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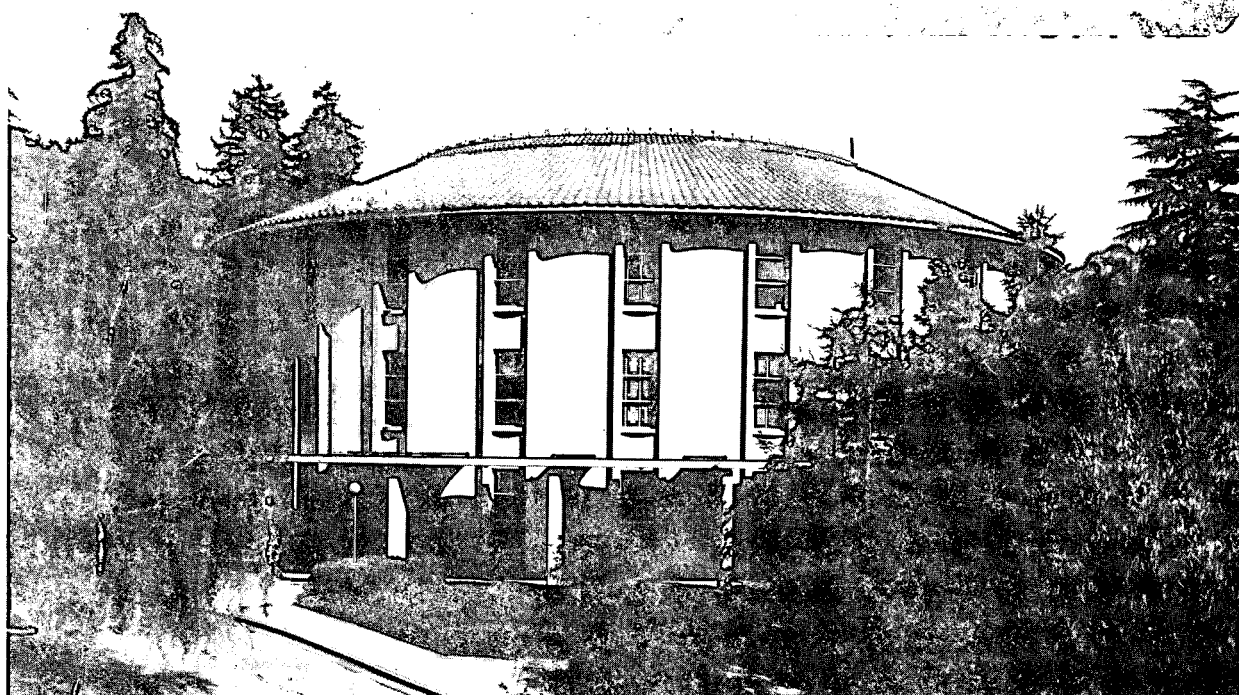
Biophysical Applications of Solid State and Tritium NMR

S. Un
(Ph.D. Thesis)

October 1987

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Biophysical Applications of Solid State and Tritium NMR

