared with the seven in his sequence). The limits of error will of course only refer to random errors and not any systematic errors which may be present. In order to obtain the true answer of eight MEA it is necessary to postulate some 10% systematic error on the above figures obtained by Payne et al. This is not at all unreasonable bearing in mind all the difficulties mentioned above and the following points should also be noted:-

1. The sequence studies of Payne et al, in common with all previous composition studies and also the sequence studies of Kiryushkin et al[3], were performed on the mixture of molecules comprising 'alamethicin', of which the F30 we are discussing here is only ca. 85%, the remainder being made up of F50 ca. 12% and other minor components 3%. The sequence of F50 is discussed in the next section and is very similar to F30, but may well have different microheterogeneities. The composition of the remaining components is absolutely unknown.
2. Since the MEA was estimated by comparison of the colour with that of GLY (after a standardisation experiment) any error in the GLY will directly reflect in the MEA result.

Taking everything into account, and particularly point 1. above an error of ca. 10% is not very surprising.

As regards other work on alamethicin, the sequence studies of Kiryushkin et al[3] obtained a similar value for the amino acid composition as Payne et al, but no details were given of how the MEA were estimated and the same criticisms, and possibly more, can be made of this work. It is interesting to note that in the original composition study performed on alamethicin by Meyer et al[1] the correct value of eight MEA was obtained.

Sequence studies

Considering the conventional sequence performed by Payne et al[4] first. They reported that after mild hydrolysis they were able to isolate the 'peptide 1', which is the PRO 1 to GLN 18 sequence without the blocking groups. This is entirely in agreement with our finding, by direct Nmr observation of a mild hydrolysis, that the two blocking groups hydrolyse off rapidly. The N-acetyl methyl alanine released in such a hydrolysis will of course go undetected, and the anomalous properties of the PHO released in that it is basic and has no carboxyl group, no doubt explain how this was missed. Once the blocking groups have been lost then the alamethicin will indeed behave as if it only contained the amino acids in the PRO 1 to GLN 18, peptide 1, and will give entirely self-consistent sequence results. Although the amino acid composition of the peptide 1 given in Payne's paper is the same as that of the intact alamethicin, for the reasons discussed earlier the analysis of the MEA would not be accurate enough to withstand the direct evidence of the N-terminal blocking group presented in this thesis.

It is interesting that Payne et al did in fact almost certainly succeed in isolating a peptide with the phenylalaninol still attached. This is the peptide 25 of Payne's paper[4] which was isolated after a mild hydrolysis of alamethicin. This peptide contained the amino acids from PRO 13 to GLN 18, but instead of having a negative charge as would be expected, the peptide was neutral. Payne et al comment on this and say 'we cannot readily explain why peptide 25 was neutral', they advanced an explanation in terms of a supposed cyclic imide intermediate involved in the breaking of the 'ring'. It is now evident that this peptide in fact still has the carboxyl group of GLU 17 blocked by the phenylalaninol and is hence neutral.

The other sequence determination was by Kiryushkin et al and involved the use of mass spectrometric techniques. However the first stage of their sequence determination was to prepare a partial hydrolysate of alamethicin and then to separate the peptides on a Dowex 50 x 8 cation exchanger, detecting the peptides by their ninhydrin-absorption at 570 nm. The cation exchanger would remove the phenylalaninol quite effectively at the pH used! The N-acetyl methylalanine would of course go undetected. Hence they again obtained the sequence for the PRO 1 to GLN 18 peptide 1 and never detected either of the blocking groups.

The above discussion clearly shows the weaknesses of both conventional chemical sequencing techniques and some physical techniques when applied to materials containing non-amino acid moieties. In the particular case of alamethicin the fact that the amino-acid involved in the complex N-terminal blocking group was a MEA was plainly unlucky, but a simple acetyl group blocking PRO 1 (or formyl) would also have been undetected by these techniques. The case of alamethicin and also the linear gramicidins mentioned earlier demonstrates the extreme care that needs to be taken with conventional and mass spectrometric techniques, especially when looking at enzymically synthetised molecules.

Membrane properties of alamethicin

The membrane conduction properties[8-11] of alamethicin are its most interesting biological properties, in particular its ability under the right conditions to mimic nerve action potentials. There are two important points raised by our sequence:-

1. The linearity of alamethicin is of obvious interest in discussing the nature of the pores which alamethicin supposedly forms to

conduct ions across lipid bilayers. In particular the alamethicin is long enough to stretch right across a lipid bilayer and so the pore may consist of alamethicin molecules laid length-ways side by side. The idea that alamethicin forms stacked rings to give a pore[4, 8] must evidently now be rejected.

2. The positive charge at the N-terminal end of the alamethicin may well be of great importance in determining the conduction properties, and the way pores are formed. The carboxyl group at the other end of the F30 molecule can provide a negative charge, though the conductance properties of alamethicin do not appear to be pH dependent.

SUMMARY

The final result for the sequence of alamethicin F30 is shown in Fig. 3.11 and the experimental evidence leading to that result is summarised below:-

Nmr spectra show the presence of two unexpected moeities in the molecule:-

(A) An aromatic material, present in 1:1 molar proportions with the F30.

(B) An 'X' moeity containing methyl groups as the most obvious anomalous peaks.

Both of these materials were shown to be covalently bound to the alamethicin and not to be impurities as follows:-

1. An extremely pure sample of F30 was prepared by column procedures and this showed all the peaks from the extra moeities absolutely unchanged in all respects.

2. It was directly shown for both of the extra moeities how they were covalently bound to the alamethicin by observation of the functional groups produced, both in the extra groups themselves and in the alamethicin when these groups were hydrolysed off the alamethicin.
3. The hydrolysis of both of these extra groups off the alamethicin was directly observed by Nmr as it was occurring in the Nmr tube in the spectrometer. It was found that both groups hydrolysed off fairly rapidly, in a matter of a few hours in 3.5 Molar DCl/75% methanol at 37^{o}C.

The extra moeities were isolated, identified and placed in the sequence as follows:-

(A) 1. The aromatic moeity was separated by solvent extraction from a complete hydrolysate of the alamethicin ($CDCl_3/D_2O$, pH = 11), and pH titrations, decoupling experiments and finally direct comparison of spectra with an authentic sample unequivocally identified this material as phenylalaninol (PHO)

(A) 2. In the intact F30 the presence of the $-NH-\underset{\displaystyle CH_2}{\underset{|}{CH}}-CH_2$ unit was established by decoupling experiments. It was also shown that the group attached to the NH was a carbonyl. The previous chemical and mass spectrometric sequence work, combined with our Nmr studies which, inter alia showed that the PHO was in the region of the GLU 17 residue, meant that the only possibility of attaching the PHO into the alamethicin was to break open the supposed GLU 17 to PRO 1 cyclising link, and attach the PHO to the GLU 17 carboxyl group.

(A) 3. In very dry DMSO the -OH proton of the PHO was observed and was decoupled from the peak from the protons of the $-CH_2-$ group to which the -OH is bound, thus identifying this peak as from the PHO -OH proton and showing that the PHO is only bound to the alamethicin by the amide link to GLU 17 and hence that alamethicin is linear.

F30 SEQUENCE

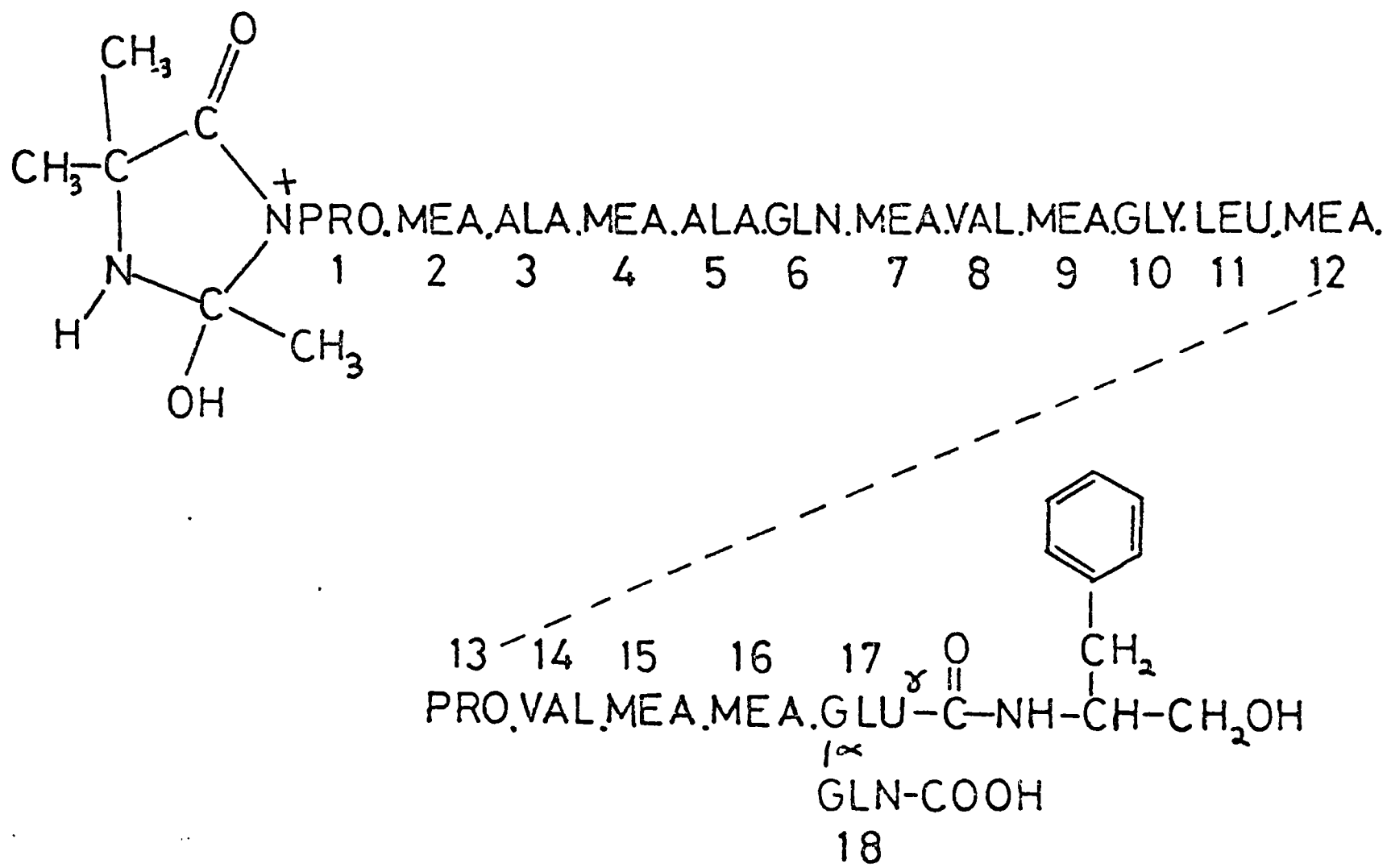


Fig. 3.11

The final sequence of alamethicin F30 showing the two blocking groups in detail and also the free carboxyl group of GLN 18. Other side-chains are not shown.

(A) 4. The PHO was undetected in all the previous composition and sequence studies because although it possesses a primary amine group for detection by ninhydrin it sticks to the amino acid analyser column .

(B) 1. Separation of the X moeity after partial hydrolysis enabled the identifcation of the hydrolysed X, by direct comparison with an authentic sample, as N-acetyl methyl alanine.

(B) 2. Previous chemical and mass spectrometric sequence determinations combined with our Nmr studies show that this group must be bound to the PRO 1 of the linear alamethicin thus blocking the N-terminus.

(B) 3. The lability of the X-PRO bond and the very strong binding of the chromicyanide ion near to X (which indicates a positively charged centre there) show that it is not sufficient to merely link the N-acetyl MEA group to the PRO 1 by a normal peptide bond. The only group which satisfies all the evidence is shown in Fig. 3.11

(B) 4. This N-acetyl methyl alanine is not seen in any of the previous sequence studies as both of them used ninhydrin staining to detect the peptides from partial hydrolysates of the alamethicin.

The methyl alanine from this group would be expected to show up, after complete hydrolysis, in the composition. In the Nmr work the integration of peak areas to obtain numbers of residues was not sufficiently accurate to make the high value obtained significant. In the chemical sequence studies the difficulty of accurately estimating the large number of very poorly staining MEA present in alamethicin (8), and the impure mixture on which the sequence studies were performed, explain the inability to find the extra residue in the composition and so there was apparent agreement of the composition with the incorrect sequence.

PART III

THE SEQUENCE OF F50

It is evident from Nmr spectra that F50 is very similar indeed to the F30 major component. A comparison of the spectra of F50 and F30 is given in Fig. 3.12, a, b, c. One important point is obvious, the aromatic group and the N-terminal blocking group are present in identical form in F50 and F30.

Many of the experiments performed on F30 to elucidate the problem of the aromatic moiety and N-terminal group were performed on F50 with identical results, these experiments will be mentioned briefly, and experiments designed to find the difference between F30 and F50 will be mentioned in a little more detail.

The experiments described below which show that F30 and F50 have the same composition (except GLU/GLN may be interchanged) and the extreme similarity of the nmr spectra of F30 and F50 and also the manner of their synthesis by Trichoderma Viride and the fact that mixtures of F30 and F50 have been worked on as 'alamethicin' for some time, all point to the sequences of F30 and F50 being virtually identical, and this assumption is made in the following discussion.

Experiments on hydrolysates

The Nmr spectrum of the fully hydrolysed F50, (65^{o}C, 12 M acid 100 hrs) is identical to that of F30 in every respect, including all the peaks from the PHO residue. Hence the

COMPARISON OF F30 & F50 SPECTRA (a) NH + AROMATIC

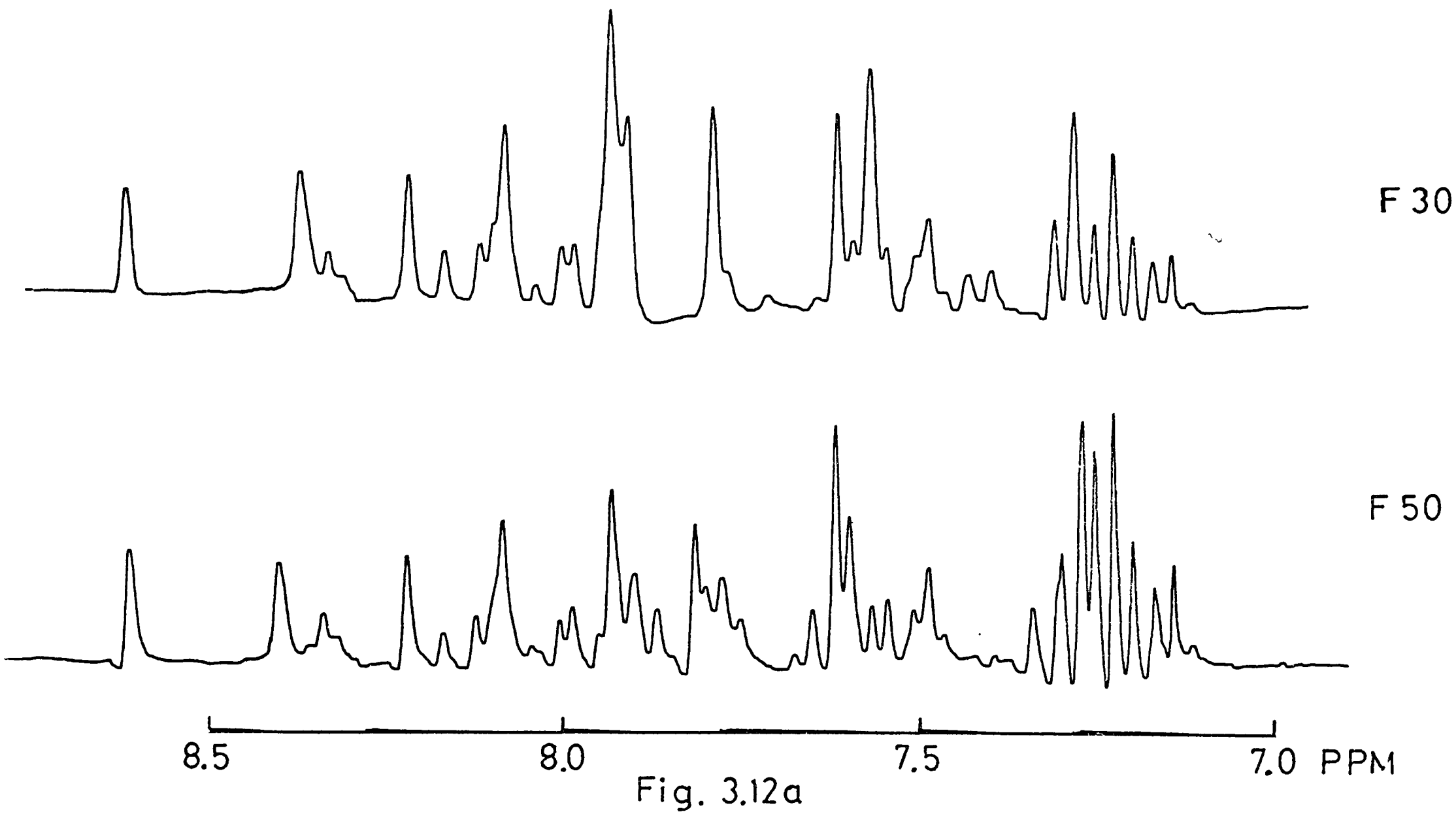


Fig. 3.12a

R.E. spectra in methanol, pH* ca 1.5, showing the great similarity of F30 and F50.

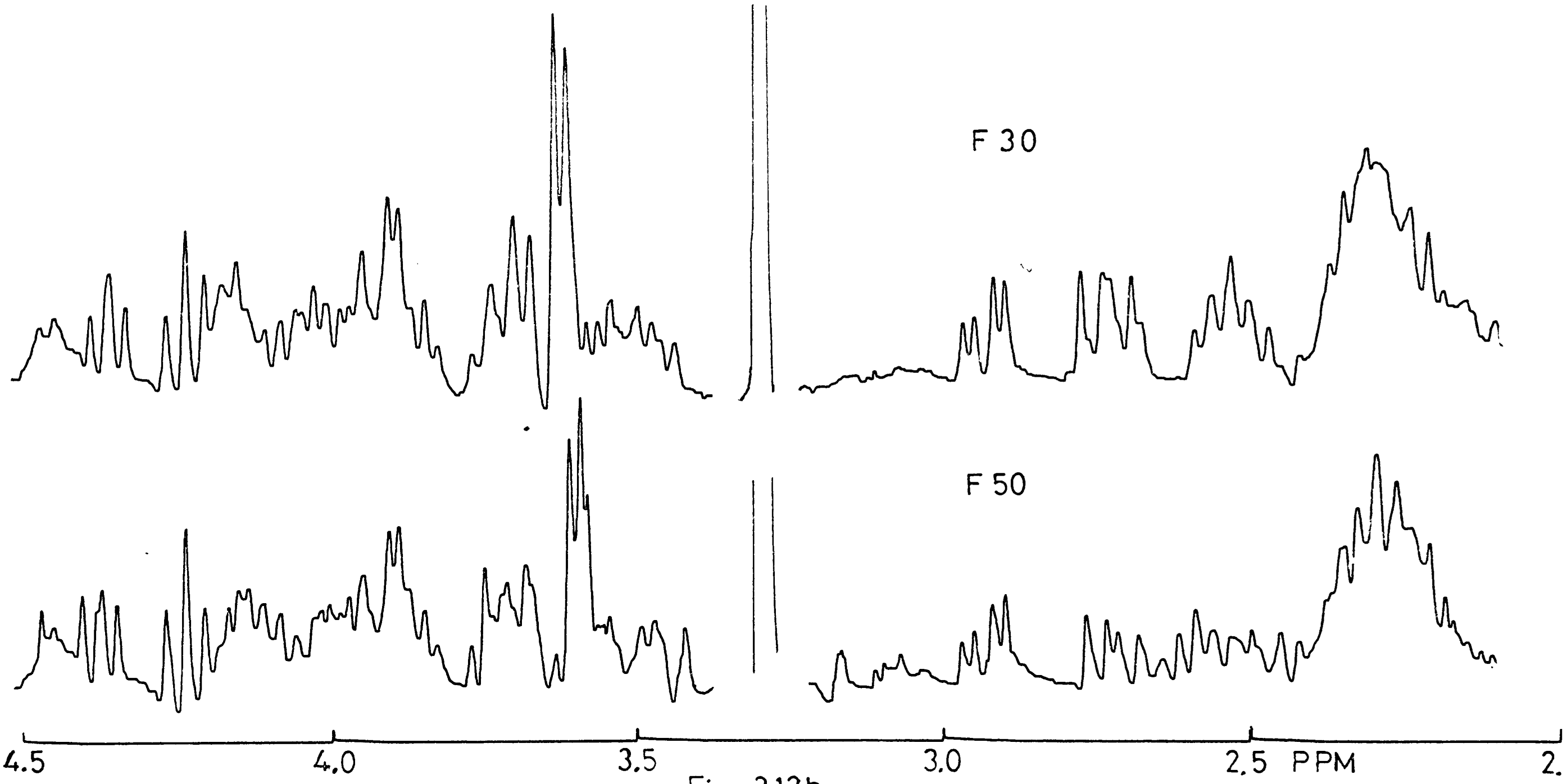


Fig. 3.12b

R.E. spectra in methanol pH* ca 1.5, showing the great similarity of F30 and F50.

(c). METHYL REGION

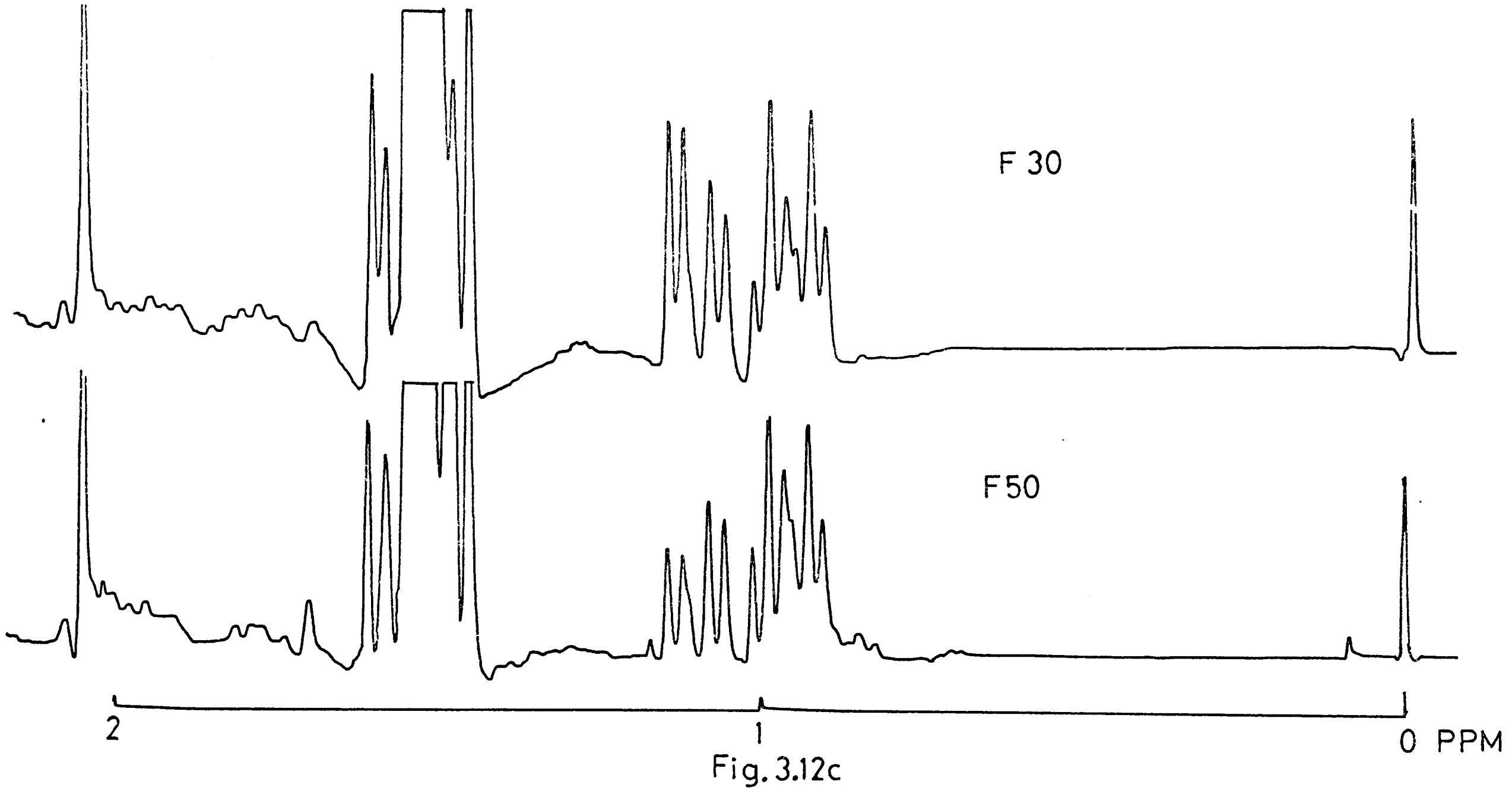


Fig. 3.12c

R.E. spectra in methanol, pH* ca 1.5, showing the great similarity of F30 and F50.

amino acid composition is identical to F30 except GLU and GLN may be interchanged, and the same phenylalaninol is present.

Partial hydrolysis experiments were conducted on F50 under identical conditions to those conducted on F30 (3M DC1, 75% CD_3OD, 37°C), and with the same results, of rapid hydrolysis of the aromatic moeity and N-terminal blocking group.

Experiments on intact F50

An Nmr pH titration shows that there are no ionisable groups, and the absence of free amino groups is confirmed by the lack of a ninhydrin reaction.

The decoupling experiments performed on the PHO residue involving irradiation of the α CH peak and observing the decoupling of the NH, β CH_2 and β' CH_2 peak were performed with the same results as F30. Hence F50 contains the same PHO bound to the GLU 17 γ carboxyl.

Experiments to find the -OH of the PHO in F50 were particularly successful. This proton exchanges less rapidly than that of F30 and hence a much sharper peak can be obtained. The experiment involving the -OH is shown in Fig. 3.13. The top spectrum shows a sample in which the -OH has been exchanged by addition of D_2O. The middle spectrum shows the spectrum which includes the -OH peak which is clearly visible as a single proton triplet. In the third, bottom, spectrum the β'-CH_2 of the PHO are irradiated hence decoupling the hydroxyl proton in a very convincing manner. In contrast to the F30 case there is no carboxyl proton visible at very low field hence directly confirming

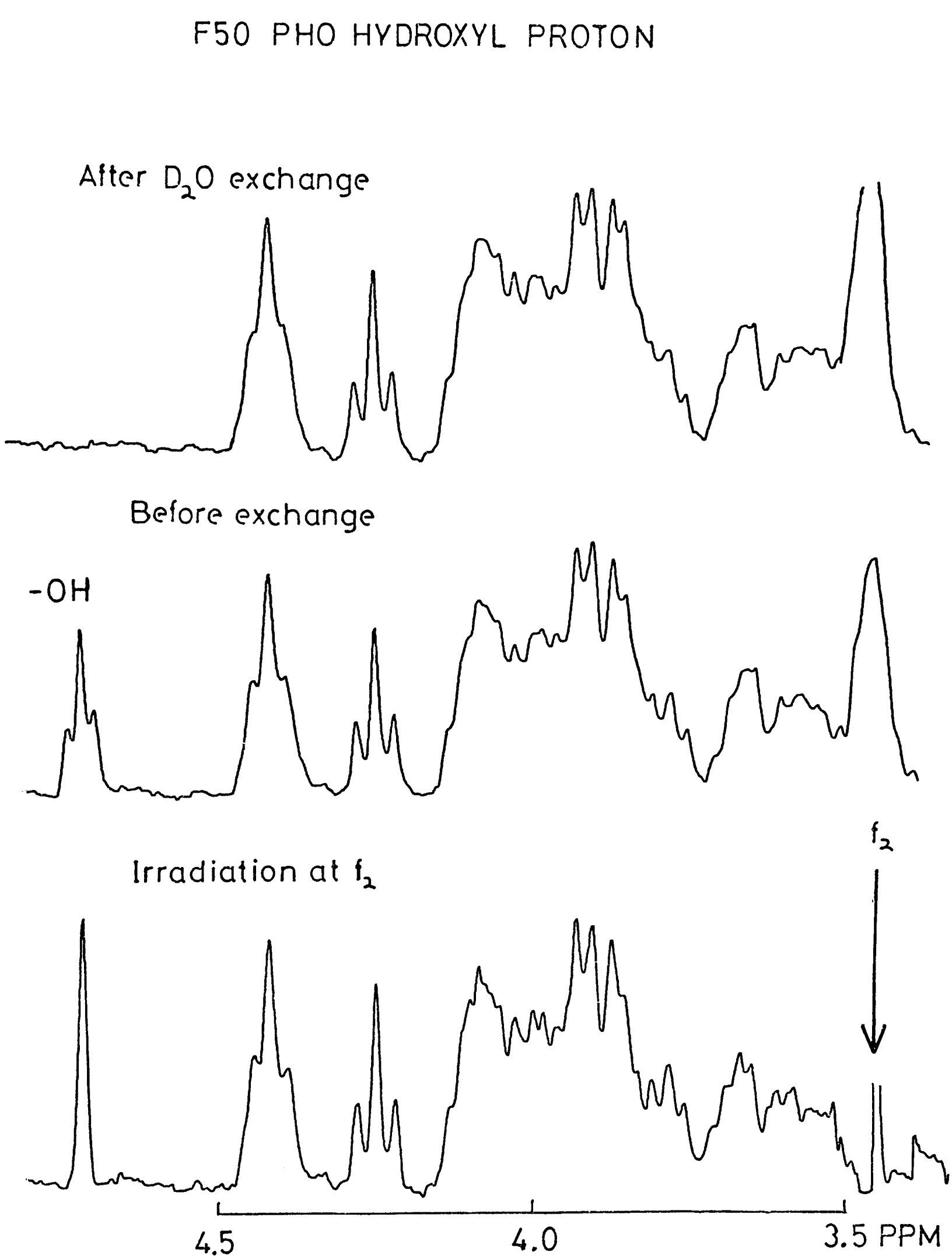


Fig.3.13

R.E. spectra in very dry DMSO at 298°K.

Top spectrum:- This shows the sample after addition of D_2O has exchanged the -OH proton.

Middle spectrum:- Normal spectrum showing the -OH proton.

Bottom spectrum:- Irradiation of the PHO β'-CH_2 peak decouples the triplet -OH peak.

the absence of the carboxyl group.

The experiment involving the chromicyanide ion was also performed on F50 with the expected result of the complete removal of the methyl peak at 2.0 PPM and a strong effect on the 1.46 PPM peak.

These experiments confirm directly that F50 possesses the linearity and positive centre of F30, and show that the C and N- termini of F50 are blocked in precisely the same way as in F30.

In order to try and elucidate the different between F30 and F50 spectra were run in DMSO, as it is possible to see amide protons as well as the peptide NH in this solvent. The anomalous low field NH of the N-terminal blocking group was present, as expected, as in F30, but there were some clear differences as regards the amide protons. The amide protons are clearly distinguishable from the peptide NH protons not only by their chemical shift but also by their much more rapid exchange with the water in the DMSO which leads to rapid broadening and large shifts of the peaks on raising the temperature. The relevant region of the spectrum and its temperature dependence are shown in Fig. 3.14 (compare Fig. 3.6 page 77). It is clear that there are six amide protons here and not four as in F30. This information along with the fact that there is no carboxyl group in F50 leads to the simple suggestion that the carboxyl of GLN 18 becomes an amide group. There is not really much possibility of a larger difference as the rest of the spectrum is so similar.

