BIOLOGICAL NMR SPECTROSCOPY

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Introduction

The founders of the field of biological nuclear magnetic resonance (NMR) spectroscopy had a clear vision of its future potential. The 65th birthday of one of these pioneers, Professor Oleg Jardetzky, provided an occasion for bringing together the small group of scientists whose research defined the field at its inception, Jardetzky and his mentors, Linus Pauling and William N. Lipscomb, and Mildred Cohn and Robert G. Shulman. Another key figure in the early days of the field, William D. Phillips who was expected to participate in the Symposium and this book died shortly before the Symposium held at Stanford University in March, 1994. The historical section of this volume (Section I) conveys the excitement of the beginnings of biological NMR spectroscopy, when every experiment was new and potentially important. Section II, which constitutes the bulk of this Volume, provides a contemporary overview of the legacy of these early experiments.

It is nothing short of astonishing to be able to say that biological NMR spectroscopy has fulfilled the dreams of its founders. Its potential as a method for determining structures and describing the dynamics of proteins and nucleic acids in aqueous solution has been realized in recent years, and the approach has become an essential part of structural biology. These capabilities, which are practically routine now, reflect the rapid pace of technological invention and the scientific context of the period, 1957 to the present. Invention refers to the continuous stream of advances in instrumentation and magnets, computers and software, pulse sequences, and experimental design for NMR studies of proteins and other biopolymers. Context refers to the advances in biology. Even the most casual reader of newspaper headlines is aware that we live in the age of molecular biology, since it is now recognized that all aspects of life reflect the linear sequence of bases in DNA that store biological information and, in turn, specify the amino acid sequences of the proteins that express biological functions. However, we also realize that biology is fundamentally both three-dimensional and dynamic. And here is where structural biology will come to play the dominant role in describing the basic principles of biology and furthering biomedical and biotechnological applications. The limits of what can be obtained from sequences alone are already at hand. The limits of what can be obtained from structural biology, in general, and biological NMR spectroscopy, in particular, have not even been approached. The frontiers of protein complexes, membrane proteins, and carbohydrates, as well as more detailed descriptions of the interplay between structure and dynamics in protein function beckon.

Oleg Jardetzky is one of the pillars of biological NMR spectroscopy. He identified at its earliest stages the importance of resolving and assigning resonances from individual sites in a protein. This enabled the unique ability of

NMR spectroscopy to differentiate among chemically identical groups within the environment of a folded protein to be fully exploited. Perhaps the major experimental tool at our disposal is the use of isotopic labeling; what is so obviously a routine laboratory method now was a totally foreign undertaking in the beginning. The same can be said for signal averaging for sensitivity enhancement. These are the procedures that led to resonances being used as monitors of the structure, dynamics, and chemistry of protein groups. The latter got its start with the titration of individual histidine residues in proteins. All of these things, and many others mentioned in the historical section of the volume, were simply not present anywhere in science in 1957. The ideas and technology could not be borrowed from any other field, they had to be invented through the process of basic research.

1995 marks the 50th anniversary of the discovery of the nuclear magnetic resonance phenomenon, recognized by the 1952 Nobel Prize to Felix Bloch and Edward M. Purcell. Biological NMR spectroscopy is generally recognized as starting in 1957 with the publication of the first NMR spectrum of a protein followed shortly thereafter by its interpretation in terms of the constituent amino acids. The second Nobel Prize in NMR was awarded to Richard Ernst in 1991, and he has contributed the Foreword for this volume. Then the story as presented in this book goes into the hands of Oleg Jardetzky, Mildred Cohn, Bob Shulman, and Joe Ackerman, who provides a moving tribute to Bill Phillips and his contributions to the field.

Readers of this book do not need to be reminded of the importance of structural biology in the scheme of science or of the importance of NMR spectroscopy to structural biology. However, we hope that this book will serve to remind all of us of the willingness to participate in unconventional research and the high level of innovation required of the founders in order to establish the field of biological NMR spectroscopy. This can be seen directly in the contributions in the historical section. It can also be seen in the successful applications of biomolecular and biomedical NMR spectroscopy described in the scientific contributions that make up the majority of the book. It has been an exciting adventure from the tentative beginnings to these latest developments in biological NMR spectroscopy.

Acknowledgments

This book is a direct outgrowth of the Symposium held at Stanford. We are grateful to all of the financial sponsors of the meeting as well as all of the participants. We especially appreciate the generous contributions from the principal sponsors, Bruker Instruments, Glaxo Research Institute, and Oxford University Press. Essential support from Varian Associates, Magnex Scientific, Martek Biosciences Corporation, Otsuka Electronics, Ajinomoto Company, Inc., Escom Science Publishers BV, IBM, Cambridge Isotope Laboratories, Inc., Tecmag, Inc., Programmed Test Sources, Inc., Molecular Simulations Inc., Intermagnetics General Corporation, Isotec Inc., and Dr. Harold Amos is also greatly appreciated.

We thank the authors of all of the manuscripts for their timely submissions. We especially thank Richard Ernst for the Foreword, the founders, Oleg Jardetzky, Mildred Cohn, and Bob Shulman, for their personal reminisces, and Joe Ackerman for the tribute to Bill Phillips.

