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Edited by

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PREFACE

The current extent of applications of NMR spectroscopy to molecular problems is indicated by the diversity of the reviews presented in this volume. Dr. H. W. E. Rattle reports on NMR of amino acids, peptides, and proteins, which brings his account in Volume 11A up to date.

It is a pleasure to welcome Dr. B. Wrackmeyer as a contributor to Annual Reports on NMR Spectroscopy. He has reviewed the field of ^{119}Sn-NMR parameters, a subject which was previously covered, *inter alia*, in Volume 8 of this series. Rotational processes involving N–X bonds are dealt with by Professors G. J. and M. L. Martin and Dr. X. Y. Sun, who are also newcomers to this series. The present account serves to extend that by Dr. I. O. Sutherland in Volume 4.

Finally, Professor W. McFarlane and Dr. D. S. Rycroft report on multiple magnetic resonance, which follows on from their previous reviews, the most recent of which appeared in Volume 9.

It is a great pleasure for me to be able to express my thanks to all of the contributors for the careful preparation of their manuscripts. Their efforts contribute significantly to the continuing success of Annual Reports on NMR Spectroscopy.

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England

G. A. WEBB
May 1984

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NMR Studies of Amino Acids, Peptides, and Proteins: A Brief Review, 1980–1982

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I. INTRODUCTION

The three years 1980–1982 were marked by a steady advance in NMR methods, improving the effectiveness of the technique particularly for the study of proteins. The improvements were principally in magnets, with 500- and even 600-MHz instruments now available for ^{1}H-NMR, probes, where signal-to-noise ratio has been slowly but steadily improved, and to a very great extent in the computation facility dedicated to each machine. Large core memories and fully interactive use of disk storage have not only made instruments more efficient in the use of time (accumulation of data and processing carried out simultaneously) but have also permitted the development of two-dimensional methods as outlined in Section II. The result of all these advances is that we are getting closer to the day when full secondary and tertiary structure analyses of small protein molecules in solution will become possible using NMR methods, with gradual extension of the method to larger molecules. Superconducting magnet technology may have reached a plateau at a corresponding ^{1}H frequency of about 600 MHz, but it will take some years to fully exploit that field and to explore the possibilities of the many new sample preparation and data analysis techniques being reported. The "eternally rosy future" of NMR is really here already.

II. ADVANCES IN NMR METHODS

The long-standing problem of peak assignment in NMR spectra takes a step toward solution, at least for smaller proteins, in the development of two-dimensional NMR spectroscopy originally proposed by Jeener. A sequence of four papers[1] presents the first examples of the full assignment of a protein ^{1}H-NMR spectrum using these methods, together with an experimental strategy which may ultimately lead to full three-dimensional structures of smaller proteins in solution. A multipulse experiment is performed, in which the sample is subjected to a pulse sequence of the general form (90° pulse) (evolution time t_1) (90° pulse) (data acquisition time t_2). Data are collected as a function of t_2, but do of course depend on the value of t_1, and if a number (several hundreds) of experiments are performed, each for a different value of t_1, a matrix of data points is obtained, each point being a function of both t_1 and t_2. This matrix is Fourier transformed twice, along first the t_2 and then the t_1 direction, to yield a new matrix which may be presented as a square "contour map" in which the normal spectrum appears along the diagonal. Any intensity away from this diagonal reveals a "connectivity" between two of the resonances on the diagonal. Such a connectivity might, for example, be

