NUCLEAR MAGNETIC RESONANCE AS A STRUCTURAL METHOD IN MOLECULAR BIOLOGY

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ABSTRACT

The discovery of nuclear magnetic resonance by F. Bloch and E. Purcell has revolutionized the field of structural chemistry. The field of molecular biology has also benefited greatly from NMR technology. In this paper, we review the contributions of this technology to the understanding of the structure and dynamics of proteins, nucleic acids, lipids, and membranes.

I. INTRODUCTION

Less than a decade after Felix Bloch's and Edward Purcell's discovery of Nuclear Magnetic Resonance, two reports appeared, one by Shaw and Elksen[1] describing its use to measure hydration of serum albumin and starch, the other by Jacobson, Anderson, and Arnold[2] examining the hydration of DNA. Although it is doubtful that the experiments actually determined the amount of water bound to protein and DNA, the reports aroused interest in the possibilities of using the new method to answer biological questions. In that sense, they inaugurated the era of biological applications of NMR, a field that now, a quarter of a century later, has its own bibliography numbering in the thousands of entries.

A brief essay cannot do justice to the many types of experiments that have been reported. Yet the time seems ripe to pose the question: What sig-

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nificant problems in molecular biology have been or can be solved by NMR?

Through the years and to this date, much of the work in the field is still best described as the pure joy of spectroscopy—the fascination with NMR signals from complex structures, focusing on attempts to find new ways of improving resolution, sensitivity, and assignment of the hundreds of distinct resonances seen in biological objects. These efforts have advanced NMR technology to a very high degree of sophistication, so that the weak signals in scarce biological materials can be detected with ease and their clustered overlapping resonances more or less readily displayed in their individuality. Perhaps more important is that hand in hand with these efforts has gone a search for the significant problems of contemporary biology onto which this wealth of information could be brought to bear. Was NMR belatedly to confirm facts already known? Was it to supplant existing methods in some areas because of its greater convenience or efficiency? Was it to open to investigation new biological questions not accessible by any other means? Not all the answers are clear, but a sufficient number are, so that we can examine the role of NMR in biological research, not from the point of view of new spectroscopic skill that is required, but in the light of its contributions to the generation of new knowledge about biological systems. Emphasis here is therefore placed on those studies in which NMR has provided direct evidence bearing on the solution of important problems in molecular biology.

II. APPLICABILITY OF NMR TO MOLECULAR BIOLOGY: HISTORY, SCOPE, AND LIMITS

The majority of biological problems to which NMR has been successfully applied concern either (a) the structure or (b) the dynamics of the constituents of living systems; (c) the nature of the interactions between them or (d) the rates and mechanisms of the chemical transformations they carry out.

The first successful attempt to observe a biological macromolecule by NMR was made in 1957 by Saunders, Wishnia, and Kirkwood,[3] who reported a 40 MHz spectrum of the enzyme ribonuclease. It was shown in the same year by Jardetzky and Jardetzky[4] that the spectrum accurately reflected the amino acid composition of the protein, but neither resolution nor sensitivity was adequate to obtain any additional information. Also in 1957, Davidson and Gold[5] first pointed out that the role of paramagnetic ions bound to macromolecules could be studied by their effects on water relaxation, and showed that the Fe^{++} in hemoglobin was effectively inaccessible to solvent.

Early spectrometers permitted no more than crude observations on larger molecules. The earliest biological NMR literature therefore focused

on three types of problems: (a) the structure and solution conformation of small molecular weight constituents of living systems, notably amino acids,[6] nucleotides,[7] and ATP-metal complexes[8]; (b) the structure of ligand-macromolecule complexes, insofar as it could be inferred from the observation of ligand spectra[9]; and (c) the use of paramagnetic probes for studying binding sites on DNA[10] and the active sites of metal-requiring enzymes.[11] An interesting early determination of the stereospecificity of an enzymatic reaction by NMR was also reported.[12] Still, a few novel insights foreshadowing a host of later findings could be derived even from the primitive observations. Thus, for example, a comparison of the spectra of native and unfolded proteins[13,14] and the study of polystyrene and poly-γ-benzylglutamate[14] led to two important conclusions[15]: (a) the existence of internal motions in proteins and (b) the dependence of the resonance positions in protein spectra on the internal organization of the protein. The latter is a fortunate quirk of nature, without which NMR would have been all but useless for the study of macromolecules.

Serious NMR spectroscopy of biological structures became possible in 1963, with the introduction of time-averaging for the collection of spectra by Jardetzky, Wade, and Fischer[16] and Klein and Barton.[17] The CW method used initially (CAT NMR) became obsolete by 1969 with the advent of Fourier transform NMR spectrometers largely through the efforts of R. R. Ernst.[18] Nevertheless, it remained in use long enough to allow the completion of several key studies. Progress was also greatly aided by the development of high field superconducting magnets. The first Varian instrument operating at 220 MHz was used for the investigation of proteins by W. D. Phillips and C. C. McDonald at DuPont in 1965,[19] and there has been a steady climb to higher fields since—to 270 MHz at Oxford in 1971, 300 MHz at Varian in 1972, 360 MHz at Stanford in 1974, 470 MHz at Oxford in 1978, and 600 MHz at Carnegie-Mellon in 1979. In addition to the general advances of chemical NMR instrumentation, many other innovations of specific interest to biological work have contributed to the rapid growth of the field. To cite but a few: biosynthetic selective isotopic labeling for the simplification of protein spectra[20]; natural abundance ^{13}C spectroscopy of proteins[21,22]; incorporation of ^{19}F into proteins[23,24]; the use of paramagnetic shift reagents for assignments and determination of distances[25,26]; the use of spin labels,[27,28] and photochemically induced dynamic nuclear polarization (CIDNP)[29,30] for the same purposes; deconvolution techniques[31,32]; two-dimensional Fourier transform spectroscopy (2DFT),[33,34] and solid state high resolution NMR spectroscopy by magic angle spinning[35,36] for resolution enhancement.

New knowledge, which has arisen from the beginnings summarized above, is most easily discussed under the separate headings of the major constituents of living systems. Each of these presents a distinct set of meth-

odological problems to the NMR spectroscopist when questions of their structure, dynamics, interactions, and function are raised: (1) biologically active small molecules, (2) proteins, (3) nucleic acids, (4) water, and (5) lipids and membranes or membrane models.

The extensive and valuable NMR literature on the structure, conformation, and dynamics of small molecules falls by convention more into the realm of chemistry than that of molecular biology, and will not be discussed. We will also omit the literature on water structure in the presence of polymers and in living tissues, because a resolution of the fundamental theoretical difficulties in the interpretation of experimental findings is not in sight.