We warmly thank Robin Holbrook whose assistance in organizing the meeting was indispensable and acknowledge Russ Altman's help with local arrangements. We especially want to thank Jennifer Wang and Linda Matarazzo Cherkassky for preparing the book in camera ready form. This took enormous patience and sophistication in software, in light of the diversity of all aspects of the contributions.

And finally we thank Oleg Jardetzky for making all of this possible.

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Foreword

My heartiest congratulations and thanks to you, dear Oleg, on the occasion of 65 intense years of invaluable contributions to science and to mankind! What would be NMR today without your foresight and your ingenuity? You foresaw the importance of biomedical NMR before many of the later contributors to the field were even born. You made attempts to this direction as one of the very first scientists and you continued to fertilize the field with ideas, concepts, critics, and valuable applications ever since the late fifties.

Indeed, the first contributions of Professor Oleg Jardetzky to biological NMR date back to 1956, when the technological development was not yet ready to successfully solve by NMR relevant biological questions. But Oleg Jardetzky never ceased to pursue his dream, and he contributed himself significantly to the advancement of NMR technology. For example signal averaging: It was the first successful attempt by Oleg Jardetzky and others to circumvent the incredibly low sensitivity of NMR and helped enormously to tackle biological systems. Probably Oleg Jardetzky was the first who recognized as early as 1965 the importance of Fourier transform spectroscopy. I still remember his enthusiastic support of this concept, in which I had difficulties myself to believe, sitting at a lake in New England on a hot summer afternoon during a Gordon Conference and discussing about the future prospects of pulse techniques. He has also picked up rapidly the two-dimensional NMR techniques for structural studies and solved many structural and functional questions of biomedicine ever since.

His early seeds have become in the mean time monumental trees which turned out to be indispensable for our understanding of biological processes. Equally valuable as his original contributions are his often rather polemic and critical discussion contributions. They are always stimulating and keep the discussion lively.

This book summarizing the 65th Birthday Symposium demonstrates vividly in how many ways you, dear Oleg, have contributed to biological NMR. It certainly creates expectations for your future productivity which I am sure you will even surpass. I hope you will continue to enjoy to actively contribute to science for many fruitful years to come. Best wishes!

R.R. Ernst
Zurich, Switzerland

The Founders Medal. Presented to Oleg Jardetzky, Mildred Cohn, and Robert G. Shulman at the XVIth International Conference on Magnetic Resonance in Biological Systems, Veldhoven, The Netherlands, August 1994. "For Outstanding Contributions to Biological Magnetic Resonance"

Section 1: History of Biological NMR Spectroscopy

1

Simple Insights from the Beginnings of Magnetic Resonance in Molecular Biology

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Birthday symposia inevitably provide an opportunity for reflection. Noting that greater minds than mine have offered an apology for their life (St. Augustine, 1853 edition; St. Thomas Aquinas; John Henry cardinal Newman, 1864), I shall attempt to answer the question: What have been the lasting contributions of my generation - the generation that began its work before Richard Ernst's epoch making development of 2D NMR, and the equally momentous development of high field spectrometers, pioneered by Harry Weaver at Varian, Rex Richards at Oxford and Günther Laukien at Bruker, revolutionized the technology and put biological applications within everyone's reach? I offer these insights in the spirit that to fully understand a subject one must understand its history.

The essence of scientific endeavor is to see something no one has seen before - or understand something no one had understood before. If there had been such a contribution, it was to understand what biological questions could be asked by NMR and to develop prototype experiments showing how. Difficult as it is to imagine this today when such understanding is taken for granted, the now obvious just wasn't obvious then. Quite the contrary: well considered expert opinion of the day held the undertaking to be of very dubious merit. Linus Pauling, with whom it was my great fortune to spend my postdoctoral year, was never much interested in nuclear magnetic resonance (and did not think much of its promise for biological applications, as he clearly pointed out at this symposium). But, Linus Pauling firmly believed in giving the young the freedom to explore, and so the first crude interpretation of a protein NMR spectrum, taken a few weeks earlier by Martin Saunders, Arnold Wishnia and J. G. Kirkwood at Yale, was based on the first amino acid and peptide spectra we

had taken at Caltech. When I got my first faculty job at Harvard, and wanted to apply for an NMR spectrometer, it was not quite as easy. I was called by the department chairman into a conference with the Dean and both tried to convince me that such a high risk request from a totally unknown young man would never be funded and I could destroy my academic career and damage the reputation of the University. My response was to take the plane to Washington the next morning and present the proposal - "NMR in Molecular Biology" - to the then director of the NSF Molecular Biology program, William V. Consolazio. With the assurance of his support and of a broadly based peer review, the proposal was submitted, reviewed and funded, and the first NMR laboratory dedicated to biological research was founded at the Harvard Medical School in 1959.