F50 -$CONH_2$ TEMPERATURE DEPENDENCE

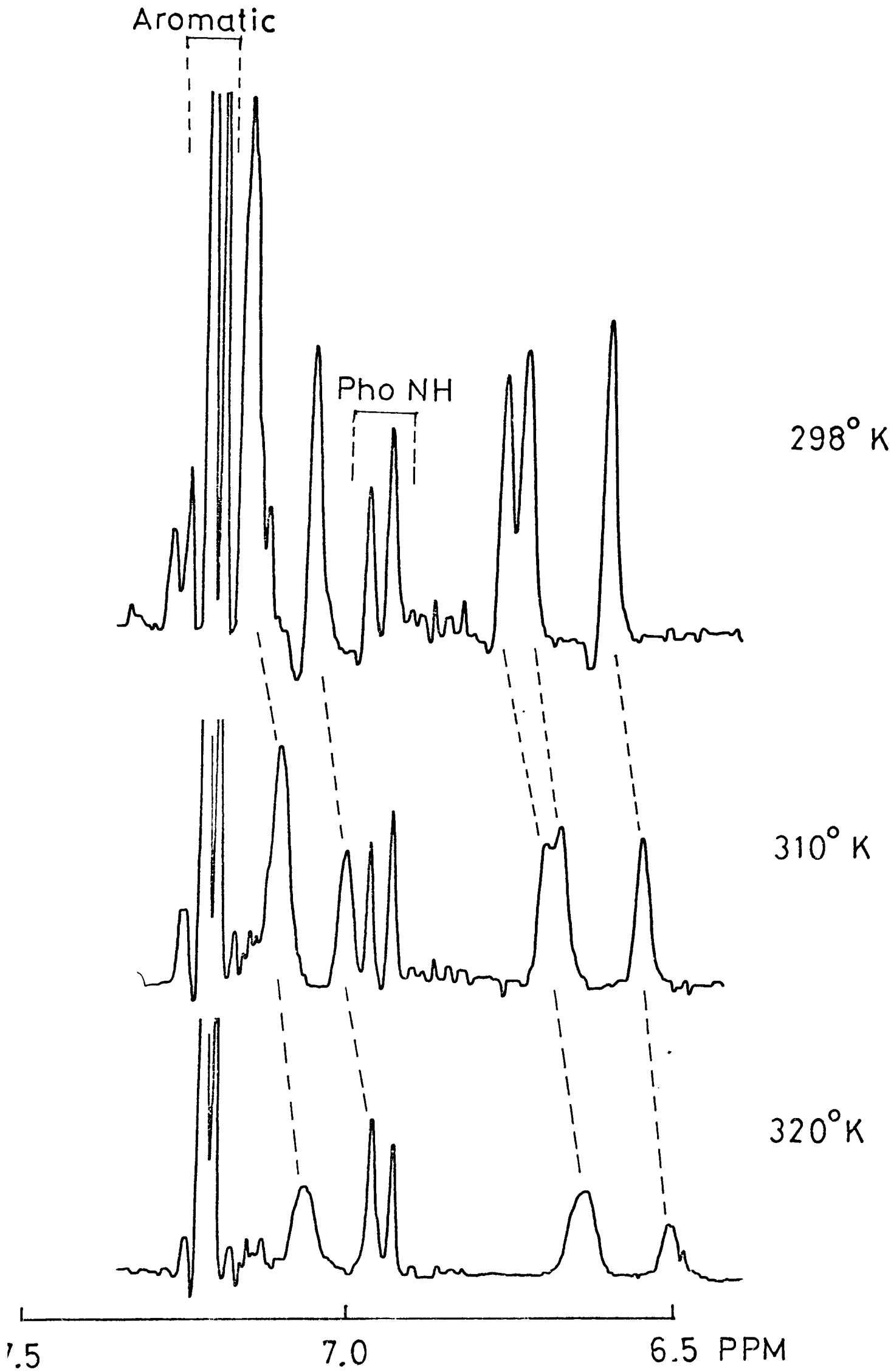


Fig 3.14

R.E. spectra in DMSO showing the six amide protons of F50

Hence the sequence of F50 becomes identical to that of F30 except the carboxyl of GLN 18 is replaced by an amide group.

REFERENCES

1. C.E. Meyer, F. Reusser — Experienta 23, 85 (1967)
2. I. V. Kozhevnikova, G. M. Smirnova, A. A. Kiryushkin and Yu. Ovchinnikov — Summaries of papers presented at the Second all-Union symposium on Peptide chemistry (in Russian) Izd. "Donish". Dushanbe (1969) p. 62
3. A. A. Kiryushkin, Yu. A. Ovchinnikov and I. V. Kozhevnkova — Journ. of General Chem. USSR 41, 2105 (1971)
4. J. W. Payne, R. Jakes and B. S. Hartley — Biochem. Journ. 117, 757 (1970)
5. A. I. McMullen — Biochem. Journ. 119, 10 P (1970)
6. D. Chapman, R. J. Cherry, E. G. Finer, H. Hauser, M. C. Phillips and G. G. Shipley — Nature 224, 692 (1969)
7. A. I. McMullen, J. A. Stirrup — Biochem. Biophys. Acta. 241, 807 (1971)
8. P. Mueller, D. O. Rudin — Nature 217, 713 (1968)
9. R. J. Cherry, D. Chapman and D. E. Graham — J. Membrane Biol. 7, 325 (1972)
10. P. Mueller, D. O. Rudin — J. Theoret. Biol. 18, 222 (1968)
11. D. A. Haydon, L. G. M. Gordon — Biochem. Biophys. Acta. 255, 1014 (1972)
12. A. I. McMullen, P. M. Bayley — Feb. Letts. 16, 278 (1971)
13. W. R. Gray, B. S. Hartley — Biochem. Journ. 89, 59 P (1963)
14. F. S. Sanger — Biochem. Journ. 39, 507 (1945)
15. "Peptides" Fifth Europium Symposium on peptides Mac millan Co. New York. Ed. G. T. Young (1963) page 41.
16. D. Chapman, H. Hauser, E. G. Finer, — J. Molec. Biol. 53, 419 (1970)

17. R.L.M. Synge — Biochem. Journ. 39, 355 (1945)

18. R.L.M. Synge — Cold spring Symp. Quant. Biol. 14, 191 (1950)

19. R. Sarges, B. Witkop — J. A. C. S. 86, 1862 (1964)

20. P.K. Glasoe — J. Phys. Chem. 64, 188 (1960)

21. 'Molecular Biol. Biochem. and Biophys. protein sequence and determination' Ed. S.B. Needleman Springer Verlage New York page 82

CHAPTER FOUR

MATERIALS AND METHOD

First the instrumentation and materials will be dealt with very briefly, and then the general problem of choice of solvent, control of pH, and water content will be discussed and finally there will be a detailed discussion of the lanthanide method for determining conformation of molecules in solution.

Instrumentation

270 MHz spectrometer

The majority of the proton spectra were run on a 'Bruker' machine with a superconducting magnet giving a field of 64,000 gauss and hence a proton resonance frequency of 270 MHz. All the spectra were run in the Fourier transform mode. The machine has facilities for homonuclear proton double irradiation experiments, and also facilities for 'gated' irradiation became available at the end of this project. The machine has a 'Diablo' magnetic disc cartridge unit fitted which enables rapid storage recall of data directly from/to the computer memory, hence enabling direct comparison of a spectrum with one run, say a year earlier.

The machine is fitted with a deuterium field frequency lock and this requires at least some 10% D in any sample. The machine is also fitted with a thermostatted probe, the temperature of which may be varied over a wide range.

90 MHz Spectrometer

Some proton spectra were runona 'Bruker' machine with a conventional magnet with a field of 21,000 gauss giving a proton resonance frequency of 90 MHz. This machine possesses all the facilities of the 270 MHz given above except for the irradiation facilities. Again all the spectra were run in the Fourier Transform mode.

Materials

Alamethicin:-

The supply of this has already been discussed in chapter 3.

Lanthanide chlorides:-

Purchased anhydrous from Research Organic/ Inorganic Chemical Corporation Ltd. Purity 99.9%.

Deuterium Oxide:-

Purchased from Norsk Hydro-elektrisk. Isotopic purity 99.8%.

Deuteromethanol, d_4:-

Purchased from B.D.H. Isotopic purity 99.0%.

Deuterated dimethyl sulphoxide d_6

Purchased from Ciba-Geigy. Isotopic purity 99.5%.

Deuterium chloride:-

Purchased as a 35% solution in D_2O, from Ciba-Geigy. Isotopic purity 99.5%.

Sodium deuteroxide:-

Purchased from Ciba-Geigy, as a 40% solution in D_2O. Isotopic purity 99%.

METHOD

Solvent

It is necessary to choose a solvent in which alamethicin is both monomeric and also reasonably soluble. Previous physical studies have shown that, as would be expected from its extremely hydrophobic nature, alamethicin aggregates strongly in aqueous media[1]. In aqueous phosphate buffer at pH 8.0 the aggregation number is approximately 16 and the critical micellar concentration 2.4 μM. In the presence of 6 M urea the aggregation is somewhat reduced but alamethicin does not become appreciably monomeric except at very low concentrations. As the pH is lowered the aggregation is reduced but the solubility is also greatly reduced. Clearly aqueous solvents are unsuitable for study of the binding of metals to the alamethicin monomer and also will provide a medium of very different dielectric constant from the lipid bilayers in which alamethicin shows its most interesting properties.

In contrast alamethicin is very soluble and monomeric in solvents such as absolute ethanol as has been shown; by sedimentation studies[1] at concentrations up to ca. 6mM. These studies have also shown that addition of e.g. 10% of water to the ethanol does not produce aggregation. Hence it was decided to work in deuterated methanol, where it was confirmed from our Nmr studies that alamethicin is indeed monomeric at the concentrations used of up to 15mM (ca. 25mg/ml.).

Some studies were also performed in deuterated dimethyl sulphoxide, as in this solvent the NH protons exchange only very slowly. The alamethicin is also monomeric in this solvent, although of course it cannot be assumed that the conformation will be the same as in methanol.

Preparation of solutions

Following the purification of alamethicin described in chapter 3, the alamethicin was lyophilised from a methanol/water mixture, i. e. frozen in liquid nitrogen and dried under vacuum. This procedure removed volatile impurities from the alamethicin (e. g. ethanol) and also served to dry it. Solutions were then prepared simply by weighing out appropriate amounts of alamethicin and dissolving in methanol. Solutions involving the lanthanides were prepared by weighing out appropriate quantities of the anhydrous lanthanide chlorides.

The method used for analysis of the data mean that quite large inaccuracies in the concentrations, say 10%, would not matter. However the above methods of making up solutions provide much greater accuracy than this.

There are a few general points about making up the solutions, firstly it is necessary to take particular care to exclude all foreign metal ions and this is done by careful washing of all glassware with EDTA. Secondly it is necessary to limit contact of the solutions and materials with the atmosphere as much as possible as contact with the atmosphere will introduce substantial amounts of water into the sample and this is undesirable for two reasons, firstly it will change the solvent system, and secondly it will give rise to a very large 'solvent' peak in the Nmr spectrum. The solid anhydrous lanthanide chlorides, in particular, are very hygroscopic and the method of handling these was to measure out a small amount of the appropriate lanthanide chloride in a dry glove box under nitrogen, and then accurately weigh out this lanthanide chloride as rapidly as possible in air.

The pH of the solutions was adjusted by addition of concentrated deuterium chloride or concentrated sodium deuteroxide or using solutions prepared by dilution of these concentrated materials in deuterated methanol. Using this method the amount of D_2O introduced into the sample by pH adjustments is extremely small (of the order of 0.1%).

Control of pH

Alamethicin F30 has a single (carboxyl) pK which is at about 5.5 in aqueous systems[1]. It was therefore necessary to devise a suitable and convenient method of monitoring pH in methanol so that the proportion of F30 which was present in the ionised form would be known, and more important when metal ions are added to the system they may complex with either the ionised or unionised F30, and it is necessary to know what proportion of these two possible complexes are present.

It is not however necessary to obtain any 'real' measure of the pH in the solutions as long as there is a reproducible measurable property of the system which can be correlated with the above proportions of species which we wish to measure. For this reason simple, practical methods were used and not lengthy rigorous procedures.

One simple method is to measure the pH with an ordinary glass electrode. However it was found that the readings obtained were highly irreproducible for no apparent reason, and further the electrode took a very long time to come to 'equilibrium'.

It was found that chemical indicators gave reproducible results and also had the advantage of being simple and

rapid, and providing a continuous monitor of the proportions of the various species present. The indicators chosen were bromophenol blue and thymol blue, these have pK values in water at 3.7 and 2.0, 8.8 respectively. It was found that by mixing these indicators in suitable proportions a continuous change of colour from pH 1.0 to around 4.5 could be obtained with an additional change at pH 9.0 in water. This provided a convenient range for monitoring a carboxylate pK.

The indicators were simply added, dissolved in methanol, directly to the sample in the Nmr tube. The concentration of the indicators required to give a good colour was so small (ca. 60μg/ml) that they gave no signals in the Nmr spectrum even when they were run at ten times the normal working concentration.

The pK values of both the indicators and the alamethicin in methanol may well, of course, be quite different from those in water, but this does not matter as long as the indicators are calibrated in terms of what we wish to measure, namely the proportions of the ionised and unionised species of alamethicin present in methanol. Several calibration experiments were performed to enable us to first set up a nominal pH scale and then to find in terms of this nominal scale both the pK and the pH at which there were equal proportions of the lanthanide ions complexed to the ionised and unionised forms of alamethicin. These calibration experiments will now be discussed.

Calibration of pH scale

To enable us to set up the nominal pH scales two pH titrations were performed by Nmr in deuterated methanol; under our normal working conditions using the mixed indicators. One titration was done on methyl alanine (water pK 2.4 and 10.2), and the other on sodium acetate (pK 4.8). These compounds were

chosen for their 'typical' carboxylate pK's which covered the range of interest well, and the amine pK of methyl alanine provided some high pH calibration.

In the Nmr spectrum a single signal is seen whose resonance position is a weighted averaged of those of the ionised and unionised forms present in solution according to the equation:-

$$\delta_{obs.} = \delta_{HA} + \frac{[A^-]}{[HA]+[A^-]}(\delta_{A^-} - \delta_{HA}) \qquad 4.1$$

where δ_{obs} is the observed shift

δ_{A^-} is the shift of the ionised form

δ_{HA} is the shift of the unionised form

Since it is easy to measure all these shifts the Nmr directly gives the proportion of ionised and unionised species present in the methanol solution and enables us to calibrate the colour of the indicators over a range extending roughly 1.5 units each side of the indicator pK.

The true value of the pK in methanol is not known but the water value will give us a nominal pH scale which is internally self consistent and relates the actual measured proportions of the carboxylate species in methanol to the indicator colours. This nominal pH scale will be used throughout the thesis and will be denoted by pH*. The above two titrations enable us to calibrate a pH range of ca. 1-6 and 9-11.

It was now necessary to directly relate the pH* scale/indicator colours to the properties of interest in our system.

The first property of interest is the pK of alamethicin F30, itself which was determined by performing a pH titration by Nmr in methanol on F30. The pK obtained was at a higher

pH* than the pK of acetate, as might be expected from the water pK's of F30 and acetate (5.5 and 4.8).

The second property of interest is the pK defined in terms of the proportions of species with lanthanide ion bound to the unionised, HA, and ionised, A^-, forms of alamethicin. These complexes are denoted M(HA) and $M(A^-)$ respectively throughout the thesis. This pK is particularly important because in the conformational studies metal (lanthanide) ions are bound to the alamethicin and the spectrum seen in the Nmr is determined by the relative amounts of M(HA), $M(A^-)$ and free alamethicin present in solution. (Lanthanide binding to both HA and A^- is in fact strong). The pK represented by the species M(HA) and $M(A^-)$, denoted pK_M^*, is of course very different from the ordinary pK* given by HA and A^-, the relationship being:-

$$pK_M^* = pK^* + \log \frac{K_{HA}}{K_{A^-}} \qquad 4.2$$

where K_{HA} and K_{A^-} are the binding constants (litre mol^{-1}) of the metal to HA and A^- respectively.

In order to find out the pH* at which pK* occurs it is necessary to do a pH titration on alamethicin with the metal present. Two such titrations were performed, the detailed results of these titrations and their somewhat complex interpretation will be considered later (chapter 5). Finding the pK from this titration is not as trivial as when there is no metal present. This is because there are now two distinct possible causes for the changes of shift with pH which are not mutually exclusive:-

(a) A change in the value of the fully bound shift from one complex to the other for a particular peak.

(b) A change in the fraction of ligand bound to the metal due to the weaker binding constant expected for the M(HA) complex.

However a detailed analysis (appendix 3) shows that for a very wide range of binding constants from 10 litre mole^{-1} upwards the value of the pK_M is very close to the pH at which:-

$$\delta_{obs.} = \tfrac{1}{2}(\delta_h + \delta_l) \qquad 4.3$$

δ_h, δ_l are the shifts at high and low pH.

This assumes that there is no change in fully bound shift between the complexes. If there is a change in fully bound shift this does not in fact matter provided that both the binding constants are sufficiently large that the fraction of complex M(HA) at low pH is the same as the fraction of complex $M(A^-)$ at high pH. Since the experiment was performed with a large metal concentration of 100 mM (alamethicin 15 mM) and since it is known (see chapter 5) that even at low pH the binding is strong (K ~ 500 litre mol^{-1}) this will be well satisfied in practice. Hence in this particular case, and under the conditions of this experiment, the equation 4.3 above will give the metal-bound pK_M with reasonable accuracy.

It was found that the two titrations, one with europium and one with praesodymium, both gave similar results for the pK_M of ca. 2.

The correlation between the indicator colour, and pH* obtained from the calibration titrations, along with the values of the pK of F30 and the value of pK_M*, are given in appendix 3.

Water/D_2O content

It was shown that in samples prepared in the normal manner, which would contain a very small amount of water, from the atmosphere there was no change of conformation on addition of a further 5% D_2O to the sample. (These experiments are discussed

in more detail in chapter 6). For this reason it was thought better to work with such samples containing very small amounts of water, rather than to attempt to obtain samples which were completely dry and might be sensitive to minute traces of water contamination. Water contamination was kept small by using samples prepared from freeze-dried alamethicin, and anhydrous lanthanide chlorides. Further, the samples were always sealed from the atmosphere after preparation, and in general, exposed to the atmosphere for as little time as possible during manipulation.

It was also found that D_2O content had a small effect on the shifts obtained with lanthanides and this is discussed in detail in chapter 5. A method was found of comparing D_2O content in the samples used in lanthanide titrations. Using the fact that some of the NH protons showed a linear shift when D_2O was titrated into a sample containing the diamagnetic lanthanide, lanthanum, which is used as the starting sample for all the constant lanthanide titrations. Using this procedure it was shown that the water content of the samples used in the shift titrations varied by less than 1%. This topic is discussed more fully at the relevant point in chapter 5.

The use of lanthanides to determine conformation

The lanthanides have several important advantages over other types of paramagnetic probes[13, 14] for determination of solution conformation.

1. They are chemically extremely similar, but with a very wide range of magnetic properties, this means that several lanthanides can be chosen which suit a particular system well, and shift and relaxation experiments performed with virtually isomorphous ions which will bind in very similar ways. This enables many important parameters to be determined with ease, for example the stoichometry of the complex, exchange properties, whether there is axial symmetry and so on.

2. Both shift and relaxation experiments can be done with isomorphous ions and the very different geometric dependences of these two processes then gives a very good check of the correctness of the conformation.
3. Because of the very poor overlap of the 4f orbitals contact effects only occur at the very nearest atoms and are much less than with say transition metal ions, and hence no separation of contact and psuedo-contact effects is required[15].
4. There are two diamagnetic ions in the series which provide the necessary blanks to enable correction for the diamagnetic effects of the paramagnetic ions in the series.

The paramagnetic lanthanide ions may be roughly divided into three groups for nmr purposes:-

1. Those ions that produce changes in chemical shift of the Nmr resonances with very little broadening. These ions have very rapid electron relaxation times and examples are Eu^{3+} Pr^{3+} Nd^{3+}.
2. Those ions that produce particularly large changes in chemical shift (10 PPM or more), but also produce a fair amount of broadening of the resonances e. g. Dy^{3+} Ho^{3+} Yb^{3+}. These ions have intermediate electron relaxation times.
3. The one (stable) ion of the lanthanide series which as an f^{7} electron configuration, namely Gd^{3+} the very high symmetry of which means that the electron relaxation time is extremely slow, 10^{-7} sec., and hence Gd^{3+} produces very considerable broadening of the resonances (relaxation of the nuclei), and virtually zero shift.

In addition to the above paramagnetic lanthanides there are the two diamagnetic lanthanides, lanthanum and lutecium.

The method uses the shifts provided by ions in groups 1 and 2 above, and the relaxation produced by the ion in group 3, Gadolinium.

There are various factors which need to be investigated using the properties of the lanthanides in order to find whether the conditions which will enable a conformational determination are met. These are as follows:-

1. Rate of chemical exchange:-

The lifetime of the ligand molecule in the bound state is denoted by τ_M and is simply $1/k_{off}$ where k_{off} is the rate constant for dissociation of the 1:1 complex. It is necessary for both shift and relaxation measurements that exchange is 'fast'.

In the case of shift fast exchange means that $\tau_M^{-1} \gg \Delta\omega_M$ where $\Delta\omega_M$ is the difference in chemical shift between the bound and unbound ligand nucleus (in radians/sec.). This may be shown to be present by showing that addition of the shift probe produces a single line whose resonance position in PPM is not frequency dependent (i. e. does not depend on the value of the main applied magnetic field). In this case it is possible to progressively shift the resonances by addition of more and more paramagnetic metal ion, and the shift depends only on the fraction of the ligand in the bound state and various geometric factors.

Fast exchange for relaxation requires that $\tau_M^{-1} \gg \Delta\omega_M, T_{2M}^{-1}$ where T_{2M} is the value of T_2 for the fully bound complex. This condition can be t ested for by frequency and temperature dependence of T_2 and this is more fully discussed in chapter 6. In the case of fast exchange the degree of relaxation will be progressively increased by addition of more paramagnetic ion and again the magnitude of the effect depends only on the fraction of ligand in the bound state and various geometric factors.

It is clear from the expressions given above that the presence of fast exchange for shift measurements does not necessarily show that there is fast exchange for relaxation, and further it is possible for different nuclei in the molecule to be simultaneously experiencing different exchange conditions for either shift or relaxation.

The detailed theory of exchange processes is given in appendix one.

2. The nature of the complex

It is necessary to show that there is a single 1;1 complex. Since under conditions of fast exchange the observed shift depends on the fraction of ligand which is present as complex, the stoichometry of the complex can be investigated by varying the metal and/or ligand concentrations and observing the effect on the shift. This is discussed in more detail,below, and in the appropriate experimental chapters. Similar data can be obtained from relaxation measurements in theory, but in practice it is more difficult.

3. Absence of contact effects

Contact effects can contribute to both shift and relaxation phenomena and in both cases are transmitted through the chemical bonds. This means that one would expect, a priori, that the very small overlap of the lanthanide 4f orbitals would make this effect very small except for atoms in the immediate coordination sphere, and there is ample experimental evidence to support this[2-10].

The collection and interpretation of the shift and relaxation data will now be considered.

SHIFT DATA

In the case of fast exchange the shift may be written:-

$$\delta_{obs} = f \ \delta_M \qquad 4.4$$

where δ_{obs} is the observed shift from the diamagnetic blank (see below)

δ_M is the fully bound shift from the diamagnetic blank

f is the fraction of ligand bound i. e. $\frac{[ML]}{[ML]+[L]}$

Thus as the paramagnetic ion concentration is increased the peaks shift progressively more. Two types of experiment were performed:-

(a) "Binding Titrations"

In this experiment progressively more paramagnetic ion is added to the ligand, alamethicin, whose concentration is kept constant so that the observed shift follows a 'titration' curve reflecting the increasing fraction of bound ligand. From this experiment the stoichometry of the complex can be determined and it can be shown whether or not there is a single 1:1 complex.

Once it has been shown that the complex possess the desired stoichometry the data can be analysed to give the relative δ_M, i. e. the relative fully bound shifts for the various nuclei, as follows.

First it is necessary to eliminate the diamagnetic effects due to binding of the paramagnetic ions. These effects are caused for example by the charge on the ion, changes of conformation on binding the metal ion, and so on. These effects are all eliminated by determining the sometimes substantial effects of the diamagnetic

ion lanthanum, at a corresponding concentration, on the spectrum, and using these chemical shift positions to give us the required diamagnetic blank shift positions.

Then after the diamagnetic effects have been corrected for this leaves the true paramagnetic effect given by the equation 4. 4 above and if ratios of shifts for the nuclei at a particular lanthanide concentration are taken then the 'f' factor is eliminated and this simply leaves a ratio of the geometric and electronic factors which determine the δ_M . The use to which these are put is considered immediately below.

(b) Constant lanthanide titrations

In the second type of experiment the total lanthanide concentration is held constant and progressively larger shifts are obtained by using a progressively higher proportion of paramagnetic lanthanide compared to the diamagnetic lanthanum in the sample. Again the alamethicin concentration is held constant. This experiment has the advantage that the shifts are linear as a function of added paramagnetic ion (in contrast to (a) above), because the fraction of paramagnetic ion bound to the ligand is simply the proportion of paramagnetic ions in solution (assuming the binding constants for the paramagnetic ion and lanthanum are the same). Also factors such as ionic strength are held constant and the experiment acts as its own diamagnetic blank.

In contrast to (a) above stoichometric information is not available from a single experiment, but the stoichometry can be investigated by doing titrations at different total lanthanide concentration or at different ligand concentrations. Again from these experiments relative values of δ_M for the various protons and a particular lanthanide may be obtained.

The detailed interpretation of the shift data from these experiments depends on whether or not there is so called 'axial symmetry'. Axial symmetry requires that as regards the magnetic properties of the complex there is one principal direction (which is by convention the z direction), which differs from the other two but that the other two directions, x and y are indistinguishable from each other. This requirement is easily understood for the case of a time-independent rigid metal-ligand complex, in terms of the effective point group symmetry of the immediate environment of the metal ion. This environment must possess a minimum of either a simple three-fold rotation axis (C_3) or a four-fold rotation reflection(S_4) for axial symmetry. However in the case of a complex with a rapidly exchanging metal ion (and also perhaps solvent molecules) it is necessary to consider the time-averaged symmetry and this is usually impossible. Fortunately as explained below there is a simple experimental test which can prove the presence of the (much simpler) case of axial symmetry.

Axial symmetry

In this case the equation for the observed shift (expanding on equation 4.4) is:-

$$\delta_{obs.} = f D_z \frac{3\cos^2\theta - 1}{r^3} \qquad 4.5$$

where f is the fraction of ligand bound

Dz is an electronic factor which is constant for any one lanthanide

θ is the angle the metal-nucleus vector makes with the symmetry axis

r is the metal-nucleus distance

If, for a particular lanthanide, the various shifts for the different protons are taken and ratioed to the shift of one (arbitrarily) chosen proton, then the 'f' and 'D_z' factors will be eliminated and the values of these various shift ratios will be dependent only on the ratio of the geometric factors $\frac{3\cos^2\theta - 1}{r^3}$ for the protons concerned. Hence for any particular proton the same ratio should be obtained if an analoguous process is repeated with any other lanthanide. If this is indeed the case for a reasonable number of protons and lanthanides then the shifts are obeying the axial shift equation 4.5 and there must be axial symmetry, (and also no contact effects, and the same conformation for the different lanthanides). These shift ratios may then be fed into a computer which can search for all possible geometric conformations of the molecule under study which will give the observed shift ratios. This method has been applied successfully to a large number of small molecules studied by our group[10, 11, 12]. Unfortunately in the case of alamethicin, the shift ratios for different lanthanides are not the same and so it cannot be said that there is axial symmetry,

Non-axial symmetry

The equation for the shift now becomes:-

$$\delta_{obs.} = f\left[D\,\frac{3\cos^2\theta - 1}{r^3} + (D_x - D_y)\,\frac{\sin^2\theta\,\cos 2\phi}{r^3}\right] \qquad 4.6$$

Other symbols as equation 4.5

and ϕ is the angle between the metal-nucleus vector and the subsidiary symmetry axis

D_x, D_y are further electronic factors which are constant for any one lanthanide

It is obvious from this equation that taking ratios of shifts will only eliminate f and not the electronic factors, so that the shift ratios will vary for the different lanthanides

It is not possible to calculate the electronic factors D_x, D_y, D_z for a complex in solution with exchanging metal and solvent, and also there is some dispute as to the precise expressions for the electronic factors (though NOT the geometric factors), so it is plainly necessary to try and analyse the experimental data to obtain D_z and $D_x - D_y$, and the geometric factors.

In fact this is not such a simple problem as it might appear. Let us rewrite the equation for the shift on the h^{th} proton with lanthanide 1, as

$$\delta_{1h} = A_1 x_h + B_1 y_h \qquad 4.7$$

where A_1 and B_1 are the unknown electronic factors (and the fraction bound) and x_h and y_h are the unknown geometric factors.

Then a detailed mathematical analysis (which is presented in appendix 2) shows that it is only possible to write such equations for any number of protons and TWO lanthanides (or any number of lanthanides and TWO protons). Any attempt to write further equations will only produce equations which are linearly dependent on the first two, and thus provide no extra information. Suppose one writes down the equations for two lanthanides and a large number, h, of protons, then there will be 2h+4 unknowns and only 2h equations. This means that there are an INFINITE NUMBER of solutions one of which is the 'conformation'. It is necessary to specify the values of FOUR of the unknowns as well as finding all the δ_{1h} (shifts) in order to be able to solve the equations.

The above analysis assumes that there is not any information relating the different geometric factors, in fact this is not so, if normal values for bond angles and bond lengths in the molecule are assumed then this obviously provides extra constraints. However if data is obtained with two lanthanides it can be shown (appendix 2) that at least five absolutely rigidly connected nuclei are needed to be able to solve the equations. It can also be shown (appendix 2) that in practice there are not enough constraints even in the whole of the rest of the alamethicin molecule (without the aromatic) to enable one to solve the system from proton shifts. The only possibility would be to determine data for two lanthanides and all five protons (or six protons) of the aromatic ring, but there are two objections to this,

1. The conformations for the whole of the rest of the molecule would depend entirely on the accuracy of the aromatic data.
2. It is highly likely that the aromatic ring is spinning round at least to some extent in solution. Our experimental results in fact show that this is indeed the case, and therefore use of the aromatic becomes impossible.

To sum up, due to a combination of the mathematical properties of the system and practical considerations it is not possible to solve the problem of non-axial symmetry for the case of alamethicin nor has it so far been solved for any molecule studied. A qualitative use of the data was possible as is explained in the relevant experimental chapter.

RELAXATION DATA

In the case of fast exchange the relevant equation becomes:-

$$\frac{1}{T_{obs}} - \frac{1}{T_{A(o)}} - \frac{1}{T_{A''}} = \frac{f}{T_M} = \frac{\text{Const.}}{r^6} \qquad 4.8$$

for either T_1 or T_2

where T_{obs} is the observed relaxation time

T_M is the relaxation at the fully bound site

$T_{A(O)}$ is the relaxation in the absence of added paramagnetic ion

$T_{A''}$ is a correction for the bulk effect of the added paramagnetic ion

Since both T_1 and T_2 give identical information it is simply a question of convenience which is used. In practice measurement of T_1 on spectra with a large number of resonances very close together is rather difficult and so T_1 measurements would only be possible after the resonances had been shifted and separated by addition of a shifting lanthanide, however the quite large broadening which shift probes gave at high pH* (see chapter 5) made it impossible to carry out such T_1 at high pH*. For this reason the majority of our relaxation data relied on T_2 measurement by line broadening as it was possible to perform these experiments at both low and high pH (on the unshifted resonances).

The quantity $T_{2A(O)}$ in equation 4.8 above is determined from the initial line-width. (Line width of a Lorentzian peak at half height is $(\pi T_2)^{-1}$ Hz). The Term $T_{2A''}$ is to allow for the outer-sphere effect of the added gadolinium, i.e. the gadolinium simply passing by in solution. This is measured approximately by measuring the broadening of a suitable standard which does not bind the gadolinium e.g. the TMS shift standard. (Note there is no comparable term in the case of shifts because here the angular dependent means that the average effect is zero).

The experiments consisted of a large number of additions of the gadolinium ion Gd^{3+} to the sample, providing values of T_{2obs} at a wide range of concentrations and hence using equation 4.8 the values of T_{2M}. No attempt was made to evaluate the rather complex constant involved in equation 4.8 so that it was not possible to obtain absolute distances. However by taking ratios of T_{2M}^{-1} approximate relative distances were obtained. The difficulties of making accurate T_2 measurements on coupled peaks are discussed in the experimental chapter 6.

SUMMARY

Deuterated methanol was chosen as the main solvent in which to work as alamethicin is monomeric and highly soluble in this solvent. The major component of alamethicin F30 has a carboxy pK and hence it is necessary to be able to measure the fractions of the species HA and A^- in solution, and also most importantly the fraction of the metal complexes M(HA) and $M(A^-)$. A nominal pH* scale was set up and correlations were obtained between the fractions of the various ionised and unionised species in solution and the colour of a simple mixed indicator system by direct methods. The D_2O/water content of the samples was carefully controlled, though measurements showed that it was not important.

The advantages of the lanthanide ions, Ln^{3+}, as conformational probes were discussed and how the lanthanides themselves could be used to determine whether the essential conditions of fast exchange, a single 1:1 complex, and absence of contact shift were present. When these conditions are met the equations for the induced pseudo-contact shift are of the following form:-

$$\delta_{obs.} = f\left[A\,\frac{3\cos^2\theta - 1}{r^3} + B\,\frac{\sin^2\theta\,\cos 2\phi}{r^3}\right]$$

In the simpler case of axial symmetry the second term (in B) vanishes. The non-axial symmetry case was shown to be basically insoluble.