the spin coupling between adjacent NH and CH protons along the peptide backbone. Starting from one known resonance, peaks may thus be assigned one by one along the entire backbone. Side-chain peaks may be assigned in the same way, ultimately leading to a full assignment of the spectrum. Spectra of this type are known by the acronym COSY (for correlated spectroscopy). An extension of this method, in which a third 90° pulse is inserted midway between the others, leads to NOESY, in which the connectivities revealed are due to the across-space nuclear Overhauser effect (NOE) between nuclei that are in close spatial proximity to one another. The majority of protons which satisfy this condition are on the same or contiguous residues; since the effect is distance sensitive, estimates may be obtained of the distances between the α-CH proton of the *i*th residue and the backbone NH of the $(i + 1)$th, between backbone NH protons of adjacent residues, and between the α-CH or β-CH of residue *i* and the NH of residue $(i + 1)$. These distances, in sets of three, are entirely equivalent to the Ramachandran angles ϕ, χ, and ψ, thus opening the possibility of an entirely NMR-based structural study of protein molecules in solution, at least for molecules of up to 60 or 70 residues which maintain a stable conformation. Examples are given[2,3] of the application of these methods to the basic pancreatic trypsin inhibitor (58 residues) in free solution and to the 29-residue peptide hormone glucagon in its membrane-bound form (Fig. 1). Further information about the methods employed and preliminary experiments is available.[4–9]

A discussion has been presented of the correlation between the stability and internal mobility of a protein, viewed as being (in solution) a dynamic ensemble of rapidly interconverting structures,[9] backed by a study of the rotational motion of buried ring structures in proteins measured as a function of applied hydrostatic pressure. Large activation volumes are observed, implying that ring flipping occurs in the unoccupied volume provided by fluctuations of the overall protein conformation.[10] Further information on internal motion in proteins may be obtained using the fact that peak intensities are affected by the application of off-resonance radio frequency (rf) fields, and that the effect is related to an induced relaxation rate which complements the usual $1/T_1$, line width, and NOE data in internal motion determination.[11] If the system under investigation is an enzyme activated by both monovalent and divalent cations, a new method for interion distance determination using relaxation effects has been described.[12] The divalent cation is replaced by a paramagnetic ion, and the resultant paramagnetic effect on the longitudinal relaxation of the monovalent ion is measured separately for two isotopes of the monovalent ion. Suitable monovalent ion pairs are $^{6}Li^{+}$ and $^{7}Li^{+}$, $^{14}NH^{+}$ and $^{15}NH^{+}$, and $^{85}Rb^{+}$ and $^{87}Rb^{+}$. Application of the Solomon–Bloembergen equation leads to unambiguous distance data.