To develop the now commonplace understanding of what NMR could do in molecular biology required thought and required experimentation, and the basic insights we now take for granted grew gradually after alternatives were carefully ruled out. It would not reflect reality to credit any one individual with having done all that was needed to develop this understanding, but we were a small group. There was an occasional chemist and an occasional physicist who did an occasional experiment of potential biological relevance, but those who persevered in exploring the potential systematically were initially Mildred Cohn, Bob Shulman, and I, along with our students, joined a few years later by Bill Phillips. Being a small group, we worked very differently from the now modern scientist, who has to protect his intellectual property and market himself, lest he remain unnoticed and unfunded and therefore unable to go on. Without a qualm we shared our guesses, our daydreams and our doubts, our experimental designs and results - far in advance of publication - and when the work came to overlap, as did Bill Phillips' and ours in later years, we often encouraged each other to do the same or similar experiments, so the results could be compared and the generality of the conclusions tested. Much of what we did we did not bother to publish, because it was not on the critical path of mapping the landscape. We argued and sometimes irritated each other by sharply pointed criticism - but there was room enough for everyone, so there was no need to compete. In a sense, it was a different phase - a different kind - of science. It was exploratory rather than exploitive science. It was not aiming to exploit existing and invent bigger and better techniques to implement ideas obvious to everyone, but to develop the basic framework of ideas to determine what could and should be done. There are areas of science where this is still necessary, but NMR is now a mature technology and is not one of them. The value of its current massive data generation phase is obvious and likely to be lasting. If anything of enduring value survives from the early phase, it is a set of a few simple, but fundamental insights, most now taken for granted, but originally wrung from nature with a primitive technology and some uncertainty of interpretation.

The high resolution NMR spectrum of a protein contains information on its secondary and tertiary structure (1961-1969).

The first glimpse of this insight came from a comparison of our protein spectra in D_2O to those in trifluoroacetic acid, first published by Frank Bovey and G. V. D. Tiers. In our first review of biological applications of NMR we therefore could categorize the applications by the type of information obtained, as "(a) the determination of primary chemical structure, (b) study of conformation or secondary and tertiary structure, (c) the study of rate processes and molecular motion, and (d) detection of interactions between molecules" (Jardetzky & Jardetzky, 1962, p. 354) and noted "....the broad lines generally observed in protein spectra may result in part from restricted motion and in part from inexact superposition of individual amino acid lines...much more extensive correlations are necessary before detailed interpretation becomes convincing" (*ibid.*, p. 363). By 1965 Bill Phillips' and our experiments, still largely unpublished, prompted the prediction: "....it will be possible to use NMR as a specific method for the study of tertiary structure" (Jardetzky, 1965, p. 3), although duly noting that "the principal obstacle to unequivocal interpretation - the extremely poor resolution resulting from the overlap of a very large number of broad peaks - has not been overcome." The point was clinched by the clear demonstration in the experiments of McDonald and Phillips published in 1967-1969 (McDonald & Phillips, 1967; 1969) and Cohen and Jardetzky (1968) that the spectrum of the completely denatured protein was to a good approximation the sum of the spectra of the constituent amino acids and much less complex than the spectrum of the native protein. In our study, incomplete denaturation, with disulphide bridges still intact, could also be distinguished from the native fold and the completely denatured chain for the first time.

Proteins undergo internal motions and the high resolution NMR spectrum of a protein contains information on protein dynamics (1961-1969).

This was fairly obvious because native proteins had broad lines, while denatured proteins has sharp lines, but the real issue was whether globular proteins were as rigid as the early crystallographers thought them to be, or whether regional differences in mobility were reflected in the NMR spectra. This could be probed by studying the binding of small molecules to proteins. In 1961 we reported: "Selective broadening of absorption in the spectra of low molecular weight molecules can be used to determine the chemical groups preferentially stabilized by the formation of specific molecular complexes in solution."

(Jardetzky & Fischer, 1961, p. 46). In 1964/1965 we showed that the three-ring compound sulfaphenazole could bind to bovine serum albumin by *either* the sulfonamide *or* the substituent phenyl ring, the difference in the relaxation rates of the bound form at the two sites being a factor of 3-4 (Jardetzky & Wade-Jardetzky, 1965, p. 228). Discussing this result, already in 1964 it was pointed out that this discrepancy could result either from a difference in the number of nearest neighbors or from a difference in the local correlation times (Jardetzky, 1964, p. 516 and Table II). For use in the study of proteins, I therefore proposed a generalization (Eq. 16) of the relaxation equations formulated by Gutowsky and Woessner, "*with the significant difference that different correlation times are assumed for different pairs of nuclei and the internuclear distances are averaged over time*," (*ibid.*, pp. 512 - 513; italics in the original) pointing out "If more than a single correlation time determines the relaxation rate of a given group or if there is a change of interproton distances, Eq. 16 must be solved in detail to obtain the desired information. In principle, this is possible by making relaxation measurements on a series of deuterium substituted analogs and thus obtaining the contribution of each individual proton to the relaxation rate of any other proton." (*ibid.*, pp. 517 - 518). This was, of course, before the invention of 2D NMR, and it was later developed by Kazuyuki Akasaka into a method for analyzing relaxation phenomena which he called DESERT (Akasaka *et al.*, 1975). Chemical shift averaging as an indication of conformational transitions of aromatic rings in proteins was first described in our spectra of selectively deuterated staphylococcal nuclease, almost in passing "...the existence of a conformational equilibrium involving a tyrosine residue seems fairly certain. The equilibrium is probably rapid on the NMR scale (see below), since a single tyrosine peak is observed for each residue." (Jardetzky, 1970, p. 120). Of greater interest and described in greater detail was the equilibrium affecting His 48 in ribonuclease, which was slow on the NMR time scale and represented the first detection of a conformational transition in native proteins by NMR (Meadows & Jardetzky, 1968). The clearest demonstration of segmental flexibility in proteins by NMR came a few years later in the study of tobacco mosaic virus prompted by the fact that crystallographers could not distinguish between static disorder and mobility in the RNA binding region (Jardetzky *et al.*, 1978).