For the relaxation the changes induced by the binding of the paramagnetic ion are;-

$$\frac{1}{T_{obs.}} - \frac{1}{T_{A(o)}} - \frac{1}{T_{A''}} = \frac{f}{T_M} = \frac{const.}{r^6}$$

Hence by use of these two principal effects of binding the paramagnetic lanthanides, geometric information about the molecule may be obtained.

REFERENCES

1. A. I. McMullen, J. A. Stirrup — Biochem. Biophys. Acta 241, 807 (1971)
2. J. M. Baker — Proc. Phys. Soc. 82, 742 (1963)
3. J. Briggs — Chem. Comm. 7, 364 (1971)
4. J. Briggs — Chem. Comm. 22, 1506 (1970)
5. R. V. Demarco — J. A. C. S. 92, 5734 (1970)
6. S. Fariol — Chem. Comm. 20, 1285 (1970)
7. D. Fiat, J. Reuben — Chem. Comm. 15, 729 (1967)
8. C. C. Hinckley — J. A. C. S. 93, 2417 (1971)
9. H. Huber — Helv. Chim. Acta 54, 913 (1971)
10. C. D. Barry, D. R. Martin, R. J. P. Williams, and A. V. Xavier — J. Mol. Biol. 84, 491 (1974)
11. C. D. Barry, R. J. P. Williams, A. V. Xavier et al — Nature, 232, 236 (1971)
12. C. M. Dobson, R. J. P. Williams in Press
13. G. N. Lamar — J. Chem. Phys. 41, 2126 (1964)
14. William deW Horrocks — In. Chem. 9, 690 (1970)
15. J. P. Jesson — J. Chem. Phys. 47, 582 (1967)

CHAPTER FIVE

PRELIMINARY EXPERIMENTS AND LANTHANIDE SHIFT EXPERIMENTS

All the work in this chapter refers to the major component F30. Precise details of many of the experiments are given in Appendix 3, except where the detailed data is of direct interest and it is then given in this chapter.

The chapter is divided as follows:-

1. The assignment of the Nmr spectrum in deuteromethanol.
2. The lanthanide shift experiments which were conducted to determine the effects of pH and D_2O content on the induced paramagnetic shifts, and which also showed that the various conditions which need to be met before the lanthanide method can be applied are in fact met.
3. The results obtained in the lanthanide shift experiments and their interpretation, particularly with respect to the problem of non-axial symmetry, and finally the conformational conclusions which can be drawn from these results.

1. ASSIGNMENT

Alamethicin is an unusual assignment problem in that it contains a large number of methyl alanine residues. The methyl groups of these residues all come under one very large peak, and assignment of these to individual residues is obviously impossible. Because of the absence of any α CH protons in the methyl alanine residues the only other protons are the NH, these are singlets and again are almost impossible to assign to specific residues.

The majority of resonances were assigned by spin decoupling experiments. The assignment irradiations were all performed at least twice on different samples. Some assignment to specific residues was possible by utilising line broadening results with the lanthanide, gadolinium Gd^{3+}, (these results are given in detail in chapter 6).

Use of spin decoupling for assignment

It is possible by careful use of spin decoupling to 'separate' and assign resonances which differ in chemical shift by only a few hundredths of a PPM (e. g. the two ALA residue methyl groups are only 0. 03-0. 04 PPM apart). In order to achieve this it is necessary to reduce the power of the double irradiation as much as possible and then to 'sweep' through the region of interest, i. e. to perform the irradiations at closely spaced intervals throughout the region of interest. In this way progressively better decoupling is observed as the actual position of the appropriate peak is reached. Initially, when the irradiation is not on target the decoupling of the peak will be very poor and there will simply be a reduction in the separation of the components of the multiplet; as the position of the irradiation is changed providing progressively better decoupling, then the separation of the components gets smaller until they are no longer properly resolved and the multiplet is replaced by an unresolved 'fat' peak, finally the peak sharpens up to a singlet when the irradiation is very close to the target peak. These effects are then seen in reverse as the irradiation goes on 'past' the peak. A graph of separation of the components of the doublet against irradiation position will then enable a really accurate determination of the position of the peak if this is required, or alternatively will clearly show whether two different peaks are decoupled 'simultaneously' by an irradiation.

Assignment results

The assignments are shown in Fig. 5.1a, b, c. Assignments which are uncertain are in brackets. All the assignments will be briefly discussed below, the details of all the observed decoupling experiments are given in appendix 3. The decoupling experiments involving the various protons of the PHO residue were discussed in some detail in chapter 3 and no more will be said here. The assignment of the protons groups belonging to the N-terminal blocking group was also discussed in chapter 3; and in Fig. 5.1 the MEA of that blocking group is referred to as MEA O, although it is really the first amino acid of alamethicin.

VAL+ LEU + ALA + GLY residues:-

These are all easily assigned by decoupling experiments. Both VAL and LEU have methyl groups upfield of the very large MeALA peak. Irradiations were performed in the β CH_2 region to decouple the peaks from these groups, and the VAL and LEU β CH peaks are easily distinguished as the VAL β CH peak occurs substantially further downfield than LEU $\beta+\gamma$. The ALA residue has its methyl group peak very close to the MEA methyl groups, and there are no β, γ CH_2 peaks in this position (1.5 PPM) so that irradiation in this region decouples the ALA α CH. The GLY residue has a pair of α CH_2 protons and hence is the only residue to give a triplet NH peak. Irradiation of this NH peak which is easily seen, decouples the CH_2 peak. In addition to the GLY NH peak, the LEU and one of the ALA NH were successfully assigned.

GLU + GLN + PRO residues:-

Comprehensive irradiations were performed on the whole β CH_2 and α CH regions to find the decoupling relationships

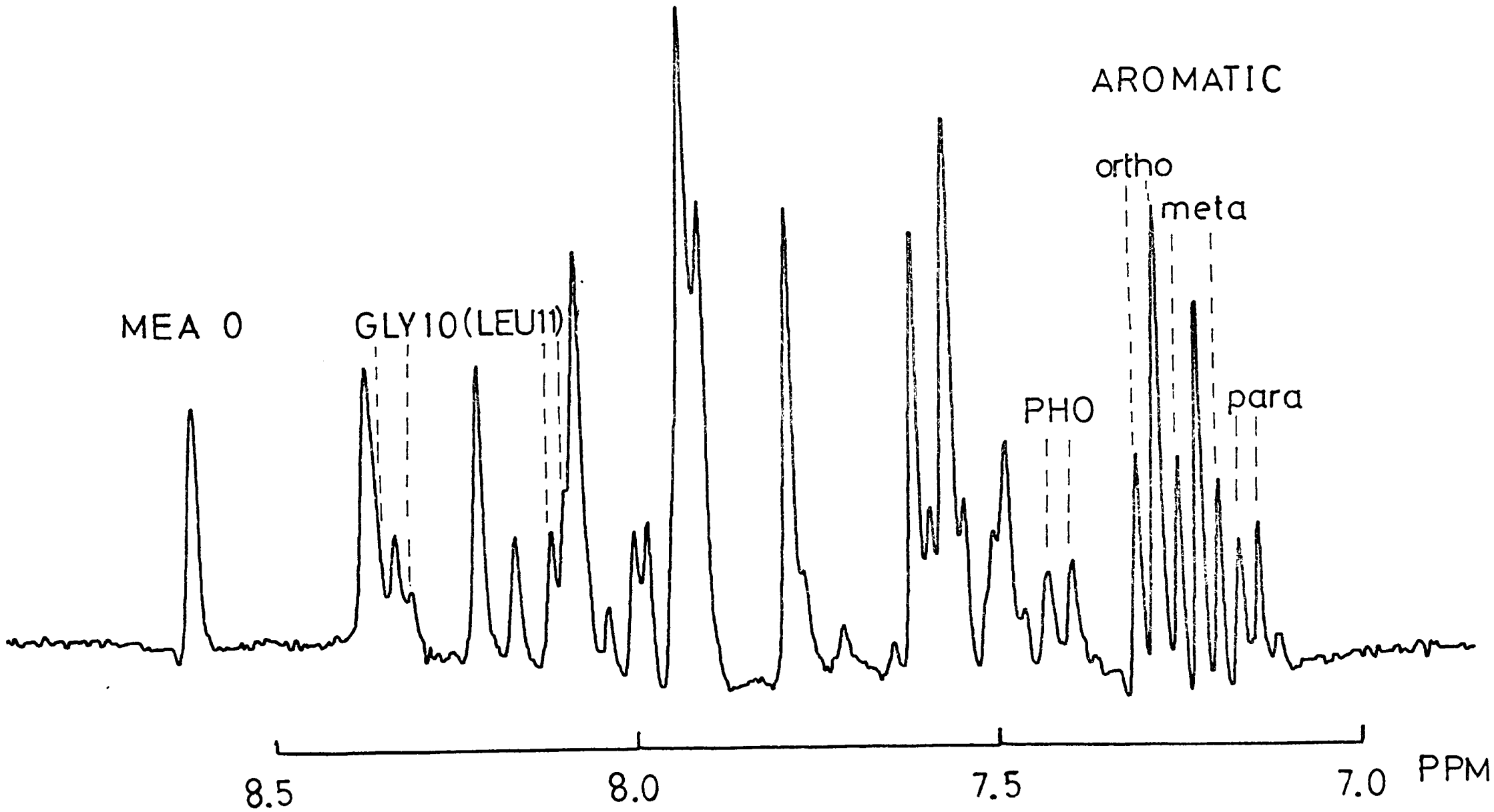


Fig. 5.1a

R.E. spectra in methanol, pH* =1.5 , assignment of the NH and aromatic protons.

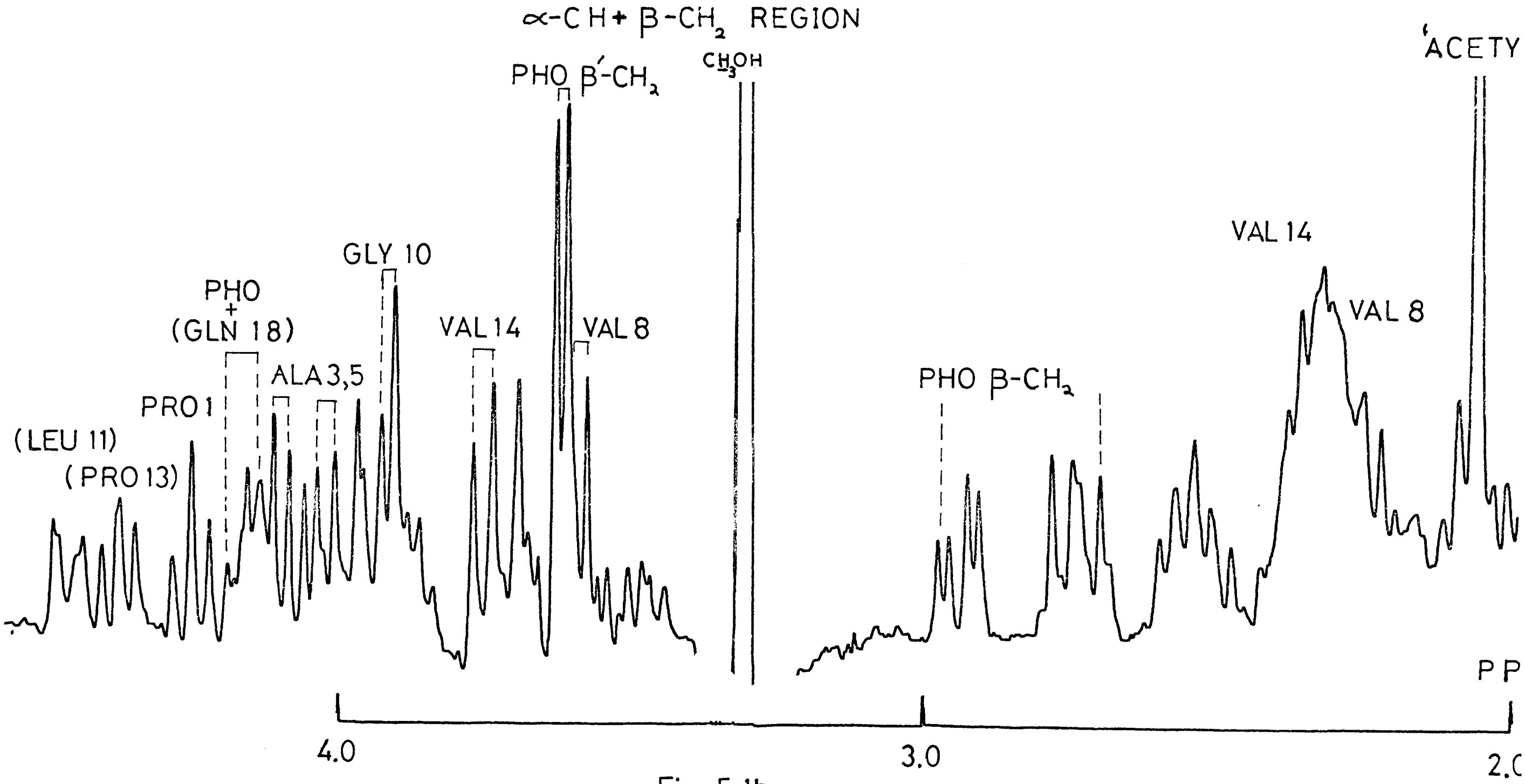


Fig. 5.1b

R.E. spectra in methanol, pH*=1.5, assignment of the ∝CH and β CH_2 region.

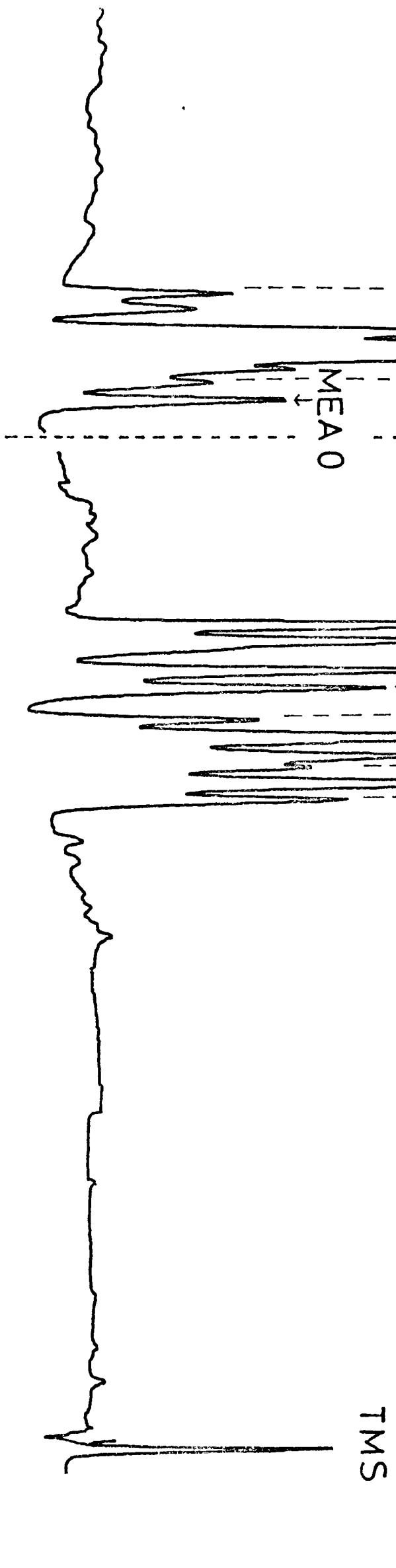


Fig.5.1c

N.E. spectra in methanol, pH*=1.5, assignment of the methyl region.

of the peaks from the remaining residues GLU, GLN and PRO. All of these amino acids have β and γ , protons and the large number of spin-spin couplings makes the peaks very complex and more difficult to perform good decoupling on. The PRO residues also have δ CH_2 protons as well which makes the spin-spin couplings even more complicated. It was not possible to distinguish the GLU GLN and PRO by the decoupling experiments. Other experiments described below were used to try and help in the assignment, there is also one other useful rule, that if an αCH peak is coupled to an NH proton peak then that α CH obviously cannot be from a PRO residue.

One experiment designed to help in the assignment, and also of general interest was an Nmr pH titration on alamethicin and this is now discussed.

pH titration of alamethicin

A titration was performed covering the range pH* 1.3-12 in the hope of finding the GLN 18 α CH proton which is next to the free carboxyl group.

The titration showed that almost all the peaks were completely unchanged in shift position to within well under 0.01 PPM. Some peaks were however affected:-

1. Various peaks showed very small shifts of the order of 0.01 PPM, these shifts were linear with pH over the whole pH range and therefore must reflect progressively changing bulk properties of the solvent as the pH changes. These small effects were reproducible with different samples. All the upfield methyl groups show these shifts, and also the phenylalaninol β CH_2.
2. There were two sets of peaks which showed a shift with pH which was in the form of a pK and these are depicted in Fig. 5.2. Both

gave a value of the pK of pH* ca. 6 a little above the value found for acetate in the calibration experiments, and obviously coming from the GLN 18 carboxyl group. This confirms the presence of a single ionisable group, the carboxyl, as has always been found from previous work and as would be expected from the sequence.

The peak showing the largest shift in this titration was the doublet at 3.709, 3.748 PPM which shifted 0.05 PPM in the wrong direction, i.e. downfield, as the carboxyl group was deprotonated, whereas on simple electrostatic grounds a peak should move upfield when a negative charge is introduced into the system. This doublet was assigned (by the decoupling experiments) to the VAL 14 α CH, and it is rather surprising that this proton should be affected by ionisation of the GLN 18 carboxyl group. Obviously the conformation of the alamethicin itself is such as to bring the carboxyl group near to this residue, or alternatively the protonation causes a change in hydrogen bonding to the VAL 14 NH which is reflected in the α CH shift. The other peak affected by the ionisation is the 4.177 PPM triplet which moves in the correct direction.

The Gadolinium relaxation data show that the 4.377 PPM triplet and the 4.177 triplet must be assigned to two out of the three from PRO 13, GLU 17 and GLN 18 α CH. In fact detailed examination of the gadolinium relaxation data confirms that the 4.177 triplet ought to be assigned to the GLN 18 α CH as this is nearer the metal than the GLU 17 α CH, as detailed examination of the conformation shows it should be (see chapter 6) and the 4.377 triplet must then be assigned to the PRO 13 residue. (The GLU 17 α CH must be hidden under some other peaks in the spectrum and therefore cannot be clearly seen.) The above assignments cannot be regarded as conclusive.

One assignment which was definite was that of the PRO 1 ∝CH peak to the triplet at 4.249 PPM. This peak is discussed in chapter 3. It will be remembered that this peak is moderately affected by the addition of chromicyanide ion to the intact F30 and is therefore near the N-terminal blocking group, in fact the only residue which could possible give a triplet ∝ CH peak and is anywhere near this blocking group is PRO 1. (See Fig. 5.2). This triplet was also strongly affected by chromicyanide ion after the partial hydrolysis which removes the N-terminal blocking group, and this is explained as hydrolysis of this blocking group will leave a normal proline which will protonate hence giving the positive centre for binding of the chromicyanide. As expected this peak is conspicuous by the very small effect which gadolinium has on it, as the gadolinium binds at the other end of the molecule near GLN 18.

BINDING SITE OF Gd^{3+}, $Cr(CN)_6^{3-}$. EFFECT OF pH.

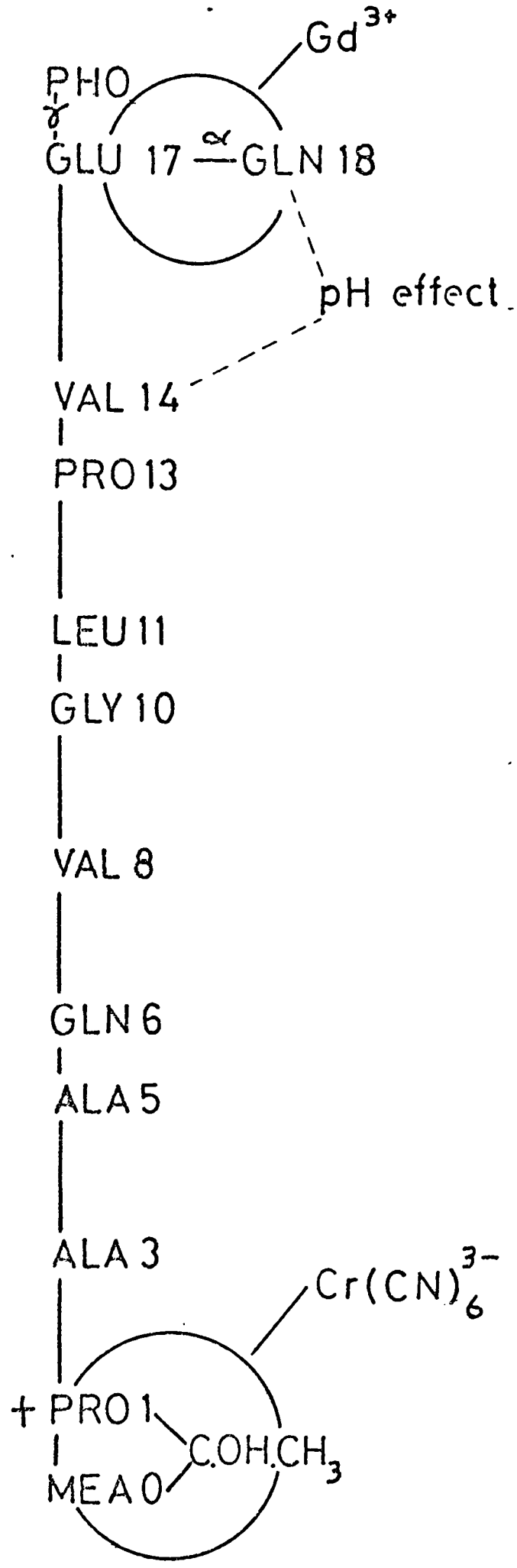


Fig.5.2

Schematic diagram showing the non-MEA residues of F30 and the binding sites of Gd^{3+} and $Cr(CN)_6^{3-}$ and the residues affected by the carboxylate pK.

2. LANTHANIDE SHIFT EXPERIMENTS

Firstly, experiments will be discussed which investigated the effects of pH and D_2O content on the induced shifts produced by binding of the paramagnetic lanthanides and thus enabled us to determine the best working conditions. Then the various conditions which need to be met before the lanthanide method can be applied (see chapter 4) will be shown to be present by appropriate experiments. Finally, the results obtained with the various lanthanides used will be presented and the detailed interpretation of the shift results obtained with the lanthanides will be discussed.

Effect of pH

It was found that in the presence of the metal ion the colour of the indicators was not exactly the same at 'high' pH as when no metal ion was present but the colour was the same with different lanthanides and reproducible so this did not matter. Further in the region of most interest the colours were the same as in the absence of metal.

This experiment consisted of taking a sample of alamethicin at the usual concentration (15mM) and containing the usual indicators and containing the concentration of paramagnetic lanthanide ion used in all the constant lanthanide titrations, (100mM). By changing the pH the various effects which occur can be correlated with the indicator colours and the metal bound pK can also be found (see chapter 4). The lanthanide initially used in these studies was praesodymium, because this ion produces reasonably large shifts (1 PPM) with little of the broadening of the peaks produced by many other lanthanides.

The starting pH* was ca 4, and aliquots of acid were titrated into this solution and the spectra measured after each

addition. The final pH* was 0.8.

The changes in shift which were moderately large (ca 25%) are shown in Fig. 5.3 for some typical peaks which were assigned from previous binding titrations with Pr^{3+}. The shifts clearly show the pK of the metal-bound species M(HA) and $M(A^-)$. One point which needs to be explained is that almost all the shifts† are smaller at low pH. The possible explanations for the changes in the shifts† of the peaks are as follows:-

1. It should first be pointed out that since the metal concentration is 100mM, the alamethicin concentration 15mM, and the binding constant, even at low pH quite strong, the fraction of ligand in the bound state at low pH is ca. 98% and thus the higher binding constant which will exist at the higher pH cannot produce more than 2% change in shift. The dilution of the sample by the addition of acid can similarly only produce a trivial effect.

2. A change in conformation and/or the electronic constants of the Pr^{3+} complex as one goes from the high pH complex $M(A^-)$ to the low pH form M(HA). In fact the relaxation measurements (chapter 6) show that there is a marked change of conformation with pH and that at low pH the molecule tends to 'unwrap' itself from the metal. This would produce a general decrease in shifts, because of the r^{-3} dependence of the shifts, though with some anomalies caused by the angular terms, and this is what is observed. Some changes of the electronic factors would also be inevitable because of the conformation change, and this would cause changes in the shifts which would be the same, percentage-wise for all the peaks.

† It should be remembered that when discussing 'shift' in the context of binding paramagnetic ions that the term 'shift' is shorthand for 'induced paramagnetic shift' i.e. the difference between the chemical shifts of the peak when the paramagnetic ion is bound and when some diamagnetic blank ion (i.e. lanthanum) is bound under corresponding conditions.

pH–TITRATION $[F30] = 15\,mM$ $[Pr^{3+}] = 100\,mM$

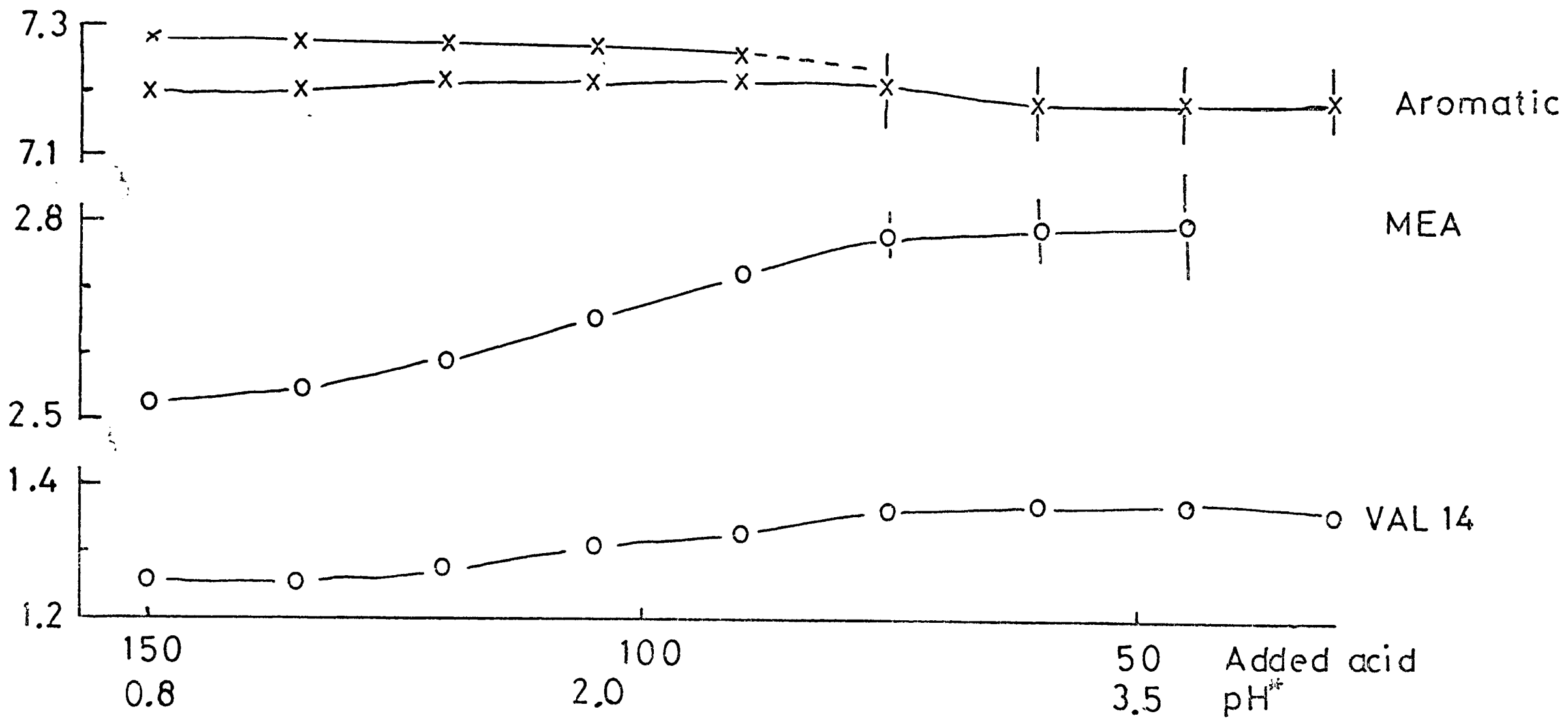


Fig. 5.3

pH titration with paramagnetic lanthanide present. The horizontal scale is linear in added acid , and included for comparison is an approximate pH* scale.

3. Intermediate exchange. This could cause a variation of shifts with pH. It was directly shown however that at all pH values the lanthanide ion is in fast exchange for shifts, this is discussed below.

In addition to the changes in shift there were also changes in line-width. The most shifted MEA methyl peak showed the following changes in line-width (resolution enhanced not conventional spectra):-

pH*	0.8	1.7	2.2	2.8	3.5	4.0
Addition of μL. acid	150	120	90	60	45	30
Half-height line-width in Hz	5.5	6.3	10	20	48	75

The changes in line-widths are relatively much greater than the changes in shift and there is one important and quite general observation, that the effect is much greater for the most shifted peaks. This latter fact points strongly towards some type of exchange phenomenom. It was found, in fact, that the data could be satisfactorily interpreted in terms of the case of intermediate exchange for line-widths. The theory of exchange for relaxation processes (line-widths) is not given in detail in the theoretical chapter but is treated in appendix 1.

It is necessary to consider the likely orders of magnitude of the relevant parameters, $\Delta\omega_M, T_{2M}^{-1}$, and τ_M^{-1} In fact the experimental results tell us that $\Delta\omega_M = 2200$ radians/sec and that $T_{2M}^{-1} \sim 5$ Hz (both of these are for the most shifted peak). The conditions for intermediate exchange are:-

$$\tau_M^{-2} \gg \Delta\omega_M^2 \gg (\tau_M T_{2M})^{-1}$$

a value of τ_M^{-1} of around 10^5 would satisfy these conditions. The equation for the case of intermediate exchange is:- ($f \ll 1$)

$$\frac{1}{f\,T_{2P}} = \frac{1}{f\,T_{2A''}} + \tau_M \Delta\omega_M^2$$

This equation has two important consequences, firstly that at low pH where the weaker binding means that the value of τ_M for the metal-alamethicin complex will be reduced, the lines should be narrower, as was observed, secondly the line-width depends on the value of $\Delta\omega_M^2$, and thus the larger the shift the larger the line-width change on going to high pH, as was observed, and more importantly the line widths in Hz will be <u>frequency dependent</u>, and this provides a further test for whether there is intermediate exchange. This test was applied by running the same sample at 270 MHz and at 90 MHz at the same temperature. The line-widths were as follows:-

	270 MHz	90 MHz
pH* = 3.5	44 Hz	13 Hz

This shows that indeed as expected for intermediate exchange the line-widths are smaller at 90 MHz than at 270 MHz. It was also observed that for the peaks with small shifts the line-widths in Hz were roughly the same at 270 MHz and 90 MHz, showing that these peaks are not in intermediate exchange because of the smaller value of $\Delta\omega_M$. The above analysis is undoubtedly an oversimplification but does provide the correct general explanation, as only an intermediate exchange type of situation can explain the $\Delta\omega_M$ and τ_M dependence of the line-widths.

The practical result of this broadening of the lines at high pH is to make lanthanide titrations much more difficult at high pH because the peaks which show large fully bound shifts ($\Delta\omega_M$) broaden rapidly as more lanthanide is added in the

titration causing an increasing fraction of bound ligand. For this reason no attempt was made to perform a set of titrations with different lanthanides at 'high' pH. Instead the results for the high-pH conformation were inferred from titrations performed at an intermediate pH when both types of complex were present and titrations performed at low pH when only the one complex was present.

It is to be stressed that the finding of intermediate exchange for the line widths in the case of praesodymium has no relevance whatever to the exchange conditions which are present with gadolinium the ion used for relaxation studies of the conformation (chapter 6), because here the value of T_{2M}^{-1} is several orders of magnitude larger, and $\Delta\omega_M$ is a few orders of magnitude smaller.

These pH experiments were also performed on samples containing 100mM europium Eu^{3+}, and the same types of changes with pH were observed, though the increase of line-width with pH was less marked because the value of $\Delta\omega_M$ are less. The value of the pK as obtained in the europium experiment was indistinguishable, within the moderate experimental error, from that of Praesodymium, both being at around pH* of 2.0.