Sun Un

Ph.D. Thesis

Lawrence Berkeley Laboratory
University of California
Berkeley, California 94720

October 1987

Biophysical Applications of Solid State and Tritium NMR

by

Sun Un

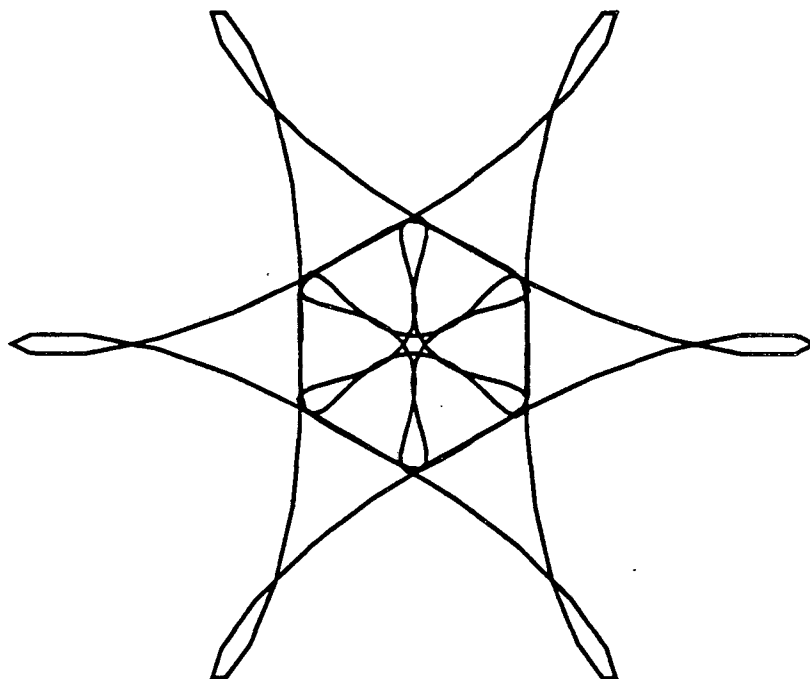
Abstract

Novel applications of Magic Angle Sample Spinning (MASS) and Tritium (^3H) NMR to the study of mammalian tissue and *in vivo* metabolism, respectively, are described. The solid state ^{31}P MASS NMR spectra of sodium, magnesium and calcium complexes of adenosine triphosphate (ATP) were recorded. These spectra indicate that the terminal phosphate group of the sodium complex is protonated in contrast to the unprotonated divalent complexes. The inductive effects of the divalent counterions are also discussed. To better understand the nature of the ^{31}P chemical shift interaction, the chemical shift tensors of a large variety of phosphates and phosphate esters were measured. The magnitude of the σ_{33} , the low field, elements of the ^{31}P chemical shift tensors in these phosphates were linearly related to the longest P-O bonds and the σ_{11} , the high field, elements to the shortest bonds. Similar relationships were found when these tensor elements were plotted against bond angles. The implications of these observations are discussed in the context of phosphorus-oxygen d-p π bonding interactions. ^{31}P and ^1H MASS NMR were applied to the study of lyophilized rat tissue. The ^{31}P spectra exhibit resonances from phospholipids, DNA, ATP and NAD. The MASS results were compared to chemical analysis data obtained by others and to calculated spectra based on MASS data of model compounds. The ^1H MASS spectra of these tissue samples exhibited lines with widths less than 40 Hz. These signals were assigned to phospholipid protons. The intensity distribution of the ^1H spectra supported this assignment. Motionally narrowed ^{31}P and ^1H resonances suggest that the phospholipids undergo varying degrees of motion with correlation times ranging from 0.5 ns to 2 μs . The degree of hydration of the DNA was also established. The metabolism of [1- ^3H]glucose in human and rat erythrocytes was monitored by ^3H NMR. The α anomer of glucose was metabolized at a higher rate than the β anomer. A glycolytic intermediate was observed and tentatively identified as 1,2-diphosphoglycerate. The total concentration

of "protons" released from the labelled positions was determined. The importance of this measurement in relation to enzyme mechanisms and the hexose monophosphate (HMP) shunt is discussed.

In memory of my grandmother

이 재 우



The trajectory of a MASS magnetization vector

I remember, in the winter of our first experiments, just seven years ago, looking at snow with new eyes. There the snow lay around my doorstep—great heaps of protons quietly precessing in the earth's magnetic field. To see the world for a moment as something rich and strange is the private reward of many a discovery. [Purcell, Science, 118, 431 (1953)]

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Preface

Preface

NMR spectroscopy has recently enjoyed a great deal of growth in its application to biology and medicine. It is obviously necessary in any spectroscopic experiment to characterize what the spectrometer is capable of "seeing". In large biological systems, this is especially important because of their highly heterogeneous nature. *In vivo* Phosphorus and proton NMR of tissue provide good examples. Much of a proton spectrum of tissue is obscured by the presence of 100 M proton from water. For reasons described later, this problem is compounded by the fact that even in the absence of water, protons on very large immobile molecules cannot be observed in routine experiments. Phosphorus spectra of tissue typically show only signals arising from small molecules, but not from the very large ones such as phospholipids and DNA. Only a small fraction of the total cell phosphorus is observed. The situation is made worse by the fact that under certain situations it is entirely possible that the observed population of a particular phosphorus bearing molecule does not constitute the total. Therefore, it is equally important to understand why certain signals are unobservable and, paradoxically, the ability to selectively observe only certain signals is also important. The two approaches which are discussed in this thesis attempt to address separately the problems of "seeing all" and "seeing only".

Some amount of explanation is needed to understand the topics covered in this thesis and their connections. The middle three chapters, Chapters 2, 3 and 4 describe experimental work which grew out of a single question: how much of the cell phosphorus can be observed using solid state NMR techniques? This question, in turn, arose from observations that not all of the phosphorus bearing molecules were observed in *in vivo* experiments (Evans, 1979). A further discussion of the importance and context of this question is covered in those chapters. The solid state technique which was chosen was magic angle sample spinning (MASS). The Chapters 2 and 3 discuss results from our first attempts to characterize phosphorus compounds which we expected to see in whole tissue and these molecules also served as a calibration experiment for both spectrometer and spectroscopist. For these experiments, ATP was an obvious first choice. As often happens, the chemical shift tensors of ATP pointed to some interesting questions about phosphorus chemical shift interactions in general. This led to a more general

study of chemical shift tensors of phosphates and their esters. The results of this study are discussed in the third chapter. Finally, in Chapter 4 results pertaining to the observation of phosphorus bearing molecules in rat tissue is presented. In this chapter, the proton data on these tissue samples are also discussed. Initial observations which eventually led to these data were obtained quite serendipitously while calibrating proton parameters for the phosphorus experiments. These experiments very nicely complemented the observations made in phosphorus experiments. Chapter 5 deals with a completely different magnetic resonance approach to studying biological systems: that of use of tritium labelled probes. These experiments on glycolysis using this technique served the dual purpose of demonstrating the applicability of tritium NMR to biological systems and obtaining basic *in vivo* information about an important enzymatic pathway.

The solid state and tritium experiments were carried out completely independent of each other. They are in many ways diametrically opposite experiments. The solid state experiments sought to obtain a "complete" spectra of tissue while the main appeal of the tritium experiments was that a highly selective "proton" spectrum could be observed. The emphasis in the MASS experiments was on the manipulation of the spins through physical compared to the essentially chemical approach of the tritium experiments where the critical element was the highly labelled pure glucose. In order for NMR to be fruitfully applied to biological problems, both approaches will be necessary.

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Evans, F. E., *Arch. Biochem. Biophys.*, **193**, 63, (1979).

Chapter 1
SU(2) and SO(3) applied to Parts of Rats
and other things.

Chapter 1. SU(2) and SO(3) applied to Parts of Rats and other things.