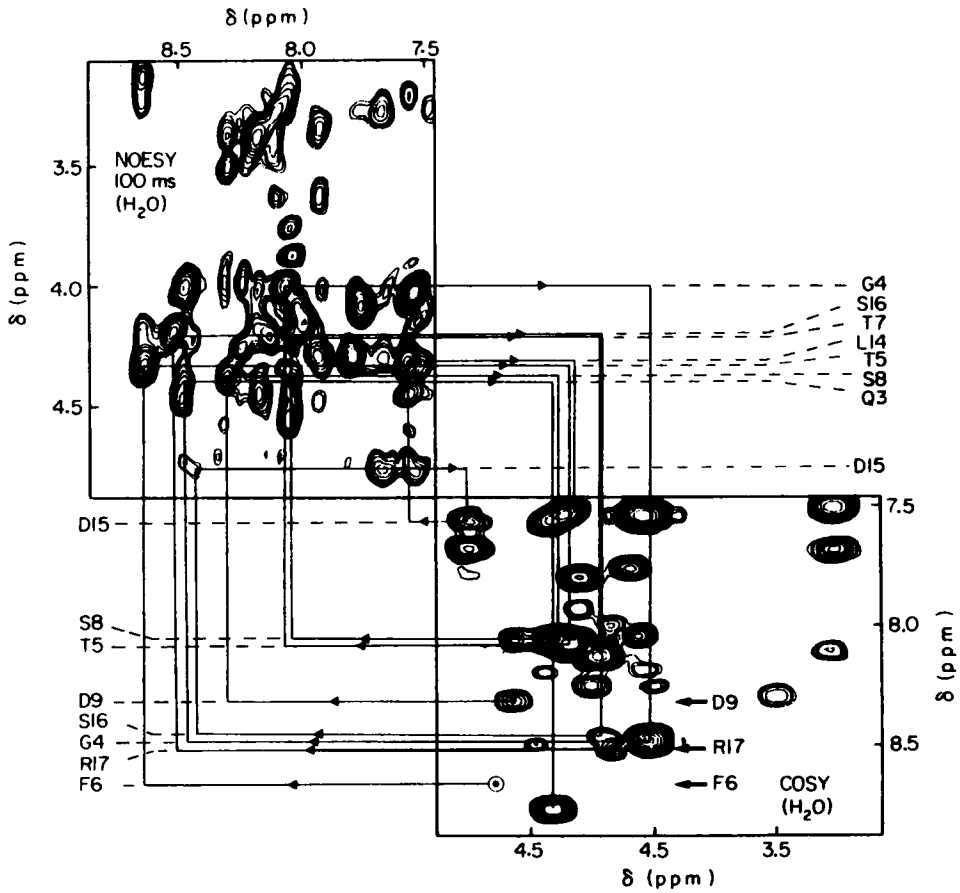

FIG. 1. Part of a two-dimensional spectrum of glucagon bound to perdeuterated dodecylphosphocholine micelles, produced by combining results from both COSY and NOESY experiments. The "normal" spectrum, not shown, would lie along the diagonal from bottom left to top right. Off-diagonal peaks above the diagonal arise from NOE effects between NH_{i+1} and α-CH_i; peaks below the diagonal arise from normal spin-coupling effects. The straight lines and arrows indicate the sequential resonance assignments obtained for residues 3–6, 7–9, and 14–17. From reference 3.

Theoretical calculations have always played an important role in the interpretation of NMR spectra, and are steadily becoming more sophisticated and more valuable. The application of ring-current calculations,[13] theories and techniques for studying the internal dynamics of proteins,[14] and the theory and applications of the transferred NOE for the study of small ligands bound to proteins[15] have been reviewed. The development of two-

dimensional NMR spectroscopy has brought the term "connectivity" into our vocabulary; connectivities between amide and α-protons in peptides and proteins may be established by selective population transfer in combination with the Redfield (2–1–4–1–2) pulse sequence.[16] Two-dimensional correlated NMR spectroscopy may be used for the unequivocal assignment of histidine residues.[17] The "normal" protein ^1H spectrum may be simplified by a related technique, in which the summation of spectra obtained with different spin-echo delay times eliminates signals from all even multiplets and collapses odd multiplets such as triplets into single lines.[18] Of course, when J values are accessible, they are very valuable in the analysis of protein spectra. Recent papers investigate the limiting couplings for side-chain rotamers,[19] the conformational dependence of the vicinal proton coupling for the α-C–β-C bond in peptides,[20] and the importance of solvent interactions on the values of the five-bond [H α-C(O) N α-CH] coupling in the peptide moiety.[21]

Another new technique is used for the assignment of NMR signals in an 18-residue neurotoxin according to the position of the amino acids in the sequence.[22] Heteronuclear decoupling of the natural-abundance carbonyl ^{13}C and the α-proton of adjacent residues is employed, with additional irradiation to suppress interactions of the carbonyl ^{13}C with protons of the same residue. The rather difficult task of assigning backbone amide proton resonances of small proteins has been approached[23] by decoupling them from α-CH resonances while exchange for deuterons is taking place; the authors term this "on-the-fly" decoupling (Fig. 2). The well-known reluctance of hydrogen-bonded peptide NH hydrogen to exchange for deuterium in D_2O solution may be of additional use here, but makes it all the more surprising that[24] hydrogen-bonded NH exchanges much more readily with chlorine than do solvent-exposed NH groups.[25]