The most important structural information obtainable by NMR comes from relaxation parameters (1961-1965).

The use of relaxation by paramagnetic ions to define distances in small molecules was demonstrated by Bob Shulman's study of the Mn-ATP complex (Sternlicht *et al.*, 1965a,b). The already cited 1964 review, before proposing the generalization of relaxation equations and pointing out that internuclear distances can be determined in proteins, summarized the understanding explicitly:

> "Most of the existing chemical correlations take into account the information obtained from the measurement of chemical shifts and coupling constants. In contrast, relaxation studies have received relatively little attention, despite the well established fact that relaxation processes are extremely sensitive to variations in the molecular environment and therefore provide the potentially most informative measurements for the study of molecular interactions." (Jardetzky, 1964, p. 500).

The application of NMR to biological problems requires signal averaging to counteract the inherent low sensitivity (1962).

The first time that a computer (the CAT - computer of average transients) was attached to an NMR instrument, we reported in Nature:

> "The unique potentialities of high resolution magnetic resonance as a method for obtaining detailed information on molecular structure and molecular interactions in solution are well recognized. However, the applicability of the method to the examination of interactions in biological systems has been hampered by its low sensitivity, requiring the use of comparatively concentrated solutions (~0.05 M or higher).
>
> "We have now applied a technique which has allowed us to perform a series of crude but informative experiments on the binding of diphosphopyridine nucleotide (DPN) to the enzyme yeast alcohol dehydrogenase (ADH) in stoichiometric proportions. Our procedure has been to couple the output of a Varian model 4300B high-resolution spectrometer operating at 60 Mc/s to a 400 channel digital average response computer, the Mnemotron model 400 computer of average transients. The sweep of the computer was triggered through a Tektronix model 535 A oscilloscope from a marker placed in the sample tube." (Jardetzky *et al.*, 1963, p. 183).

This was our one and only contribution to NMR technology.

The complexity of protein NMR spectra is generally so high as to require isotopic spectral editing (1965).

This was first reported at the memorable meeting in Tokyo in 1965:

"Detailed comparison of the chemical shifts in a large series of amino acids and their derivatives, obtained by Fujiwara and collaborators, ourselves as well as others has convinced us that the degree of resolution required for a direct, complete interpretation of protein NMR spectra is not to be expected even at the highest now attainable NMR frequencies of 300-400 Mc. The close similarity in the magnitudes of the relaxation times of amino acids in peptide linkage, precludes the alternative possibility of a detailed assignment of lines by selective saturation.

"A complete interpretation of a protein NMR spectrum is therefore contingent on the preparation of partially deuterated analogs. The feasibility of this approach is suggested by the recent success of Katz and coworkers in isolating a completely deuterated enzyme. Given the possibility of observing the proton resonance spectrum of an individual amino acid in peptide linkage, against the background of a completely deuterated polypeptide chain, nuclear magnetic resonance becomes decisively superior to any existing method in the wealth of detailed structural information which it can provide about a protein in solution." (Jardetzky, 1965, pp. 1-2).

The idea was shared with J. J. Katz during a visit to Argonne, who was generous enough to provide deuterated amino acids for my initial deuteration experiments, carried out in Cambridge in 1965/66. The ultimate realization of the proposal was published in Science in 1968 (Markley *et al.*, 1968).

Contributions of secondary and tertiary structure to the NMR spectrum are at least in part separately identifiable (1967).

In the paper by Markley *et al.* (1967) on the helix-coil transition of polyamino acids we noted:

"The peak corresponding to the proton on the α-carbon of the polypeptide backbone shifts upfield on helix formation. The magnitude of this shift, which is attributed to the magnetic anisotropy of the peptide bond, appears to be a sensitive measure of per cent helicity. The chemical shift on helix formation of the peak corresponding to the proton on the peptide nitrogen is determined by two opposing factors: (1) differences in hydrogen bonding; (2) magnetic anisotropy of the adjacent peptide bond. Side chain resonances are not shifted appreciably on helix formation." (Markley *et al.*, 1967, p. 25).

The paper by Nakamura and Jardetzky (1967) summarized the available information: "....the chemical shifts accompanying the incorporation of a given amino acid into a polypeptide chain can be systematized in terms of a few, relatively simple rules. The findings offer little hope for the use of NMR for the analysis of the primary sequence of large peptides, but underscore its usefulness in the study of secondary and tertiary structure." (Nakamura and Jardetzky, 1967, p. 2212). The conclusions reached in these early studies have been amply confirmed by the massive statistical analysis of chemical shifts in proteins by Wishart *et al.* (1991). Their importance however lies not so much in the information they contain on the origin of structural shifts, but in the already noted simple fact that without the secondary and tertiary structure giving rise to a separation of lines for identical amino acid residues, there would be no way of extracting structural information about proteins from NMR spectra.

High resolution NMR in principle allows a complete description of protein structure, dynamics and interactions (1970).

Summarizing the state of knowledge in the field in 1970, Gordon Roberts and I wrote about the possibilities offered by NMR:

"1. The complete definition of the conformation of a protein in solution. It should be noted that this is in principle possible on the basis of high resolution NMR data alone. However it is an extremely difficult and laborious task and has thus far not been attempted.