I. History

First some historical background is in order. Andrew (Andrew, 1959) and Lowe (Lowe, 1959) independently realized that by spinning a sample at an angle of 54.7356 degrees with respect to the magnetic field axis one could remove certain broadening interactions in solids. This angle is often called the “magic angle” and the technique, magical angle sample spinning (MASS). The details and importance of these observations are discussed below. Much of the subsequent early work was carried out by Andrews and coworkers (Andrews, 1959,1962,1966). The coupling of the cross polarization (CP) experiment developed by Pines, Gibby, and Waugh (Pines, 1972) with MASS by Schaefer and Stejskal (Schaefer and Stejskal, 1977) delivered the MASS experiments from the clutches of the physicists into those of the chemists. The cross polarization experiment allowed enhanced detection of low magnetogyric ratio nuclei, such as ^{13}C and ^{31}P . MASS, in conjunction with CP and dipolar decoupling, made it possible to detect a high resolution “liquid-like” spectra in solids which endeared this experiment to chemists. In the category of having-your-cake-and-eating-it-too, Mariq and Waugh (Mariq and Waugh, 1979) pointed out that with slow spinning one could obtain not only high resolution, but also, recover the spatial information about the interaction which are averaged by sample spinning. For those only interested in obtaining the highest possible resolution very recent technical developments have seen spinning rates of 20 kHz! The combination of these efforts over the past 28 years has made MASS and in particular CP-MASS a workhorse technique for studying material in the solid state.

II. Quanta of Quantum Mechanics

There have been many good reviews on MASS and its application. In particular, the reviews by Andrew (Andrew, 1971) and Yannoni (Yannoni, 1982) are noteworthy. This section, as well as Appendices B, C and D, reviews the basic quantum mechanics and other bits and pieces of chemical physics which are necessary to understand how magic angle sample spinning (MASS) works and how a MASS spectrum can be calculated. The ability to calculate MASS spectra will be much exploited in the subsequent chapters. The comparison of experimental and theoretical spectra will provide the basis for analyzing the MASS data.

A. A Quantum Mechanics Primer

The centerpiece of this discussion is the familiar energy operator, the Hamiltonian. For a given particle, in our case a nuclear spin, the Hamiltonian is given by the equation

$$\hat{H}|\Psi\rangle = E|\Psi\rangle \quad 1.1$$

where $|\Psi\rangle$ is the state vector, or eigenfunction, and it represents the quantum state of the particle. The $\hat{H}$ is the Hamiltonian and E , the eigenvalue, is a real number equal to the energy of the particle. The time evolution of the state is given by Schrödinger's equation

$$i\hbar \frac{d}{dt}|\Psi(t)\rangle = \hat{H}(t)|\Psi(t)\rangle \quad 1.2$$

where $\hat{H}(t)$ and $|\Psi(t)\rangle$ are labelled explicitly with the time variable to indicate possible time dependences. For the remainder $\hbar$ will be equated to unity. Formal integration of Eq 1.2 yields

$$|\Psi(t)\rangle = \hat{U}(t)|\Psi(0)\rangle \quad 1.3$$

where

$$\hat{U}(t) = \hat{T} \exp\left(i \int_0^t \hat{H}(t') dt'\right). \quad 1.4$$

$\hat{T}$ is the Dyson time ordering operator. The detailed derivation of Eq. 1.4 and the significance of the time ordering operator can be found in Baym (Baym,1978). For situations with which we will be concerned, the time-ordering operator may be rigorously dropped.

Alternatively, the time dependence of the system may be relegated to the operators instead of the state vectors. In this case, the equation of motion for an explicitly time independent operator, $\hat{M}$, is

$$\frac{d}{dt}\hat{M}(t) = i [\hat{H}, \hat{M}(t)], \quad 1.5$$

the solution to which is

$$\hat{M}(t) = \hat{U} \hat{M}(0) \hat{U}^\dagger \quad 1.6$$

where $\hat{U}$ is defined by Eq. 1.4 and $\hat{U}^\dagger$ is adjoint operator of $\hat{U}$. Eq. 1.5 and 1.6 describe the system in the Heisenberg representation.

Often in magnetic resonance, it is convenient to endow both the operator and the state vector with time dependences. A general treatment is given in standard textbooks (Baym, 1974; Davydov, 1976). For the present, it is sufficient to observe the following. The Hamiltonian in many cases may be broken up in the following manner

$$\hat{H} = \hat{H}_0 + \hat{H}_1. \quad 1.7$$

The motivation for this division will become clearer in the following discussion. In the Heisenberg representation, the time dependence for an operator, $\hat{M}$, is given by Eq. 1.6

$$\hat{M}(t) = e^{i(\hat{H}_0 + \hat{H}_1)t} \hat{M}(0) e^{-i(\hat{H}_0 + \hat{H}_1)t}$$

A new operator, $\hat{\tilde{M}}(t)$, and state vector, $\tilde{\psi}(t)$, defined by

$$\hat{\tilde{M}}(t) = e^{i\hat{H}_0 t} \hat{M}(t) e^{-i\hat{H}_0 t} \quad \text{and} \quad |\tilde{\psi}(t)\rangle = e^{i\hat{H}_0 t} |\Psi(t)\rangle \quad 1.8$$

is now considered. If $\hat{\tilde{M}}(t)$ does not explicitly depend on time, differentiation of expression Eq. 1.8 with respect to time yields

$$\frac{d}{dt} \hat{\tilde{M}} = i [\hat{\tilde{H}}_0, \hat{\tilde{M}}] \quad 1.9a$$

and

$$\frac{d}{dt} |\tilde{\psi}(t)\rangle = -i \hat{\tilde{H}}_1 |\tilde{\psi}(t)\rangle \quad 1.9b$$

In this representation, the state evolves under $\hat{\tilde{H}}_1$ only, while the operators evolve under $\hat{\tilde{H}}_0$. If $\hat{\tilde{M}}(t)$ commutes with $\hat{\tilde{H}}_0$, then $\hat{\tilde{M}}(t)$ will remain constant in time. This representation is called the interaction representation and $\hat{\tilde{H}}_1$ is commonly referred to the interaction Hamiltonian, the reason for which is made clearer in the following discussion.