Methods continue to advance in other areas of protein NMR as well, of course. The synthetic introduction of ^{17}O into various sites in oxytocin[26] and of deuterium into the egg white proteins of Japanese quail[27] is described: > 80% incorporation of selected deuterated amino acids into lysozyme is achieved using a synthetic diet. A $^{13}C/^{15}N$ double-label method has been used to estimate a protein half-life of some 30 days in soybean leaves,[28] while a simple multinuclear multipulse technique[29] is described which enables the collection of the spectrum of only those protons which are directly bonded to ^{13}C atoms in ^{13}C-enriched samples. A review has been given of high-resolution solid-state ^{13}C NMR in biopolymers (including proteins and whole viruses) using magic-angle spinning.[30] The transfer-of-saturation method is of increasing importance; theoretical calculations of the effects to be expected in a three-site exchange situation are presented.[31]

Among other new techniques reported we may note a method for the quantitative determination of the total protein content of natural products

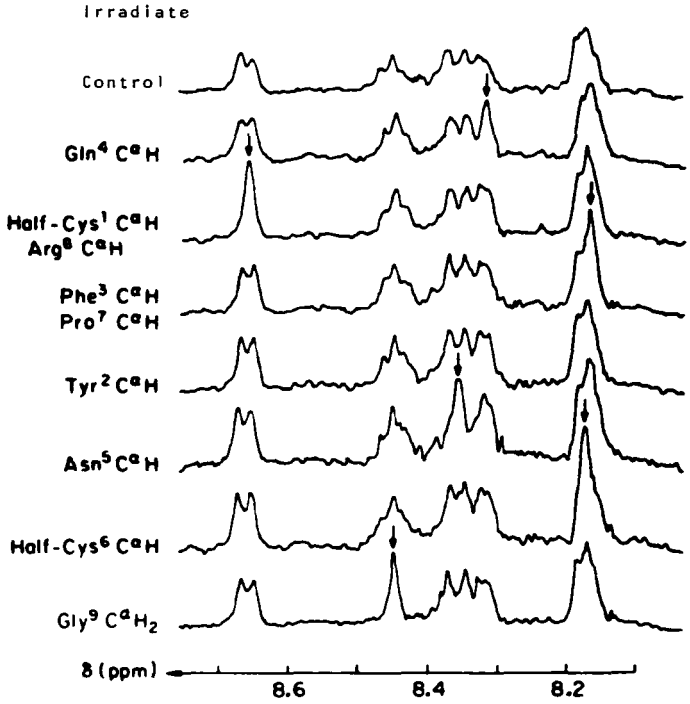

FIG. 2. Assignment of the NH protons in the 360-MHz spectrum of arginine vasopressin by spin decoupling, with irradiation at the resonances indicated on the left. Arrows denote multiplet collapse. As all the protons are exchangeable, the entire data set for the spectra was collected within 3 minutes, using a concentrated solution. From reference 23.

using a copper relaxation reagent[32] and (rather the opposite) the suppression of the total haemoglobin spectrum in ^{1}H-NMR spectroscopy of intact erythrocytes by using selective transfer of saturation by spin diffusion, in order to reveal the spectra of other components of the system.[33] A new possibility for the study of enzyme mechanisms involving phosphorus is opened by confirmation that for most phosphate derivatives of biochemical interest, a broadening effect due to the presence of a neighbouring ^{17}O nucleus is detectable. This effect can be combined with direct ^{17}O-NMR measurements to study the interaction of diamagnetic enzyme-bound metal ions with nucleotides.[34]

Workers engaged in labeling studies may also be interested in a strategy for uniform ^{15}N labeling of both nucleic acids and proteins for subsequent solid-state NMR,[35] and in a paper on the use of special strains of *Escherichia coli* to produce specifically ^{13}C-labeled amino acids for subsequent biosynthetic

incorporation into proteins.[36] The characteristics of ^{13}C-labeled peptides have been discussed[37] with particular reference to the relation between information content and labeling pattern.