Two new biological applications of NMR do not properly fall in our chosen domain of molecular biology, but deserve mention for their promise in physiology: NMR studies of metabolism in live tissues, pioneered by the Oxford group,[37] and Lauterbur's zeugmatography-NMR imaging of tissues and organs.[38]

III. STRUCTURE AND DYNAMICS OF PROTEINS

Problems in protein chemistry that have been successfully studied by NMR can be grouped into five categories: A. solution structure; B. behavior of individual residues; C. structure of binding sites; D. dynamics and internal motion; and E. conformational transitions and problems of protein folding.

A. Solution structure of proteins

Two questions are fundamental to our study of protein function: (1) Is the structure of a protein the same in solution as it is in the crystal? and (2) Is the structure of an isolated protein the same as it is when the protein is part of a functioning macromolecular assembly?

A wealth of circumstantial evidence, chemical and spectroscopic, supports the generally accepted affirmative answer to the first question. Yet there are observations that cast doubts on the universal validity of this answer: for example, the occasional ready accessibility to chemical reagents of residues located in the interior of a protein in the crystal structure. Much less is known about the second question. In the one case, of tobacco mosaic virus, where the structure of both the assembled virus and its building block, a double disc, has been determined, both significant similarities and significant differences are apparent.[39] The differences may well be due to the fact that viral RNA is present in the first and not in the second. But in general, the answer is unknown.

NMR is a method that permits a critical test and a definitive answer to both questions.

The basic reason for this is that the NMR spectrum of a protein reflects to only a minor extent its primary structure, i.e., the specific amino acid sequence, and to a large extent its folded (secondary and tertiary) structure.[40] The NMR spectrum of the unfolded protein can be closely approximated by the sum of the spectra of the constituent amino acids, whereas in the folded protein the resonances of chemically equivalent but positionally nonequivalent amino acids are shifted with respect to each other.

If a solution spectrum of a protein—containing dozens of individually resolved lines—is compared to an equally well-resolved solid state spectrum and the two are found to be identical, it can be argued that the protein structures in the two phases are identical. Conversely, spectroscopic differences can be taken to reflect differences in structure or dynamics or both. The probability that identical spectra in solution and in the solid could arise merely by accident is, roughly speaking, inversely proportional to the number of well-resolved lines in the spectra: The less resolved the spectrum, the less well-founded the conclusion.

Our first attempt to make such a comparison is shown in figure 1 for the enzyme lysozyme. The similarity of the two pairs of ^{13}C spectra is striking, but the resolution of the solid state spectrum (~ 7 Hz) is adequate only for a comparison of gross features. The nature of the discrepancies remains to be established, yet they are seen at once not to be large. The answer to the question that has been debated by protein chemists since the publication of the first protein structure (are the solution and the crystal structure identical?) therefore seems to be: almost. Similar experiments can be done with proteins in organized assemblies, except that the background arising from proteins other than the unit of interest would have to be blanked out by selective isotopic substitution.[20,40]

NMR has also provided an abundance of less direct evidence leading to the same conclusion. Essentially, four types of experiments have contributed to it: (1) The structure of substrate and inhibitor binding sites, insofar as it can be inferred from NMR data, is consistent with that found by x ray diffraction. Conversely, the assumption that the structure of an enzyme-inhibitor complex as determined by x ray diffraction also exists in solution accounts for the observed NMR parameters of the complex. This type of study is more fully discussed below (section III,C) and provides strong evidence that *at least* the binding sites and their immediate vicinity are the same in the two phases. (2) Anomalous chemical shifts of methyl groups in the solution NMR spectra of proteins, e.g., such as are observed in lysozyme and hemoglobin, can be explained by the proximity of the observed methyls to aromatic rings, assuming the known crystal structure of the protein. Very good agreement between the observed chemical shift and that calculated from ring anisotropy shielding has been obtained for lysozyme by McDonald and Phillips[41] and for several other proteins by others. (3) Experi-

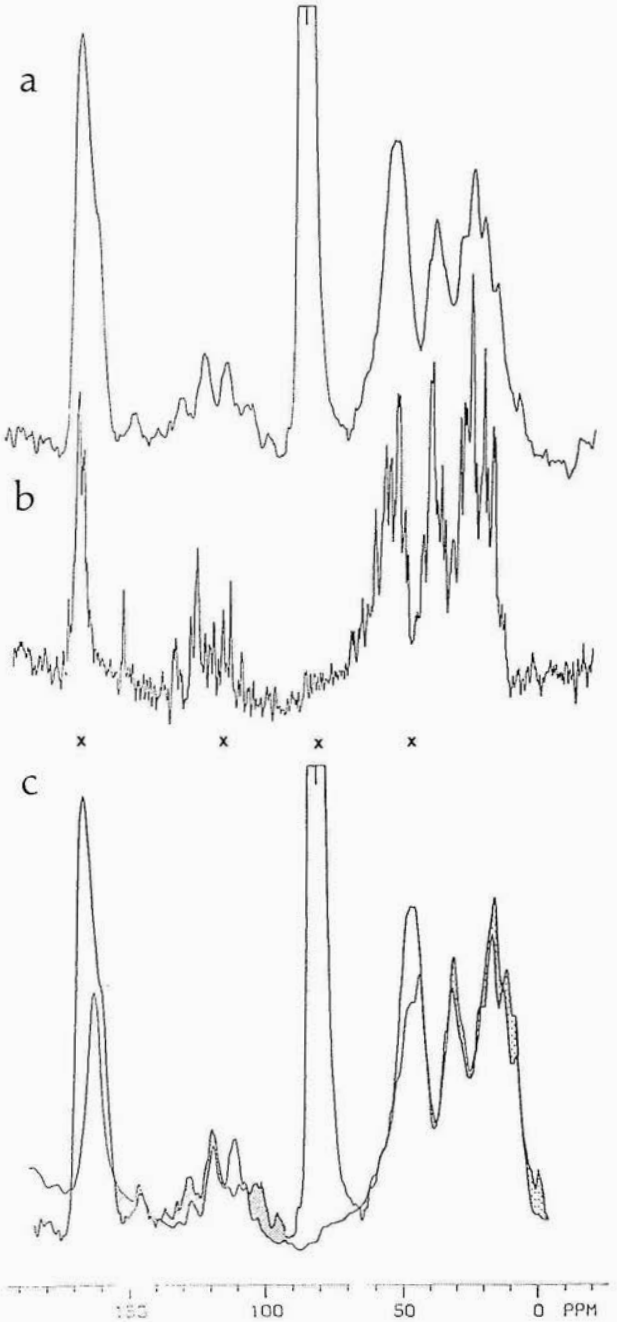

FIG. 1. ^{13}C NMR SPECTRA OF LYSOZYME AT 35.2 MHz. (a) Crystal powder spectrum with magic angle spinning, 25°C. (b) Aqueous solution (250 mg/ml, pH 7) spectrum, 25°C. (c) Aqueous solution spectrum with 200 Hz line broadening superimposed on solid state spectrum, 25°C. Dotted: difference spectrum. Dense dots: excess area in solid state spectrum. Sparse dots: excess area in solution spectrum. X: rotor peak (center) and side bands.