The final concept to be considered in this quantum mechanics primer is the density operator. The density operator is defined as

$$\hat{\rho} = |\Psi\rangle\langle\Psi| \quad \text{where} \quad |\Psi\rangle = \sum_i c_i |\chi_i\rangle \quad 1.10$$

where the eigenfunction has been expanded as a linear combination of a complete set of basis functions, $|\chi_i\rangle$, and the coefficients, c_i , are in general complex. If the density operator is expressed in matrix form using the $|\chi_i\rangle$ basis then the elements of the matrix are

$$\rho_{ij} = \bar{c}_i c_j = \langle \chi_i | \hat{\rho} | \chi_j \rangle. \quad 1.11$$

The expectation value of an operator, $\hat{A}$, is given by

$$\begin{aligned}\langle \hat{A} \rangle &= \langle \Psi | \hat{A} | \Psi \rangle \\ &= \sum_{ij} \langle \chi_j | \hat{\rho} | \chi_i \rangle \langle \chi_i | \hat{A} | \chi_j \rangle \\ &= \text{Tr}(\hat{\rho} \hat{A})\end{aligned}\tag{1.12}$$

where Eq 1.10 and the closure property (Cohen-Tannoudji, 1977) have been used. The last equality of Eq. 1.11 is an alternative expression for the expectation value of an operator. Finally, the time dependent behavior of the density operator is considered. Explicit inclusion of the time evolution into Eq. 1.10 yields

$$\hat{\rho}(t) = |\Psi(t)\rangle\langle\Psi(t)| = e^{-i\hat{H}t}|\Psi(0)\rangle\langle\Psi(0)|e^{i\hat{H}t} = e^{-i\hat{H}t}\hat{\rho}(0)e^{i\hat{H}t}.\tag{1.13}$$

and differentiation with respect to time gives

$$\frac{d}{dt}\hat{\rho} = i [\hat{\rho}, \hat{H}].\tag{1.14}$$

When the density matrix is transformed into the interaction representation using Eq 1.9 followed by substitution into Eq 1.14, the resulting equation of motion is

$$\frac{\partial}{\partial t}\hat{\tilde{\rho}}(t) = i [\hat{\tilde{\rho}}(t), \hat{\tilde{H}}_1]\tag{1.15}$$

where

$$\hat{\tilde{H}}_1(t) = e^{i\hat{H}_0t}\hat{H}_1(t)e^{-i\hat{H}_0t}.$$

It is also possible to show that

$$\langle \hat{\tilde{A}} \rangle = \text{Tr}(\hat{\tilde{\rho}} \hat{\tilde{A}}).\tag{1.16}$$

For problems in magnetic resonance an ensemble of particles must be considered. This is where the density matrix has its greatest utility. The value of $\bar{c}_i c_i$ or ρ_{ii} simply gives the probability of the particle being in state $|\chi_i\rangle$. Therefore, if the ensemble averaged values of these quantities are known, one can apply Eq. 1.16 to calculate the ensemble averaged expectation value of an operator. Therefore, the statistics of the ensemble is built into the density matrix. The treatment of the density operator and quantum statistics discussed here is far from being either complete or rigorous. Such treatment on the topic can be found in textbooks by Feynman (Feynman, 1981), Pathria (Pathria, 1972) and reviews by Fano (Fano, 1957) and Ter Haar (Ter Haar, 1961).

B. The Hamiltonians

The Hamiltonian which describes the basic nuclear spin interactions can be divided into extrinsic and intrinsic components

$$\hat{H}_{total} = \hat{H}_{ext} + \hat{H}_{int}. \quad 1.17$$

The first term on the right side of the equality represents the interaction of the nuclear spins with the externally applied magnetic fields. This term can be further divided to give

$$\hat{H}_{ext} = \hat{H}_s + \hat{H}_{rf}.$$

$\hat{H}_s$ is the Zeeman interaction of the spin with the large field produced by the superconducting magnet and $\hat{H}_{rf}$ describes the interactions between the spins and the various fields which the spectroscopist can apply using radio-frequency (rf) irradiation. The second term on the right side of Eq. 1.17 represents the intrinsic interactions of the spins. Depending on what nuclei are part of the system, there can be a wide variety of terms which contribute to the intrinsic part of the Hamiltonian. We will limit the discussion to a small subset which is appropriate for future discussions. The Hamiltonian which describes these interactions is given by

$$\hat{H}_{int} = \hat{H}_I + \hat{H}_S + \hat{H}_{II} + \hat{H}_{SS} + \hat{H}_{IS}.$$

The first two terms represent the chemical shift interaction of two different types of nuclei. This interaction originates from the magnetic shielding by the electron density surrounding the nuclei. A related phenomenon in metals was observed first by Knight (Knight, 1949) and shifts in chemical compounds were observed by Proctor and Yu (Proctor and Yu, 1950) for ^{14}N nuclei. The last three terms of Eq. 1.8 represent the interactions between two nuclei, the various dipole-dipole interactions. Now, we shall discuss the specific forms of the interactions.