These experiments show that the value of the pH affects the magnitude of the induced paramagnetic shift, and also has a substantial effect on the line-widths of some of the peaks. These experiments show that it would be difficult to work at a high pH when all the complex is present as $M(A^-)$ because of the very large line-width of the most shifted peaks. From these experiments the metal-bound pK can be found, and this point is discussed in more detail in chapter 4.

Effect of D_2O content

These experiments were intended to find out whether there is a substantial conformational change caused by the presence of a few percent of D_2O, (further experiments on this are discussed in chapter 6), and also to see if the presence of D_2O would change the shifts by causing changes in the electronic factors of the lanthanides. Three experiments were performed, the first two consisted of the addition of D_2O to samples of alamethicin by itself and alamethicin with 150mM lanthanum La^{3+}. These experiments will show whether there are any substantial conformational changes caused by addition of the D_2O. The third experiment is to add D_2O to a sample containing alamethicin and 100mM of the paramagnetic lanthanide praesodymium. This experiment will investigate both conformational changes and changes in electronic factors of the praesodymium.

These experiments will be discussed separately:-

1. A sample of alamethicin of concentration 15mM and pH* 1 was titrated by addition of D_2O in 1% aliquots up to 8%, the pH* remaining constant. At this pH* most of the NH only exchange very slowly, however the GLY, PHO, and MEA O NH protons exchange very rapidly and these were not visible in this experiment. The effects on the spectrum were very small, the main effects being in the NH region, where there were very small linear shifts, the largest of which was 0.06 PPM. These shifts are probably due to small hydrogen bonding changes. There were no changes in line-widths, and the remainder of the spectrum was virtually unaffected, and this is therefore an indication that any conformational changes accompanying the addition of D_2O are small, and there is no tendency for aggregation.

The shift of the CD_3OH solvent peak was found to vary very slightly and in linear fashion with D_2O content, and the shifts were the same as in another experiment which measured CD_3OH shifts with nothing else present and also showed that the shift was independent of pH over a wide range. The shift of this solvent peak can thus be used to monitor the approximate D_2O content of CD_3OD either by itself or containing alamethicin. Details are in Appendix 3.

2. A sample of alamethicin 15 mM and lanthanum, 150 mM at pH* = 1 was titrated in the same manner as 1. above up to a concentration of D_2O of 8%. (The addition of lanthanum, a diamagnetic lanthanide, has large effects on the spectrum which are discussed below, and which suggest a definite conformational change on the binding metal). The addition of D_2O to this La^{3+} containing sample produces the same type of effects as for alamethicin alone. However this time the NH peak shifts, which with a single exception are invariably upfield, are a little larger and in particular one doublet shows a shift of ca. 0.2 PPM by 8.5%. This doublet could be a GLU, GLN or ALA NH, and this large change must represent a substantial change in hydrogen-bond strength. This peak and also other peaks in the spectrum can be used to monitor the D_2O content of sample.

The experiments 1. and 2. above show that there is little effect on the shifts and hence on the conformation on addition of D_2O to the sample.

3. In this experiment the starting sample contained 100 mM Pr^{3+}, 15 mM alamethicin and was at pH* 0.8. Aliquots of D_2O were added up to a final concentration of 6%. There were only very small changes of the line-widths but there were substantial changes in the shifts. The most shifted MEA methyl peak had shifted by 0.4 PPM (ca 40%) in the 6% D_2O sample. The values of the chemical shift, and hence of the induced paramagnetic

shifts, varied linearly with the concentration of D_2O in the sample. Also the changes in shifts were such as to take the peaks back to their diamagnetic positions as more D_2O was added.

However it was found that the shift ratios were fairly constant for all the peaks except the aromatic protons of the PHO. The shift ratios of some methyl groups are given in table 5.1, where the shifts are all ratioed to the shift of the most shifted VAL 14 methyl group, the standard which is used for all the shift data.

Table 5.1

	MEA	MEA	VAL8	VAL14	VAL8	VAL14
0.8%D_2O	2.28	0.99	(0.16)	0.42	(0.15)	1,000
6% D_2O	2.20	0.89	(0.08)	0.42	(0.13)	1,000
	LEU	LEU	o-	m-	p- of PHO aromatic	
0.8%D_2O	0.27	0.27	-0.15	-0.08	0.06	
6% D_2O	0.21	0.21	-0.42	-0.28	-0.07	

Innaccurate values are given in brackets ().

In order to consider these results in a little more detail it is necessary to consider the shift equation for the most general case, and to remember that in this experiment changes in the shift may be caused by two factors, namely a change in conformation and/or a change in the electronic factors for the lanthanide. This is obvious from the equation which is:-

$$\delta_{obs} = f(Ax+By) \qquad 5.1$$

Where δ obs is the observed induced paramagnetic shift.

f is the fraction of ligand in the bound form

A and B are electronic factors for the particular lanthanide

x and y are geometric factors for the particular proton and in fact:-

$$x = \frac{3\cos\theta^2 - 1}{r^3} \qquad y = \frac{\sin^2\theta\cos 2\phi}{r^3}$$

In fact experimental evidence, presented later, shows that in the case of praesodymium the 'B' factor is not very large (i.e. symmetry is not far from axial) hence the contribution from this term is usually small. This is important as otherwise it would be difficult to explain the fact that the ratios were constant whilst the absolute magnitude of the shifts was changing. However, since 'B' is small, the experimental observations can be explained by a change in 'A', caused by the different solvent conditions around the metal ion. This will not cause a change of shift ratio's provided that the geometric factors x, and hence the conformation, are not changed by addition of D_2O. Obviously such a change in 'A' would however change the absolute magnitude of the shifts.

Referring back to equation 5.1 the only other variable is the fraction bound, but the change in binding constant required to produce the observed reduction in absolute value of shifts would be over two orders of magnitude (assuming starting value of K $\sim$ 500 litre mol^{-1} found from experiments described in the next section), and this is clearly impossible.

Thus these results show that the conformational changes on addition of D_2O are generally very small, and although only a few residues are given in table 5.1 they are spread throughout the molecule and so 'monitor' the conformation of the whole molecule.

The ratios for the aromatic protons of the PHO do however show a more substantial change. There are two possible explanations for this,

1. It is known from the relaxation measurements that the aromatic is extremely close to the metal and thus the very small shifts observed in 0.8% D_2O show that the angular part of the geometric factor x must be close to zero and so it is possible that the shift for the aromatic protons is substantially determined by the By term as well as the Ax term in equation 5.1. In this case there is no reason why the ratios should remain constant if A and B change on addition of D_2O even if x (and y) are unchanged:
2. The second explanation is that there is a movement of the aromatic group of the PHO which changes the angular term and thus the shifts. (It is known from other experiments that the distance of the aromatic group does not depend on D_2O content). Since the aromatic group is near the metal a small movement could cause a large change in angle and so in the shift.

These two explanations are not mutually exclusive. The important point is that neither involve a large conformational change.

The above three experiments are strong evidence that the conformations of alamethicin itself and of the metal-alamethicin complex are largely unchanged when D_2O is added even up to 8% or so, and in chapter 6 further experimental evidence is given which shows that the conformation does not change with D_2O content. Nevertheless the D_2O content of all the starting samples in the constant lanthanide titrations was monitored by making use of the known variation of NH shift in lanthanum samples with D_2O content. This showed that the variation in D_2O constant was less than 1%.

The experiments will now be described which show that the various conditions for application of the lanthanide (shift) method are met. These conditions are discussed in chapter 4 and are:-

1. Fast exchange:-

In all the lanthanide titrations only one peak is seen whose position changes according to the fraction of ligand in the bound state. (This is discussed under heading 2.) Also the shifts were shown to be frequency independent by taking a 100mM Pr^{3+} sample and running it under identical conditions at both 270 MHz and 90 MHz when the shifts were found to be identical to within less than 0.01 PPM. This was true at both pH* 1 and 5, i.e. the exchange is fast for shift for both metal complexes.

2. A single 1:1 complex:-

Two types of experiment were performed to show this (a) a binding titration; (b) a constant lanthanide titration;

(a) A binding titration type of experiment (see chapter 4) was performed in which praesodymium chloride was added in increasing concentration to alamethicin (at 5mM), the alamethicin concentration being kept constant (pH* = 1). The equation for the variation of the shift for the case of a one site 1:1 complex can then be written down and the experimental data can be tested against the theoretical binding curve. The equation is the usual one:-

$$\delta_{obs} = f \cdot \delta_M \qquad 5.2$$

where δ_M is the fully bound shift (= Ax + By see equation 5.1)

and f is simply, $\frac{[ML]}{[L_t]}$ $[L_t]$ = total ligand concentration in all forms.

Hence there are two basic unknowns in this equation, the fully bound shift and the binding constant which determines f, (total ligand and metal concentrations being known). It is therefore necessary to try and fit the data to give the best fit whilst varying the fully bound shift and the binding constant independently. In order to do this a program was written for a Wang 600 programmable calculator which performed a least squares fit to the curve determined by the above equation, with both the fully bound shift and binding constant as variables (see appendix 3). This program was then applied to a total of eight peaks from various regions of the spectrum, and the best value of the binding constant for each peak (and the fully bound shift) determined. A mean value of K was then evaluated and then the program was modified to find the best least squares fit with the assigned mean value of K and changing only the fully bound shift. The experimental shifts were then 'normalised' to the same fully bound shift of 1 unit and plotted with the theoretical curve for the mean value of K and the fully bound shift. This is shown for some of the eight peaks in Fig. 5.4 and it can be seen that very good agreement is obtained between the experimental points and the theoretical curve for a single 1:1 complex with K = 475 litre mol.$^{-1}$.

The eight peaks for this analysis vary in shift by a factor of eight, and this is important because it means that some of these peaks must be from groups a moderate distance from the main binding site. These protons would be expected to show anomalous effects if a second metal ion started to bind at some different site with a different fully bound shift (possibly even of opposite sign). The good agreement obtained for all the protons with the one site 1:1 theoretical curve is hence good evidence that there is indeed only one major site and that the observed shifts of these groups is caused almost entirely (say 95%) by the metal bound at the major site.

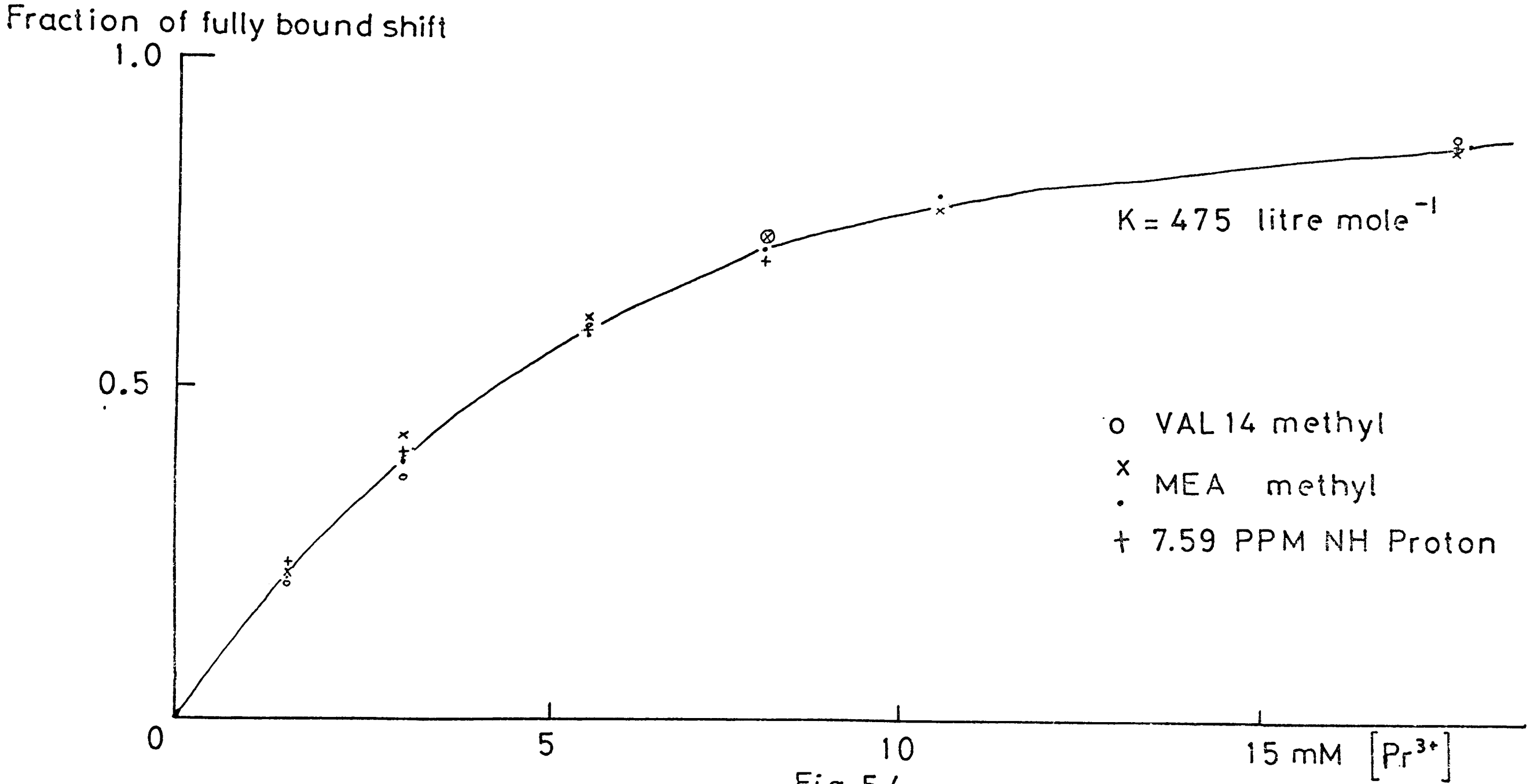


Fig. 5.4

Fit of experimental points, o x . + , with the theoretical curve for 1:1 binding K=475 litre mol^{-1}

It should also be pointed out that all the peaks in the NH region (except GLY, PHO, and MEA O) were observed and there were no anomalous effects in the observed shifts of the peaks. Since the NH are obviously evenly spread throughout the molecule, this is again strong evidence in favour of the one site 1:1 complex.

(b) The second experiment to test the stoichometry of the complex consisted of a constant lanthanide titration at a concentration of alamethicin much lower than usually used namely 3mM instead of the usual 15mM. Both titrations were performed with the total lanthanide $La^{3+} + Pr^{3+}$ = 100 mM. If there is only a 1:1 complex equation 5.2 applies and hence the shift ratios should be the same for the two titrations, and this was found to be so, within experimental error. If there is more than one complex, say complexes 1 and 2 then the equation becomes:-

$$\delta_{obs} = f_1 \delta_{1M} + f_2 \delta_{2M} \qquad 5.3$$

obviously in this situation the shift ratios will not be the same if the relative values of f_1 and f_2 are different as they will be for constant lanthanide titrations at different ligand concentration.

Both titrations were performed at pH* ca 1.5 at which low and high pH complexes will be present, and so this experiment will test whether either the low or high pH complex departs from 1:1 binding. In fact it was found that the shift ratios were fairly constant between the two titrations. The data for some peaks is given in table 5.2 :-

Table 5.2

Chemical shift	Assignment	Ratio 15mM	Ratio 3mM	Chemical shift	Assignment	Ratio 15mM	Ratio 3mM
8.617	MEA O	(.03)	(.031)	1.53	MEA	2.00	2.14
8.378		0.33	0.29	1.56	MEA	0.91	1.03
8.11	LEU	(0.65)?	0.50	1.13	VAL 8	0.33	0.31
8.086	-	0.32	0.29	1.07	VAL 14	0.30	0.23
8.043	-	(0.07)	(0.06)	1.01	VAL 8	0.20	0.18
8.00	-	(0.31)	0.24	0.97	VAL 14	1.000	
7.792	-	1.02-1.21	1.08	0.94	LEU	(0.37)	(0.26)
7.55	-	1.3	1.46	0.91	LEU	(0.20)	(0.17)
7.28	PHO o-	(0.0)	(0.0)	2.05	acetyl	(0.05)	(0.05)
7.226	PHO m-	(-.05)	(0.0)	1.48	MEA O	(0.0)	(0.0)
7.15	PHO p-	(0.07)	(0.1)				

Many more peaks were observed and although these could not all be followed for the whole titration there were no discernable differences in the shift patterns for the two titrations.

This titration would be particularly useful for detecting complexes of the type M_nL, because the proportion of these would be much higher in the titration with alamethicin at 3mM than the titration at 15mM, the metal concentration being very high relatively at 100mM.

Looking back at equation 5.3 it can be seen that shift ratios will not be the same, if there is more than one complex, at different metal concentrations (as f_1 and f_2 will change by different amounts). Hence in a binding titration type experiment as in (a) above ratios which do not vary with metal concentration in the titration are a further proof that there is only one complex. It was indeed found that ratios were not dependent on the concentration of metal.

In summary there is very strong evidence that there is only one major binding site and that the complex has 1:1 stoichometry. Although all the experiments described above were for Pr^{3+}, the other lanthanides would be expected to bind in identical manner and this was found to be the case from the experiments with the other lanthanides.

3. The absence of contact shifts

The best way to demonstrate the absence of contact shift is to be able to show that all the shift ratios for the protons are independent of lanthanide, but unfortunately this also requires that there is axial symmetry which there is not in the case of alamthicin. In the case of alamethicin the presence of contact effects cannot be ruled out for those atoms in the immediate coordination sphere of the lanthanide. However, for reasons discussed later, the shift data were not used quantitatively and so a small contribution from contact for the nearest atoms would not matter anyway. There is an abundance of experimental evidence from other systems to show that, as expected from the very poor overlap of the 4f orbitals of the lanthanides the contact effects are negligible for all but the very nearest atoms. (See discussion and references given in chapter 4).

3. DETERMINATION OF THE CONFORMATION BY SHIFT EXPERIMENTS.

Now that it has been demonstrated that the various conditions for application of the lanthanide shift method have been met the detailed lanthanide experiments will be discussed, first the experiments with the diamagnetic lanthanum, and then the experiments with the paramagnetic lanthanides.

Lanthanum results

Lanthanum was the diamagnetic blank used in all the studies. Several lanthanum titrations were performed at various pH*, the results were always similar and the details are given in appendix 3. The effect of lanthanum on the spectrum was marked, particularly in the NH, aromatic and α CH regions, and the results will be considered for each region separately:-

(a) NH and Aromatic region:

The addition of lanthanum shifts many of the NH peaks usually by small amounts, well under 0.1 PPM, and almost invariably down-field. One of the NH peaks is shifted by about 0.1PPM downfield. The PHO aromatic peaks are shifted and split up to some extent by the lanthanum, again shifts are less than 0.1 PPM. The PHO NH peak also shows a very large effect.

(b) α CH and β CH_2 region:-

The result on this section of the spectrum is shown in Fig. 5.5 where it can be seen that the protons of the PHO residue are particularly strongly affected, in fact the PHO α CH and β' CH_2 peaks are shifted downfield by some 0.2 PPM. The GLN 18 α CH peak also shows a big shift. Also of interest is the clear increase in line-width for some of the peaks e.g. PHO β CH_2.

(c) Methyl region:-

Here the shifts were generally much smaller, of the order of 0.01 PPM or less.

These above effects show that the lanthanum is binding very close to the PHO and also the GLN 18 (see fig. 5.6) and that the binding produces some restrictions of motion, particularly of the PHO, but also for the rest of the molecule, hence giving rise to the increases in line-widths which were observed over much of the spectrum. Most of the shifts of the protons other than the NH will simply be caused by the charge on the lanthanum ion and this will of course tend to move the peaks downfield as was observed. For the

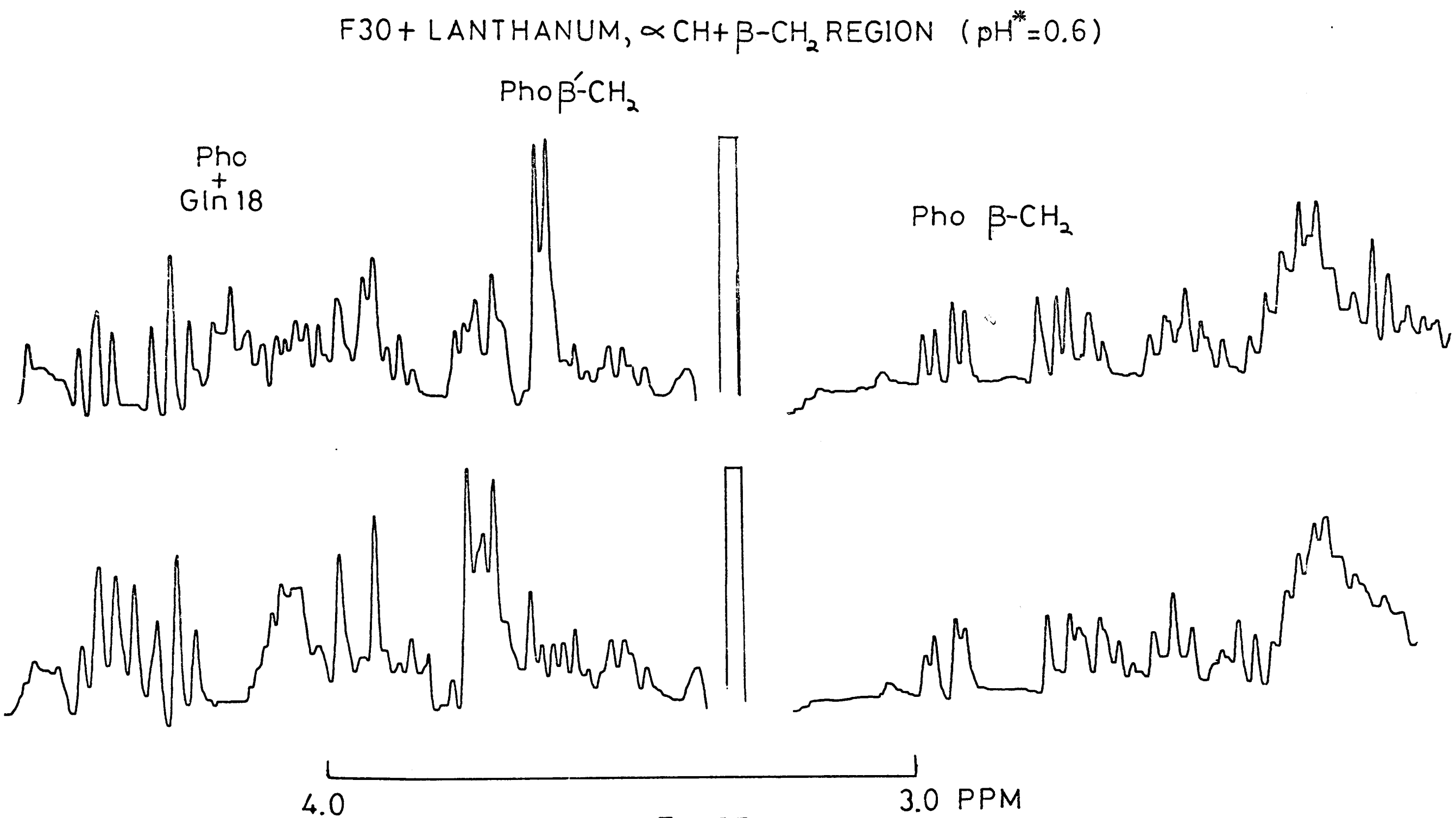


Fig. 5.5

R.E. spectra in methanol showing the effect of 100 mM La^{3+} .Note the large effect on the PHO.

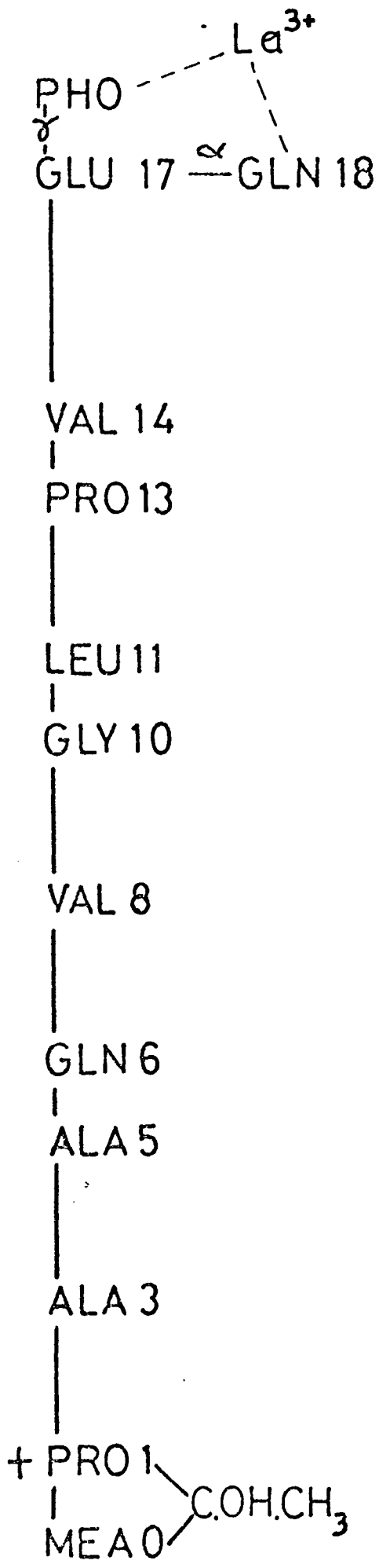


Fig. 5.6

Schematic diagram showing the non-MEA residues of F30 and the residues whose peaks are shifted substantially by La^{3+}.

NH peaks the shifts may also arise as a result of changes in the hydrogen-bonding on binding the lanthanide, though in fact virtually all the shifts are again downfield. The shifts follow the characteristic binding curve pattern with increasing metal concentration (see fig. 5.4), and though it is not possible to obtain an accurate value of the binding constant because of the small shifts the calculation for the most shifted peak gave a value of $\sim$ 500 litre mol.$^{-1}$ in close agreement to the values with the other lanthanides as would be expected.

The titrations reported above were used for determining the diamagnetic blank corrections required for calculation of paramagnetic effects in the binding titrations of the paramagnetic lanthanides reported below, and also those titrations already considered. In constant lanthanide titrations lanthanum is present throughout the experiment and the titration therefore has an 'internal' diamagnetic blank.

Paramagnetic lanthanide experiments

The first experiments with paramagnetic lanthanides, involving praesodymium, europium, and erbium were performed at a pH* where there were both the high and low pH complexes present. These experiments showed that the shift ratios were different for the different lanthanides and it was therefore not possible to say that there was (the much simpler case of) axial symmetry. These were followed by experiments at low pH where there was just the complex of metal and protonated alamethicin, and these experiments again gave different ratios for the different lanthanides. The differences were shown to be real and in some cases substantial.

No attempt was made to perform experiments at high pH, because of the extensive broadening, especially of the more

shifted peaks (and the exchange of the NH protons), further the results at low pH and intermediate pH are sufficient to show that the symmetry is non-axial for both of the individual complexes.

The experiments provided data which is suitable for qualitative interpretation in terms of the conformation both the low pH complex and the high pH complex.

The experiments at intermediate pH will be discussed first, giving the experiments performed with each lanthanide and then bringing the results together for interpretation. Then the low pH experiments will be discussed, and there will be a detailed discussion of the problem of interpreting the results, in particular showing that the difference in ratios for different lanthanides is due to non-axial symmetry and not some other cause. Finally, there will then be a discussion of what can be deduced about the conformations of the two complexes from these results.

Intermediate pH experiments

These experiments were all constant lanthanide titrations and the pH and D_2O content were held as constant as possible by preparing the samples in identical manner for each of the titrations. Further the large and constant lanthanide concentration in these experiments (100 mM) compared with the relatively small and constant alamethicin concentration (15 mM) should help to ensure a fairly constant pH, and does keep other factors such as ionic strength constant. The pH* was not measured directly in these experiments, though it was found for some of the titrations after the detailed pH titrations reported earlier had been performed. Titrations were performed for praesodymium, europium, erbium and dysprosium, and these will now be discussed.

The results for the praesodymium titration performed with the concentrations given above (precise details are in appendix 3) and at a pH* of 1.5, are given in Fig. 5.7a, 5.7b. These show that the shifts vary in a linear manner with the fraction of praesodymium present in the solution. This linearity demonstrates that the binding constant of lanthanum and praesodymium are similar, but more important shows that factors such as pH and D_2O content are being held constant. This is because the linear variation of the shifts means that the shift ratios are constant, and the detailed pH and D_2O experiments discussed earlier showed that shift ratios (of at least some peaks) were pH and D_2O dependent.

It will be seen that results are only given for peaks in the NH + aromatic and methyl regions. This is because following the shift of the peaks in the α CH and β CH_2 regions is made very difficult by the large coupling and overlap of many of these peaks, and further the coupling, and hence mutliplet structure, changes as more paramagnetic ion is added causing relaxation as well as shift of the nuclei. However data was obtained for some protons in these regions. Data for the VAL β CH_2 protons was obtained by finding their position by double irradiation by observing the decoupling of the methyl protons. Qualitative effects were also observed on some peaks in the α CH region.

Similar experiments were performed with europium and again showed that shifts were linear with added fraction of paramagnetic ion. The europium titration was important from another point of view, in that it shifted the aromatic peaks into three clear groups with areas 2:2:1 and with the coupling still present. Decoupling experiments were performed on these peaks hence enabling assignment of the peaks to the ortho-, meta-, and para-proton (s). It is worth pointing out that in <u>all</u> titrations which were ever performed

CONSTANT LANTHANIDE Pr^{3+} TITRATION

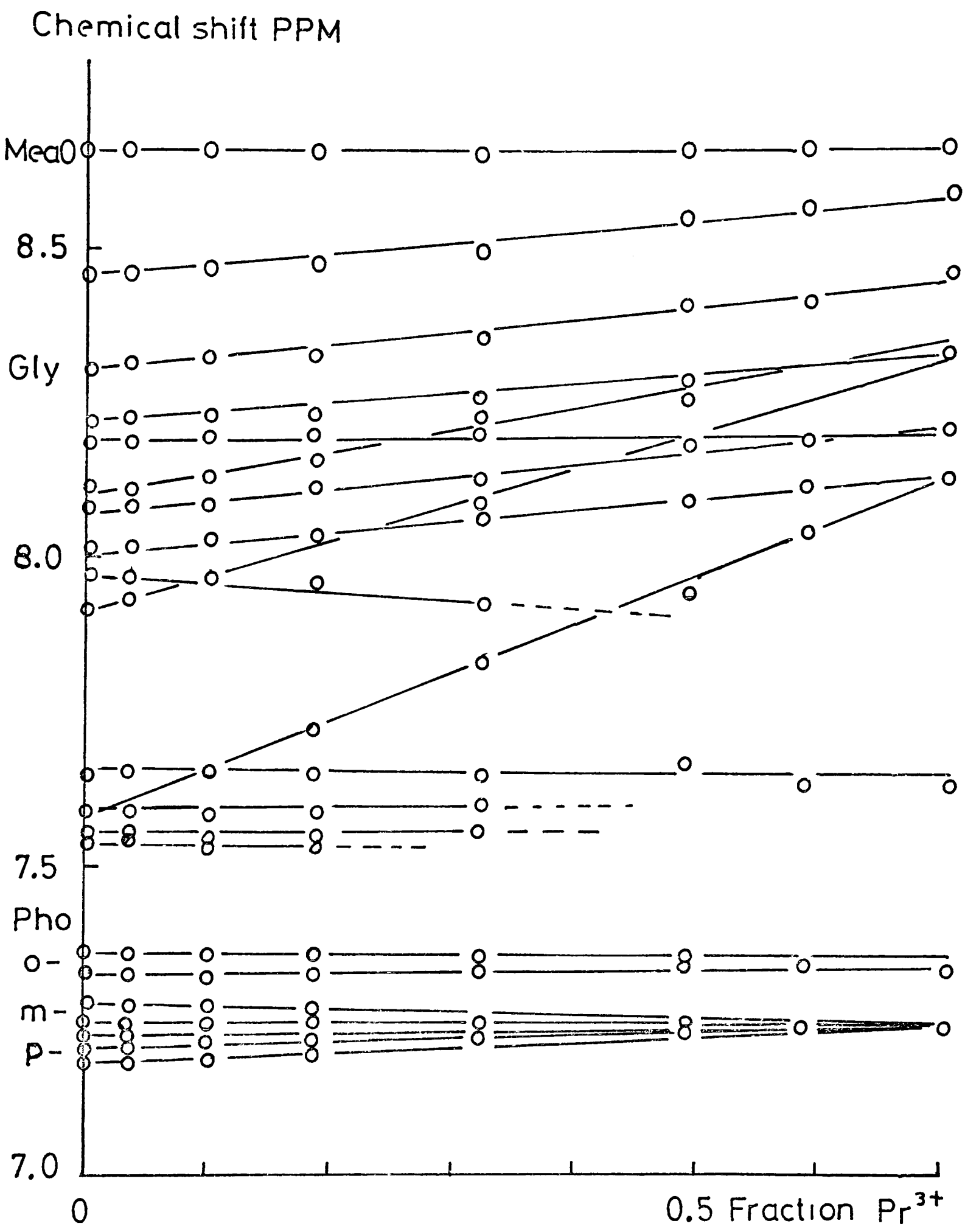


Fig. 5.7a

Intermediate pH* Pr^{3+} titration. A few peaks were ommitted from this diagram to preserve clarity. This diagram shows peaks in the aromatic + NH region of the spectrum .