III. AMINO ACIDS AND SYNTHETIC PEPTIDES

A. Amino acids

As always, the mainstream of amino acid studies concentrates on their use as simple systems for the testing of new techniques or theories. Cross-relaxation effects in the photochemically induced dynamic nuclear polarisation (photo-CIDNP) spectra of *N*-acetyltyrosine and *N*-acetyltryptophan have been used, for example,[38] to assess the possibilities for observing population transfer between amino acids in proteins. Trials of the methods and the effects of isotopic labeling have been reported using deuterium in phenylalanine[39] and ^{17}O in glycine, alanine, glutamic acid, and aspartic acid[40] while the more familiar ^{13}C labeling, this time biosynthetically accomplished in *Spirulina maxima* and *Synechococcus cedrorum*, is shown to be neither random nor statistical.[41] Carbon–carbon couplings are reported for labeled tryptophan[42] and ^{13}C–^{15}N vicinal couplings for a number of other amino acids.[43] The further development of ^{15}N labeling as a usable technique is also exemplified in studies of the stereospecificity of the polymerisation of DL-leucine and α-OMe-DL-glutamic acid anhydrides[44] and of the acid–base and tautomeric equilibria in solid histidine.[45] Even closer to our ultimate biological goals is the use of ^{15}N relaxation times and NOE data to probe the intracellular environment in intact *Neurospora crassa*, yielding microviscosity data unobtainable by any other technique.[46] More conventional conformational studies have been reported for 5-adenosyl-L-homocysteine[47] and for the 5-cis and 5-trans isomerism in a number of acylproline analogues.[48] The use of relaxation times of ^{13}C nuclei as a probe of proline ring conformations has been discussed.[49] High salt solvent conditions can induce conformational changes in aspartate, stabilising the conformers with gauche carboxylates at the expense of trans conformers.[50]

The use of a UV excimer laser will enable a number of new photoreagents to be used in chemically induced dynamic nuclear polarisation (CIDNP) experiments, and has been tested using solutions of histidine, tyrosine, and tryptophan,[51] while more normally excited CIDNP measurements on tryptophan[52] reveal details of the unpaired spin-density distribution in the Trp radical cation. Analysis of coupling data, NOE data, and lanthanide perturbations reveals no less than six conformers in solutions of DL-tryptophan,[53] while the solvent dependence of tyrosine and tryptophan side

chain conformations has been discussed.[54] Detailed studies of the interactions of aqueous lanthanide ions with various amino acids are discussed,[55,56] while the modes of binding of Ca^{2+} and Mg^{2+} to aspartic acid and asparagine are also covered[57]; both cations interact with the carboxyl groups of the amino acids, but only Mg^{2+} binds to the amino group.

B. Synthetic polypeptides

Polypeptides are no longer the vital protein models they once were; however, they can still prove useful in the study of some aspects of protein origin and behaviour. A comparison of poly(aspartic acid), prepared by common methods[58] and by thermal polycondensation,[59] reveals that the latter has β-peptide bonds in a mole fraction of about 0.8, which may have some significance in the study of protein evolution. The relaxation behaviour of poly(γ-benzyl-L-glutamate) shows some interesting features which can only be explained in terms of internal rotations about α-C—H and α-C—β-C bonds.[60,61] The relative stabilities of the poly(proline II) helix formed by poly[Gly–(Pro)$_n$], with $n = 3$ or 4, have been determined; the polypeptide is a model for a proline-rich human salivary protein.[62] Solid-state NMR techniques are used to determine conformation-dependent ^{13}C shifts in polyvaline, polyisoleucine, and polyleucine in the α-helical and β-sheet forms.[63] Analysis of the ^{13}C spectra of poly(aspartic acid) samples, prepared by hydrolysis of polysuccinimide under various conditions, reveals a random distribution of α- and β-bonds in all samples.[64] However, the stereoselectivity of polymerisation of DL-valine and DL-leucine monomers, also investigated by ^{13}C NMR, reveals the expected preference for isotactic sequences but with no isotactic block longer than six units.[65] Experiments with ^{15}N NMR[66] show that separate signals are detected from the central residue in each of the four possible triads L–L–L, L–D–L, L–L–D, and D–L–L. An interesting amphiphilic block copolypeptide, with hydrophilic termini and hydrophobic central block, alters the liquid crystal–gel phase transition in a deuterium-labeled dipalmitoylphosphatidylcholine membrane.[67]