"2. The mechanisms of folding and unfolding of protein chains. Little detailed work on this question has been done. However, the high information content of a protein NMR spectrum should allow one to distinguish between a two-step and a multistep mechanism of denaturation and to describe precisely the sequence of structural changes.

"3. Changes in conformation involving individual amino acid residues or entire regions of the polypeptide chain. Both conformational equilibria in the protein itself and conformational changes produced by ligand binding have been detected by NMR and will be discussed below. In addition to providing estimates of the rates of such changes, NMR makes it possible to define the amino acid residues involved." (Roberts and Jardetzky, 1970, p. 487).

Everything we have done since - perhaps even what the field as a whole has done since - was to exploit these basic insights: the first partial NMR structures

of enzyme binding sites (Meadows *et al.*, 1969; Markley *et al.*, 1970), the first NMR detection of protein conformational changes (Meadows *et al.*, 1969; Nelson *et al.*, 1974), the detection of mobile segments in larger proteins (Jardetzky *et al.*, 1978), model-free analysis of relaxation data on proteins (King & Jardetzky, 1978), the first NMR determination of the three dimensional fold of a protein fragment, the lac-repressor headpiece, in solution (Ribeiro *et al.*, 1981 and Jardetzky, 1984), the critical analysis and development of methods for protein structure calculation (Lane & Jardetzky, 1987; Altman & Jardetzky, 1989; Zhao & Jardetzky, 1994), and finally the detailed NMR analysis of the structure and dynamics of a complete allosteric system (Zhao *et al.*, 1993; Zhang *et al.*, 1994; Gryk *et al.*, 1995). It was of course Richard Ernst's own and his students' monumental contribution to teach us how to exploit them best (Ernst *et al.*, 1987). And, it was Willie Gibbons' insight in 1975 that a combination of coupling constant and nuclear Overhauser effect measurements along the peptide backbone could be used as a scheme for the sequential assignment of protein resonances which made a systematic and complete interpretation of protein NMR spectra possible (Gibbons *et al.*, 1976). This approach was extended and refined by Kurt Wüthrich and Gerhard Wagner and their colleagues in Zurich into a practical procedure for assigning the spectra of small proteins. Publication of several protein structures determined by NMR followed, but it was the successful result of Robert Huber's challenge to Wüthrich to do a double-blind structure determination of tendamistat, a protein whose structure had not been determined previously, by x-ray diffraction and NMR in parallel (Billeter *et al.*, 1989; Braun *et al.*, 1989) that did much to convince the world that NMR was a useful structural tool.

In retrospect it is of course easy to say that all these general ideas and paradigms were obvious all along. *A priori* these ideas did not necessarily have to be true. Thirty years ago, there were those who thought that only primary structure should be reflected in the spectra, other influences being too weak. Others thought that relaxation parameters of macromolecules would forever remain uninterpretable, and thus of no use in the study of either structure or dynamics. There were those, as Amory Lovins, who since became a famous consultant on environmental affairs, who in 1965 proposed to Ed Purcell and to me to improve the sensitivity of NMR by a factor of at least 1000, using unstable vacuum tubes - a project he never had time to complete, but which would have rendered signal averaging obsolete. Then there were those who thought isotopic labeling of macromolecules would be impossible, and if not, far too expensive. There were many who thought that it was proof of scientific immaturity to even think of a protein structure determination by NMR. It was our extreme fortune that this thinking dominated neither the peer review panels of the NSF and NIH, nor the executive boards of Merck, Bell and DuPont, who underwrote some of our more expensive daydreams. Yet, we also had to learn

that seeing something others did not see before does not always earn admiration - sometimes quite the contrary. As the possibility of protein structure determination by NMR was first discussed in public, at a CIBA Foundation symposium in 1970 (Porter and O'Connor, eds., 1970, p. 130), peer disapproval was recorded for all posterity:

> "F. M. Richards: Without reference to other techniques, how much information could you specify....? Are you able to specify distances and orientations to the other groups whose signals you can measure, or put certain limits on them?
> O. Jardetzky: ....In principle you can get this information if you measure relaxation times and the dependence of relaxation times on each neighbor in the vicinity.
> H. M. McConnell: That is an exaggeration!
> O. Jardetzky: It is (only) an exaggeration in the sense that it is a diabolically difficult undertaking. It is a very hard thing to do, but if you did have just two protons, you could make a good guess about the distance between them. Admittedly nobody has tried to do this."

Reflecting on how the now obvious was not always obvious, one is tempted to ask the question: what is it that is now not obvious that will be obvious later? The caveat referred to above and some of its consequences immediately come to mind, and we might consider them as additional insights:

Wherever there is conformational averaging, NMR solution structures calculated from averaged NMR parameters have no physical meaning.

For some time, the dominant creed has been that all proteins can be treated as rigid in solution. This prompted a veritable race to report more and more precise coordinates for NMR structures and to develop more and more sophisticated refinement methods for this purpose. Only recently is one beginning to see doubts on this point, and, with increasing frequency, references to my 1980 paper (Jardetzky, 1980) in which the nature and implications of conformational averaging were described - a paper which I thought for 17 years to be too self-evident and too trivial to publish. The paper was a summary of the comments I made at a 1963 Gordon conference in response to Bob Shulman's report of the structure of the Mn-ATP complex. They greatly angered him at the time, but they are true - and in the long run did not wreck our friendship.

