Biological Magnetic Resonance

Volume 24

Biomedical EPR, Part B: Methodology, Instrumentation, and Dynamics

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Dedication:

To the students whom we hope to stimulate to become the next generation of biomedical EPR researchers.

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PREFACE

There has not been an attempt to cover the full scope of biological EPR in a single volume since *Biological Applications of Electron Spin Resonance* edited by Swartz, Bolton, and Borg in 1972. In three decades there have been enormous changes in the field. Our original plan for one volume expanded into two. A stimulus for an updated book at this time was the 70th birthday of James S. Hyde (May 20, 2002), one of the leaders in the development of EPR instrumentation and methodology applied to biological problems. To symbolically tie this book to Jim Hyde's efforts, we choose the title "Biomedical EPR", which is the name of the NIH-funded National Biomedical EPR Center founded by Harold Swartz and James Hyde at the Medical College of Wisconsin in 1975. This Center has been funded continuously since then, and has been a focal point of new developments and applications in biomedical research. Many of the authors of chapters in this book have been close associates of Jim Hyde, and several have been long-term members of the Advisory Committee of the Center.

There is a long history underlying most of the topics in these books. Some of this history was surveyed in *Foundations of Modern EPR,* edited by Eaton, Eaton, and Salikhov (1998). It is helpful to keep in mind that theoretical and experimental studies of spin relaxation preceded the development of EPR and NMR. The early work of Waller and of Gorter, for example, focused on spin relaxation (see *Foundations of Modern EPR*). Long development periods, and indirect paths from initial concept to biomedical application are the norm. Even new instrumentation or methodology developments, with few exceptions, require of the order of 10 to 15 years from "invention" to general application. No one could have predicted that the attempt to make a better measurement of the deuterium magnetic moment would lead to functional magnetic resonance imaging (fMRI), and if such a prediction had been made, it would have been dismissed as ridiculous. Those who sponsor research, and nurture researchers, enrich humanity by not demanding proof of relevance. We each pursue goals that inspire us, and hope that they will be of benefit. This book is part of a story as it unfolds.

Contributors were asked to make this book more "pedagogical" than "review." The goal is a multi-author introduction to biomedical EPR with up-to-date examples, explanations, and applications, pointing toward the future. Thus, the book is aimed not just at readers who are EPR experts, but at biomedical researchers seeking to learn whether EPR technology and methodology will be useful to solve their biomedical problems. The derivation and explanation of the underlying theory and methodology for many of the topics presented would require separate books. The authors

were asked to keep the background and theory to a minimum, referring whenever possible to other texts and reviews to lead the reader to additional information. The referencing in most chapters is thus to be tutorial and helpful, rather than to be comprehensive or to reflect priority of discovery. There is a focus on papers with a biological orientation. Thus, for example, although the fact that oxygen in solution broadens CW EPR spectra has been known since 1959 (see the chapter by Hauser and Brunner in *Foundations of Modern EPR*), the citations in the oxymetry chapter in this book to biologically relevant literature about oxygen broadening start about twenty years later. The perspective in each chapter is presented from the viewpoint of people involved in cutting-edge research.

Chapters, including our own, were peer-reviewed, usually by at least two referees in addition to the editors. We thank the referees for their assistance in improving the pedagogy of the chapters. The editors have added cross references between chapters.

In these volumes, we did not include some topics that had been reviewed recently. Spin Labeling I (1976) and II (1979), and the two volumes in this series that are successors to these, volumes 8 (1989) and 14 (1998), emphasize nitroxyl radicals. Volume 13 (1993) emphasizes paramagnetic metals, especially in enzymes, and transient EPR and spin trapping. Volume 18 (2004) describes *in vivo* EPR. Volume 19 (2000) is about measuring distances between unpaired electrons. Volume 21 of the Biological Magnetic Resonance series includes chapters on instrumentation (Bender), sensitivity (Rinard, Quine, Eaton, and Eaton), and a survey of low-frequency spectrometers (Eaton and Eaton). Other chapters of interest can be found in the list of contents of related prior volumes, at the end of each of these volumes. Some volumes in the series Metal Ions in Biological Systems, edited by Sigel focus on EPR. See, for example, Volume 22 (*ENDOR, EPR, and Electron Spin Echo for Probing Coordination Spheres,* 1987).