C. Synthetic linear peptides

A review of structural studies of peptides, including many using NMR, may be found in the Proceedings of the 6th American Peptide Symposium.[68] The development of ^{15}N-NMR spectroscopy for peptide and protein studies continues, and some of the advantages of this relatively new probe into the peptide backbone are now becoming apparent. In proline-containing peptides, the ^{15}N nucleus is very sensitive to conformational changes induced by cis/trans isomerism of the proline. These effects are long range and depend on both the amino acid side chains and the solvent.[69] Strong neighbouring residue effects have been seen in random copolymers of Gly, Leu, and Val; the

spectra resemble a superposition of the corresponding binary copolymers.[70] In a series of tripeptides of the form Gly–Gly–L-X a combination of double resonance and difference NMR spectroscopy gives values for $^1J(^{15}N-^1H)$ and ^{15}N chemical shifts, though not yet sufficient for a systematic analysis of their behaviour.[71] Attempts to improve structure analysis using shift reagents on ^{15}N samples have not yet been entirely successful.[72] Solvent effects have proved to be more useful. An attack on the sensitivity problem for ^{15}N using NOE[73] and the INEPT (insensitive nuclei enhanced by polarisation transfer) pulse sequence, to transfer spin polarisation from amide protons to ^{15}N, produces an improvement over unenhanced spectra by factors of 8 for ^1H decoupled and 15 for ^1H coupled spectra, a very worthwhile improvement.[74–76]

Other studies involving small synthetic peptides include a series[77–79] on the binding of various divalent cations to the tripeptide Asp–Ala–His–*N*–methylamide, the N terminus of the human serum albumin molecule, with clear evidence for metal coordination in each case. Another study[80] involves a combination of transfer of saturation and selective saturation recovery methods to estimate amine H exchange rates, and hence to some extent conformational mobility, in a pentapeptide that represents the active fragment of thymopoietin. The pentapeptide is found to be in a very mobile conformational equilibrium between several conformations.

The stereoselectivity of oligopeptide syntheses can be slightly affected by the solvent and activating agents. This effect is shown in the formation of di- and tripeptides.[81] Conformational and dynamic studies of Ala–Trp and Gly–His show that their internal motions are slow compared to overall tumbling[82] while a type II β-turn is detected in Me_3 CCO–Pro–Aib–NHMe (Aib = aminoisobutyric acid) in solution[83] in line with X-ray studies of the crystalline form. Comparison has also been made between the crystal and solution conformations of (Ac-Asp–α-Abu) (Abu = aminobutyric acid).[84] The N-terminal tripeptide of human serum albumin (HSA), Asp–Ala–His, shows a marked preference for binding to Zn(II) rather than to Pr(III).[85] This may or may not account for the ability of HSA to bind transition metal ions in the presence of Ca^{2+}.

A number of one-bond α-^{13}C—H couplings for amino acids and small peptides, with variations of substituents and pH, are presented for use in spectrum prediction and assignment.[86] Conformational studies are also reported for histidine-containing peptides,[87] Ac–Ala–Ala–NHMe,[88] and the methylamides of the four lysine and/or tyrosine dipeptides.[89] An NMR investigation has been presented of the racemisation of benzoyl dipeptide methyl esters.[90]

An interesting example of a β-turn locked by a salt bridge has been presented.[91] The peptide is Boc–Arg–Ala–Gly–Glu–NHEt (Boc = butoxycarbonyl) and the Arg–Glu hydrogen bonds forming the β-turn are considerably reinforced by the Arg^+–Glu^- interaction (Fig. 3).