This caveat and a host of other observations have given rise to a still more general insight about the role of NMR in Molecular Biology, which is still far from being generally accepted - in fact it is being zealously disputed by some -

but which undoubtedly will be, once the dust has settled. It was summarized in Gordon Roberts' and my book "NMR in Molecular Biology" in 1981:

> "Thus, NMR does not derive its importance as a method in molecular biology from being a technique in which one can routinely proceed from the measurement by straightforward calculation to a definite answer.... The importance of NMR rests rather on the fact that it provides a much greater wealth of different clues on questions of structure, dynamics and function than other methods. The individual clues may not always be unequivocal, but interpreted in the context of carefully designed chemical and biological, as well as spectroscopic, experiments, can lead to important discoveries" (Jardetzky & Roberts, 1981, p. 9).

Once this becomes generally understood, it will be appreciated that the primary role of NMR in Molecuar Biology is not as a - necessarily second rate - method for structure determination, but as a unique tool for the study of *dynamics* and *function*.

Epilogue

What we did *was* exploratory science. The essence of exploration is to try something, show that it can work by a prototype experiment and then go on to the next question and do the same, rather than exploit the first finding to its fullest, as is now commonly done. The freedom to pursue exploration rested on the free, permissive, unpressured, scientific climate which we had when we began, but which has all but disappeared in our lifetime.

In today's climate favoring utilitarian science, public accountability, narrowly conceived hypothesis testing, setting and achieving specific goals, mass production of data by proven methods, and cut-throat competition for resources and funds, Pauling's readiness to grant a postdoctoral fellow freedom to explore, Consolazio's visionary funding of a vague proposal with an uncertain outcome, the free bouncing back-and-forth of ideas in the early days of NMR without concern for priority and credit have become virtually unthinkable.

We may not have had an NMR in Molecular Biology today, perhaps not even 2D NMR and high field magnets, if a few of us had not had the opportunity to dream and play with it in the early days, groping in the dark and sorting out soluble and unsolved problems. After all, no lesser a group of NMR experts than the management of Varian declared in the mid-sixties that NMR had gone as far as it could go and stopped the development of high field magnets after pioneering it. Had everyone shared their views and the views of the few distinguished physicists who already in 1958 advocated abolishing the Gordon

conferences on Magnetic Resonance, since nothing significant was left to be done in the field - what did happen could not have happened. If one looks closely, no technology has developed without the stepwise development of its use going hand in hand. It should not be forgotten that the giant steps of accomplished craftsmen cannot be taken if not preceded by the modest and halting - and yet seminal - steps of those who dream. To be sure, there is an inevitability to the course of science and at some time, somewhere, someone would have discovered that there was more to be done, and done it - but it would have been later rather than sooner and somewhere where the freedom to explore had not become extinct.

The freedom and the opportunity to daydream are important. The freedom and the opportunity to explore paths that others don't appreciate, or even disapprove of, are important. Taking the time to understand generalities and to develop a perspective is important. The freedom not to have to compete is important. The freedom to arouse - and survive - controversy is important.

Exploration has its dangers. Tackling the unknown can easily end in failure. Explorers rarely get their name attached to a specific contribution, because they jump from one thing to another before everyone sees their point and because it is easier to remember those who exploit an idea in tens or hundreds of different variations than those who originate it only once. Today this can be deadly in funding and tenure decisions. It is a tribute to those who set the climate at the time we were young that we did not unduly suffer for it.

Yet, it is not nostalgia for the "good old times" that prompts me to underscore this point. It is simply the recognition of a simple fact. If we forget that to find and shape an idea before it becomes common property is at least as important as its implementation after it does and that a perspective is at least as important as a specific result, we as a scientific community - perhaps we as a society - are on a self-defeating path. A society that increasingly thinks only in terms of directing and channeling craftsmanship in the pursuit of clearly visible goals is cutting itself off from the source of all innovation.

I was fortunate to have lived when and where I did. There is a feeling known only to those who have climbed a mountain at the break of dawn, long before it became crowded by afternoon hikers who must step on each other to move up the last inch. The exhilaration of even minor discovery in a field that still lies in the dark is something akin to it. It was a rare - now nearly unimaginable - privilege to have belonged to the generation that entered this branch of science - Molecular Biology - at its dawn. Perhaps it was even a greater privilege to have been part of a generation that understood and had not yet forgotten that science, as medicine, is not a trade, but a calling, that it is not about wealth, power, public attention and personal glory, not even about the good life and social standing, but above all, about the simple love of understanding the unknown.

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2

Choice of Problems in the Early Days of Biological NMR Spectroscopy

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It was a mere ten years after the discovery of NMR that Oleg Jardetzky under the mentorship of the physical chemist John Wertz (Wertz and Jardetzky, 1956) began using ^{23}Na NMR with the aim of studying Na^+ transport in biological systems as suggested by William Lipscomb. Jardetzky found that Na^+ NMR provided a unique method for following the binding of Na^+ in weak complexes. Advantage was taken of the sensitivity of quadrupolar nuclei to their chemical environment as reflected in their relaxation rates which could be readily observed at a field of 7,030 gauss available at the time (Jardetzky and Wertz, 1956). From the very first, Jardetzky limited his choice to those problems that could be investigated uniquely or most effectively by NMR spectroscopy.