Although the focus of this book is on biomedical applications of EPR, and the examples used in this book therefore are largely from the biomedical field, an analogous treatise could focus on materials science, traditional small-molecule chemistry, or solid state physics. There are, of course, unifying theoretical, instrumental, and experimental methodologies that cross disciplinary applications. EPR has the great power of specificity for unpaired electron spins, and as Jim has said more than once, "there are spins everywhere."

Biological applications of EPR encompass measuring metal ion environments in proteins at liquid helium temperature and measuring NO production in living animals. The variety of technologies and methodologies required is so wide that a researcher who is expert in one may be almost

unaware of another. The landscape is rich and the horizons extend as far as we can see. These two volumes, which should be read as a single treatise, have the goal of helping biomedical researchers see a little further.

Some potential users will need a more extensive basic introduction to EPR. The reader unfamiliar with EPR may want to start with the Introduction to the chapter by Subramanian and Krishna in Part B (Volume 24), which includes a concise survey of the basic principles of EPR. The Swartz, Bolton and Borg book (1972) mentioned above also is a good place to start. Among the several complete texts on EPR, those by Carrington and McLachlan (1967), by Weil, Bolton and Wertz (1994), and by Atherton (1993) are particularly appropriate for beginners who have a good physical chemistry background. Eaton and Eaton (1997) present an introduction to CW and pulsed EPR, with an emphasis on practical experimental aspects for the novice. Experimental and instrumental aspects of EPR are treated in Fraenkel (1959) and Reiger (1972), but the two major and most highly recommended sources are Alger (1968) and Poole (1967, 1983). Jim Hyde also wrote a brief summary of instrumental aspects of EPR (1995). It is hoped that some readers will enjoy learning some of the historical background of the field. Some of the chapters in this book provide a glimpse, and Foundations of Modern EPR (1998) captures the thinking of pioneers in the field on the occasion of the 50^{th} anniversary of the discovery.

Pictures of experimental EPR spectra beyond those in these books may help the reader's understanding. Many spectra are reproduced in the texts cited above, and in Yen (1969), McGarvey (1966), Goodman and Raynor (1970), Drago (1992), Gerson (1970), and Gerson and Huber (2003). Some early reviews of spin labeling remain very useful introductions to the fundamentals of CW EPR of nitroxyl radical line shapes (Griffith and Wagoner, 1969; Jost, Wagoner, and Griffith, 1971; Jost and Griffith, 1972; Gaffney, 1974).

There is not enough space in these two volumes to teach the underlying principles of pulsed EPR in depth, nor to illustrate the wide range of applications. Readers are directed to several other books for more on these topics: Kevan and Swartz (1979), Keijzers et al. (1989), Hoff (1989), Kevan and Bowman (1990), Dikanov and Tsvetkov (1992), Schweiger and Jeschke (2000), and Berliner, Eaton, and Eaton, (2000) (volume 19 in this series).

For those readers unfamiliar with the practical methodology of EPR, it is reasonable to ask "how long will it take to run an EPR spectrum?" The answer depends strongly on what one wants to learn from the sample, and can range from a few minutes to many weeks. Even the simple question, are there any unpaired electrons present, may take quite a bit of effort to answer, unless one already knows a lot about the sample. Column fractions of a nitroxyl-spin-labeled polymer can be monitored for radicals about as fast as

the samples can be put in the spectrometer. This is an example of an application that could be automated. On the other hand, the spins may have relaxation times so long that they are difficult to observe without saturation or so short that they cannot be observed except at very low temperature where the relaxation times become long enough (e.g., Co(II) in many environments). If one wants to know the concentration of Co(II) in a sample, need for quantitative sample preparation, accurate cryogenic temperature control, careful background subtraction, and skillful setting of instrument parameters lead to a rather time-consuming measurement.