i. The extrinsic Hamiltonians

The dominant interaction, in most NMR experiments, is the Zeeman interaction given by

$$\hat{H}_s = \gamma_I \mathbf{B}_0 \cdot \mathbf{1} \cdot \hat{\mathbf{I}} \quad 1.18$$

where $\mathbf{1}$ is a 3 by 3 unit matrix, $\hat{\mathbf{I}}$ is the spin angular momentum operator, γ_I is the magnetogyric ratio, $\mathbf{B}_0$ is the static applied field vector. The frequency determined by $\gamma_I B_0$ is called the

Larmor frequency. The magnitude of this field, for all of the experiments described in this thesis is about 63 kG. At this point, a simple example is in order. For a proton spin ($I=1/2$) in a magnetic field aligned along the z -axis, Eq. 1.8 becomes

$$\hat{H}_z = \gamma_I B_0 \hat{I}_z \quad 1.18a$$

which is a simple scalar equation. All of the quantum mechanics resides in the operator $\hat{I}_z$. The two eigenfunctions and eigenvalues of $\hat{I}_z$ are well known and the states are denoted $|+\rangle$ and $|-\rangle$ with the eigenvalues $+1/2$ and $-1/2$, respectively (Cohen-Tannoudji, 1977). The eigenvalues of $\hat{H}_z$ are, therefore, $+1/2\gamma_I B_0$ and $-1/2\gamma_I B_0$, respectively. This constitutes one of the simplest two-level systems and is depicted in Figure 1.1. At 63 kG, for protons, the difference in the energies between the two states is about 270 MHz, 1.79×10^{-25} J, 1.79×10^{-18} erg, 1.12×10^{-6} eV or 4.27×10^{-26} cal. The selection rule for NMR transitions is $\Delta m = \pm 1$; therefore, the NMR spectrum of a sample of such proton spins would be a single line at 270 MHz. For a large ensemble of isolated spins in thermal equilibrium at room temperature, the ratio of the populations of the two states is 1.0000438. The states are essentially equally populated with slightly more spins in the $+1/2$ state. The poor sensitivity of NMR spectroscopy (for example compared to electronic and infra-red spectroscopy) arises from the fact that the signal is proportional to this population difference.

The other contribution to the extrinsic Hamiltonian is the interaction of the nuclear spins with radio-frequency irradiation. The spins are irradiated by applying a radio-frequency voltage to a set of coils placed orthogonal to the static field axis. The coils in turn produce an oscillating magnetic field, the two components of which precess in the x - y plane in opposite sense. The interaction of a spin with this magnetic field is given by

$$\hat{H}_{rf} = \gamma_I B_1 \left[\hat{I}_x \cos(\omega t + \phi) + \hat{I}_y \sin(\omega t + \phi) \right] \quad 1.19$$

where $\gamma_I B_1$ is the strength of the r.f. field and ω and ϕ are the frequency and phase of the oscillation (Schlichter, 1980). It will be assumed that B_1 can be made arbitrarily large, smaller than the applied static field, B_0 , and much larger than any intrinsic interactions (see below). However, this may not be the case in practice.