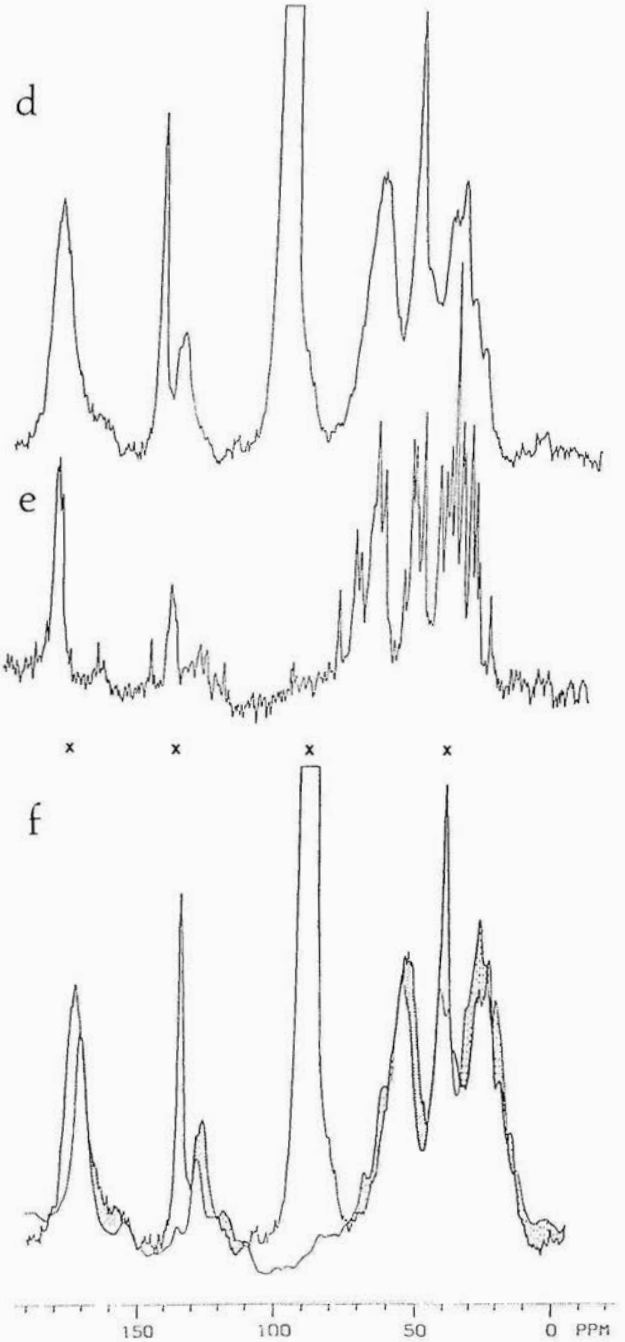

FIG. 1. (d) Crystal powder spectrum with magic angle spinning, 70°C. (e) Aqueous solution (250 mg/ml, pH 7) spectrum, 70°C. (f) Aqueous solution spectrum with 200 Hz line broadening superimposed on solid state spectrum, 70°C. Dotted: difference spectrum. Dense dots: excess area in solid state spectrum. Sparse dots: excess area in solution spectrum. X: rotor peak (center) and sidebands.

ments on spin labeled proteins, such as those of Morrisett, Wien, and McConnell on lysozyme[28] and Dwek et al. on myeloma proteins,[42] yield distances between the spin label at a known site and a proton at another known site (e.g., His 15 and Trp 123 in lysozyme) that are in good agreement with known crystallographic distances. (4) Perturbation of nuclear resonances in proteins by paramagnetic ions bound at known sites sometimes permits the calculation of a distance between the ion and the perturbed group. In a pioneering study, Navon, Shulman, Wyluda, and Yamane[43] calculated the distances in an inhibitor-Mn^{++}-carboxypeptidase complex. Many similar calculations for other enzymes are now recorded in the literature. A bold proposal for the complete quantitative determination of the structure of lysozyme in solution, using lanthanide ions, has been made by R. J. P. Williams and coworkers,[32] and the initial reports suggested that the distances between the lanthanide ion and the amino acid residues in its immediate vicinity were the same in the crystal and in solution. However, serious problems exist in the theoretical interpretation of perturbations of protein resonances by paramagnetic ions.[44,45] Not all the distances calculated by this method are in agreement with those obtained from crystal structure determinations.[46] It would be hazardous to assert on this basis that the solution and crystal structures are different. The discrepancies tend to increase with the distance between the ion and the perturbed group. This reflects not only the increasing error but also the increasing role of paramagnetic ions bound at secondary binding sites, which most proteins have in abundance. If this approach were to succeed completely, it would succeed for a metalloprotein with a unique strong metal binding site and no paramagnetic ions in solution. So far, where the condition of a dominant binding site is even approximately satisfied, the calculated short-range distances are fairly consistent with those seen in the crystal.

The question of the accessibility of exchangeable protons in proteins to solvent is a special aspect of the problem of the solution structure. In a rigid structure or in hydrogen-bonded segments, NH and OH protons in the interior would be essentially inaccessible to solvent; exchangeability would reflect exposure to solvent, although the exchange rates are also known to depend on the character of the X-H bonds involved. Slow exchange of some protons in proteins with solvent protons has been known since the classical isotope experiments of Linderstrøm-Lang in the early 1950s, but NMR has proven to be a particularly easy and convenient method for the study of this question. Glickson, McDonald, and Phillips[47] were the first to report this type of experiment on the NH resonances of tryptophan in lysozyme, and exchangeable protons have since been studied in ribonuclease, chymotrypsin, heme proteins, azurin, and others.[48,49] Assignments of specific Trp and imidazole NH resonances have been made in lysozyme, ribonuclease, and azurin. The essential conclusion emerging from such studies on proteins

whose crystal structure is known is that there are marked similarities between the structures in solution and in the crystal. In the case of the copper protein azurin, a cage formed by the peptide segment Tyr 108-His 117 around the Cu^{++} ion making the ion inaccessible to solvent has been proposed by Ugurbil and Bersohn[49]; it remains to be established whether the proposal, based entirely on NMR data, correctly predicts part of the crystal structure.

B. Behavior of individual residues

A unique feature of high resolution NMR of proteins is that it permits the observation of resonances from individual amino acid residues and thus the study of the behavior of these residues for many different purposes. Other spectroscopic methods will occasionally allow the observation of individual residues—for example, in a protein containing a single tryptophan, the specific tryptophan residue and its environment can be studied by fluorescence. But in general, spectra of positionally different, chemically identical residues cannot be resolved, and non-aromatic residues cannot be seen at all, as they can be by NMR. This, in principle, will permit the identification of an individual residue—or constellation of residues—as, for example, the trigger of a functionally significant conformational change, much in the same way as it has permitted the identification of specific residues as part of a binding site (see section III,C). Although no specific instance of such an identification has been reported, it doubtless will be in the near future. At the same time, the feasibility of simultaneously observing resonances from many residues opens the possibilities of a very detailed description of binding interactions and structural transitions. The mechanisms of protein function that will emerge from such descriptions rank for the present among the major unsolved problems of molecular biology.