Other reasonable questions include "how much will this cost?" and "how/where can I do this?" EPR measurements require a significant investment in instrumentation, but spectrometer systems are available from several vendors. The largest manufacturers, Bruker BioSpin EPR Division, and JEOL, market general-purpose spectrometers intended to fulfil most analytical needs. The focus is on X-band (ca. 9-10 GHz) continuous wave (CW) spectrometers, with a wide variety of resonators to provide for many types of samples. Accessories facilitate control of the sample temperature from <4K to ca. 700 K. Magnets commonly range from 6-inch to 12-inch pole face diameters. Smaller, table-top spectrometers are available from Bruker, JEOL, and Resonance Instruments. Some of these have permanent magnets and sweep coils for applications that focus on spectra near g = 2, and others have electromagnets permitting wide field sweeps. Bruker makes one small system optimized for quantitation of organic radicals and defect centers, such as for dosimetry. Bruker and JEOL market pulsed, time-domain spectrometers as well as CW spectrometers. Bruker and JEOL market spectrometers for frequencies lower than X-band, which are useful for study of lossy samples. Bruker markets high-frequency (95 GHz), high-field EPR spectrometers that require superconducting magnets, not electromagnets.

Volume 23 begins with an appreciation of the contributions that Jim Hyde made to biomedical EPR, with some historical perspective by Helmut Beinert and Harold Swartz of the mutual stimulation of Jim, the NIH Research Resource "Center" funding program, and the collaborations it spawned.

Among the common analytical tools available to those who study the properties of matter, whether biological or non-biological, ESR has the special feature that it is very sensitive to the anisotropy of the environment of the unpaired electron. The CW EPR spectral line shape is strongly influence by motions that are of the order of the anisotropies in hyperfine couplings and in g-values. Electron spin relaxation times are also sensitive to molecular motions. These effects give rise to the ability to measure rates

and anisotropies of molecular motions, and stimulate the extensive field of spin labeling. One of the first physical parameters of spin labels to be exploited, the incomplete averaging of anisotropic g and hyperfine values, remains central to many uses of nitroxyl spin probes and spin labels. Freed (volume 24 chapter 9) explores the motions reported in great detail by nitroxyl EPR spectra. The saturation transfer technique developed by Jim Hyde and Larry Dalton (1979) is crucial to learning about the dynamics of biological membranes (Marsh et al., volume 24 chapter 11, and Beth and Husted, chapter 12). Beth and Husted show the sensitivity of Q-band (ca. 35 GHz) and W-band (ca. 95 GHz) EPR for analyzing complex anisotropic rotational dynamics, and emphasize the utility of global analysis of spectra obtained at two or more microwave frequencies. Basosi in volume 23 chapter 13 illustrates the kinds of information that can be learned about motions of metal ions.

There are contributions to the CW lines shape and some relaxation properties from electron-nuclear and electron-electron couplings. The dipolar part of the interaction is the basis for distance measurements. Electron-electron distance measurements were the topic of Volume 19 in this series (Berliner, Eaton, and Eaton, 2000), and the Eatons have presented a concise summary of this topic in chapter 8, and in Eaton and Eaton (2002). Because the electron dipole is larger than the nuclear dipoles, EPR measures distances that are larger than the distances measured by NMR. Multiple resonance techniques provide more detail about the spin environment than do "normal" EPR techniques. ENDOR is a very important tool for resolving hyperfine structure. ENDOR of species in frozen glassy solutions is described by Mustafi and Makinen in volume 24 chapter 4 and ENDOR of radicals in fluid solution is described by Gerson and Gescheidt in chapter 5. Next, Lowell Kispert (chapter 6) describes CW, pulsed, and multiquantum ELDOR as ways of probing electron-electron spin-spin interactions.