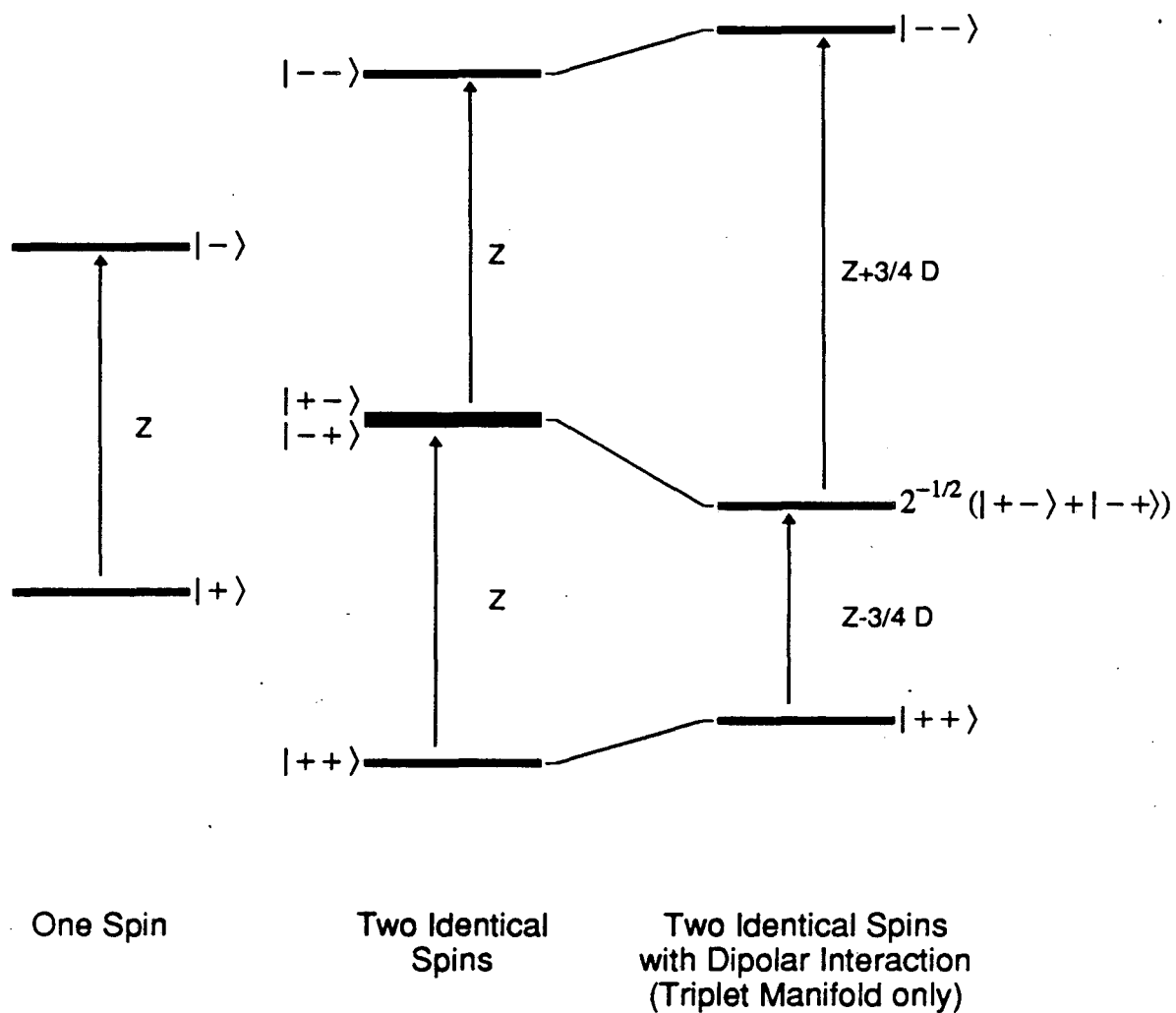


Figure 1.1. The energy levels of one and two spin systems. The symbol, Z , represents the Zeeman interaction and is equal to $\gamma_I B_0$ and the symbol, D , represents the dipolar interaction and is equal to $\gamma_1 \gamma_2 / r^3 (1 - 3 \cos^2 \theta)$. The spectrum of the dipolar system is consists of two lines separated by $3/2D$ centered about Z .

ii. The intrinsic Hamiltonians

The first intrinsic term of the nuclear spin Hamiltonian to be considered is the chemical shift interaction. The chemical shift interaction results from the modification of the applied magnetic field at the nucleus due to the current induced in the surrounding electron density. A theoretical treatment can be found in the text by Slichter (Slichter, 1980) and some more practical aspects of ^{31}P chemical shifts are discussed in subsequent chapters. The chemical shift interaction Hamiltonian has the same form as Eq 1.18 and is given by

$$\hat{H}_c = \gamma_I \mathbf{B}_0 \cdot \underline{\sigma} \cdot \hat{\mathbf{I}} \quad 1.20$$

where $\underline{\sigma}$ is a tensor which can be represented as a 3 by 3 matrix. The tensorial nature of Eq. 1.20 reflects the anisotropic (*i.e.* orientation dependent) nature of the interaction which arises from the non-spherically symmetric electron density about the nucleus. To first order, the tensor, $\underline{\sigma}$ may be considered symmetric (see Appendix B). A discussion detailing the manifestations of a small antisymmetric component of the chemical shift is found in a review by Haeberlen (Haeberlen, 1976). In the special case where the electron density is spherically symmetric, $\underline{\sigma}$ reduces to scalar (a multiplicative constant) and is given by

$$\hat{H}_c = \gamma_I \sigma \mathbf{B}_0 \cdot \hat{\mathbf{I}}. \quad 1.20a$$

The distinction between chemical shift and Zeeman interactions is somewhat arbitrary. The chemical shift interaction is usually measured relative to a chemical standard which is assumed to have a vanishing chemical shift interaction and undergoes just a Zeeman interaction. The quantum mechanics of the chemical shift interaction is exactly the same as in the Zeeman case discussed above, the difference being a small anisotropic modification of the eigenvalues (*i.e.* energies). This would result in a small orientation dependent shift of the NMR resonance from its Zeeman value. The manifestations of the anisotropy will be discussed in the following sections. The range of this interaction is on the order of 20 kHz for ^{31}P nuclei in phosphates, the Zeeman interaction being about 109 MHz at a field of 63 kG.

Finally, the quantum mechanical expression for the interaction between two nuclear dipoles is given by

$$\hat{H}_d = \frac{\gamma_1 \gamma_2}{r^3} \left[\hat{\mathbf{I}}_1 \cdot \hat{\mathbf{I}}_2 - 3 \frac{(\hat{\mathbf{I}}_1 \cdot \mathbf{r})(\hat{\mathbf{I}}_2 \cdot \mathbf{r})}{r^2} \right]. \quad 1.21$$

or alternatively

$$\hat{H}_d = \hat{\mathbf{I}}_1 \cdot \underline{D} \cdot \hat{\mathbf{I}}_2 \quad 1.21a$$

where

$$\underline{D} = \frac{\gamma_1 \gamma_2}{r^3} [1 - 3\mathbf{R}]$$

and where the subscripts refer to the two nuclei which may be of the same type or different; $\mathbf{r}_{12}$ is the internuclear vector which connects the two spins and $\mathbf{R}$ is a tensor, the elements of which are

$$R_{ij} = r_i r_j, \quad \text{where} \quad i, j = x, y, z$$

The magnitude of the pre-factor, $\gamma_1 \gamma_2 / r^3$ for two protons separated by 1.78 Å (typical for protons bound to the same carbon) is 21 KHz. A good general value for the pre-factor for two protons is $1.20 \times 10^{-25} \text{ Hz} \cdot \text{m}^3$. It is often useful to re-express Eq. 1.21 as the famous "dipolar alphabet". To do this, $\mathbf{r}_{12}$ is expressed in polar coordinates and the angular momentum vector operators are expanded into their components, expressed in terms of raising and lowering operators. With some algebraic manipulation, the following results