CONSTANT LANTHANIDE Pr^{3+} TITRATION

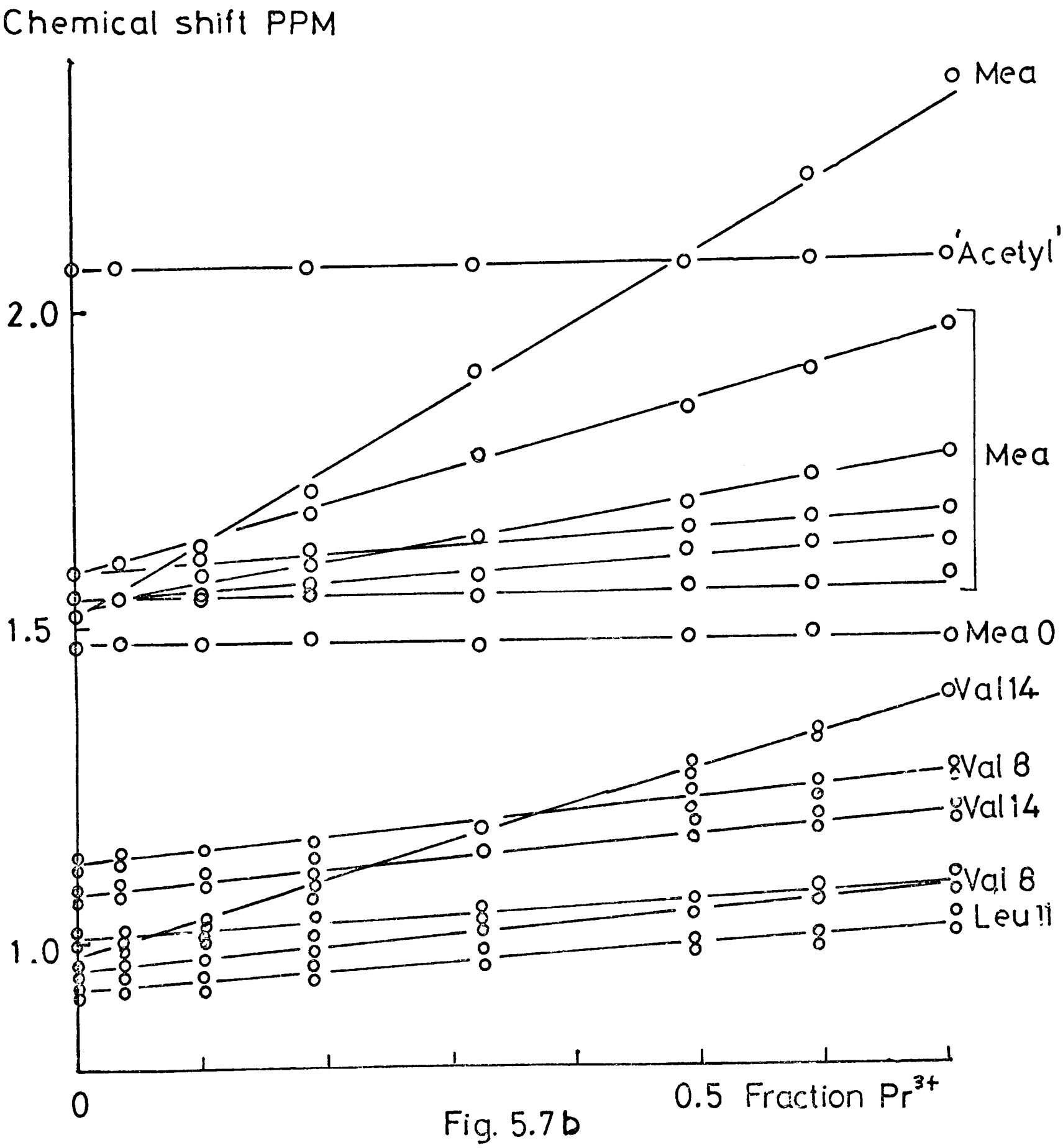


Fig. 5.7b

Intermediate pH* Pr^{3+} titration, showing the peaks in the methyl region. A few MEA peaks were omited to preserve clarity.

on the alamethicin the aromatic split up into only three groups of peaks of areas 2:2:1, corresponding to the ortho, meta and para protons. The only possible reason for the fact that the two ortho (and two meta protons) were not giving different shifts with any of the lanthanides (and shifts of the aromatic protons with some lanthanides were very substantial) is that the aromatic is 'spinning' about its axis at a rate greater than the 'Nmr time scale', so that the average environment seen by the two ortho protons is the same. The Nmr 'time scale' in this case would only be some 500 Hz which is extremely slow (corresponding to an energy barrier of some 20 Kcal at 25^{o}C), so this is by no means a surprising result.

Titrations were also performed with erbium and dysprosium, both gave linear shifts as expected. In the case of dysprosium the broadening was so large that interpretation was difficult and these results will not be considered further.

The shift ratios obtained in the europium, praesodymium and erbium titrations are given in full in table 5.3 along with those of some other titrations discussed below. The most important point is that the ratios are clearly different for the different lanthanides. However bearing in mind that the pH* of these experiments was not measured at the time, and also the variability of the aromatic peak shifts with D_2O content, it is necessary to show that the observed differences are real and not due to variation in one of these factors. The differences were shown to be real by the following:-

1. The ratios for erbium are so very different from those of europium and praesodymium that the effect cannot possibly be due to pH changes of D_2O content, as the small effect these have is known.
2. To test the variability of samples prepared by the usual procedure, two other samples containing 100 mM praesodymium were made up, and comparison was then made between the pH* of these various

Table 5.3

'Shift'.	Assignment.	Ratio					
		1.	2.	3.	4.		
8.617	MEA O	-	(0.03)	-0.29	-		
8.378		0.56	0.33	0.20	0.08		
8.342T	GLY 10	-	0.35	0.59	-		
8.222		0.40	0.26	0.50	0.44		
8.177		(0.4)	0.04	0.53	-		
8.11 D	LEU 11	0.22	0.65?	-	-		
8.086		0.45	0.32	1.33	-		
8.043		0.21	(0.07)	-0.21	-		
8.00 D		0.10	(0.31)	-0.05	-		
7.921		0.07	-0.22	-	-		
7.792		1.29	1.10±.1	-0.40	0.95		
7.618		0.21	-	-0.55	0.32		
7.596		0.12	-	-0.38	-		
7.572		0.21	-	-	-		
7.547		L	1.3	0.60	-		
7.506-7.465		S	0	-	-	5.	6.
7.29*	PHO ortho	-0.05	0	-1.4	-0.41	-0.10	-0.42
7.23*	PHO meta	0.10	-0.05	0.15	0.14	-0.05	-0.28
7.17*	PHO para	0.23	(0.07)	1.14	0.59	0.08	-0.07
4.377T	PRO 13	L	L	L	0.35	-	-
4.249T	PRO 1	vS	vS	vS	0	-	-
4.177T	GLN 18 +PHO	vL	vL	vL	vL	-	-
2.93 Q	PHO βCH_2	vL	vL	-	-0.43	-	-
2.73 Q		vL	vL	-	1.5		

Continued overleaf

Table 5.3 continued.

'Shift.'	Assignment.	Ratio					
		1.	2.	3.	4.	5.	6.
2.34*	VAL 14 β CH	(0.7)	(-0.1)				
2.24*	VAL 8 β CH	(0.2)	(0.1)				
2.05	'acetyl'	-	0.05	-0.26	0.16	0.02	-0.01
1.53	MEA	2.69	2.00	2.72	3.48	2.29	2.20
1.56	MEA	1.22	0.91	-1.95	-	1.02	0.89
1.48	MEA O	0	0	0.21	-	0	-0.07
1.13 D	VAL 8	0.16	0.33	0.25	0.23	0.17	(0.08)
1.07D	VAL 14	0.07	0.30	-0.43	0.18	0.42	0.42
1.01 D	VAL 8	0.18	0.20	0.33	0.24	0.15	0.13
0.98 D	VAL 14	Standard peak 1.000 in all titrations.					
0.93 D	LEU 11	0.27	(0.37)	0.43	-	0.28	0.21
0.91 D	LEU 11	0.15	(0.23)	0.36	0.28	0.28	0.21
Actual shifts of 0.98D VAL 14 in PPM.		0.286	-0.525	0.540	0.129	-0.435	-0.280

'shift' is the chemical shift at pH*= 1 and with no lanthanides.

D,T,Q,* denote doublet, triplet, quartet and complex fine structure

() denote imprecise values, ? denotes doubtful assignment .

L , S, denote 'large' and 'small' effects, v denotes 'very' .

1. Ratios for the constant lanthanide titration, Eu^{3+} pH* ca 1.5
2. Ratios for the constant lanthanide titration, Pr^{3+} pH* 1.5
3. Ratios for the constant lanthanide titration, Er^{3+} pH* ca 1.5
4. Ratios for the europium binding titration at 100mM Eu^{3+} pH* 0.6
5. Ratios for 100 mM Pr^{3+} sample pH* 0.8 no D_2O (extrapolated)
6. Ratios for 100 mM Pr^{3+} sample pH* 0.8 6% added D_2O .

In all above the alamethicin concentration was 15 mM.

samples and the pH* of the constant lanthanide praesodymium titrations, both the 15 mM alamethicin titration discussed above and the 3 mM alamethicin titration discussed previously. It was found that the pH* of these samples, as determined by comparison with the detailed praesodymium pH titration, were all very similar, (though the 3 mM alamethicin samples had slightly lower pH*).

3. The D_2O content of the three titrations (praesodymium, europium and erbium) was found by mea suring the shift of one of the NH which is particularly sensitive to the D_2O content in the 100 mM La^{3+} starting sample. The shifts showed that the D_2O content was the same to less than 1%.
4. Detailed examination of table 5.3, which gives data collected at other pH and also shows the effect of added D_2O on the ratios, does in fact show that even the comparatively small europium-praesodymium differences cannot be explained by variation of pH/D_2O, this is particularly clear for peaks such as the 1.53 PPM or 1.56 PPM MEA and the 1.07 PPM VAL 14.

Hence it can be seen that the small pH and D_2O content variation which did occur between the various titrations cannot account for the observed differences in shift ratios, so that there is a real and significant difference in the shift ratios for the different lanthanides at intermediate pH values. However it is still true that in general the same residues are the onesshowing large shifts, and this can be seen in Fig. 5.8 which shows the assigned residues with large shifts (i.e. shift ratio greater than ca 0.8).

At the intermediate pH at which these experiments were performed both complexes are present in significant concentrations. It was therefore decided to investigate the situation at low pH when only the protonated complex is present. The low and

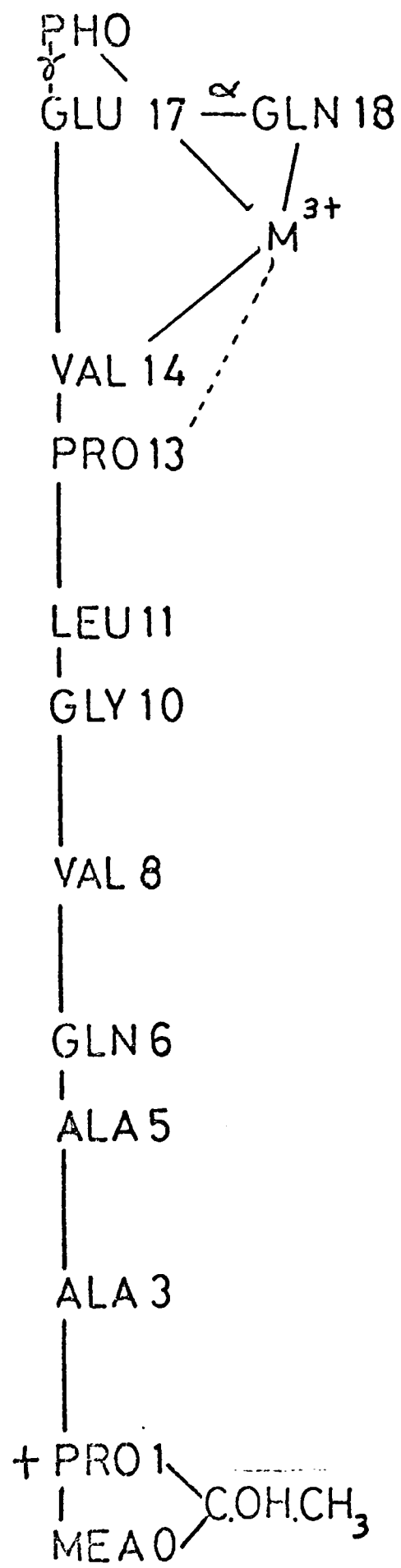


Fig. 5.8

Schematic diagram showing the non-MEA residues of F30 and the residues whose peaks have large shift ratios greater than ca. 0·8 .

high pH experiments combined do in fact provide sufficient information to show whether the ratios for the high pH complex could be the same. (Direct experiments at high pH were not undertaken because of the broadening of the peaks at high pH and also the fact that the NH protons exchange much more rapidly at this pH and so cannot provide any data).

Low pH experiments

A Europium titration was performed at pH* 0.6 and the ratios at 100 mM Eu^{3+} evaluated (see table 5.3). The ratios were also evaluated for the praesodymium pH experiment, at the pH* of 0.8 (see table 5.3) and these again showed that there were differences between europium and praesodymium. However the slightly different pH* of these experiments and also the small amount of added D_2O (0.6%) in the praesodymium experiment made it desirable to confirm beyond all possible doubt that the ratios were different, by performing a direct experiment. This consisted of using the two paramagnetic lanthanides europium and praesodymium as the two lanthanides in a constant lanthanide titration. Thus a 100 mM solution of europium with alamethicin 15mM, and pH* 0.6 was used as the starting sample, and then a solution of 100 mM praesodymium, 15 mM alamethicin, pH* 0.6, was titrated into this in increasing amounts. In this way the total lanthanide concentration (and the alamethicin concentration) are kept constant and most important, all extraneous variables are 'eliminated' as both lanthanides as present in the same sample. If the shifts for such a titration are plotted as a function of say fraction of praesodymium in the sample, then they should vary linearly, and will start at the 100 mM europium value and proceed towards the 100 mM praesodymium value. However the important point is that if the relative shifts, i.e. the shift ratios, are the same for the two lanthanides, then at some point in the titration the peaks should all (simultaneously) be in their diamagnetic positions (i.e. the positions in 100 mM La^{3+} at pH* 0.6), if the shift

ratios are different the peaks will reach their diamagnetic positions with different fractions of praesodymium in the sample.

The results of this experiment were that good linear variation of shifts with fraction of praesodymium present were indeed obtained, but the peaks passed through their diamagnetic positions at fractions of praesodymium in the sample varying from 0.14 to 0.54, again clearly demonstrating that the shift ratios are not the same and are lanthanide-dependent.

From the results for europium and praesodymium at both low pH and intermediate pH it can be shown that the shift ratios at high pH must also be different. This was done by a graphical method, and does not require any knowledge of the proportions of complexes present in the intermediate pH experiments (though of course it must be the same for the two lanthanides). This is described in detail in appendix 3.

Interpretation of results

The above experiments have conclusively shown that the shift ratios are different for different lanthanides, and that this is true at both low pH, for the complex M(HA) and at high pH for the complex $M(A^-)$, and this means that it cannot be said that there is axial symmetry for these complexes. There is also strong evidence that the conformation is different at the two pH though the major binding site is not greatly changed, this point will be returned to later, first the possible reasons for variation of shift ratio with lanthanide must be considered so that the data can be correctly interpreted. These reasons are as follows:-

1. Change in the number and position of solvent molecules surrounding the metal, leading to change in the direction of the symmetry axis in an axially symmetric complex. The conformation of the

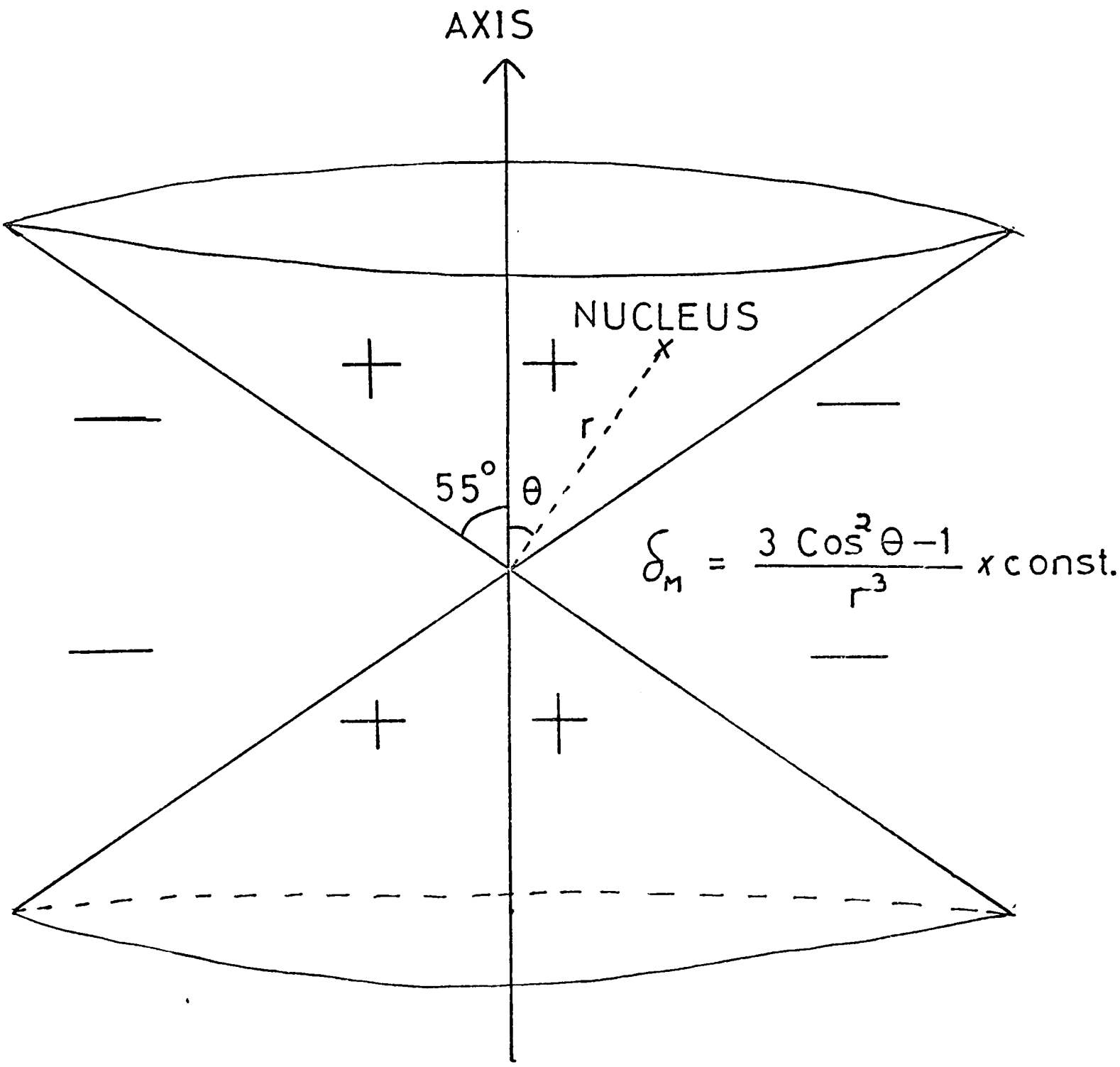
different alamethicin-lanthanide complexes being identical.

2. Change in the conformation of the complex (which will also lead to change in direction of the symmetry axis in an axially symmetric complex).
3. Non-axial symmetry, with or without changes in conformation/ symmetry axes etc.

These above three causes can obviously be combined in various ways, but the important point is that if causes 1 and/or 2 above are operative the symmetry could still be axial and if this were the case the conformation could be determined (for each lanthanide if necessary) using the usual equation and the methods already developed for axial symmetry (see chapter 4). If the symmetry is non-axial then the system is fundamentally insoluble even if the conformation and direction of the symmetry axes are identical for the different lanthanides (this is also explained in chapter 4). It is therefore important to know which of these causes is operative.

It is convenient to start by first considering cause (2) above, namely a change of conformation from one lanthanide complex to the next. It is very likely a priori, that the conformation of the alamethicin-lanthanide complex with the various different lanthanides bound is going to be very similar because the lanthanides are so similar in their chemistry, and in particular they have the same charge, and they have very similar ionic sizes (particularly europium and praesodymium) and in confirmation of this it is evident from the results with the diamagnetic lanthanum, and the titrations with all the lanthanides that the same groups are near the metal and this is also shown directly by the gadolinium results (chapter 6). A detailed look at the ratios in table 5.3 also shows that different conformations of the complexes cannot explain the praesodymium/ erbium differences for example.

Before proceeding to discuss the various explanations involving changes in direction of the symmetry axis for an axially symmetric complex it will be useful to introduce a simple concept concerning the surface of zero shift in an axially symmetric complex. This is simply given by the condition that the angular term in the expression for the shift is zero and this condition gives the surface of a double infinite cone which is sketched below, the sign of the shift of course reversed on the 'inside' as compared with the 'outside' of the cone as indicated. Also the symmetry axis of the complex is the axis of the cone.



It is convenient to first consider the differences between praesodymium and erbium and then the praesodymium/ europium differences.

Praesodymium v. Erbium

It is quite easy to show that a reorientation of the symmetry axis in an axially symmetric complex cannot explain the different ratios for these lanthanides.

The argument uses the cone idea introduced above. A cone of known angle is defined completely in space by the apex of the cone (i.e. the metal position) and three points on the surface of the cone, i.e. three residues with zero shift. It has already been argued that the metal position is very similar for both praesodymium and erbium (and all the other lanthanides as well) so this gives the apex of the cone. The three points through which the surface of zero shift passes in both erbium and praesodymium are the aromatic moeity, the VAL 14 and the PRO 1 blocking group, as it can be seen from table 5.3 that there is a reversal of sign of the shift or almost zero shift for different parts of the moieties. Since the apex is the same and the surface of the cone passes through the same three residues the cone must be similarly placed in both erbium and praesodymium. This would mean that erbium and praesodymium ought to give very similar shift ratios (because the axis must also be in almost the same direction). This is evidently not so and in particular the disagreement is a large one as there are many peaks giving shift ratios of opposite sign. Since the 'conclusion' that erbium and praesodymium should give very similar shift ratios is evidently false our starting 'premise' must be wrong, this premise is that the complex has axial symmetry. If the symmetry is non-axial the cone argument cannot be applied at all as the surface of zero shift in non-axial symmetry is more complex.

The above argument is only valid because the praesodymium/erbium differences are large. It does show that there must be at least a contribution from non-axial symmetry to explain the different ratios for praesodymium and erbium.

Praesodymium v. europium

This case is much more diffcult because the differences are comparatively small. There is one important point about the europium and praesodymium results, namely that the signs of the shift ratios for europium and praesodymium are almost without exception the same. This means that there cannot be a large difference in the degree of non-axial symmetry (if there is non-axial symmetry), because the exact shape of,the surface of zero shift for non-axial symmetry depends on the degree of non-axiality and if this were to change substantially between the two lanthanides then the surface would move and some peaks would change the sign of their shifts. Since also it is unlikely that both europium and praesodymium would show similar large degrees of non-axial symmetry, the most likely explanation of the results is that in fact for these two lanthanides the symmetry is close to axial. The possibility that both are in fact of rigorous axial symmetry and that the differences between them are caused by small conformation/axis changes cannot be ruled out, though equally there is no evidence to show that this is so.

Conclusions concerning the conformation

The above evidence shows for erbium that there is a substantial degree of non-axial symmetry and that there may well be non-axial symmetry for europium and praesodymium. As discussed in detail in chapter 4 the case of non-axial symmetry is fundamentally insoluble, and so the data cannot be analysed using the non-axial symmetry shift equations. There is no justification for assuming that

either europium or praesodymium show rigorous axial symmetry and any attempt to obtain a detailed conformation with the assumption of axial symmetry using this data would be greatly over-interpreting the data. It is however possible to draw somequalitative conclusions from the data which confirm the more detailed conformational information which was obtained from an analysis of the rel axation data presented in chapter 6.

Three criteria will be used to analyse the data:-

1. If there is a large difference in the shifts for two different groups of the same residue with a particular lanthanide, this is a certain indication that the residue is near the lanthanide, as only if the residue is near the metal can different parts of the same residue subtend substantially different angles and/or be at different relative distances from the metal, and hence give rise to substantially different shifts.
2. If a group shows large shifts with all the different lanthanides it is very likely to be close to the metal.
3. If a group from a residue through which the surface of zero-shift passes shows even only moderate shifts, then that residue must be near the metal as the angular part of the geometric factor is known to be very small for that residuc.

Let us first consider the data for the intermediate pH. Using the above criteria then the aromatic moiety, the VAL 14 GLN 18 . and at least one methyl alanine are very close to the metal, also at least one NH (7.792) which is unassigned is very close. In contrast VAL 8 and LEU 11, for which the shifts of the different methyl groups in each residue are always similar for all the lanthanides and under various conditions are probably a comparatively long way away from the metal. This places the metal, as would be expected, binding up next to the GLN 18 residue (with its carboxyl group) and the phenylalaninol blocking the C-terminus, and would indicate that the last four or five residues of the chain are

wrapping themselves around the metal. A space-filling model of alamethicin shows that it is possible to place the last few residues around the metal in such a way as to provide a good binding site. The remainder of the chain can then possibly form an α helix, certainly the data shows that the blocked N-terminus is a long way from the metal and does not fold back in any way.

At low pH the conformation is broadly similar, the main difference being that the metal gets much nearer the PHO residue, this is shown by the much larger shifts on the aromatic protons, which cannot be explained by a change in angular factor without a change in distance, because the surface of zero shift still passes through the aromatic. This point is emphasised by the large shifts of opposite sign for the PHO β CH_2 protons which again show that the metal must be very close to the PHO residue. This group with its free hydroxyl seems to be one of the main binding groups at low pH when the carboxyl becomes protonated.

The above conclusions are in complete agreement with the more detailed results obtained from the relaxation studies present in the next chapter and so a more detailed discussion of the conformation will be left until the end of the next chapter. Although the conclusions obtained from the shift results are not quantitative nevertheless the shift work is of importance because the very different geometric dependence of the shift affects as compared to the relaxation effects is an important check on the correctness of the conformation, and also the shift probe, because of its different geometric dependence is effective at greater distances than the relaxation probe. There is also the point that the shift work provided important proofs that various important conditions, such as a single site 1:1 complex, were being met.

SUMMARY

This chapter was broadly divided into three sections:-

1. Assignment

The assignment of the spectrum was mainly achieved using decoupling, but some results were obtained from gadolinium relaxation experiments. Most of the residues were assigned but some of the GLU, GLN and PRO residues proved difficult.

2. Lanthanide shift experiments

The effect of changing the pH on the shifts and line-widths with 100mM lanthanide present was investigated. It was found that the (induced paramagnetic) shifts were smaller at low pH, and that the line-widths were much larger at high pH for the most shifted peaks. For this reason (and also NH exchange) titrations were not performed at high pH.

The effect of D_2O on the conformation was investigated in experiments with alamethicin by itself, with lanthanum present and with praesodymium present. These showed that the conformation was almost unaffected by up to 6% D_2O in the sample.

It was then shown that the various conditions necessary for application of the method were all present at both low and high pH, these conditions are fast exchange, a single site 1:1 complex and no contact effects.

3. Determination of the conformation by shift experiments

The detailed experiments to determine the conformation were then described. First the diamagnetic blank experiments with lanthanum. Then the experiments with praesodymium

europium and erbium. These showed that the shift ratios were different for the various lanthanides at both low and high pH. It was shown that there was a contribution from non-axial symmetry for the case of erbium and possibly praesodymium and europium as well and hence it was not possible to perform a quantitative analysis of the results. The results were analysed in a qualitative fashion and showed that the metal binds in the region of the GLN 18, PHO, MEA 15, 16 and GLU 17 residues at high pH, but that at low pH there are less residues binding the metal which now binds nearer to the PHO end of the molecule. These results agree with the relaxation results discussed in the next chapter.

CHAPTER SIX

DETERMINATION OF CONFORMATION BY RELAXATION STUDIES

This chapter is divided into three, first there will be a discussion of the method then there will be a discussion of the determination of the conformation of the F30-lanthanide complex using gadolinium relaxation studies for both the high pH, $M(A^-)$ and low pH, M(HA), complexes. Finally there will be a discussion of the determination of the conformation of the minor component of alamethicin, F50 and a comparison of the conformation with that of the F30. Again the conformation was determined by gadolinium studies.

Method

This is discussed in detail in chapter 4 but here some difficulties of the relaxation method will be discussed, and also the experiments showing that the various necessary conditions are met.

First there will be a discussion of the general difficulties of determining conformation by relaxation using any relaxation probe. There are two main difficulties:-

1. There is always the problem of accurately correcting for the outer-sphere effects of any relaxation probe. Most relaxation probes have a geometric dependence of their effect which is simply r^{-6} (where r is the distance) and this means that relaxation effects are caused by <u>all</u> probe ions (or molecules) and not just those which are actually bound to the molecule in

question. Hence ions which are free in solution but happen to pass near a particular group will cause a relaxation of that group.

This effect can be approximately corrected for by observing the relaxation of nuclei of some molecule in the sample which does not bind the relaxation probe at all. This method was adopted in our experiments, where the relaxation of the tetra-methyl silane standard was observed. However this can only be an approximate correction, as the true correction depends on how close the relaxation probe can get to a particular group in the molecule under study when the relaxation probe is free in solution. Also there may be 'sticky' collisions which do not involve formation of an inner sphere complex but which mean the relaxation probe spends more time in that particular region.

The above effects will be small for a group which is anywhere near the main binding site of the relaxation probe. However for groups further than say 10 Å from the main site these effects rapidly become progressively more important.

The above difficulty does not arise at all for shift probes because here the angular dependence of the shifts and the random orientation of the symmetry axes for an unbound probe ion means that the average effect is zero.

2. The extremely steep dependence of the relaxation effect on distance means that even an extremely small amount of a second binding site can contribute a substantial proportion of the relaxation effect for a group a long way (i.e. $>$ ca 10 Å) from the main binding site.

This effect is not important for shifts because of the much less steep dependence of the shift effect

on distance (r^{-3}). It should be made clear though that it is possible in theory to determine whether or not the observed effects, shift or relaxation, are coming from the major binding site, in practice this is more difficult with relaxation measurements than shift measurements.

The important conclusion to be drawn from the above is that determination of accurate relaxation data becomes more difficult as the observed group gets further from the binding site of the probe. It has been found in practice, both as a result of the studies on alamethicin and previous experience, that with the gadolinium ion as the relaxation probe, distances much above 10 - 12 Å present rapidly increasing difficulties.

In all of the studies to be reported the relaxation probe used was the gadolinium ion Gd^{3+}, used as the chloride.

Before the conformation can be determined from studies using the gadolinium ion Gd^{3+}, it is necessary to show that various conditions are met. These conditions are similar to those for the shift experiments:-

1. A single site and a 1:1 complex:-

The presence of this condition was proved using shift probes (see chapter 5), and since the gadolinium ion will bind in an almost identical manner to the other lanthanides used in these shift experiments it will be true for the Gd^{3+}. However the greater sensitivity of relaxation experiments measurements to small amounts of a second site make direct confirmation necessary. It can in fact be seen from the gadolinium experiments that there is no substantial contribution from a specific second site as the relaxation effect increases in an approximately linear manner with gadolinium

concentration, and falls off continuously from the main binding site.

2. The absence of contact effects:-

The same comments apply here as to the shift probe case in chapter 5 and in particular there may well be a contact contribution for those atoms in the immediate coordination sphere of the bound lanthanide.

3. Fast exchange:-

Here the conditions which have to be satisfied are different from those of the shift. The condition is that $\tau_M^{-1} \gg T_{2M}^{-1}, \Delta\omega_M$. $\Delta\omega_M$ is very small for gadolinium because of the extremely small zero field splitting (no shift at all is observed during experiments of course because such a minute concentration of gadolinium is used, ca 10^{-5} Molar). This leaves T_{2M}^{-1} to consider. It can be calculated from our experimental results that T_{2M}^{-1} is of the order of 10^4 Hz for the most affected nucleus. It is therefore necessary that τ_M^{-1} is greater than this. A crude estimate of τ_M^{-1} can be obtained by remembering that it is simply the off rate constant for the binding equilibrium and since the binding constant is ca 1000 litre mol^{-1}, if the one rate constant is assumed to be diffusion controlled this gives τ_M^{-1} of 10^7 or so. This shows that we would indeed expect the exchange to be fast.