Many fundamental studies are directly relevant to biomedical science, but the goal of it all is to understand function and malfunction of living systems. It is important to perceive the relevance to human studies of early explorations in plants, for example. The chapter on free radicals and medicine (volume 23 chapter 3) surveys many of the motivations for investigating free radical phenomena. We thank Hal Swartz for coordinating the several contributors to this chapter, and for writing the introduction that give his overall perspective on this important area of science. How far we have come toward studies of animals and humans is reflected by several chapters. Hal Swartz and Nadeem Khan in volume 23 chapter 9 discuss the achievements to date and future possibilities in EPR spectroscopy of function *in vivo.* Depending on your point of view, Hal's perspective could be described as realistic or pessimistic. Maybe some reader will be

stimulated to demonstrate clinical importance of tools that Hal says are unlikely to have major application. The major focus of research for *in vivo* EPR is the development of methodology to measure O_2 concentrations for medical purposes. Modern instrumentation facilitates a new focus on measurement of relaxation times, and use of relaxation properties to measure O_2, distances, etc. (see, for example, volume 24 chapters 1 and 8 by Eaton and Eaton). Benjamin Williams and Howard Halpern in volume 23 chapter 11 and Sankaran Subramanian and Murali Krishna in chapter 12 describe the fundamentals *in vivo* EPR spectroscopy and imaging by CW and pulsed low frequency EPR, respectively. Both of these chapters relate to measurement of O_2 in vivo. Oximetry is also the topic of the very detailed chapter 10 by Subczynski and Swartz.

Reactive free radicals, including superoxide (see volume 23 chapter 4 by Vásquez-Vivar, Martásek, and Kalyanaraman), are studied by the spin-trapping technique. In chapter 5 Ron Mason and Maria Kadiiska describe trapping of reactive radicals *in vivo,* with *ex vivo* detection of the EPR signal. Then, in chapter 6 Keszler and Hogg demonstrate linear regression analysis of multiple spin-trapped spectra to obtain kinetic information.

Application of EPR to understanding complex biological systems is illustrated by the examples of melanin (Sarna and Plonka, volume 23, chapter 7) and photosynthesis (Tikhanov and Subczynski, chapter 8).

As is emphasized in the introductory perspectives by Beinert and Swartz, many of Jim Hyde's contributions were innovations in instrumentation. The tight coupling to biomedical applications, first via visitors to Varian Associates and then with his colleagues at the Medical College and visitors to the National Biomedical ESR Center, focused Jim's instrumentation and methodology development on biomedical problems. Saturation recovery (Eaton and Eaton, volume 24 chapter 1) is applied in several biological investigations, including the oximetry measurements mentioned above. Loop-gap resonators (volume 24 chapter 2 by Rinard and Eaton) were the enabling technology for some of the recent developments in stopped-flow and rapid mixing EPR (Scholes, volume 24, chapter 3) and for the ability to study small spin-labeled protein samples, which gave the impetus to rapid application of site-directed spin labeling (Feix and Klug, Volume 24 chapter 10). In volume 24, chapter 13, Jim presents a perspective on the role of instrumentation in biomedical research. One of the trends foreseen, increased use of computer capability for fast digitization and post-processing, is illustrated by volume 24 chapter 7 (Hyde et al.). Having all frequencies in a magnetic resonance spectrometer phase-locked to a single master oscillator, then using fast digital detection and time-locked

subsampling permits, for example, study of multiple harmonics of the field modulated signal.

We thank the authors for contributing to this book, and we also thank the many anonymous referees whose attention to both large and small matters helped improve the chapters. Beverly Ventura, Biophysics Research Institute, Medical College of Wisconsin, helped several authors with grammar and layout of their chapters. The final formatting of all chapters was done by one of the editors (SSE). We add special thanks to Hal Swartz, whose recruitment of Jim Hyde from Varian to the Medical College of Wisconsin set the stage for much of what is presented in these books, and who somehow could not say no to repeated entreaties to write on yet another topic. He ended up writing the introductory chapter on the background of Jim Hyde and the ESR Center in Milwaukee, and three chapters covering *in vivo* spectroscopy, oximetry, and free radicals in medicine. The last of these was a major effort, since we persuaded him to take contributions from many co-authors and assemble them into the overall chapter, which was probably more work than just writing it all himself!

Overall, even though we introduced these two books as a successor to the Swartz, Bolton, and Borg 1972 book, these books are still just a preface to the future of biomedical applications of EPR.

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