One of Jardetzky's principal goals was to elucidate, at least in part, the three-dimensional structures of biological macromolecules in aqueous solution, a distant goal in the late 1950's. He realized that before attempting to tackle the structure of these complex molecules, proteins and nucleic acids, by NMR it was essential to initially characterize the spectra of their components, amino acids and nucleosides. In 1957, he published a note in the Journal of Chemical Physics (Takeda and Jardetzky, 1957) on a few amino acids, not only reporting the chemical shifts of all the protons but also showing that in a dipeptide, for example, glycylglycine, the two CH_2 groups are non-equivalent. In 1958, he published an NMR paper, a systematic study of the proton NMR spectra of amino acids, in the Journal of Biological Chemistry (Jardetzky and Jardetzky, 1958), thus introducing many facets of NMR spectroscopy to the biochemical community. This seminal paper included: 1) the chemical shifts of the protons of 22 amino acids and their dependence on pH, concentration and ionic strength

and 2) the effect of rate processes on the NMR spectrum as exemplified by the exchange of the guanidino protons of arginine with water. Increased structural information from peptide NMR spectroscopy attracted many investigators to this area of research. Nakamura and Jardetzky (1967) and Saunders, Wishnia and Kirkwood (1957) were among the first to specify systematically the effects of peptide bond formation and of primary structure on the proton spectra of amino acids, thus making it possible to distinguish these effects from changes due to secondary and tertiary structural features of proteins.

The first NMR spectrum of a protein, ribonuclease, consisting of four broad peaks, was reported in 1957 by Saunders, Wishnia, and Kirkwood (1957). Soon thereafter, Jardetzky and Jardetzky (1957) analyzed the magnitude of each peak of this low resolution spectrum demonstrating that the summation of the proton chemical shifts of the constituent amino acids of the protein determined by them (Jardetzky and Jardetzky, 1958) could account in first approximation for the observed protein peaks. Subsequently, Cohen and Jardetzky (1968) pointed out that only the completely denatured protein could be represented by the sum of the spectra of its constituent amino acids.

Following the study of amino acid spectra, an investigation in 1960 of the proton NMR of the components of nucleic acids (Jardetzky and Jardetzky, 1960), purines, pyrimidines and nucleosides, led to the discovery of base stacking which laid the basis of future investigators' interpretations of nucleic acid spectra. Conformational information could also be gleaned from the nucleoside spectra, since the α and β anomers could readily be distinguished. In further studies (Jardetzky, Pappas and Wade, 1963), the lifetimes of the base stacking and hydrogen-bonded interactions were determined

In the mid-1960s, Jardetzky really hit his stride and introduced many applications of NMR to biochemical problems. Although the ability to analyze protein spectra was very limited, Jardetzky pointed out that interactions of proteins with small molecules could be profitably studied by NMR. Thus he opened up the area of identifying the groups in the ligand molecule involved in the ligand-protein interaction by NMR spectroscopy. Such interaction was predicated on observing chemical shift changes and/or selective broadening of individual protons of the ligand upon binding, the former due to change in the environment of the observed nucleus and the latter due to a decrease in the motional freedom upon binding to protein. This approach was suggested by Jardetzky, Fischer and Pappas (1961) and implemented in a study of the binding of penicillin to bovine serum a few years later (Fischer and Jardetzky, 1965). Many other protein-ligand interactions including enzyme-inhibitor complexes were subsequently investigated by Jardetzky's group and other investigators in that decade (Roberts and Jardetzky, 1970).

Serious attempts to assign resonance peaks to individual amino acids began in the second half of the '60s initially with those proton resonances that fell

outside the general envelope of the protein spectrum. In 1966, Bradbury and Scheraga (1966) observed three resolved C2 H histidine resonances in the spectrum of bovine pancreatic ribonuclease at 60 MHz. The next year, Jardetzky and his collaborators (Meadows *et al.*, 1967) with a 100 MHz spectrometer, were able to resolve the C2 H resonances of all four histidine residues of RNase as well as those of staphylococcus nuclease and lysozyme. For the purpose of resolving other aromatic residues and eventually assigning all the amino acids, in 1965 Jardetzky suggested (Jardetzky, 1965) selective deuteration to simplify the usual complex spectrum with many overlapping peaks. Protein could be isolated from organisms grown on deuterated amino acids with the exception of one or two protonated amino acids, thus eliminating most of the resonances in crowded regions of the spectrum. This ingenious strategy was realized experimentally by his group in 1968 (Markley *et al.*, 1968). Many variations of the strategy of isotopic substitution have proven useful for the determination of protein structure by NMR spectroscopy.