It can be shown that because of the small value of $\Delta\omega_M$ exchange cannot possibly be intermediate. Hence the two possibilities are slow or fast exchange. These two possibilities are distinguished experimentally by the fact that in slow exchange all the peaks from nuclei not a long way from the metal are broadened equally and also as the temperature increases so will the broadening of these peaks. In fast exchange the broadening has an r^{-6} dependence and gets smaller as the temperature is increased. It was shown experimentally for both the low and high pH complexes that there was fast exchange, because the broadening of peaks near

the metal was not equal and also broadening was decreased as the temperature was increased. This hence confirms the rough calculation above.

Now that it has been shown that the required conditions have been met it is possible to write down the equation for the observed relaxation time as:-

$$\frac{1}{T_{obs}} - \frac{1}{T_{A(\omega)}} - \frac{1}{T_{A''}} = \frac{f}{T_M} = \frac{const}{r^6}$$

This equation applies equally to T_1 and T_2 and so the choice now has to be made between measuring T_1 or T_2 to obtain the relaxation information and precisely which technique is to be used for the measurement of either T_1 or T_2.

The determination of absolute distances with any sort of precision, even for groups near the main binding site, requires a considerable number of very careful experiments using pulse T_1 measurements (and T_2 measurements). This is very difficult for the case of alamethicin, because it was desired to obtain the conformation of both the high and low pH complexes and the only way this could be done would be by making measurements on the unshifted alamethicin spectrum. However this spectrum contains a large number of closely spaced peaks and pulse T_1 measurements on such a spectrum would be very difficult in practice because of the inevitable phase problems with pulse measurements. Pulse T_2 measurements would be even worse because of the phase problems caused by the spin-spin coupling present. Because of these difficulties it was decided that the best approach was to use a method which did not require measurement by pulse sequences. The best method then is to measure semi-quantitative relative distances by measuring T_2 from the line-widths of the peaks. It is still necessary

to overcome the problem caused by the spin-spin coupling of the peaks which means that the line-width is not simply $(\pi T_2)^{-1}$. This problem will only exist for part of the experiments, because the relaxation of the nuclei removes the spin-spin coupling and once this has happened the $(\pi T_2)^{-1}$ expression is valid again. The method adopted to overcome this problem was to choose peaks with very similar coupling and then to compare the gadolinium concentrations required to produce the same effect on the two different peaks. In this way an approximate estimate of the relative values of T_{2M}^{-1} was obtained for peaks whilst they were still coupled.

The detailed experimental results will now be considered:-

CONFORMATIONS OF THE F30 COMPLEXES

The conformation of the two different complexes, the high and the low pH complexes will be considered separately:-

1. The high-pH complex $M(A^-)$.

These experiments were conducted at pH* 3.5, and at an alamethicin concentration of 15 mM. A total of five additions of gadolinium chloride were employed with concentrations ranging from a total of 2.2×10^{-5} up to 6.3×10^{-4} Molar. The results of these experiments are shown in table 6.1 which lists the various peaks in distance order with an approximate relative distance quantification. It is at once evident that the nearest residues are the

Table 6.1

High pH gadolinium results.

Distance	T_{2M}^{-1}	Residues/peaks
0.8	380	PHO α CH,NH, β CH_2 β' CH_2 GLN 18 α CH
1.00	100	PHO aromatic ortho, PRO 13 α CH
		PHO aromatic meta, 2.538 set,2.313 set,2.239 set.
		7.792,
1.16	40	VAL 14 α CH ,methyl
		7.920,7.904,7.879
		PHO aromatic para
		7,572,8.122,8.378
1 .3	20	LEU 11 methyl ALA α CH
		3.859, ALA α CH
		7.618,8.086,7.506-7.465
1.47	10	8.222,8.171
		3.963 GLY 10
1.52	8	VAL 8 methyl
v Large	v small	MEA O NH, methyl. 'acetyl' , PRO 1 α CH .

This table gives the residues in distance order from the gadolinium. If a particular peak is not assigned the chemical shift in PPM in the absence of lanthanides at pH* 1 is given, except for the 7.936,7.912 NH peaks which split at the pH* of these experiments into peaks at 7.939,7.920,7.904,7.879 PPM.
The relative distances and T_{2M}^{-1} values are very approximate especially for those residues/peaks not near to the gadolinium.

PHO, various peaks not assigned to numbered residues which will come from the GLU 17 and GLN 18 and also VAL 14 and PRO 13 are fairly close. The MEA 15 and 16 will not show up very easily because the methyl groups from all the MEA are under one very large peak, and the NH protons are not assigned to specific numbered residues but the 7.792 PPM NH peak might well be the NH of MEA 15 or 16.

A technique which clearly shows up those residues near the metal is illustrated in Fig. 6.1 and Fig. 6.2. This is the technique of difference spectra and is shown in Fig. 6.1 for a simulated set of peaks. The top spectrum shows the normal spectrum, in the second spectrum some gadolinium ion has been added to the sample and this has broadened out the peak from a nucleus which is near the bound gadolinium. If these two spectra are simply subtracted in the computer memory then the unaffected peaks are subtracted to zero and the affected peak is the only one left in the spectrum as shown in the bottom spectrum. This is shown for an actual sample in Fig. 6.2 where the spectra correspond to those of the simulated case, namely normal, with gadolinium ion added and the difference between these two. Only the α CH and upfield methyl residues are shown. In the α CH region the peaks which show up are the PHO + GLN 18 α CH, the PHO β' CH_2, and also the PRO 13 α CH to a small extent. In the upfield methyl region, the VAL 14 pair of doublets clearly shows up (with a small signal from the LEU 11 and almost nothing from the VAL 8). In the regions of the spectrum not shown the peaks that show up are again those of the PHO, GLU and GLN 18. In particular it can be clearly seen that the distance order of the protons of the phenyl ring of the PHO is ortho (nearest), meta, para a result which has been confirmed in various experiments.

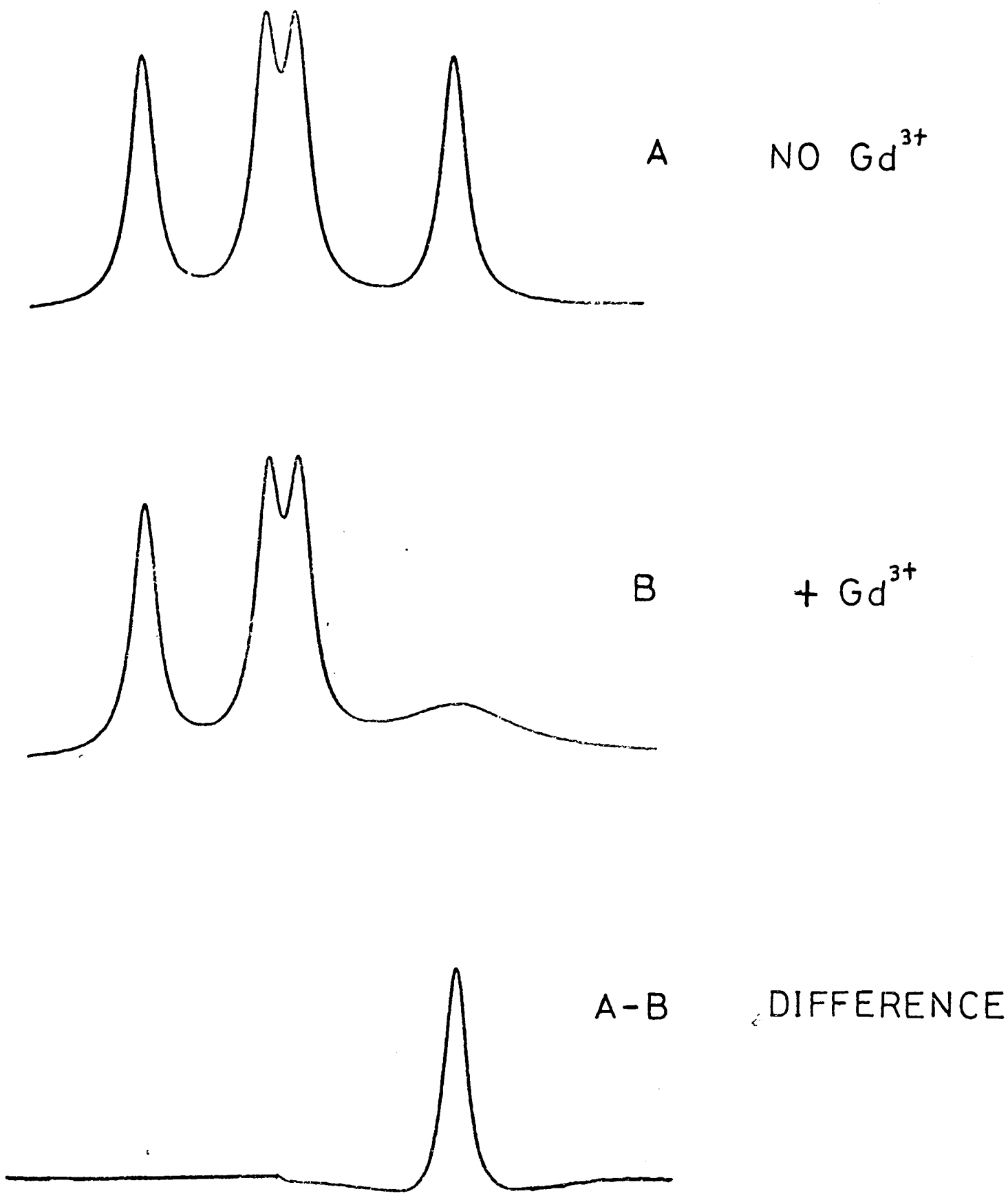


Fig. 6.1

A gadolinium difference spectrum on simulated peaks. The peak on the far right comes from a nucleus near the bound gadolinium ion and is hence considerably broadened by the gadolinium addition and appears in the difference spectrum. The other peaks are from nuclei a long way from the bound gadolinium.

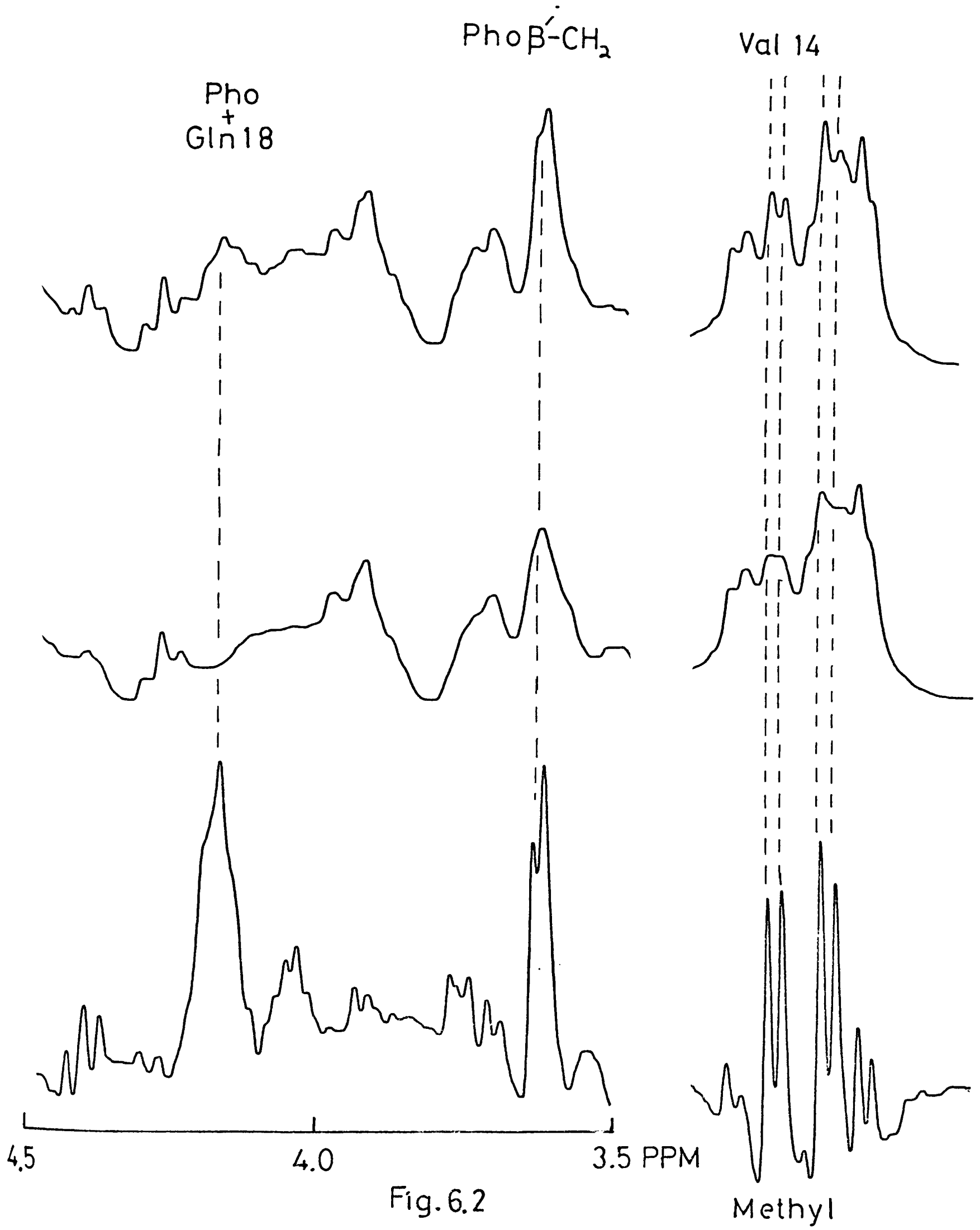


Fig. 6.2

Gadolinium difference spectrum of F30 in methanol at 298°K

Top spectrum:- normal spectrum without gadolinium.

Middle spectrum:- spectrum with gadolinium added.

Bottom spectrum:- the difference, top - middle, x4 vertical scale.

These results all confirm that the lanthanide ion is binding close to, PHO, GLN 18, GLU 17, VAL 14, PRO 13. Using the distances given in table 6.1 it is possible to construct a model of the likely conformation of the alamethicin using space filling models. There is a considerable amount of steric hindrance in alamethicin because of all the MEA residues and so comparitively little information about the distances is sufficient to provide considerable constrains on the conformation. The result is shown in Fig. 6.3. The conformation of the part of the molecule beyond say around GLY 10 can not be determined with any accuracy at all from the gadolinium data for the reasons discussed at the beginning of the chapter. However it can be stated with some confidence that the N-terminal blocking group of the molecule does not fold back in any way as it is quite clear from the gadolinium data that this is indeed a long way from the metal, and the weaknesses of the method previously discussed mean that it must be further away than it will appear to be from the relaxation data.

There are various other methods that might have been used to study the conformation of that part of the molecule which is a long way from the metal but in the case of alamethicin these methods would not be practical. Hence study of coupling constants of the NH protons to the α CH protons is very little use because so many of the residues are MEA which do not have any α CH protons. It is not really possible either to learn much about hydrogen bonding in this molecule, because it is impossible to assign NH protons to specific MEA residues and so there are only a few assigned NH protons. However there is a fair spread of chemical shifts of NH protons from the free amino acid chemical shift positions and this might indicate a fair amount of hydrogen bonding in the molecule.

HIGH pH CONFORMATION

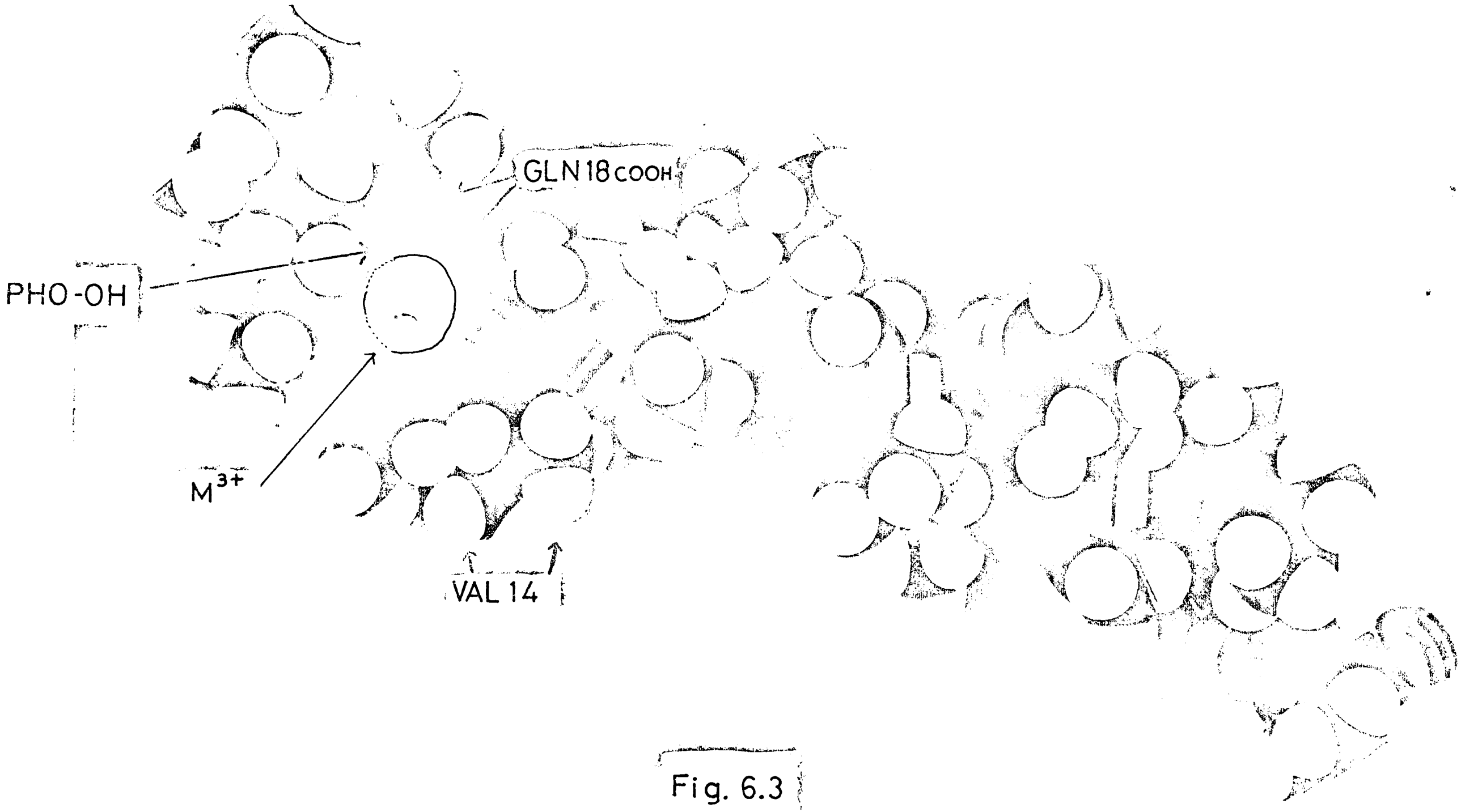


Fig. 6.3

It is possible to place the residue from PRO 1 to GLY 10 into an α helix and this is the arrangement which is shown in Fig. 6.3. The hydrogen-bonding in this helix might well stabilise it over other possible conformations, although the entropy will be unfavourable,however it must be stressed that there is no evidence from our studies to support (or negate) this idea.

It will be seen that the qualitative conclusions which were drawn from the shift data (see chapter 5) are in complete agreement with the above conformation and this is important because the geometric dependence of the shift effect is very different from that of the relaxation effect and also as discussed earlier the relaxation method has various disadvantages that shift measurements do not possess and hence their agreement is a good crosscheck.

2. The low pH complex M(HA)

These experiments were performed at pH* 0.6 and with an alamethicin concentration of 15 mM. The experiments considered in detail here consisted of seven additions of gadolinium with total concentrations of from 1.1×10^{-5} to 2.8×10^{-4} Molar, and these experiments were performed on precisely the same lyophilised batch of F30 and using the same gadolinium solutions as the results considered in the previous section. The experiments were also performed on other similar samples with very similar results.

A typical spectrum obtained in such an experiment is shown in Fig. 6.4 where there is also a normal spectrum for comparison. The spectra are R.E. to make the effects clearer. It can be seen that the various peaks from the PHO

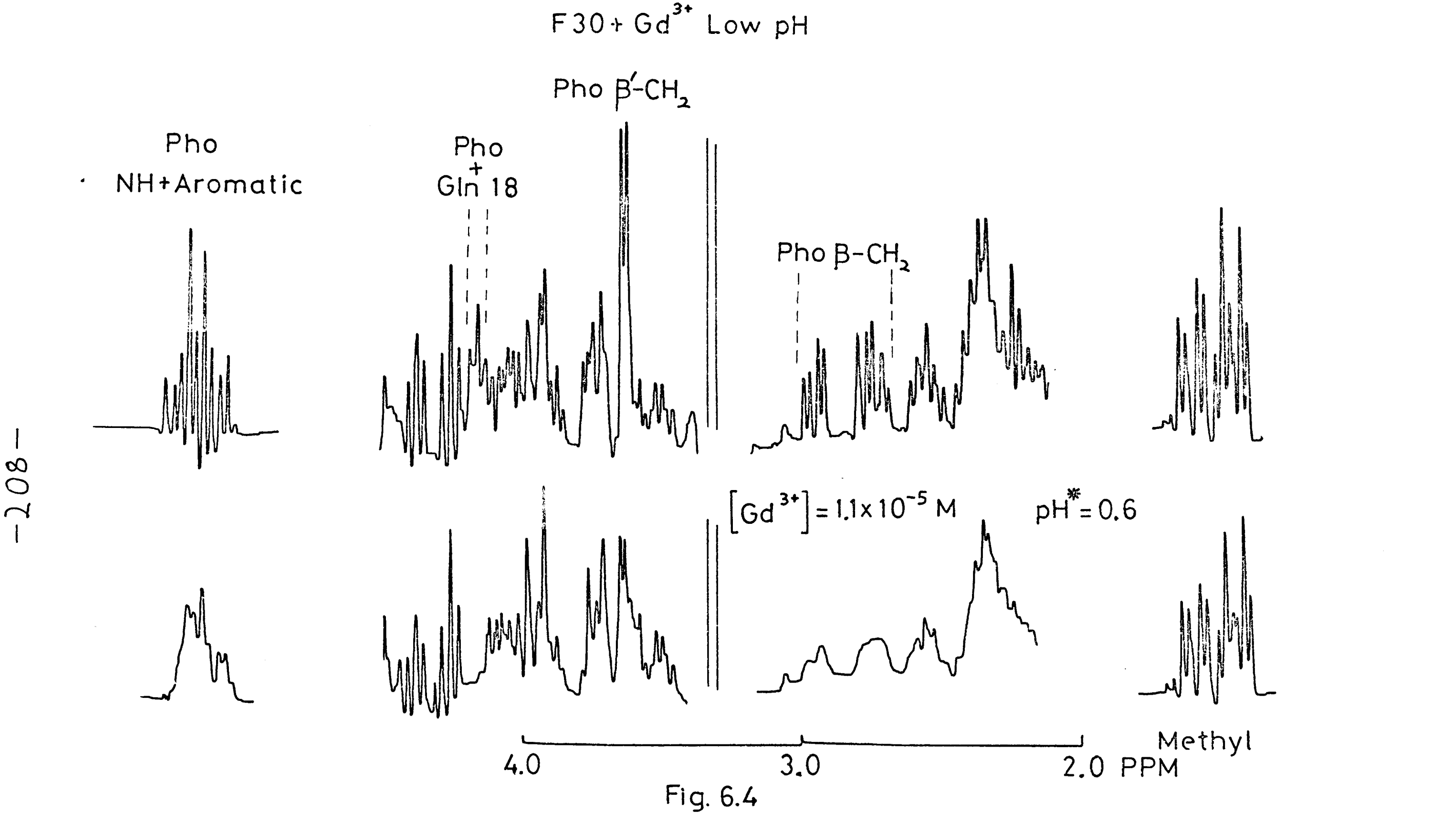


Fig. 6.4

R.E. spectra in methanol showing the effect of adding gadolinium . Normal spectrum at the top.

Table 6.2

Low pH gadolinium results

Distance	T_{2M}^{-1}	Residues/peaks
0.8	380	PHO αCH, NH, GLN 18 α CH
		PHO β, β' CH_2
1.0	100	PHO aromatic ortho
1.1	67	PHO aromatic meta
1.5	33	PHO aromatic para 7.792
		2.538 set, 2,33 set, 2.21 - 2.24 set
		7.572,7.618
		7.912, 8.1 D
		3.748 set
1 .8	3	VAL 14 methyl
		PRO 13 α CH
		8.222 ,3.859 T, 4.061
2.0	1.5	LEU 11 methyl
2.1	0.8	GLY 10 α CH_2, 3.974, ALA α CH
2.25	0.4	VAL 8 methyl
		7.506,7.487,7.465

v large v small MEA 0 NH,methyl. ' acetyl' PRO 1αCH.3.559-3.443

This table gives the residues in distance order from the gadolinium. If a particular peak is not assigned the chemical shift in PPM in the absence of lanthanides at pH* 1 is given. The relative distances and T_{2M}^{-1} values are only approximate, especially for those residues a long way from the gadolinium.

residue and also the GLN 18 α CH are particularly strongly affected showing that these residues are very close to the lanthanide binding site. Many other peaks are completely unaffected at this gadolinium concentration. The detailed results of these experiments are given in table 6.2. Difference spectra were also performed as for the high pH complex and again very clearly showed all the peaks of the PHO and the α CH of GLN 18. (It should be remembered that the complex coupling patterns of the β and γ protons of GLU and GLN make their peaks much more difficult to observe).

There are however clear differences between the results at low pH and those at high pH, and this is evident from Fig. 6.5 which shows the spectrum of the low pH experiment at the top and the high pH at the bottom. It can be seen that whilst the aromatic peaks in the low pH spectrum are more broadened, the 4.377 Triplet (PRO 13) and the VAL 14 α CH peak and methyl peak doublets are much less broadened; this clearly means that the PHO is relatively nearer the lanthanide at low pH.

The conformation deduced from the results in table 6.2 is shown in Fig. 6.6 and comparing this with the high pH result it can be seen that there are many less residues near the metal and in particular residues such as VAL 14 and PRO 13 have moved away and now the lanthanide is being bound only by the last few residues the MEA 16 GLN 18 and PHO. It is not certain that the GLN 18 carboxyl group is any longer bound to the lanthanide now that it has become protonated. The same comments concerning the part of the molecule a long way from the bound lanthanide discussed for the high pH complex apply here as well.

The shift results at low pH also showed that the lanthanide bound nearer to the PHO residue and in general agreed with the conformation presented in Fig. 6.6 and again this

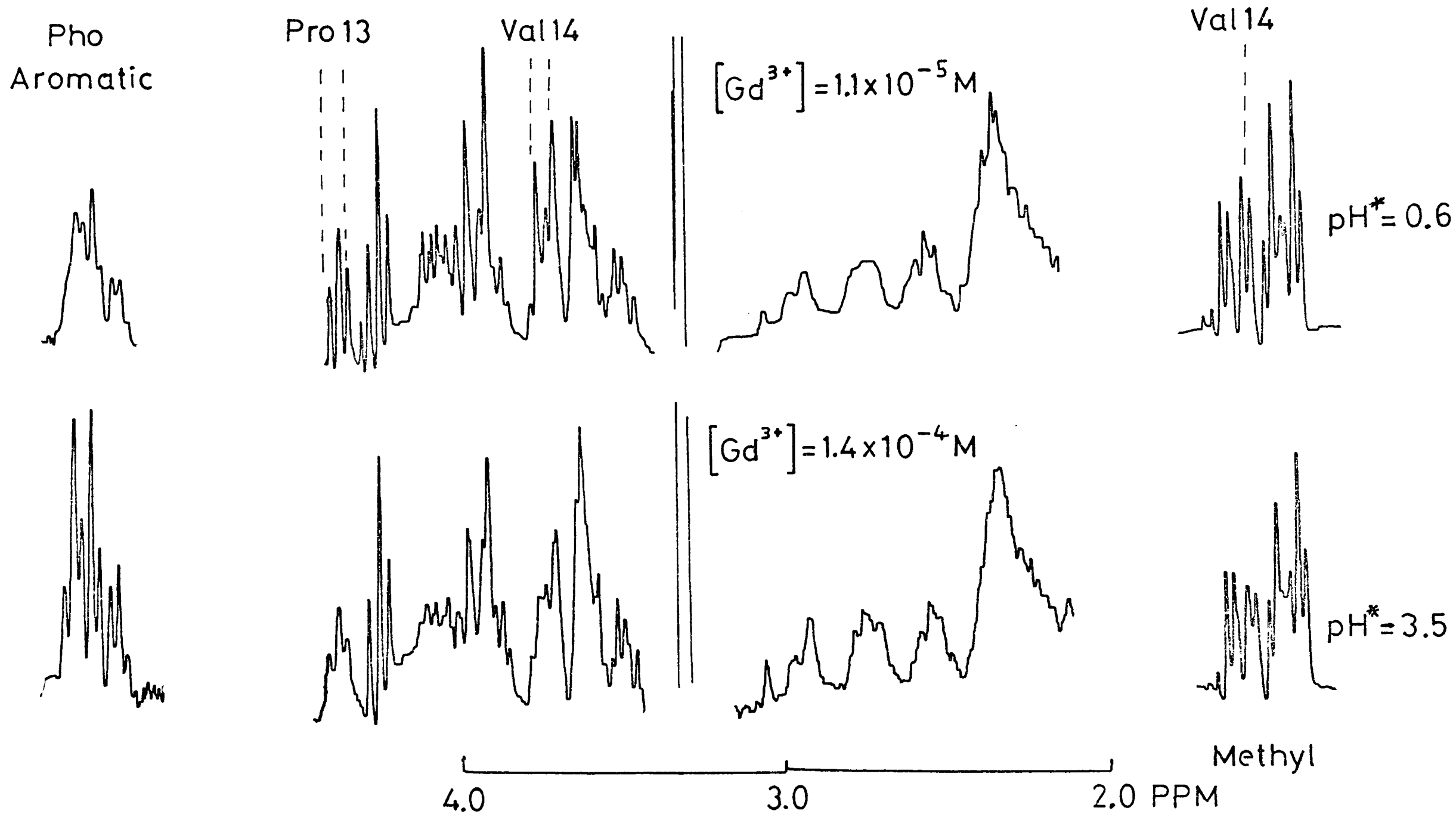


Fig. 6.5

R.E. spectra in methanol showing the different conformations of the two complexes.

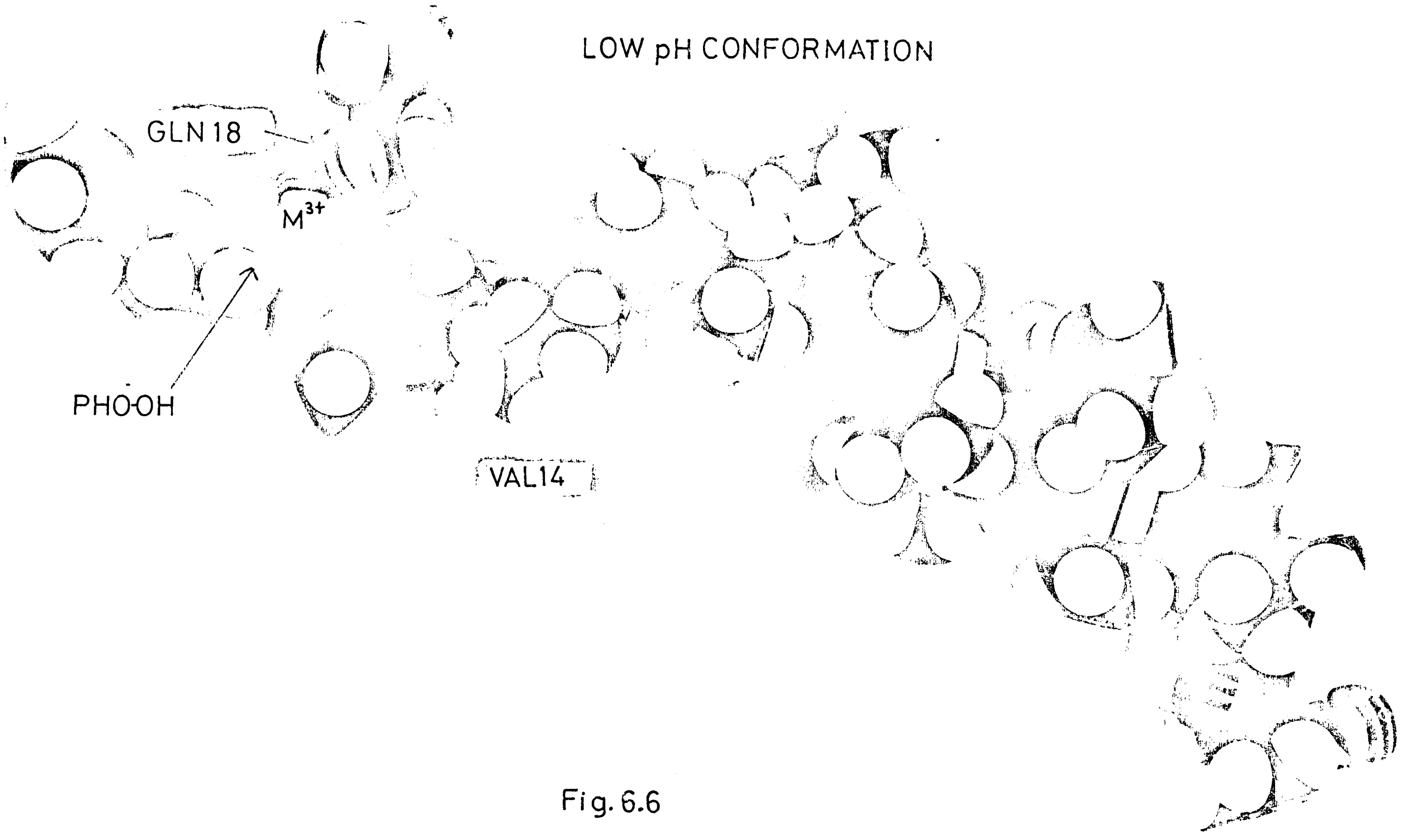


Fig. 6.6

agreement is important confirmation of the correctness of the result.