$$\begin{aligned} A &= (1 - 3 \cos^2 \theta) \hat{I}_{1z} \hat{I}_{2z} \\ B &= \frac{1}{2} (1 - 3 \cos^2 \theta) (\hat{I}_{1x} \hat{I}_{2x} - \hat{I}_1 \cdot \hat{I}_2) \\ C &= -\frac{3}{2} \sin \theta \cos \theta e^{-i\phi} (\hat{I}_{1x} \hat{I}_{2+} + \hat{I}_{1+} \hat{I}_{2x}) \\ D &= -\frac{3}{2} \sin \theta \cos \theta e^{i\phi} (\hat{I}_{1x} \hat{I}_{2-} + \hat{I}_{1-} \hat{I}_{2x}) \\ E &= -\frac{3}{4} \sin^2 \theta e^{-2i\phi} \hat{I}_{1+} \hat{I}_{2+} \\ F &= -\frac{3}{4} \sin^2 \theta e^{2i\phi} \hat{I}_{1-} \hat{I}_{2-} \end{aligned} \quad 1.22$$

Eq 1.21 can now be rewritten,

$$\hat{H}_d = \frac{\gamma_1 \gamma_2}{r^3} [A + B + C + D + E + F] \quad 1.21$$

This form of Eq. 1.21 has the advantage that the spatial parts of the interaction are separated from the spin operators. The spatial terms are related to the spherical harmonics and have useful rotation properties. Consider a system composed of two identical spins ($I=1/2$) which are dipolar coupled to each other and also interact with a large applied field. Since two spins

are involved there must be four states, the energies of which are predominantly determined by the applied field (*i.e.* Zeeman interaction). In the absence of the dipolar interaction the simplest set of eigenstates would be $|++\rangle, |-+\rangle, |+-\rangle$ and $|--\rangle$. Taking the dipolar interaction as a perturbation, the "A" and the $\hat{I}_{1x}\hat{I}_{2x}$ part of the "B" term leave all states unchanged and modify just the energies. The second part of the "B" term mixes the middle levels only. The rest of the terms mix states of different energies and therefore they do not contribute to either the eigenfunction or energies. The perturbed states are divided into two manifolds, the singlet

$$|\Psi\rangle_s = \frac{1}{\sqrt{2}} [| - + \rangle - | + - \rangle] \quad E_s = \frac{3\gamma_I^2}{4r^3}$$

and the triplet

$$\begin{aligned} |\Psi\rangle_1 &= |++\rangle & E_1 &= \gamma_I B_0 + \frac{\gamma_I^2}{4r^3} R(\theta) \\ |\Psi\rangle_0 &= \frac{1}{\sqrt{2}} [| - + \rangle + | + - \rangle] & E_0 &= -\frac{\gamma_I^2}{2r^3} R(\theta) \\ |\Psi\rangle_{-1} &= |--\rangle & E_{-1} &= -\gamma_I B_0 + \frac{\gamma_I^2}{4r^3} R(\theta) \end{aligned}$$

where

$$R(\theta) = (1 - 3 \cos^2 \theta)$$

The triplet manifold is depicted in Figure 1.1. The difference in these manifolds is that they are of different total angular momenta. The singlet state is isolated and need not be considered. The NMR spectrum, in this case, is a pair of lines separated by $3\gamma_I^2/2r^3$ and centered about the Zeeman frequency. When $\theta = \cos^{-1} \sqrt{\frac{1}{3}}$, $R(\theta)$ vanishes, the dipolar interaction is zero and only a single line corresponding Zeeman frequency is observed. For the case where the two spins are not identical, the situation is simpler. Only the $\hat{I}_{1x}\hat{I}_{2x}$ terms are important. The $\hat{I}_{1\pm}\hat{I}_{2\mp}$ contribution to the "B" term have no effect, since in this case they are non-energy conserving. The eigenstates are just the four unperturbed states listed above with the appropriate changes in energies.

IV. The calculation of the Bloch decay spectrum

Having reviewed the quantum mechanics, we can now calculate the NMR spectrum from the response of the ensemble of spins to a pulse of radio frequency excitation. The response is measured by the same set of coils with which the excitation pulse is applied. The detection

is based on Faraday's Law. The changes in the magnetic field in the plane orthogonal to the axis of B_0 are measured by the voltage induced in the coils. The problem becomes one of calculating the ensemble averaged expectation values of the magnetic moment operators.

As indicated earlier, the Hamiltonian for an isolated spin ($I=1/2$) in a magnetic field, in our case 63 kG, applied along the z -axis is simply the Zeeman Hamiltonian which is given by Eq 1.18a. An isolated spin in this field can only be in either one of two eigenstates, $|+\rangle$ and $|-\rangle$. Consequently, the values of $\bar{\epsilon}_{ij}$ which define the density matrix (Eq. 1.11) are zero when $i \neq j$ making the density matrix diagonal. When the spins are in thermal equilibrium, the population of the states is determined by the Boltzmann distribution law. For an ensemble of isolated spins under the influence of a Zeeman interaction, the density matrix which describes the system is given by

$$\hat{\rho} = Z^{-1} \begin{bmatrix} e^{+\frac{1}{2}\beta\gamma_I B_0} & 0 \\ 0 & e^{-\frac{1}{2}\beta\gamma_I B_0} \end{bmatrix} \quad \beta = \frac{1}{kT} \quad 1.23$$

where

$$Z = e^{+\frac{1}{2}\beta\gamma_I B_0} + e^{-\frac{1}{2}\beta\gamma_I B_0}$$

In most situation, $\beta\gamma_I B_0$ is small enough compared to kT so that the first two terms of the expansion for exponentials are adequate. Under these conditions the density operator reduces to