Although this ultimate use of the observation of resonances from individual amino acid residues in proteins has not yet been achieved for a variety of technical reasons, several more modest but unique contributions to the understanding of protein structure have resulted from it. Among these, one can name (1) identification of titrating groups; (2) determination of microscopic pK values; (3) identification of points of contact; and (4) identification of N- and C-terminal residues.

The prototype study of individual resonances was reported in 1967 by Meadows, Markley, Cohen, and Jardetzky,[50] following up on a report by Bradbury and Scheraga,[51] and involved the determination of the microscopic pK values of the four histidine residues of pancreatic ribonuclease A. Since all four histidines could be assigned by chemical modification and isotopic replacement, identification of individual pK values with specific residues in the sequence became possible. Extensive subsequent work (cited in references 52 and 53) has shown that the assignments were valid for the

EDTA-containing enzyme used in the original study, but otherwise the assignments of the active site His 12 and His 119 were to be reversed.[52,53] This underscored the extraordinary sensitivity of NMR line positions in proteins to the presence of ligands, but the usefulness of the method for the examination of individual resonances remained established. Many studies of histidine residues in proteins have been reported since.

Individual tyrosine resonances have been resolved and at least partially assigned in staphylococcal nuclease,[54] ribonuclease, lysozyme,[45] bovine pancreatic trypsin inhibitor,[55] the N-terminal peptide of the *lac*-repressor, ferricytochrome C and several other proteins. Titration of tyrosine residues is frequently associated with the denaturation of the protein, since the tyrosine pK values fall in the pH range of 10-11. For this reason, microscopic pK values of tyrosines are of lesser interest for studies of enzyme mechanisms than those of the histidines. Tyrosine residues have, on the other hand, attracted attention as frequent constituents of binding sites and indicators of internal mobility, and more will be said about them in these contexts.

Individual tryptophan resonances in the ^{1}H spectra of lysozyme have been assigned by Campbell et al.,[46] and the corresponding resonances in the ^{13}C spectra by Allerhand and his colleagues.[56] The most interesting result of such assignments was the detection of an interaction between Trp 180 and Glu 35, with the spatial relationship between the two residues being altered by the ionization of the carboxyl of Glu 35. The proximity of two tryptophan residues in dihydrofolate reductase had been demonstrated by ^{19}F NMR before the crystal structure of the enzyme became known.[57]

A study of the ^{13}C resonances of the carboxyl group of Glu 81 and the guanido group of Arg 75 in the muscle calcium binding protein parvalbumin led to the conclusion that the two groups formed a salt bridge in the native protein structure.[58]

Identification of N- and C-terminal residues by titration of their NMR resonances has also been repeatedly reported.[40]

The study of individual residues is often difficult, especially in proteins of larger molecular weight, because of the extensive overlap of spectral lines. The difficulty can be largely overcome by a combination of selective isotopic substitution and the use of high frequency spectrometers. Experiments of this type were first done by complete biosynthetic deuteration of the protein, except for the specific amino acid residues to be observed by ^{1}H NMR. The protonated residues were leucine and the protein phycocyanin in the work of Crespi, Rosenberg, and Katz,[59] and tyrosine and tryptophan in staphylococcal nuclease in the study by Markley, Putter, and Jardetzky.[54,60] The power of this approach for simplifying the NMR spectra of macromolecules was clearly established in both studies. In addition, the fortunate choice of tyrosine as the selectively protonated amino acid in our study

provided detailed information on the structure of the binding site in staphylococcal nuclease and on the probable folding pathway of the enzyme. The approach was subsequently extended to the selective ^{13}C-^{2}H labeling of histidines in tryptophan synthetase and aspartic transcarbamylase,[61] and to the biosynthetic ^{15}N labeling of hemoglobin.[62]

The full potential of NMR in conjunction with selective isotopic labeling for the study of biological macromolecules has not yet been realized, mainly for reasons of cost. Yet there is little doubt that the combination of these techniques permits the solution of many structural and dynamic problems in molecular biology not otherwise accessible to experimental study.

C. Mapping of binding sites

NMR is a powerful method for the determination of the structure of ligand binding sites in enzymes and other proteins. This is a natural consequence of the fact that individual resonances can be observed for different groups on both the ligand and the protein, and that the spectral parameters of these resonances are very sensitive to interactional effects. Nearly half of the entire literature on protein NMR published in the past decade addresses itself to this problem in a wide variety of proteins.

The first NMR study of the structure of binding sites observing ^{1}H resonances of both the protein and the ligand was that of Meadows, Roberts, and Jardetzky in 1969[63] on the active site of pancreatic ribonuclease A. In this study, information on the geometry of the active site, available from x ray crystallography, was combined with information on chemical shift changes in the spectra of the active site histidines and of the three inhibitors, 2′CMP, 3′CMP, and 5′CMP, to deduce the structure of the enzyme-inhibitor complexes. Differences in the conformations of the inhibitors at the active site were inferred from spectroscopic data, providing an example of "induced fit" in the inhibitor. The consistency of the observed spectroscopic changes with those predicted from the crystal structure of the complex also led to the conclusion that the structures of the active site were identical in the crystal and in solution.

The extensive use of crystallographic information in that study, as well as in many other studies of proteins by NMR (cited in reference 64), has tended to obscure the important fact that interpretation of NMR data in terms of the structure of protein-ligand complexes does not depend on the knowledge of the crystal structure. It is merely simplified by it. The internal consistency of the spectral changes on a series of inhibitors bound to the same site is often sufficient to define the points of contact. This was first demonstrated in the study of the binding of the inhibitor 3′5′ thymidine diphosphate (pdTp) to selectively deuterated staphylococcal nuclease by Markley and Jardetzky.[65]

Exact distances, on the other hand, are not readily obtained from

NMR experiments on diamagnetic species, except for groups strongly shielded by aromatic rings. In the study of ligand-protein complexes, this is a much less severe handicap than is generally recognized, because a large number of constraints is already given by the van der Waals radii of atoms. The correct geometry can often be found by model building from the knowledge of the matrix of pairwise interactions and the assumption of van der Waals contacts. In the case of the pdTp-staphylococcal nuclease complex, the qualitative arguments alone defined a sufficient number of constraints on the possible structure of the enzyme-inhibitor complex to allow only minor deviations of the solution structure from that found in the crystal. A different approach to the study of the same complex was taken by Furie et al.[66] Taking advantage of the fact that the active site of the enzyme contains a calcium ion, and substituting the lanthanide Gd(III) for it, they mapped out the distances from Gd to the pdTp protons in the complex by NMR relaxation measurements. The distances calculated from NMR were consistent with those determined by x ray diffraction. The structure of the complex is shown in figure 2.