To summarise, alamethicin F30 binds lanthanide ions strongly whether or not its carboxyl group is ionised. In both cases the lanthanide ion binds near the PHO end of the molecule, but there is a clear difference in the conformation of the $M(A^-)$ and M(HA) complexes. In the $M(A^-)$ complex more groups are involved in binding the lanthanide than is the case of the lanthanide complexed with unionised alamethicin. In both cases the results of these relaxation studies agree with the conclusions from the shift studies.

Effect of added D_2O on the conformation

This subject has already been dealt with from the point of view of the shift experiments in chapter 5, and there the conclusion was that additions of D_2O up to,ca. 10% produced minimal changes in the conformation. This conclusion was directly confirmed by additions of D_2O to gadolinium-containing samples to see whether there were any changes in relative broadenings. These experiments were conducted on both the high and low pH samples. To the pH* 0.6 sample containing 2.8×10^{-4} Molar gadolinium chloride two additions of D_2O were made corresponding to a total of 3.3% and 9.9%. It was found that these additions resulted in a very slight sharpening of all the peaks in the spectrum but there were only very small changes in relative broadenings of the peaks. The same result was found on adding 3.3% of D_2O to the pH* 3.5 sample (gadolinium concentration 6.3×10^{-4} Molar). These experiments confirm that the conformation is not sensitive to D_2O content.

DETERMINATION OF THE CONFORMATION OF THE F50 COMPLEX

It was shown in chapter three that the sequence of F50 is almost certainly the same as that of F30 except that the carboxyl group of GLN 18 is converted to an amide group. There are no ionisable groups in F50 and so the conformation would not be expected to be radically pH dependent. As F50 is so extremely similar to F30 in almost every way, it was assumed that the extensive work done on F30 concerning the presence of only one binding site, the insensitivity to D_2O content, and the presence of fast exchange could be directly applied to the F50.

A gadolinium experiment was performed on F50 under usual conditions i.e. with an F50 concentration of 15 mM, at pH* ca 1. It was found that very similar results were obtained with F50 to those obtained with F30 at low pH when the carboxyl group is protonated. A spectrum showing that for F50 the PHO is again, along with GLN 18, very close to the lanthanide binding site is shown in Fig. 6.7. The full results are given in appendix 3. Hence it can be seen that as might be expected, the conformation of the F50 complex is very similar to that of the F30 M(HA) complex.

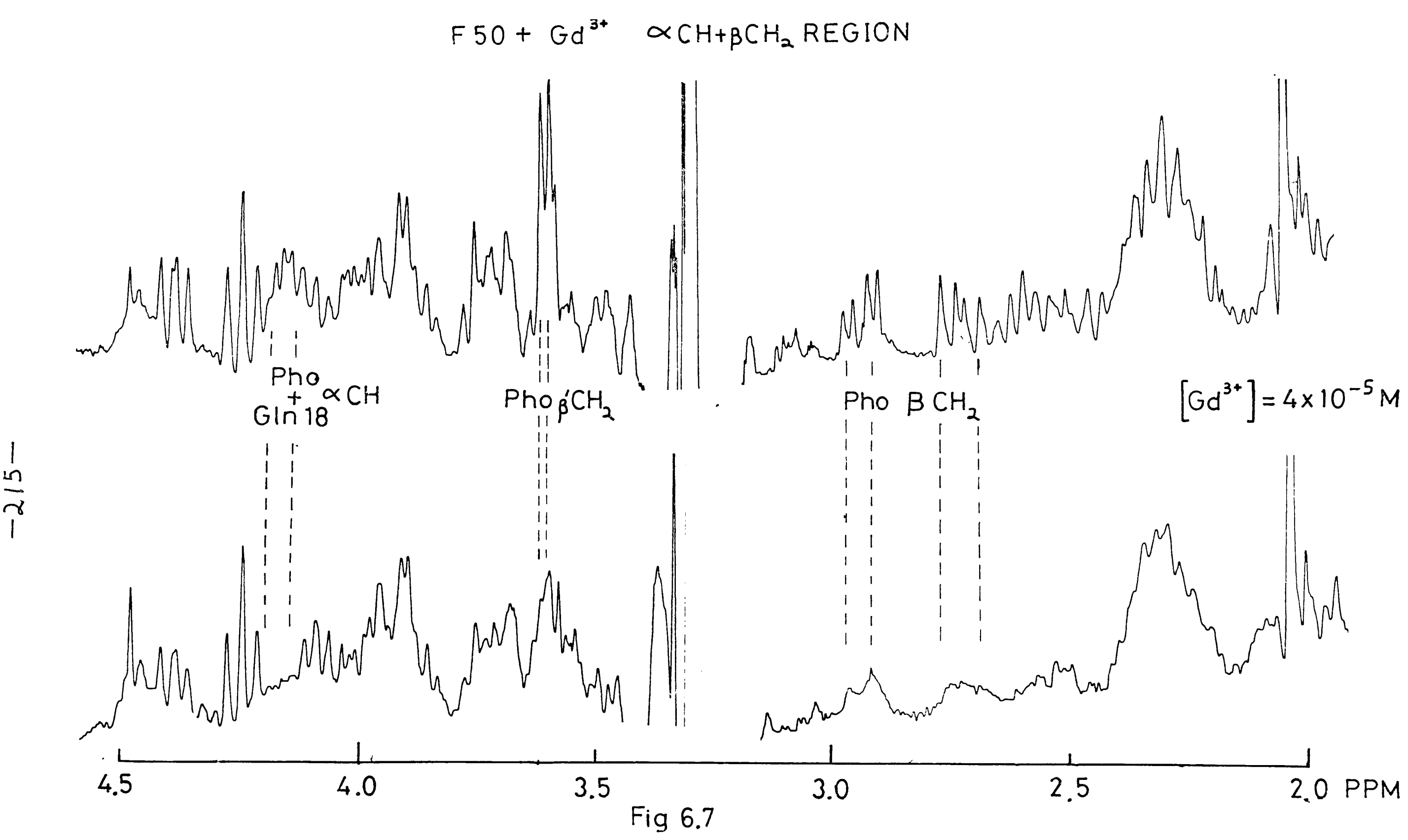


Fig 6.7

R.E. spectra in methanol showing the effect of adding gadolinium. Normal spectrum at the top.

APPENDIX ONE (THEORETICAL)

INDEX

PART I (General)

The Nuclear Transition ... 217
(a) continuous wave method ... 220
(b) Fourier Transform method ... 221
Resolution Enhancement ... 223
Measurement of relaxation times ... 224

PART II (Paramagnetic)

Pseudo-contact shift ... 226
Effect of exchange on the shift ... 227
Paramagnetic relaxation effects ... 229
Effect of exchange on relaxation ... 231

The format is the same as in the theoretical chapter itself, dividing the material into 'general' and 'paramagnetic' effects. The order and classification of material is also the same as in the theoretical chapter. Where a detailed understanding of the material in this appendix is required for interpretation of experimental results, this has been indicated in the experimental chapter concerned.

PART I (GENERAL)

The Nuclear Transition

A very simple derivation of the basic equation for the transition, equation 2.1.1 will be given and then more detailed theory of the transition will be given leading on to the two different modes of measuring Nmr. spectra, the continuous wave method (which will be treated extremely briefly as it was not used) and the Fourier transform method which will be treated in some detail.

The magnetic moment μ of a 'simple classical' particle cf mass M charge e, and angular momentum P is:-

$$\hat{\mu} = \gamma_{class}\ \hat{P} \qquad \gamma_{class.} = \frac{e}{2Mc}$$

(where $\hat{j}$ denotes a vector j)

This is found experimentally to be incorrect and it is necessary to introduce a 'g-factor' (which for electrons may sometimes be calculated), and we may then write, for a nucleus

$$\hat{\mu} = g_N \gamma_{class}\ \hat{P}$$
$$= g_N \frac{e}{2Mc} \hbar . \hat{I} \qquad \hat{P} = \hbar . \hat{I}$$

$\hat{I}$ is the spin angular momentum vector

In fact I has magnitude $\sqrt{I(I+1)}$, putting $\frac{e\hbar}{2Mc} = \beta_N$

$$\mu = g_N \beta_N \sqrt{I(I+1)}$$

The energy of interaction of the nucleus with the applied field, with which the nuclear magnetic moment makes an angle θ is,

$$= \mu H_0 \cos\theta$$
$$= g_N \beta_N H_0 \cos\theta \sqrt{I(I+1)}$$
$$= g_N \beta_N H_0 I_z$$

The selection rule is $\Delta I_z = \pm 1$ thus the energy of transition, and the frequency of the radio-frequency radiation causing the transition is given by;

$$\underline{h\nu_0 = g_N \beta_N H_0} \qquad \text{A.1.1.1.}$$

The above simple derivation gives no indication that precession will occur, a more sophisticated treatment is as follows[1],

Consider the magnetic dipole making an angle with the applied field $\hat{H}_0$ (always assumed along the z axis), there will be a torque on this dipole which must equal the rate of change of momentum $\hat{p}$

$$\frac{d\hat{p}}{dt} = \hat{\mu} \wedge \hat{H}_0$$

Where $\wedge$ denotes a vector product.

using the standard expression for the vector product and $\hat{\mu} = \gamma \hat{p}$

$$\frac{d\hat{p}}{dt} = \gamma \begin{vmatrix} \hat{i} & \hat{j} & \hat{k} \\ p_x & p_y & p_z \\ H_x & H_y & H_z \end{vmatrix}$$
$$= \gamma H_0 (p_y \hat{i} - p_x \hat{j})$$

$\hat{i}$, $\hat{j}$, $\hat{k}$, are unit vectors along the x, y, z, axes respectively.

Differentiating again and solving the resultant equation;

$$\frac{d^2 \hat{p}_x}{dt} = \gamma H_0 \frac{d\hat{p}_y}{dt}$$
$$= -\gamma^2 H_0^2 \hat{p}_x$$

$$\therefore p_x = p \sin\alpha \sin\gamma H_0 t \qquad \text{A.1.1.2}$$

This variation of P_x corresponds to a precession

of $\hat{p}$ and thus of $\hat{\mu}$ of frequency in radians/sec,

$$\omega_o = \gamma H_o$$

This frequency of precession is easily shown to be the same as that of the electromagnetic radiation whose energy corresponds to transitions between the various orientations of the magnetic moment.

Let us now consider how transitions may occur. It is convenient to use a system of coordinates rotating about the z axis at frequency W_o, equal to the Larmor precession frequency. A radio-frequency field H_1 is applied, a component of which will rotate with the nuclei. In time dt the angular momentum vector $\hat{p}$ will travel a distance $d\hat{p}$, but to an observer in the rotating coordinate system it will travel $d\hat{p}^*$ where,

$$d\hat{\rho}^* = d\hat{\rho} - \hat{\omega} \wedge \hat{\rho}\, dt$$

$$\therefore \frac{d\hat{\rho}^*}{dt} = \frac{d\hat{\rho}}{dt} - \hat{\omega} \wedge \hat{\rho}$$

$$\text{Since } \frac{d\hat{\rho}}{dt} = \hat{\mu} \wedge \hat{H}_o = \gamma \hat{\rho} \wedge \hat{H}_o$$

It follows that:-

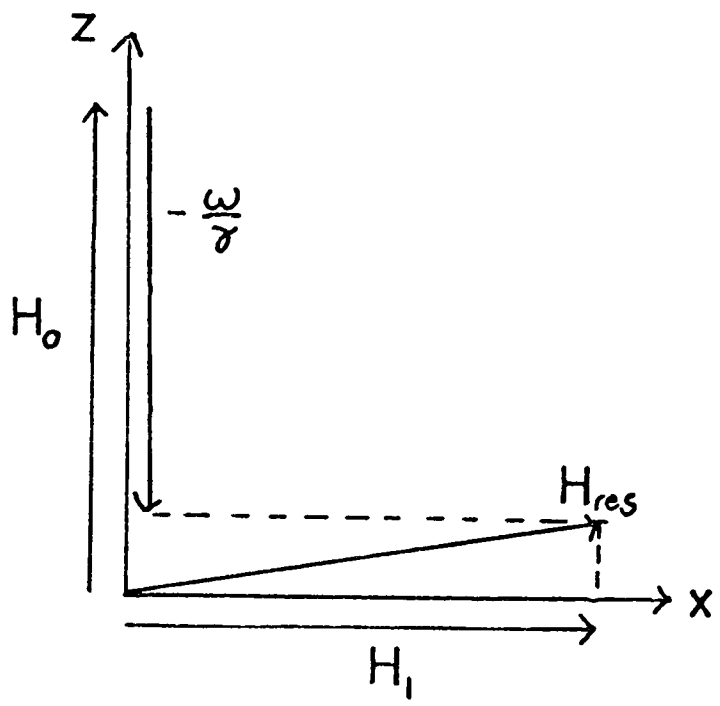
$$\frac{d\hat{\rho}^*}{dt} = \gamma \hat{\rho} \wedge \left(\hat{H}_o + \frac{\hat{\omega}}{\gamma}\right)$$

Comparing the above two equations, it can be seen that in the rotating coordinate system the nuclei experience an effective magnetic field given by-

$$\hat{H}_{rot} = \hat{H}_o + \frac{\hat{\omega}}{\gamma} \qquad \text{A.1.1.3}$$

When $\hat{\omega} = \hat{\omega}_o = -\gamma \hat{H}_o$ (i.e. the Larmor frequency), there is no effective field and the nucleus behaves as a constant vector in the rotating coordinates. Larmor first showed the equivalence of a rotating coordinate system and a magnetic field[2].

If the radio-frequency field, H_1 of frequency ω is now applied then the effective field has components H_1 in the x direction and $H_o - \frac{\omega}{\gamma}$ in the z direction. The resultant field H_{res} will then be the vector sum of these as:



With the above diagram in our minds, the two modes of measuring Nmr. spectra, will now be considered.

(a) Continuous wave mode

When H_1 is applied then the initial total magnetisation M_o (which will start along the z axis) will precess about H_{res} at a rate γH_{res}, if $W \sim W_o$ then H_{res} will nearly be along the x direction. If $W = W_o$ then H_{res} is along the x direction and the precession will mean that nuclei pass back and forth between the two spin states. The magnetisation along the z direction will reach an equilibrium value which may be determined by application of the; full Bloch equations. However it is sufficient to note that the component of the magnetisation in the xy plane which is 90^o out of phase with the applied field H_1 is directly proportional to the rate of change of M_z, and gives the absorption mode signal usually measured in nmr. The intensity I (ω) of this signal at frequency ω being given by, (small H_1)

$$I(\omega) = \frac{\text{Const. } H_1 T_2}{1 + T_2^2 (\omega_o - \omega)^2} \qquad \text{A.1.1.4.}$$

ω_o is the resonance frequency

T_2 is the transverse relaxation time.

This is the equation of a Lorentzian peak with a half height width of $(\pi T_2)^{-1}$ Hz.

(b) Fourier Transform Mode

The detailed mathematics involved[3, 4], will not be given but instead a simple picture of what is happening.

In this mode a powerful radio-frequency field is applied at a frequency ω_{rf} higher than all the resonance frequencies to be observed. Since a strong r.f. H_1 field is applied, H_{res} (see above diagram) will still be almost along the x axis for all nuclei which have 'resonant' frequency within a wide frequency range (say 10,000 Hz) of the applied r.f. frequency, and thus the magnetisation of ALL nuclei in this range will start to precess about H_{res}. When the magnetisation has precessed through 90^o, i.e. from the z direction where it started into the xy plane (in fact pointing in the y direction), the radio-frequency field is turned off. Thus a 90^o pulse has been applied to the system, (the length of which would typically be 40 μsec.), and now the magnetisation of ALL the nuclei is in the xy plane. In the absence of the radio frequency field 'precession' is once again about H_o.

The detector measures the magnetisation in the xy plane, and may be considered to be 'rotating' at the frequency of the applied radiation ω_{rf}. Thus if the ith nucleus precesses at ω_i about the main field H_o then the signal in the detector will have a frequency $\omega_{rf} - \omega_i$, in addition the magnetisation from this nucleus will decay in the xy plane exponentially with time, t, as

exp $(-t/T_{2i})$. The signal in the detector $M_{det.}(t)$. is the sum of such signals from all the nuclei in the sample, and thus may be written (equation 2.1.3),

$$M_{det.}(t) = \sum_i \sin(\omega_{rf} - \omega_i)t \cdot \exp(-t/T_{2i})$$

This exponentially decaying magnetisation is the free induction decay

The free induction decay in time f(t) as given above and the conventional Lorentzian line shape which is in the frequency domain F(w) are a Fourier Transform pair i.e. in general:-

$$F(\omega) = \int_{-\infty}^{\infty} f(t) \exp(-i\omega t)dt$$

$$f(t) = \frac{1}{2\pi} \int_{-\infty}^{\infty} F(\omega) \exp(i\omega t)\, dt$$

i is the usual complex number symbol

and in this particular case:-

$$\frac{T_2}{1 + T_2^2(\omega - \omega_i)^2} = \int_0^{\infty} \exp\frac{-t}{T_2} \cdot \cos(\omega_i - \omega_{rf})t \cdot dt$$

Thus after a suitable number of free induction decays have been added together to average out the noise (each decay measurement lasts only a matter of 1 sec or so), their sum is Fourier transformed by the computer and yields a 'conventional' spectrum made up of Lorentzian-shaped peaks.

The free induction decay is fed directly into an on-line digital computer and this means that the magnetisation can only be sampled at discrete time intervals. This is important because obviously a certain number of points within one cycle of a sine wave are required to define the frequency of the sine waves of which the magnetisation is the sum. If a frequency is very high compared with

the sampling time it will evidently not be defined. Consideration of the detailed mathematics shows that the highest frequency Υ_{max} which is unambiguously defined is given by:-

$$\Upsilon_{max.} = \frac{1}{2\delta t}$$

δt is the sampling time or 'dwell' time.

Thus the 'sweep width' of the Fourier Transform experiment is determined by the highest defined frequency and so is determined by the sampling rate, any peak which falls outside this sweep will still 'appear' within the sweep but will be out of phase and obviously at the wrong frequency it may also be attenuated.

It has been shown that in the Fourier transform mode the radio-frequency field excites ALL the nuclei simultaneously, and this is the reason for the considerable saving in time compared with the continuous wave mode where each nucleus is excited individually. The reduction in time for a given signal to noise ratio is roughly the total sweep width Υ divided by the average line-width in the spectrum $\delta\Upsilon$. Thus the improvement in signal to noise in a given time is the square root of this, $\sqrt{\frac{\Upsilon}{\delta\Upsilon}}$.

Resolution Enhancement

Let us consider the free induction decay due to a single nucleus of resonant frequency ω_o this will be of the form $\exp(-t/T_2)\cos(\omega_o-\omega_{rf})$. If this decay is multiplied by the function $\exp(-t/\tau_1)$, then the free induction decay becomes $\exp(-t(T_2^{-1}+\tau_1^{-1}))\cos(\omega_o-\omega_{rf})$ which on Fourier Transformation yields a (broader) Lorentzian line with line-width $(\pi T_2')^{-1}$ where $(T_2')^{-1} = T_2^{-1} + \tau_1^{-1}$, instead of $(\pi T_2)^{-1}$. If however the free induction decay is multiplied by the function

$$\exp(-t/\tau_1) - K\exp(-t/\tau_2)$$

a <u>new line shape</u> is generated[5] as:-

$$F_{RE}(\omega) = \frac{T_2' - KT_2'' + \Delta\omega^2\left[T_2'(T_2'')^2 - KT_2''(T_2')^2\right]}{1 + \Delta\omega^2\left[(T_2')^2 + (T_2'')^2\right] + \Delta\omega^4(T_2'T_2'')^2}$$

where $\Delta\omega = \omega - \omega_0$

T_2' is given by $(T_2')^{-1} = T_2^{-1} + \tau_1^{-1}$

T_2'' is given by $(T_2'')^{-1} = T_2^{-1} + \tau_2^{-1}$

This should be compared with the equation for a Lorentzian line:-

$$F(\omega) = \frac{T_2}{1 + \Delta\omega^2 T_2^2}$$

As previously noted the important point about this line-shape is that it eliminates the long wings of the Lorentzian peak (see diagram 2.1.2 on page 17). Several other points should be noted:-

1. There is a loss in signal to noise ratio.
2. The loss of intensity is worse for relatively broader peaks, and so the normal area relationships are not preserved, i.e. all n-proton peaks are not of the same area.
3. All the operations required for the process will already be programmed into any Fourier transform system.

The various constants τ_1, τ_2, K are adjusted empirically to give the desired line shape, line width and signal to noise. Typical values for a fairly sharp spectrum such as that of alamethicin are; $\tau_1 = 0.194$ sec. $\tau_2 = 0.272$ sec. $K = 1.02$.

Measurement of relaxation times

T_1 measurement

This is best performed by pulse methods. Consider again the rotating coordinate frame considered on page 219 . If a radio-frequency pulse is applied for the right length of time along

the x axis, then the magnetisation will precess through 180^{o} i.e. from the + z to -z direction and finish pointing against the field H_o. This is a $\underline{180^{o}\text{ pulse}}$. When the pulse is removed the magnetisation will decay exponentially along the z direction at a rate determined by T_1. If after allowing the magnetisation M_z to decay for a certain time, the delay time $\underline{t}$, we then apply a 90^{o} pulse, the magnetisation will be turned into the xy plane and the signal in the detector will have an initial magnitude $M_z(t)$. By varying the delay time t a set of values of $M_z(t)$ as a function of t is obtained, and T_1 can then be obtained from the relation:-

$$M_z(t) = M_o - 2M_o \exp(-t/T_1)$$

Where M_o is the initial equilibrium value of M_z.

In fact after application of the 90^{o} pulse the magnetisation is in the xy plane and the situation is the same as that in a straight Fourier Transform experiment, (where there is just a 90^{o} pulse). Thus the free induction decay can be accumulated and transformed in the usual way, and the $\underline{\text{intensity}}$ of each peak in the high resolution spectrum is proportional to $M_z(t)$. The T_1 of all the (resolved) peaks in the spectrum can thus be measured in one experiment.

To summarise the sequence used is:- $(180^{o}\text{-}t\text{-}90^{o})$. In practise it is necessary to sum a large number of free induction decays and care must be taken to see that the magnetisation decays fully before the next pulse sequence is started. Thus a very long delay (i.e. perhaps 5-10 sec.) is placed at the end of each sequence.

T_2 measurement

This may also be done by pulse sequences,[6] though there are various difficulties. In our experiments we simply measured the line-widths, which for a Lorentzian line are $(\pi T_2)^{-1}$ Hz.

PART II (PARAMAGNETIC)

Pseudo-contact shift

Bleaney's theory[15] for the pseudo-contact shift induced by lanthanides, will be briefly considered.

For the lanthanides the ground state, except for Europium may be accurately specified by a state with a well defined J value, which splits on application of a magnetic field. The ground J state may also be split by the crystal field into sets of closely spaced levels which may have highly anisotropic g values (such as are measured in esr. experiments). However the lifetime of an ion in any one of these states is very short at room temperature compared with the time of an nmr. experiment, so that the g-value is effectively isotropic. There is no doubt that the electron relaxation times are very short $< 10^{-11}$ sec. except for f^7 ions.

Thus in an nmr. experiment it is only necessary to consider the magnetic moment (of the unpaired electrons) averaged over a time long compared with the electron relaxation time, and then take the mean of the local magnetic fields at the neighbouring nucleus averaged over all orientations of the complex with respect to the main field H_o, this allows for the rapid rotation of the complex. The time averaged magnetic moment is simply given by the susceptibility, to first order this is isotropic and can therefore give no shifts but to second order anisotropic terms appear which are responsible for the shift. The second order terms arise from anistropic zero field, splitting. The final expression which Bleaney derives for the general case of non-axial symmetry is:-

$$\delta = -\frac{g^2\beta^2 S(S+1)(2S-1)(2S+3)}{60\,(kT)^2} \cdot \frac{D_z(3\cos^2\theta - 1) + (D_x - D_y)\sin^2\theta\cos 2\phi}{r^3}$$

where Θ is the angle the lanthanide-nucleus vector makes with the principal axis, and ϕ the angle it makes with the subsidiary symmetry axis

r is the distance from the lanthanide to the nucleus concerned

D is the zero field splitting

T is the temperature, and other symbols have their usual meaning.

In the case of axial symmetric when $\chi_x = \chi_y$ then the term in $D_x - D_y$ disappears and so the shift depends on only one angle Θ. The matter of axial symmetry is considered in some detail in appendix 2, page 234.

In contrast with the calculations based on anisotropic g-values, Bleaney predicts a $1/T^2$ variation of shift with temperature. It is possible to calculate the relative shifts which should be given by the different lanthanides, and Bleaney's predictions have been confirmed experimentally, except for thulium[7].

Gadolinium is a special case because of its f^7 configuration. It has a very slow electron relaxation time and thus causes very considerable relaxation of the nuclei. Bleaney predicts a very small shift caused by high order terms, but in practice the very large broadening (relaxation) it produces would render these shifts quite unobservable.

Effect of exchange on the observed shift

The various theories for the pseudo-contact shift all give the shift of the fully formed complex, in which the metal is bound 1:1 on to the complex with no exchange. In practise the metal exchanges on and off the ligand molecules at a rate which is reflected by the binding constant, and in addition there will be, at any one time,

appreciable concentrations of unbound ligand molecules present. The signals observed in the Nmr depend on the relative magnitudes of various factors, principally the value of the fully bound shift (from the unbound position) $\Delta\omega_M$ radians/sec., and the value of the lifetime of the ligand nucleus in the bound state τ_M.

The equation for the shift $\Delta\omega_A$ from the unbound resonance position is:-

$$\Delta\omega_A = \frac{f\,\Delta\omega_M}{(1+\tau_M/T_{2M})^2 + \tau_M^2\,\Delta\omega_M^2} \qquad \text{A.1.2.1.}$$

w here f is the fraction of ligand bound and

T_{2M} is the value of T_2 in the fully bound complex.

Three cases may then be distinguished:-

(1). Slow exchange:-

If $\tau_M^2\,\Delta\omega_M^2 \gg 1$ then it can be seen from the above equation that $\Delta\omega_A = 0$. Thus separate signals are seen for the bound and unbound species with values of the chemical shift corresponding to those which would be given in the absence of exchange, and with intensities which depend on the relative concentration of the bound and unbound species in solution.

(2) Intermediate exchange:-

Here the rate of exchange τ_M^{-1} is of the same order as $\Delta\omega_M$ and so the above equation does not simplify. The separate resonances seen in slow exchange then start to shift towards a weighted average position.

Whether or not two separate resonances are seen depends on whether the system is on the slow or fast side of intermediate exchange. However two important points may be made:-

(a). The shift $\Delta\omega_A$ is proportional to the fraction of ligand bound as in fast exchange (see below).

(b). The shift $\Delta\omega_A$ expressed in PPM of the proton resonance frequency ω is <u>frequency dependent</u> ie; if the experiment is performed at a different value of the main applied field H_o, and hence at a different value of the proton resonance frequency then $\frac{\Delta\omega_A}{\omega}$ in PPM will be different. This becomes apparent on inspecting equation A.1.2.1 and remembering that $\Delta\omega_A$ is in radians/sec and is therefore directly proportional to H_o, whereas τ_M and usually T_{2M} are independent of H_o.

(3). Fast exchange.

Here $\tau_M^{-1} \gg \Delta\omega_M, T_{2M}^{-1}$ and in this case the above equation simplifies to :-

$$\Delta\omega_A = f\,\Delta\omega_M$$

(this is equation 2.2.4 of chapter 2). Here there is only one peak whose position is a weighted average of the positions of the unbound and fully bound positions. Here the shift $\frac{\Delta\omega_A}{\omega}$ in PPM is not frequency dependent.

<u>Paramagnetic relaxation effects</u>.

The degree of relaxation caused by a paramagnetic ion depends, inter alia, on the electron relaxation time, and appreciable relaxation only occurs for long relaxation times. In the lanthanide series the Gd^{3+} ion has an f^7 configuration and thus a much longer relaxation time than the other ions ca. 10^{-7} sec. and gives considerable relaxation of the Nmr nuclei

The bound and unbound sites will first be considered separately, and then the effect of exchange on the relaxation at the <u>unbound</u> site will be considered .(In relaxation experiments the concentration of bound species is extremely small and thus it is the unbound species which is ' observed ').

Bound site

The equation for the relaxation at the bound site, (denoted by M) are the Solomon-Bloembergen equations[9-13],

$$\frac{1}{T_{1M}} = \frac{2}{15}\frac{\gamma_I^2 g^2 S(S+1)\beta^2}{r^6}\left[\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_S^2\tau_c^2}\right] + \frac{2}{3}S(S+1)\left(\frac{A}{\hbar}\right)^2\left(\frac{\tau_e}{1+\omega_S^2\tau_e^2}\right)$$

$$\frac{1}{T_{2M}} = \frac{1}{15}\frac{\gamma_I^2 g^2 S(S+1)\beta^2}{r^6}\left[4\tau_c + \frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{13\tau_c}{1+\omega_S^2\tau_c^2}\right] + \frac{1}{3}S(S+1)\left(\frac{A}{\hbar}\right)^2\left(\frac{\tau_e}{1+\omega_S^2\tau_e^2} + \tau_e\right)$$

where $\tau_c^{-1} = \tau_S^{-1} + \tau_M^{-1} + \tau_R^{-1}$ A.1.2.2.

and $\tau_e^{-1} = \tau_S^{-1} + \tau_M^{-1}$

τ_S is the correlation time for electron relaxation

τ_R is the correlation time for molecular rotation

τ_M is the correlation time for exchange, and is the ligand lifetime in the bound state

ω_S, ω_I are the Larmor frequencies of the electron and the nucleus

A is the hyperfine coupling constant

r is the distance from the metal ion

The second term in these equations is the contact contribution which for most of the nuclei in our system is negligible.

These equations are derived on the basis of the following assumptions:-

(1) No exchange.

(2) $\omega_S \gg \omega_I$ this is obviously valid.

(3) The rotation of the paramagnetic ion is isotropic

(4) There is only one electron relaxation time

(5) The zero field splitting is much less than ω_S (for Gd^{3+} the zero field splitting is $\sim 10^3$ Hz, so this is easily satisfied).

(6) The ion concerned has an isotropic g-value, equations for anistropic g-values have been derived[14].

The above assumptions have been found to be experimentally justified.

Unbound site (no exchange)

This is denoted by A, and here the relaxation is given by

$$T_{1A}^{-1} = (T_{1A(0)})^{-1} + (T_{1A''})^{-1} \qquad A.1.2.3$$

$T_{1A(0)}$ is the value in the absence of added paramagnetic ion.

$T_{1A''}$ is the effect of the bulk susceptibility of the added paramagnetic ion.

$T_{1A''}$ may be calculated by averaging the dipolar interaction over the volume between the sphere of closest approach and infinity. Note that this outer-sphere effect is NOT present in shifts because the angular dependence averages to zero over the surface of a sphere.