The next problem Jardetzky undertook to solve was the assignment of resonances to specific residues of a given amino acid in a protein for which the amino acid sequence is known. For example, in RNase, pH titrations of the histidine residues revealed four pK values, each associated with an individual histidine. In a landmark paper, Jardetzky and Scheraga and their coworkers (Meadows *et al.*, 1968) used several strategies to assign each of the four histidine residues characterized by their pKs to a specific histidine residue in the RNase sequence. These strategies included 1) minor chemical modification of a single known histidine residue 2) specific cleavage of the 20-21 peptide bond of RNase (recombination of fragments yielded an active enzyme) and 3) deuteration of the single histidine in the small fragment allowing it to be unequivocally assigned in the spectrum of the recombined complex. It was the first time the pKs of amino acids in a protein had been identified with specific residues in that protein. Further developments for assignment ensued in Jardetzky's group; for example, the comparison between wild type and mutant forms of staphylococcal nuclease (Figure 15, Roberts and Jardetzky, 1970) and the identification of the amino acid residues at the active site of enzymes deduced from the spectral changes accompany complex formation upon complexation with specific inhibitors (Jardetzky and Wade-Jardetzky, 1971)

Only a few highlights among Jardetzky's many contributions to NMR spectroscopy before 1970 have been included in this brief discussion. In retrospect, the application of NMR was in its infancy from 1956 to 1970; nevertheless the number of papers related to biochemical problems had grown from 1 to 800 during that period (Jardetzky and Wade-Jardetzky, 1971). Oleg Jardetzky had the vision and dedication to nurture this emerging technique's applicability to biochemical problems and by developing ingenious strategies kept it viable in the face of skepticism of both biochemists and NMR experts.

And he welcomed those who joined him in this arduous pursuit. What could be regarded at the time only as his undue optimism when expressed in a review in 1971 (Jardetzky and Wade-Jardetzky, 1971), may be regarded today as truly prophetic:

> "And thus today high-resolution NMR has emerged alongside X-ray diffraction as one of the two most powerful methods for the study of the structure and conformation of macromolecules, of molecular interactions, and of the time course of molecular processes. It is unique in the combination of high information content, sensitivity to both molecular dynamics and molecular geometry, and applicability to molecules in solution."

The maturation of NMR spectroscopy's ability to solve biochemical and biological problems as manifested by the papers presented at this symposium and the high regard in which this method is now held, owes much to Oleg Jardetzky's vision and dedication in the period before high magnetic field spectrometers, Fourier, transform and multidimensional techniques became available.

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3

Early Days of Biochemical NMR

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It was my pleasure to participate in Oleg's 65th birthday celebration and to reminisce about the early days of Biochemical NMR. Oleg was always there. I remember in the summer in the early 1960s sitting on lawn chairs at a Gordon Conference and discussing the need for a meeting on biochemical NMR. This was to convene those with common interests, and out of this grew the 1964 meeting in Boston, which was the first International Conference on Magnetic Resonance in Biological Systems. In organizing the 1964 meeting Oleg was stalwart, in charge of the local arrangements at the old mansion, home of the American Academy of Arts and Sciences. The venue was much appreciated by the more than 100 attendees, and the smooth arrangements and elegant, although somewhat dowdy locale, contributed to the sense, generated by the meeting, that the field had a coherent scientific core and a meaningful future.

In the early days of the 1960s the field of magnetic resonance in biological systems, brought together biannually by the society, had a coherence that was nurtured by the society. In those days the NMR and ESR methods were much less developed than they soon became, so that any reasonably competent spectroscopist could understand all the methods employed. Additionally, because the earlier studies concentrated upon the better understood biological molecules or processes, the breadth of the applications did not baffle a slightly informed biochemist. The rapid advances in definite understanding were thrilling to practitioners in the field, and individual efforts were motivated by a sense that the field was going to grow. By that time NMR was firmly established as a

quantitative method in chemistry, solid state physics, and other material sciences so that with the results in hand it was logical to extrapolate to a future in which magnetic resonance could be central to biological research.

These high hopes, however, required considerable confidence in extrapolation, because the individual findings were sometimes slight when compared to the exciting cutting edges of biological research. When Oleg and Jim Fisher, for example, made their pioneering studies of relaxation changes in the ^{1}H resonances of penicillin as it bound to BSA (and we initiated parallel changes in molecules like ATP bound to paramagnetic ions) structural information of high quality was obtained but it was not comparable with rich detailed structural information being derived at the time from the early x-ray protein structures. These early individual hopes needed and received support from the excitement of more organized interplays, of which the ICMRBS was particularly helpful.

Oleg made two particularly personal contributions to the ICMRBS. First as mentioned above for the Boston meeting he worked continually to support its activities. In addition to organizing the Boston meeting, he participated in the organization of several succeeding meetings. He often wrote grants for funding and contributed his own support to the organizational matters. He kept records and kept track in a guardian way of the worldwide activities. This sense that he was available to back up the organization was particularly important because of the loose organization of these meetings in which a new group arranged all aspects of each meeting. Oleg helped form an advisory council system in which former organizers were represented on a committee whose composition turned over with time, and he was involved in the other continuing activities.

Beyond his steady hand at the oar, Oleg contributed a more formalized historical perspective both to the Society and the field by a sense of place and moment. As one can see it in retrospect, if this field were really to grow in strength, as we believed, then it truly represented an important historical flow. Oleg, perhaps because his earlier life was in a more formalized environment, became the embodiment of the more ceremonial aspects of the society and the field. He kept and preserved the records, he organized meetings to supplement the biannual conference and he spoke of the past, present and future of the field. Gradually over the years the future happened, and it is a full and rich time for NMR as we hoped.

Oleg's scientific contributions in retrospect had the same seminal values as the more historical perspectives I have been describing. His early experiments on ^{23}Na *in vivo*, his characterization in the early days of high-resolution NMR of the nucleic acids and the amino acids, his relaxation studies, and his introduction of isotopic labels were all original findings which have been the basis, not always acknowledged, of future research. Of his research at that time two directions stand out in my mind as the cutting edge, years ahead of their time.