An elegant use of NMR in combination with other spectroscopic techniques to define an antibody binding site was recently reported by Dwek et al.[42] High resolution NMR, paramagnetic probes, fluorescence transfer, chemical modification, and the assumption of the invariance of the immunoglobulin fold yielded a structure for the DNP-antibody complex that has not yet been determined by x ray diffraction. If solution and solid state structures of proteins prove generally similar, at least a partial prediction of a protein crystal structure from spectroscopic measurements should be possible. This study may provide a prime example.

Many other examples of the identification of individual residues and ligand groups in enzyme-inhibitor complexes by NMR could be cited, but detailed NMR studies mapping such sites are still relatively few.

In contrast, the use of paramagnetic centers to probe binding sites of enzymes by NMR enjoys continued popularity. It is difficult to assess what lasting contribution to molecular biology will ultimately result from this approach. Its two principal pitfalls, the multiplicity of ion binding sites in most cases, and the use of unverifiable assumptions in the theoretical analysis,[44,45] are well stated by Morris and Dwek[67]: "If there is to be a moral to this article, it is that the perturbations caused by paramagnetic ions on NMR parameters may be severe, and therefore easily measured. While measurement is easy, the interpretation is often difficult."

The reverse might be said of NMR studies on unperturbed diamagnetic protein-ligand systems.

D. Internal motions and protein dynamics

High Resolution NMR is the only method available to the molecular

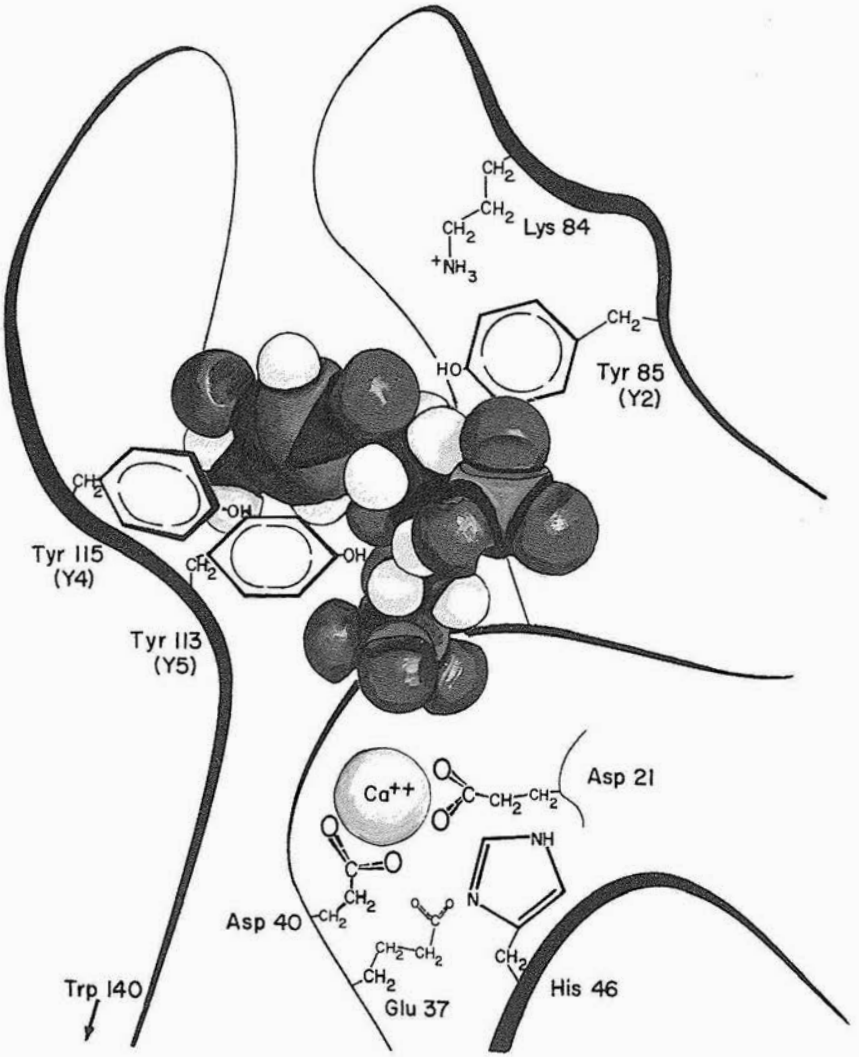

FIG. 2. STRUCTURE OF THE 3′,5′-pdTP-NUCLEASE COMPLEX, showing proximity relationships, which can be inferred from magnetic resonance data.[65,66]

biologist capable of providing detailed, accurate information on macromolecular dynamics. Very little is known about the internal dynamics of proteins, apart from the fact that structural rearrangements are important in their function. The motions by which such rearrangements occur, their extent, and their time scales are for the most part unexplored. Several spectroscopic methods—notably nanosecond fluorescence and depolarized

light scattering—have provided evidence for the existence of rapid internal motions and regions of varying flexibility in proteins. Yet the serious drawback of such methods is that they rest on the observation of only a few isolated chromophores and do not permit the reconstruction of a detailed picture of the internal dynamics of a macromolecule. The combination of the feasibility of observing a multitude of individual resonances and the dependence of NMR spectral parameters on the frequency and amplitude parameters of molecular motion makes NMR a method uniquely suitable for this purpose.

It cannot be said at the time of this writing that a molecular dynamic problem of interest in molecular biology has been completely solved by NMR—or by any other technique. This would require not merely the detection of internal motions, but a detailed understanding of their interrelationships and functional significance. Nevertheless, several fundamental developments have taken place that make the success of such an undertaking highly probable in the near future.

Three types of observations pertinent to protein dynamics have been made by NMR: (1) rotation of aromatic rings in the interior of well-defined protein structures; (2) a wide range of rates for amino acid side-chain rotations in different proteins; and (3) occurrence of unusual segmental mobility in some proteins.

The rotation of aromatic side-chains in protein structures is apparent from two types of NMR measurements: (a) averaging of chemical shifts and (b) deviation of the measured relaxation parameters from those predicted from a rigid sphere model.

In several proteins, aromatic resonances do not show a complex splitting pattern, which would be expected in the absence of internal rotation, but reflect motional averaging of chemical shifts. A detailed study of rotational averaging of tyrosine chemical shifts in the bovine pancreatic trypsin inhibitor has been reported.[55,68] Observations in ribonuclease, lysozyme, parvalbumins, and alkaline phosphatase[24] also indicate that ring rotation is a common phenomenon. The rate constant for this rotation has been estimated to be $\sim 10^4$ sec^{-1} from the temperature dependence of the multiplet collapse.[68] On the other hand, much faster rotational motions ($\sim 10^8$ sec^{-1}) have been found by ^{13}C relaxation for phenylalanine rings in parvalbumin.[58] Analysis of the relaxation data suggests that these are librations of relatively low amplitude that have a relatively low probability of exceeding 180° (required for chemical shift averaging).