Effect of exchange on relaxation at the unbound site

The general equations for the paramagnetic contribution to the observed relaxation T_p are:- $(f \ll 1)$

$$(f T_{1p})^{-1} = (f T_{1A''})^{-1} + (T_{1M} + \tau_M)^{-1}$$

$$(f T_{2p})^{-1} = (f T_{2A''})^{-1} + \frac{1}{\tau_M} \frac{T_{2M}^{-1}(T_{2M}^{-1} + \tau_M^{-1}) + \Delta\omega_M^2}{(T_{2M}^{-1} + \tau_M^{-1})^2 + \Delta\omega_M^2} \qquad A.1.2.4.$$

Where T_{1P} and T_{2P} are given by:

$$(T_p)^{-1} = (T_{obs})^{-1} - (T_{A(0)})^{-1} \qquad A.1.2.5.$$

There are again several possible cases to consider depending on the relative magnitudes of T_{2M}, τ_M, $\Delta\omega_M$.

(1) Slow exchange

The conditions for this are

$$\Delta\omega_M^2 \gg (T_{2M}\tau_M)^{-1}, \tau_M^{-2}$$

or else

$$T_{2M}^{-1} \gg \tau_M^{-1}, \Delta\omega_M^{-1} \quad \text{or} \quad T_{1M}^{-1} \gg \tau_M^{-1}$$

In this case the relaxation is determined by τ_M

$$\frac{1}{f}\cdot\frac{1}{T_P} = \frac{1}{f\,T_{2A''}} + \frac{1}{\tau_M} \qquad \text{A.1.2.6}$$

so that it is notpossible to obtain any geometric information.

(2) Intermediate.

This region only occurs for T_2, not T_1. In this region

$$\tau_M^{-2} \gg \Delta\omega_M^2 \gg (\tau_M T_{2M})^{-1}$$

and T_{2P} is determined by τ_M and $\Delta\omega_M$

$$\frac{1}{f T_{2P}} = \frac{1}{f T_{2A''}} + \tau_M \Delta\omega_M^2 \qquad \text{A.1.2.7}$$

Again this will not give any geometric information. Note also that in this region T_{2P} will be frequency dependent. (cf. shift case page 228).

(3) Fast exchange

$$\tau_M^{-1} \gg T_{2M}^{-1}, \Delta\omega_M \text{ or } \tau_M^{-1} \gg T_{1M}^{-1}$$

and the relaxation is

$$\frac{1}{f T_P} = \frac{1}{f T_{A''}} + \frac{1}{T_M}$$

Here the relaxation is determined by T_{1M} or T_{2M}, and looking back at equation A. 1. 2. 2 and neglecting the negligible contact term (the second term in the equation), then the equation becomes:-

$$T_M^{-1} \propto r^{-6}$$

Thus using equation A. 1. 2. 5:-

$$\frac{1}{T_{obs}} - \frac{1}{T_{A(0)}} - \frac{1}{T_{A''}} = \frac{f}{T_M} = \frac{f.\,\text{const.}}{r^6}$$

This is equation 2. 2. 6. and measuring all the quantities on the left hand side enables determination of the relative distances for the nuclei concerned.

REFERENCES

1. R.E. Richards — Adv. in spectroscopy 2, 102 (1961)
2. J. Larmor — 'Aether and matter' C.U.P. New York 1900 page 341
3. R.R. Ernst — Rev. Sci. Instr. 37 no. 1, 93 (1966)
4. T.C. Farrar, E.D. Becker — 'Pulse and Fourier Transform Nmr' Academic press 1971
5. I.D. Campbell et al — J. Mag. Res. 11, 172 (1973)
6. E.L. Hahn — Phys. Rev. 80, 580 (1950)
7. B. Bleaney et al — Chem. Comm. 791 (1972)
8. T.J. Swift, R.E. Connick, — J. Chem. Phys. 37, 307 (1962)
9. I. Solomon — Phys. Rev. 99, 559 (1955)
10. N. Bloembergen — J. Chem. Phys. 27, 572 (1957)
11. N. Bloembergen, L.O. Morgan — J. Chem. Phys. 34, 842 (1961)
12. T. Luz, S. Meiboom — J. Chem. Phys. 40, 1058 (1964)
13. A.R. Peacocke, R.E. Richards, B. Sheard — Mol. Phys. 16, 177 (1969)
14. H. Sternlicht — J. Chem. Phys. 42, 2250 (1965)
15. B. Bleaney — J. Mag. Res. 8, 91 (1972)

APPENDIX TWO

NON-AXIAL SYMMETRY

The assumption will be made throughout the following that all the lanthanides bind in an identical manner to the molecube under investigation, and hence that the directions of the two symmetry axes is the same for all the lanthanides, andalso that the conformation of the alamethicin itself is identical for the different lanthanides.

Suppose that data is collected for a large number of protons and various lanthanides, it will then be possible to write down equations relating the measured shifts δ_{lh} with the electronic factors A_l, B_l for the various lanthanides and the geometric factors x_h, and y_h for the protons. It will be first shown that it is mathematically impossible to solve these equations in the absence of further constraints. Then the number of further constraints required will be considered in detail and it will be shown that in practice the molecule alamethicin does not possess these constraints.

It should also be realised that even if the complete solution of these equations were possible this would not be sufficient to determine the conformation, because the x_h and y_h which would be obtained contain three unknowns:-

$$x_h = \frac{3\,\mathrm{Cos}^2\theta - 1}{r^3} \qquad y_h = \frac{\mathrm{Sin}^2\theta\ \mathrm{Cos}2\phi}{r^3}$$

and all of these unknowns are required to determine the position in space of the relevant nucleus. So that it would be necessary to use extra information such as is provided by bond angles and bond lengths to obtain the final answer.

Insolubility of the basic equations

The notation used is as follows :-

$\hat{z}$ is vector 'z' .

$\hat{\delta}_{1H}$ denotes the vector $(\delta_{11}, \delta_{12}, \delta_{13} \cdots \delta_{1h} \cdots \delta_{1H})$

$\hat{x}_H$ denotes the vector $(x_1, x_2, x_3, \ldots x_h, \ldots x_H)$

similarly for y_h

δ_{lh} is the shift with the l th lanthanide and the h th proton.

A_l, B_l are the electronic factors for the lth lanthanide.

x_h, y_h are the geometric factors for the hth proton.

Then the equations for the shifts with lanthanides 1,2 and 3 and all H protons would be :-

$$\hat{\delta}_{1H} = A_1\hat{x}_H + B_1\hat{y}_H \qquad \text{A.2.1}$$

$$\hat{\delta}_{2H} = A_2\hat{x}_H + B_2\hat{y}_H \qquad \text{A.2.2}$$

$$\hat{\delta}_{3H} = A_3\hat{x}_H + B_3\hat{y}_H \qquad \text{A.2.3}$$

The key point as mentioned in chapter 4 is that it is only possible to write down linearly independent equations for TWO lanthanides and H protons, this means that if we write down a third equation that third equation gives no more information about the unknowns

in the first two equations. This means that all the information there is about the vectors x_H and y_H is contained in equations A.2.1 and A.2.2 above and that equation A.2.3 gives no more real information about x_H and y_H. This is easily proved, first the definition of linear dependence is that two constants α and β must exist such that :-

$$Q_3 = \alpha Q_1 + \beta Q_2 \qquad \text{A.2.4}$$

Then Q_3 is linearly dependent on Q_1 and Q_2 . Here the Q are any 'quantities', such as vectors or whole equations.

However it must always be possible to find at least two constants α and β such that :-

$$\begin{aligned} \alpha A_1 + \beta A_2 &= A_3 \\ \alpha B_1 + \beta B_2 &= B_3 \end{aligned} \qquad \text{A.2.5}$$

(where the A and B are also constants)

and if this is so it is evident that one can write (using equations A.2.1 , A.2.2, A2.3, A.2.5) :-

$$\begin{aligned} \alpha \hat{\delta}_{1H} + \beta \hat{\delta}_{2H} &= (\alpha A_1 + \beta A_2)\hat{x}_H + (\alpha B_1 + \beta B_2)\hat{y}_H \\ &= A_3 \hat{x}_H + B_3 \hat{y}_H \\ &= \hat{\delta}_{3H} \end{aligned}$$

Hence:-

$$\text{A.2.3.} = \alpha\,(\text{A.2.1}) + \beta\,(\text{A.2.2.})$$

Then looking at the above equation and at the definition

of linear dependence given in A.2.4 it is obvious that the third equation A.2.3 is linearly dependent on equations A.2.1 and A.2.2 .

This means that all the information that can ever be obtained (from measuring shifts δ_{lh}) about the vectors $\hat{x}_H$ and $\hat{y}_H$ is contained in the two equations:-

$$\hat{\delta}_{1H} = A_1 \hat{x}_H + B_1 \hat{y}_H$$
$$\hat{\delta}_{2H} = A_2 \hat{x}_H + B_2 \hat{y}_H \qquad \text{A.2.6.}$$

These two equations have six unknowns , A_1, B_1, A_2, B_2 $\hat{x}_H$ and $\hat{y}_H$ and evidently cannot be solved unless four of the unknowns are specified eg; A_1, B_1, A_2, B_2, . They do in fact have an INFINITE number of solutions, one of which corresponds to the conformation we are looking for.

The same conclusion is obtained if we try and write explicit equations for the unknowns using A.2.6. It is possible to write equations for the x_H and y_H unknowns separately. Consider protons 1,2 and h and the two lanthanides 1 and 2, then the following equation may be derived :-

$$\begin{vmatrix} \delta_{12} & \delta_{22} \\ \delta_{1h} & \delta_{2h} \end{vmatrix} x_1 + \begin{vmatrix} \delta_{11} & \delta_{21} \\ \delta_{1h} & \delta_{2h} \end{vmatrix} x_2 + \begin{vmatrix} \delta_{11} & \delta_{21} \\ \delta_{12} & \delta_{22} \end{vmatrix} x_h = 0$$

$$\text{where} \begin{vmatrix} \delta_a & \delta_b \\ \delta_c & \delta_d \end{vmatrix} = \delta_a \delta_d - \delta_b \delta_c$$

Only one such equation may be written for any particular set of three protons. It is then evident that specification of the ratio x_2/x_1 allows us to find the ratios x_h/x_1 for all the remaining

H protons. Similar equations may be written down for the y_h unknowns. Alternatively if x_1 and x_2 are known then the absolute value of all the x_h can be found. Similarly for the y_h unknowns, so that it is again necessary to specify a total of four unknowns to solve the equations completely.

If it were possible to obtain the ratios x_h/x_1 for all the H protons then the situation would be precisely the same as if there were axial symmetry when the terms in $B_1 y_h$ are zero. This case has been successfully solved in all such cases that our group has studied to date, by using the ratios x_h/x_1, in a computer program along with the bond lengths,and angles and Van der Waals radii, to find the conformation. (See page 139).

Extra information needed for solution of the equations.

It is necessary to consider what extra information there is,apart from the shift data obtained with the lanthanides, which might enable us to solve the equations.

It has been assumed in the above treatment that there is no relationship between the various geometric factors, but this is not so as the values of the bond lengths and angles are known and this information is not included in the equations presented above. Unfortunately even this information is not sufficient in practice. Let us consider how many rigidly connected points (ie; nuclei) are needed to enable complete solution of the system.

If there are h rigidly connected nuclei, a plane may be drawn through any three of them and these three points specified relative to each other by three distances. The remaining h - 3

points can each be specified by the distances of the three to each base point. Thus a knowledge of the complete geometry of the h rigidly connected nuclei corresponds to $3(h-2)$ equations. The total number of unknowns in the system is the 3h coordinates of the h nuclei and the four electronic factors of the two lanthanides. The total number of equations is the 2h equations relating the measured shifts δ_{1h} and the $3(h-2)$ geometric equations above. In order to be able to solve the system there must be at least as many equations as unknowns ie; $5h-6 \geqslant 3h+4$, and hence $h \geqslant 5$. This means that at least five <u>rigidly</u> connected nuclei, such as is provided by the protons of an aromatic ring, are needed. In fact consideration of the alamethicin molecule shows that only two such types of unit occur, the aromatic and the proline residues. The use of these is ruled out in practice by the following :-

(1). The complexity of the proline spectrum which arises from the large number of spin-spin couplings between the protons makes it impossible to obtain accurate data for the individual nuclei.

(2). The experimental data show that the aromatic ring is 'rotating' rapidly in the Nmr time scale so that only three types of proton can be seen and a precise knowledge of the motion of the aromatic would be needed to analyse the data.

(3). Even if such a unit could be used to solve the system the conformation for the rest of the molecule would depend entirely on the accuracy of the data for the unit. This is because as shown below the rest of the molecule does not possess in practice sufficient constraints to enable solution of the conformation.

The above analysis is for absolutely rigidly connected nuclei. If the analysis is performed for nuclei connected by free

rotations the number of nuclei needed to solve the system rises rapidly, and although there are are probably sufficient protons in alamethicin to allow the system to be solved in theory,in practice the problem is insuperable, one of the reasons being that almost half of the residues are MEA the singlet resonances from which occur under one very large peak and assignment of which to specfic residues would constitute an almost impossible task, so that it is not possible to obtain sufficient accurate data from the assignable resonances to solve the system.

There is another constraint that we have not so far considered, namely Van der Waals contact distances. The usefulness of this is extremely difficult to quantify, but again the main difficulty in the use of this information would be practical. Unlike a geometric constraint, the Van der Waals gives only a minimum distance, so that inclusion of this in an analytical computer program would be difficult.

Another possible parameter which could provide information is NH-αCH coupling constants. However the large number of MEA (which do not have any αCH) and prolines (no NH) leaves only a few residues where this would be useful, so this gives only a very few extra constraints.

The final remaining possibility is to determine the value of the distances of the nuclei from relaxation measurements and to use this information to try and help solve the system. However determination of the distances does not alter the fundamental insolubility of the shift equations, it only reduces the number of other external constraints such as bond lengths etc required to

solve the system. Thus for example if distances can be determined for all the nuclei concerned, shift measurements on four rigidly connected nuclei (instead of five) will enable solution of the system.

To sum up, the basic equations are mathematically insoluble and require a large number of extra constraints to be placed on the system before solution becomes possible. Analysis of the possible extra constraints for the case of alamethicin shows that the molecule does not possess sufficient of these in practice and so the problem of non-axial symmetry is insoluble in any quantitative manner for alamethicin.

APPENDIX THREE

EXPERIMENTAL

INDEX

Chapter 3

Details of PHX α CH decoupling experiments. 243

Chapter 4

Analysis of M to HA and A^- binding. 244

Indicator colours 247

Chapter 5

Assignment. Effect of lanthanum 247

Decoupling experiments 249

Effect of D_2O 251

Constant lanthanide titrations 252

High pH ratios 252

Binding constant fit 254

Chapter 6

F50 relaxation results 255

Chapter 3

Details of PHXαCH decoupling experiments

These were the decoupling experiments on intact F30 in methanol and here the experiments will be described which show that the decoupling of the extra doublet at 3.64 PPM was due to irradiation of the PHXαCH and not some other peak.

Looking at the position in the spectrum corresponding to the PHX αCH irradiation there is a triplet peak and a complex peak underneath this. The triplet was decoupled to a singlet by an irradiation in another part of the β CH_2 region of the spectrum so this peak cannot be coupled to the 3.64 PPM doublet. The complex peak underneath the triplet is probably the PHX α CH (the complexity of the peak is due to the large number of protons to which it is coupled). It was necessary to be sure however that there was not a furtherαCH close to the PHX α CH position which was coupled to the αCH doublet. This was shown by shifting the PHX αCH peak by use of the diamagnetic lanthanum ion. It was found that the lanthanum produced relatively large shifts of the PHX αCH and the triplet at that position, and also the 3.64 PPM doublet whereas most of the rest of the peaks in the αCH region were unaffected. This enables us to do several experiments:-

(1). The double irradiation was repeated on the lanthanum shifted sample . Thus it was shown that the β CH_2 and the α CH doublet are decoupled simultaneously, to within a few hundreths of a PPM by irradiation of the PHX αCH, in the lanthanum shifted sample as well as in the original sample. The PHX αCH was shifted by approximately 0.2 PPM by the lanthanum addition. It is very unlikely that if there were another αCH coupled to the 3.64 PPM doublet that it would

show almost precisely the same change in shift with the lanthanum as the PHX α CH.

(2). It is possible to perform difference spectra. Thus the spectrum for the sample with lanthanum is simply subtracted from the spectrum for the sample without lanthanum (no irradiations). All the unshifted peaks, and this means almost all the α CH, subtract to zero, but the peaks which do shift (this includes the PHX α CH and the 3.64 PPM doublet) give rise to two peaks, one where the peak has shifted 'from' and one where it has shifted 'to '. These peaks may be integrated (provided they do not overlap) without interference from most of the other peaks in the α CH region which subtract to zero. This experiment showed that the PHX α CH and the triplet starting at nearly the same position were two protons only and hence there cannot be another peak moving with the PHX α CH. This experiment also gives an area for the 3.64 PPM doublet though this area measurement was not quite so accurate. The result was an area corresponding to two protons .

Hence the above experiments show that the PHX α CH is indeed coupled to the doublet at 3.64 PPM as well as the pair of β CH_2 quartets at ca 3.0 PPM and the NH proton.

Chapter 4

Analysis of M to HA and A^- binding

It is assumed that the proton binds to A^- with a binding constant such that the pK is 'p', and that the metal binds to

HA with a binding constant of K_H and to A^- with a binding constant K_{A^-} . This set of equilibria is easily analysed to give the fraction of ligand present as M(HA), M (A^-) , and the total amount of complexed ligand. These are denoted by f_H , f_{A^-} , and f_T respectively. It will be remembered in chapter four that the quantity that we are trying to determine is the metal bound pK_M ie; when f_H f_{A^-} , and that it was stated that to a good approximation in the conditions of our experiments this was given by the pH when

$$\delta_{obs} = \frac{1}{2} (\delta_h + \delta_l) \qquad \text{A.3.1}$$

Below is a table of values of the various f for a metal concentration of 100 mM and a ligand concentration of 15 mM and for various values of the two binding constants. Note that the true value of pK_M is always of course given by:-

$$pK_M = p - \log_{10} (K_{A^-} / K_H)$$

The value of the pK as determined by equation A.3.1 above is indicated in the table, and it can be seen that even for values of the binding constants which are known to be less than the true values the approximation holds quite well.(The approximation gets worse as the binding constant gets smaller) . The value of the pK determined from equation A.3.1. depends to a small extent on the values of the fully bound shifts for the two complexes and in the table the values are given for the two extremes of the fully bound shift of M(HA) being twice, and half that of those of $M(A^-)$. It can be seen from the table that the approximation represented by A.3.1. is certainly valid when K_H is aslarge as the 500 Litre mol^{-1} found experimentally. The equation for the observed shift used to

perform the relevant calculations is:-

$$\delta_{obs} = f_H \Delta_H + f_{A^-} \Delta_{A^-}$$

where Δ_H is the fully bound shift of the peak in the M(HA) complex

Δ_{A^-} is the fully bound shift of the peak in the $M(A^-)$ complex

δ_{obs} is the observed induced paramagnetic shift of the peak

	K_A - 100 litre mol^{-1} K_H 10 litre mol^{-1}			K_A - 10,000 litre mol^{-1} K_H 1,000 litre mol^{-1}		
pH. - p	f_H	f_{A^-}	f_T	f_H	f_{A^-}	f_T
2	.00089	.89471	.89560	-	-	-
1	.00879	.87957	.88837	.00988	.98883	.99872
0	.07528	.75280	.82808	.09071	.90715	.99786
-1	.31120	.31120	.62241	.49678	.49678	.99357
- 2	.45616	.04561	.50177	.89939	.08993	.98933
-3	.47863	.00478	.48342	.97871	.00978	.98849

The true value of the pK_M is given by pH. - p = -1 as is obvious from the above table. The value of pK_M calculated from equation A.3.1 and the above values of f and fully bound shifts of the complexes in the ratio 2:1 and 1:2 are:-

-0.83 and-0.68 -0.9942 and -0.9938

It can be seen that the approximation is better for higher binding constants (the values on the right), but is still quite good even for the lower binding constant values under the conditions of the experiments.

Indicator colours

Below are given the colours of the indicators in methanol under the following conditions; the 'stock' solution of bromophenol was 5.5 mg per ml of deuterated methanol, and the stock solution of thymol was 7.4 mg per ml of deuterated methanol these were then added in the proportion of 5 μLitre per 400 μlitre of actual sample. The volume of a standard Nmr sample being 400μlitre. The colours of such a concentration of indicators in the nmr tube are given below for the mixed indicators (5 μlitres of each in 400μL).

Colour	pH*	pK values
strong blue	10 and above	
blue	9 - 4.3	F30 ca 6.5
green-blue	4.3 - 3.2	
yellow-green	3.2- 2.0	pK_M ca 2.0 for both Eu^3 ,Pr^3
yellow/trace green	2.0 - 1.5	
yellow	1.5 - 1.0	
weak orange	1.0 -0.7	
red	0.7 and below	

Chapter 5.

Assignment and effect of lanthanum

In the table below are given the chemical shifts of all the peaks in the alamethicin F30 spectrum in deuterated methanol at pH 1, and also the assignment of the peaks,if any . Also given in

this table for convenience are the effects of 100 mM La^{3+} at pH* 1 on the peaks. The chemical shift under these conditions is given, although it was not possible to follow all the peaks.

Chemical shift	Assignment	Effect of La^{3+}	Chemical shift	Assignment	Effect of La^{3+}
8.617	MEA O	8.642	7.293 D	PHO o-	7.348 D
8.378		8.459	7.226 T	PHO m-	7.247 T
8.342 T	GLY 10	8.326	7.149 D	PHO p-	7.193 D
8.222		8.222			
8.171		8.176	4.480	LEU 11	4.494
			4.431		4.453
8.10 D	(LEU 11)	8.145D			
			4.407		4.412
8.086		8.130	4.377	PRO 13	4.384
		8.108	4.350		4.358
8.043		8.083			
			4.281		4.287
7.998 D		8.028D	4.249	PRO 1	4.254
			4.219		4.224
		7.996			
7.934	] ALA ?	7.975	4.200		4.358
7.909		7.961	4.170	PHO + GLN 18	4.325
		7.945	4.143		4.287
7.792		7.879			
			4.107	ALA (1.508)	4.096
7.618		7.631	4.080		4.083
7.596		7.607	4.066		4.058
7.572		7.590	4.053		4.020
			4.034	ALA(1.547)	
7.547		7.566	4.009		
			3.974		3.982
7.506		7.533	3.930		3.943
7.487		7.512	3.911	GLY	3.922
7.465		7.490			3.900
			3.884		3.881
7.435	] PHO		3.862		3.859
7.403		???	3.838		3.829
			3.748	VAL 14	3.764
7.443 †	GLN		3.709		3.718
7.302 †					
6.754 †	-$CONH_2$		3.636	PHO $\beta'CH_2$	3.737
6.626 †			3.617		3.718
			3.592	VAL 8	3.600

Chemical shift	Assignment	Effect of La^{3+}	Chemical shift	Assignment	Effect of La
					1.618
3.543		3.560			1.593
3.505	PRO δ CH_2 ?	3.500	1.62		1.523
3.483		3.482	1.59		1.541
			1.547	MEA	1.522
2.974		2.988	1.508		1.490
2.955		2.971	1.48	MEA O	1.479
2.925	PHO β CH_2	2.936			
2.903		2.920	1.147		1.141
			1.125	VAL 8	1.117
2.775		2.783			
2.743	PHO β CH_2	2.745	1.084		1.092
2.726		2.729	1.059	VAL 14	1.070
2.694		2.694			
			1.019	VAL 8	1.019
2.598			0.994	VAL 14	0.994
2.574			0.967		0.969
2.538			0.953		0.937
2.511		small	0.929	LEU 11	0.912
2.476			0.907		
2.359		"			
2.34	VAL 14 β				
2.312					
2.24	VAL 8 β	"			
2.211					
2.184					
2.157					
2.041	'acetyl'				
2.018					
1.988		"			
1.961	LEU $\beta+\gamma$				
1.936					
1.862					
1.835					
1.813		"			
1.790					
1.762					
1.741					

† only observable in protonated methanol.

Decoupling experiments

Below the full details of the comprehensive decoupling experiments carried out on F30 in methanol are given, the chemical shift of the irradiated peak and the effects of the irradiation.

First the irradiations of the αCH and β CH_2 regions are given, and then various irradiations designed to assign the NH protons.

Irradiation position PPM	Result
4.453	Decouples 1.988 -1.936
4.373	Decouples 1.835 , 1.813 peaks
4.112,4.072	No effect
4.022	Changes at 2.359 to 2.27
3.969 - 3.885	changes peaks at around 3.5 PPM
3.754	No effect
3.721	Changes around 2.33 PPM
3.641	Changes at 2.27 PPM
3.627	No observable effect.
3.609,3.577,3.534	No effect
2.581	No effect
2.436	No effect
2.399,2.378	Partial decoupling of VAL 14 methyl and αCH , decoupling of 4.377 T and 4.249 T (PRO 13 and 1)
2.354 -2.330	Good decoupling of VAL 14 peaks.
2.309	Partial decoupling of VAL 14 peaks
2.27,2.24,2.22	Decouples VAL 8 methyl and αCH and produces changes in the 2.598 - 2.476 set of peaks.
2.128,2.092	Produce changes in GLN 18, and 3.543 -3.483 peaks.
2.044,2.002	Decouples 3.862 T .
1.961,1.918	Decouples LEU methyl and αCH.
1.864 -1.763	Changes in PRO 13 and PRO 1 α CH.
1.692,1.652	No effect.

Irradiation position	Result
1.547	Decouples ALA αCH centred at 4.023 PPM
1 .508	Decouples ALA α CH centred at 4.093 PPM.
8.341	Decouples GLY 10 α CH_2
8.116	Decouples LEU 11 α CH
7.99	Changes peak at 3.94 PPM
7.936,7.912	Decouples ALA at 4.02 PPM
7.599	No clear effect
3.940	Decouples 7.99 D

Effects of D_2O

These are the effects of D_2O on the shift of the solvent peak in deuterated methanol and also the effects of D_2O on NH-peak shifts with 100 mM lanthanum present.

It was found that the shift of the hydroxyl solvent peak of CD_3OD was dependent on D_2O content as follows:-

% added D_2O	0	1.2	2.5	3.7	5	6.2	7.5
Chemical shift.	4.859	4.856	4.856	4.851	4.848	4.845	4.842 PPM

These shifts are the same if there is 15 mM alamethicin present as well.

In the presence of 100 mM lanthanum the 8.459 NH peak can be used to monitor D_2O content as it shows a shift of 0.011 PPM upfield from its starting position of 8.459 for each 1% of added D_2O.

Constant lanthanide titrations.

Below the details of the various constant lanthanide titrations are given:-

	F30 conc.	La^{3+} conc.	F30 conc.	Paramagnetic conc.
Europium titration:-	15.0	101.6	14.5	101.8
Praesodymium :-	14.5	102.0	15.2	99.7
Erbium :-	15.0	102.3	15.2	99.9
		Eu^{3+}		Pr^{3+}
Europium/praesodymium	14.2	100.2	14.2	102

All concentrations are in mM.

High pH ratios.

The method adopted for showing from the results at low and 'intermediate' pH that the ratios at high pH cannot be the same is as follows. It is assumed that in the intermediate pH titrations performed with the various lanthanides the same proportion of complex was present as $M(A^-)$ in all the titrations, though it is not necessary to know exactly what that proportion of complex was.

Let us suppose that the fully bound shifts of nucleus 1 , in the low pH complex M(HA) is Δ_{1H} and in the complex $M(A^-)$ it is Δ_{1A^-} and similarly for nucleus h, and let all shifts be ratioed to that of nucleus 1 . Then at low pH the shift ratio for the hth proton nucleus will be $\frac{\Delta_{hH}}{\Delta_{1H}}$ and at intermediate pH, where f denote the fractions of the appropriate complexes the ratio will be :-

$$\frac{f_H \Delta_{hH} + f_{A^-} \Delta_{hA^-}}{f_H \Delta_{1H} + f_{A^-} \Delta_{1A^-}}$$

and denoting the ratios by ρ_H, the intermediate pH ratio ρ_i will be:-

$$\rho_i = \frac{f_H \Delta_{IH}}{f_H \Delta_{IH} + f_{A^-} \Delta_{IA^-}} \rho_H + \frac{f_{A^-} \Delta_{IA^-}}{f_H \Delta_{IH} + f_{A^-} \Delta_{IA^-}} \rho_{A^-}$$

$$\therefore \rho = \chi \cdot \rho_H + (1 - \chi) \rho_{A^-}$$

$$\underline{\rho = (\rho_H - \rho_{A^-}) \chi + \rho_{A^-}}$$

Hence ρ is a linear function of χ which is determined in a complex fashion by the various fractions of complex present ie by the <u>pH</u> . In particular when $\chi = 1$, at low pH when $f_{A^-} = 0$ then $\rho = \rho_H$ and similarly when $\chi = 0$ then $\rho = \rho_{A^-}$. The value of χ at the pH at which the intermediate pH experiments was done is denoted by χ_i .

The proof that the ratios cannot be equal for the different lanthanide at high pH consists of working out what would happen if this were the case and then showing that this does not happen.

A graph is plotted of ρ against χ . Only two points are 'known' for each peak. When $\chi = 1$, ie; at low pH then $\rho = \rho_H$ and when $\chi = \chi_i$ at intermediate pH then $\rho = \rho_i$. If a straight line is drawn through these points it must pass through the high pH value of ρ_{A^-}, when $\chi = 0$. The point at which this occurs on the χ axis is not known as the value of χ_i is not known ie: the 'scale' of the χ axis is not known. Suppose that these points are plotted and the lines drawn for a particular peak ratioed to a standard peak for two different lanthanides then where these lines meet the ratios are plainly equal. If this process is repeated for many more peaks and all the lines intersect at the same value of ie at the same pH (as χ is determined by pH). Then it is <u>possible</u> that this common point of intersection is where $\chi = 0$ and that hence all the ratios are equal at high pH. However if all the pairs of lines intersect at different values of χ ie; at different pH values then it is plainly impossible that the ratios for the different lanthanides

are equal at high pH (or any other pH for that matter). The results of applying this test to the experimental results are that the points of intersection vary wildly from values of χ close to $\chi = 1$ and corresponding to a much lower pH than χ_i and also physically meaningless cases of lines which would only meet at a value of $\chi > 1$. The severe nature of these disagreements is hence a conclusive proof that the ratios cannot be equal at high pH, and in fact are not all equal at any pH between low and high ie; they are not equal for any mixture of the high and low pH complexes.

Binding constant fit

This consisted of a least squares fit program written for a Wang 600 programmable calculator. In order to obtain approximate values of the binding constant and the fully bound shift in a short time the first section of the programme calculated a least squares fit by varying only one parameter, namely the fully bound shift and using the relation:-

$$K = \frac{R}{(1-R)(M_t - RL_t)}$$

where $R = M_t/L_t$

M_t is the total metal concentration

L_t is the total ligand concentration.

Hence values of K were calculated for each point, and the fully bound shift was varied to obtain the best least squares fit with the theoretical curve calculated for the fully bound shift and the average value of K. This process is repeated for successive cycles of the program until the changes in average K for successive cycles is less than a certain percentage amount. At this point the program moves on to its second section.

In the second section of the program the approximate values of the binding constant and the fully bound shift are used as the starting values for the proper least squares fit in which the binding constant and the fully bound shift are varied independently of each other.(This is a lengthy process which is why it is useful to use a short-cut procedure to obtain approximate starting values.) Hence the program varies first one variable and then the other to obtain the best least-squares fit, and will continue for as long as desired. When the final best fit binding constant and fully bound shift have been obtained the program prints out the theoretical observed shifts which may then be compared with the experimental values.

The actual equation determining the observed shift is given in chapter 5. The program does of course assume simple 1:1 binding.

The program itself uses standard programming techniques and since it is rather lengthy (some 800 steps) it will not be listed.

Chapter 6

F50 relaxation results.

These were determined with a F50 concentration of 15 mM and at pH* ca 1 . The results for the relative distances of the peaks are given below :-

Distance	T_{2M}^{-1}	Residues/peaks	
0.8	380	PHO NH, αCH, 'GLN' 18 αCH	
		PHO β' CH_2	
		PHO β CH_2	
1.0	100	PHO aromatic ortho-	
		PHO aromatic meta-	
1.5	40	PHO aromatic para-	7,800,7.779
		2.65 - 2.195	
		3.761 -3.693	
1.75	3.5	VAL 14 methyl	
		PRO 13 αCH	
		LEU 11 methyl	
2.0	1.5	GLY 10 $\alpha$$CH_2$, ALA αCH	
		VAL 8 methyl	
v large	v small	LEA 0 NH, methyl 'acetyl'	PRO 1 αCH

The assignments of the peaks are assumed to be the same as those of F30, though some of them have been confirmed, eg all the PHO assignments and those of the N-terminal blocking group.