Since the advent of Fourier transform NMR spectrometers, which make relaxation time measurements comparatively easy, a wealth of experimental relaxation data for both ^1H and ^{13}C spectra of proteins has been accumulated. Qualitatively, two facts may be considered as established: (1) The overall diffusional rotation of the protein rarely determines the ob-

served NMR parameters for side-chain resonances; (2) there are differences between relaxation rates of different side-chain groups that could be interpreted as differences in mobility. The quantitative interpretation of such measurements, however, represents a formidable theoretical problem, more fully discussed elsewhere.[69]

Most reported data on protein relaxation have been analyzed using simple motional models—a spinning top on a rigid sphere, or an ellipsoid of revolution. Order of magnitude estimates of correlation times can be made using such models, but for a detailed study of protein dynamics, they are too simple to do justice to the problem. A general theoretical formulation suitable for the analysis of NMR relaxation data on macromolecules with internal motions has only recently been developed by King and Jardetzky.[70] The formulation permits a rigorous definition of the limits of the analysis of relaxation data on systems undergoing complex motions, the systematic testing of specific motional models, and, as an alternative, the treatment of relaxation as an eigenvalue problem with semi-empirical eigenfunctions as amplitude parameters and eigenvectors as rate constants.[71] Analysis of NMR relaxation data on the bovine pancreatic trypsin inhibitor indicates that, on the time scale of motions that such data reflect, convergent solutions of the equations are possible. Thus, internal motions can be described in sufficient detail to permit an understanding of their contribution to protein function.[72,73]

Even a qualitative interpretation of differences in relaxation parameters between residues in different segments of a given protein has led to the discovery of segmental motions and regions of unusual flexibility in several proteins. E. M. Bradbury and colleagues have inferred the existence of random coil, globular, and mixed structures containing either a globular N-terminal half and a flexible C-terminal or a globular core with two "sticky" ends for several histones,[74] and have proposed that a local helix → coil transition involving residues 60-102 occurs in histone F2b. Jardetzky et al.[75] have described segmental flexibility in a region of the isolated coat protein of tobacco mosaic virus, which normally exists as an α-helix (the V-helix) in the assembled virus. Since the V-helix separates the viral RNA from the water in the interior of the virus cylinder, the probable functional role of this local helix → coil transition is to facilitate the embedding of the RNA in the interior of the protein in the course of virus assembly. Myosin (M.W. 450,000) and its high molecular weight fragments were found to contain highly flexible segments.[76] The DNA-binding N-terminal "headpiece" of the *lac*-repressor protein has been shown by NMR to be a distinct, highly mobile domain in the protein structure.[77]

A report on the use of NMR for the study of protein dynamics ten years hence may well contain some of the method's most significant contributions to molecular biology: The fact that internal motions occur in pro-

teins may have been obvious from NMR data for a long time, but the discovery of the diversity of such motions and the development of adequate experimental and theoretical methods for their systematic study are of a very recent origin.

E. Study of conformational transitions and the problem of protein folding

The detection of conformational changes in proteins and a description of their nature is one of the most important applications of protein NMR. Again, ribonuclease was probably the first protein for which a conformational equilibrium involving His 48 was described.[63] No study has yet appeared describing a protein conformational change in a degree of detail comparable to that found in the mapping of binding sites. The most detailed characterization of a conformational change attempted from NMR data is probably that in the calcium binding muscle protein parvalbumin.[58] When one of the two Ca^{++} ions chelated by the protein is removed from its binding site at Lys 96, the salt bridge between Glu 81 and Arg 75 is broken, and Tyr 2 becomes free to rotate. Both effects presumably result from a slight displacement of the E and F α-helices on the hydrophobic core. The slowness with which studies of this type are appearing in the literature reflects the still considerable difficulty of assigning individual resonances to specific amino acid residues in the amino acid sequence. Constituents of binding sites are usually more reactive and therefore more easily identified by any one of several chemical and physical techniques. The direct observation of individual resonances has nevertheless been used to detect an inhibitor-induced local conformational change involving Trp 62, 63 and Ile 98 in lysozyme,[45] as well as a temperature dependent transition involving Trp 108. Similar observations have been reported on ribonuclease and hemoglobin. Many more studies have used extrinsic nuclear spin or paramagnetic probes to detect conformational changes. Among the first was the work of Huestis and Raftery[23] on ribonuclease, lysozyme, and hemoglobin. Radda and his colleagues[78] have monitored structural changes in phosphorylase *b* by a combination of magnetic resonance and fluorescence methods. A less direct method—assessing solvent accessibility to a paramagnetic ion in the interior protein by the measurement of solvent relaxation—has suggested the existence of a conformational change in concanavalin A.[79] Many other suggestions implicating individual residues in conformational changes in different proteins have been published. More detailed descriptions of conformational transitions on the basis of NMR data can be expected as the techniques for resolving and assigning individual resonances improve.

The principal contribution NMR has made thus far to the thorny problem of protein folding is in the detection of kinetic and equilibrium intermediates or alternative structures, especially in ribonuclease and staphylo-

coccal nuclease. Subtle local pre-denaturation changes in ribonuclease have also been found. The major part of the problem, however, remains unsolved. The usefulness of NMR in its study has recently been discussed by Baldwin.[80]

IV. STRUCTURE AND DYNAMICS OF NUCLEIC ACIDS

Applications of high resolution NMR to the study of nucleic acids have been relatively fewer than applications to proteins, although a very large literature exists dealing with the conformation and solution interactions of mono- and oligonucleotides. Three kinds of problems have been investigated with considerable thoroughness: (1) the dynamics and the unwinding of DNA; (2) intercalation of dye and drug molecules into both DNA and RNA; and (3) the solution structure and dynamics of tRNA.

By a combination of ^{1}H, ^{13}C, and ^{31}P NMR measurements on monodisperse DNA fragments 260 and 140 base pairs long, it has been shown that within the rodlike DNA helix the base planes, the deoxyribose sugar, and the sugar-phosphate backbone all experience large fast internal motions.[81,82] The amplitudes A and correlation times τ for these motions could be calculated on a two-state model. For the base planes, $A = \pm 20°$ and $\tau = 1 \times 10^{-9}$ sec; for position 2′ of the deoxyribose sugar, $A = \pm 20°$ to $\pm 33°$ and $\tau = 1 \times 10^{-9}$ sec; for the P-H vector of the sugar-phosphate backbone, $A = \pm 27°$ and $\tau = 2.2 \times 10^{-9}$ sec. Intercalation of ethidium bromide was found to quench the motions of the two base pairs at the intercalation site.[83]

Studies of the interaction of Gene 5 protein from bacteriophage M13 with DNA fragments by Garssen et al.[84] and Coleman et al.[85] using ^{1}H and ^{31}P NMR have led the former group to propose a mechanism of the unwinding of DNA by the protein. This mechanism—tested in detail on the self-complementary tetranucleotide d(pC-G-C-G) with the hydrogen bonded proton resonances of the Watson-Crick base pairs as a probe—consists of two steps: (1) rapid opening and closing of double helical DNA, and (2) subsequent trapping of the single-stranded species by the protein. Stacking of nucleotide bases with tyrosine residues in the protein has been inferred from upfield shifts in the complex. The second step is rate determining, and the rate constant has been estimated to be approximately 10^{8} sec^{-1} M^{-1}. The proposed mechanism depends on the existence of very rapid, spontaneous fluctuations in the DNA structure, so that the double helix opens and closes many times before the single-stranded tetranucleotide interacts with the protein. The evidence for the existence of such fluctuations in this case is kinetic. In a tetranucleotide, the proposed mechanism may hold. Yet in the only double-stranded nucleic acid structure ($tRNA_1^{phe}$), for which the rate of base-pair opening has been measured by saturation transfer, the rate is much slower, and of the order of 10-100 sec^{-1}.[86] The mechanism found in

the short nucleotide sequence may not apply to longer double strands, but the usefulness of the method for the study of DNA unwinding, replication, and repair is clear.

Intercalation of Actinomycin D between nucleotides has been studied by Krugh[87] and others, and the intercalations of both Actinomycin D and ethidium bromide into short self-complementary double-stranded DNA fragments, by Patel.[88] The geometry defined by NMR data in solution has proved to be identical to that found in the earlier crystallographic studies of Sobell. Base specificity of Actinomycin D and ethidium bromide for dC-dG sites, selectivity for a pyrimidine 3′-5′ purine sequence, and an increase in the melting temperature of the helix as a result of intercalation, all found previously by other methods, have been confirmed.

Very extensive NMR work has been done on the solution structure and dynamics of tRNA, following the pioneering study by McDonald, Phillips, and Penswick.[89] The sometimes controversial literature has been summarized by Reid and Hurd[90] and by Kearns.[91] Most of the studies rely on the observation of exchangeable protons in the hydrogen bonds of the helical regions. It has been established by careful area measurements that there are 27 such protons in the tRNA from *E. coli*. They yield a well-resolved ^1H NMR spectrum at 360 MHz, since the chemical shift of these lines is strongly sequence-dependent (figure 3). This implies that there are 7 ± 1 hydrogen bonded protons in excess of those expected from the known crystal structure of $tRNA_1^{phe}$. These excess protons have been attributed to tertiary base-pairing interactions. It has also been shown that the seven tertiary structure protons disappear at lower temperatures than the secondary structure, indicating that the melting of $tRNA_1^{phe}$ begins with the destruction of the tertiary structure. The sequence of the melting of the secondary structure, which occurs in steps, was found to be rT helix → h_2 U helix − anticodon helix → acceptor helix. Comparisons between the observed NMR spectra of $tRNA_1^{val}$ and spectra calculated on the assumption that its structure is identical to the crystallographically determined structure $tRNA_1^{phe}$ (allowing for differences in base sequence), suggests that the structures are indeed identical, and that there is a universal pattern in the structure of different tRNA's.

V. STRUCTURE AND PHYSICAL PROPERTIES OF MEMBRANES

Biological membranes are known to consist of proteins and phospholipids, arranged, as the bulk of evidence suggests, in a phospholipid bilayer with proteins embedded in it or traversing it. The general features of this so-called "fluid mosaic" model may be regarded as established, but the organization of proteins and lipids in the plane of the membrane remains in essence unknown. Several key problems of membrane organization and

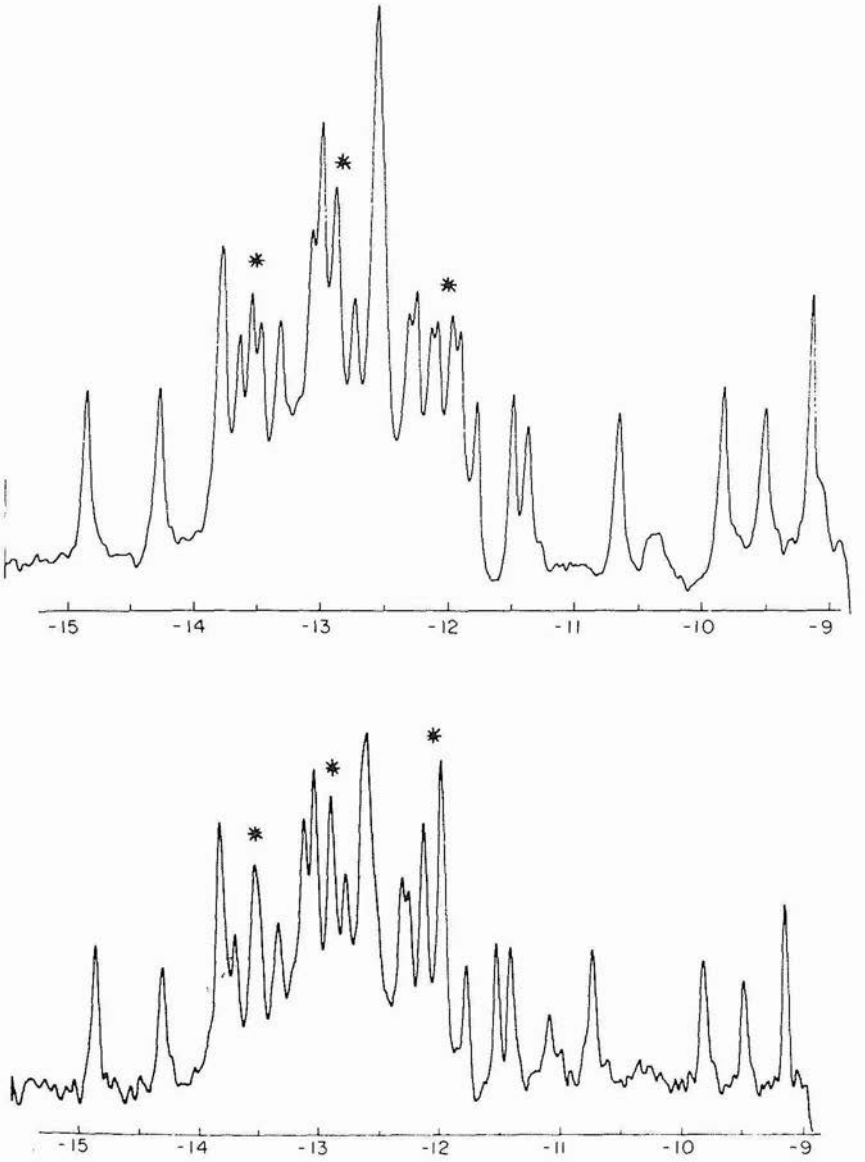

FIG. 3. THE LOW-FIELD 360 MHz NMR SPECTRUM of *E. coli* $tRNA_1^{val}$ under minimal magnesium (upper) and no magnesium (lower) conditions at 35°C. The tRNA concentration is 1 mM; the no-magnesium solvent contains 10 mM sodium cacodylate, 10 mM EDTA, pH 7.0, and the minimal magnesium spectrum is the same sample supplemented by 2-μL additions of magnesium chloride to a final concentration of approximately 8.8 mM magnesium. The resonances that shift with respect to the excess magnesium spectrum are indicated with asterisks.[90]

dynamics have been investigated—in most cases on model membranes—by ^{1}H, ^{13}C, ^{31}P, and ^{2}H NMR. Among the most prominent are: (1) orientation of hydrocarbon chains in phospholipid bilayers; (2) conformation of the polar headgroup of the phospholipids; (3) amplitudes and rates of segmental motions in the hydrocarbon chains; (4) lateral diffusion of phospholipids between the two layers in phospholipid vesicles; (5) rates of transmembrane exchange ("flip flop") of individual phospholipid molecules between the two layers; (6) interaction of small molecules, especially local anesthetics, with lipid bilayers; (7) stabilization of hydrocarbon side chains by proteins, mostly in reconstituted systems; and (8) intermembrane phospholipid exchange.

All of the foregoing structural features and processes can be detected by NMR. At present not all interpretations of experimental findings are clear or consistent, however, and there has probably been more occasion for controversy in this area of biological applications of NMR than in any other. Since our aim is to illustrate the contributions of the method, we will confine ourselves to a few findings where the interpretation is less problematic without attempting a review of the voluminous literature.

Fluidity in membranes was seen by ^{1}H NMR in the early experiments of Chapman[92] without, however, yielding details of the internal order deduced from the study of spin-labeled membranes by ESR.[93] Differential mobility, increasing from the polar head toward the interior of the bilayer, was first observed by Metcalfe and co-workers[94] using ^{13}C NMR. The dominant effect of incorporating cholesterol into bilayers was found to be a decrease in mobility. The most rigorous NMR study of the ordering of the interior of phospholipid bilayers has been made possible by the use of deuterium resonance on selectively deuterated phospholipids, introduced by Seelig.[95] Deuterium resonance experiments have shown that the order parameter, defined from deuterium quadrupole splittings, remains constant along most of the chain and decreases toward the methyl end. This result differs from that obtained by the use of spin labels, which show a continuous gradient of flexibility along the chain. The difference has been attributed to the perturbation introduced by the bulky nitroxide groups used for spin labeling, since the perturbation is minimal in the case of simple isotopic substitution. The origin of chain flexibility is thought to be in the rapid isomerization between trans and gauche conformations of carbon-carbon bonds.

The structure and orientation of the polar phosphocholine or phosphotidylethanolamine head group has been studied by both deuterium and ^{31}P NMR.[96] The simplest interpretation of the results is that the head group is aligned parallel to the bilayer plane, rapidly oscillating between two conformations in the case of phosphoethanolamine. A different conformation had been proposed on the basis of experiments with lanthanide ions.[97] In criticism, it has been pointed out[95,96] that ionic charge may alter and fix the

conformation. In counter-criticism, it could be said that ions are ubiquitous in biological systems, and that the "natural" polar head group conformation in the membrane is more likely to be that of the metal ion complex rather than that of the pure model system. The important conclusion is, probably, that different polar group conformations will be found, depending on the ambient microenvironment.

Asymmetry in the distribution of lipids has been demonstrated in several ways, notably using paramagnetic ions. Since the permeability of phospholipid bilayers and membranes to cations is small and the rate of transmembrane exchange is slow, adding a shift probe to the outside will split the overlapping resonances of the polar head group into two, and adding a paramagnetic relaxation reagent will broaden the resonances of the outer layer. This effect has been demonstrated by ^{1}H, ^{13}C, and ^{31}P NMR, and detailed mapping of membrane sidedness in phospholipid vesicles of different composition has been carried out by Bergelson and Barsukov, who also present an interesting discussion of its possible biological relevance.[98] A combination of magnetic resonance measurements has been used to measure the transmembrane exchange of phospholipids ("flip-flop"), following the classic experiments of Kornberg and McConnell,[99] and values ranging from 0.5-24 hours have been reported for different systems. The significance of the differences is not entirely clear, and the differences may in part reflect artifacts of preparation. But the basic fact that these rates are slow by comparison with the rates of lateral diffusion in the plane of the membrane—estimated as diffusion coefficients $D_L \approx 10^{-7} - 10^{-8}$ cm^2/s—has been established by magnetic resonance methods.

Lau and Chan[100] have observed the facilitation of fusion of lecithin vesicles by the antibiotic alamethicin. The process is related to the phenomenon observed as protein-mediated lipid exchange from one membrane to another.[98] In the protein case, only lipids in the outer layer appear to be exchanged, implying that the transfer protein interacts only with the outer layer. Alamethicin—and possibly other smaller molecules—on the other hand, penetrate both layers.

Many observations on the interaction of ions, drug molecules, and proteins with phospholipid vesicles have been reported, but very few NMR studies have so far progressed to the point of shedding light on the structure of a ligand-phospholipid complex. Lowering of the phase transition temperature seems to be a general feature of increasing the heterogeneity of membrane composition. In many cases, NMR results also indicate decreased mobility in the mixed fluid phase. This suggests that motional rigidity in the fluid phase and the tendency to solidification are not necessarily related in any simple way. The local rigidity, monitored by NMR, may or may not require short-range order, but is compatible with long-range disorder. Solidification, on the other hand, depends on the possibilities of cre-

ating long-range order. Comparing the effects of cholesterol and lanosterol on phospholipid vesicles by ^{13}C NMR[101] permits the conclusion that a planar sterol α-face is important for an effective interaction of steroids with hydrocarbon chains, which leads to decreased mobility. Other structural requirements for effective complex formation will undoubtedly be found by similar comparative studies.

The future, however, still holds the answer to the usefulness of NMR in the most difficult area to which it has been applied—that of phospholipid-protein interactions in membranes.

VI. CONCLUSION

There was a time, not so long ago, when writing on NMR in biology consisted of a recital of the physical principles and prophetic praise for its potential in biological research. Barely thirty years after its discovery, and barely fifteen years after the beginning of its serious applications to the study of biological systems, this is no longer necessary. The record of accomplishment, briefly outlined here, is in large measure solid. NMR, viewed as a structural method in molecular biology, illustrates the simple perennial truth that all natural science is ultimately physics and that hence basic discoveries in physics have a profound impact on other fields.

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