$$\hat{\rho} = \frac{1}{2} \left[\mathbf{1} + \frac{1}{2}\beta\gamma_I B_0 \sigma_z \right] = \frac{1}{2} \left[\mathbf{1} + \beta\gamma_I B_0 \hat{I}_z \right] \quad 1.24$$

where $\mathbf{1}$ is the identity operator (a 2 by 2 unit matrix) and σ_z is a Pauli matrix. The identity operator will have no effect on further calculations and can be safely ignored. The density matrix and the Hamiltonian are both functions of $\hat{I}_z$ only; therefore, by Eq. 1.5, the density matrix is time invariant. Finally, the expectation value of the magnetic moment can be calculated. The magnetic moment operator is defined as $\hat{\mu}_j = \gamma_I \hat{I}_j$ where $j = x, y, z$. When Eq. 1.12 is used to calculate the ensemble averaged magnetic moment, it is found that only a static z -component of the magnetic moment (or magnetization) is present and no signal can be detected by the excitation/detection coils.

The effect of a radio-frequency excitation pulse applied on an ensemble of spins at thermal equilibrium can now be calculated using the density matrix given above. First, a transformation

into the interaction representation is made where in Eq. 1.15, $\hat{H}_1 = \hat{H}_{rf}$ and $\hat{H}_0 = \hat{H}_z$. In this case the radio-frequency Hamiltonian is given by

$$\hat{H}_{rf} = \gamma_I B_1 e^{i\gamma_I B_0 \hat{I}_z t} \left[\hat{I}_x \cos(\omega t + \phi) + \hat{I}_y \sin(\omega t + \phi) \right] e^{-i\gamma_I B_0 \hat{I}_z t}. \quad 1.25$$

If $\omega = \gamma_I B_0$, then

$$\hat{H}_{rf} = \gamma_I B_1 \left[\hat{I}_x \cos \phi + \hat{I}_y \sin \phi \right]. \quad 1.25a$$

Eq. 1.25 shows that in the interaction representation, when $\omega = \gamma_I B_0$, the magnetic field which oscillates at the Larmor frequency transforms into a static one. In order to calculate the time evolution of the density matrix in this interaction representation, Eq. 1.15 is used. First, for simplicity, the phase of the oscillating field is assumed to be $-\pi/2$ (i.e. $\phi = -\pi/2$) which reduces Eq. 1.25a to $\hat{H}_{rf} = \gamma_I B_1 \hat{I}_x$. If the initial density matrix given by Eq. 1.24 (ignoring the unit operator) is substituted into Eq. 1.15, the time dependent density matrix is given by

$$\begin{aligned} \hat{\rho}(t) &= K e^{-i\gamma_I B_1 \hat{I}_x t} \hat{I}_x e^{i\gamma_I B_1 \hat{I}_x t} \\ &= K \left[\hat{I}_x \cos \gamma_I B_1 t + \hat{I}_y \sin \gamma_I B_1 t \right]. \end{aligned} \quad \text{where } K = \frac{\gamma_I B_0}{2kT} \quad 1.26$$

and where $\gamma_I B_1$ is the magnitude of the oscillating field. The second equality is obtained from the fact that operators of the form $e^{i\theta \hat{I}_j}$, where $j = x, y, z$, act as rotation operators about the j -axis (Cohen-Tannoudji, 1977). This can be proven by expansion of the exponential operator into standard series form. If the rf induced magnetic field is left on for a time, t_0 , such that $\gamma_I B_1 t_0 = \frac{\pi}{2}$, then the density matrix at the end of the interval would be proportional to $\hat{I}_y$. Immediately following the pulse, the evolution of the spin ensemble is controlled by the Zeeman interaction and the density matrix in the interaction representation will remain constant (the interaction Hamiltonian being zero). In the Heisenberg representation, the density matrix oscillates at the Larmor frequency. This can be seen by simply applying Eq. 1.13 and by defining the density matrix immediately after the rf pulse as the initial density matrix, denoted $\hat{\rho}(0+)$. This yields

$$\begin{aligned} \hat{\rho}(t) &= K e^{-i\gamma_I B_0 \hat{I}_z t} \hat{I}_x e^{i\gamma_I B_0 \hat{I}_z t} \\ &= K \left[\hat{I}_x \cos \gamma_I B_0 t - \hat{I}_y \sin \gamma_I B_0 t \right] \end{aligned} \quad 1.27$$

The expectation value of the magnetization is calculated from Eq. 1.27. The result is a magnetic field produced by the spin which oscillates at the Larmor frequency. This oscillating field will

not persist indefinitely. The spins, excited by the radio frequency irradiation, will eventually relax through spin-spin and spin-lattice interactions and the density matrix for these spins will again become proportional to $\hat{I}_x$. The dampened oscillatory magnetic response of the spins to the radio-frequency pulse is called the Bloch decay.

Now we consider the case where the spin ensemble also possesses an isotropic chemical interaction, where $H_c = \gamma_I \sigma B_0 \hat{I}_x$, in addition to, the Zeeman interaction. At the end of the pulse, the density matrix will not remain constant, since an interaction Hamiltonian is present. Evolution due to the chemical shift interaction during the pulse is ignored, since the radio-frequency interaction dominates the Hamiltonian and the time evolution dominates the behavior of the spins. Eq 1.15 can again be used to calculate the evolution of the density matrix immediately following the rf-pulse. This calculation is simplified by the fact that the chemical shift and Zeeman interactions commute and the form of chemical shift interaction remains unchanged when transformed into the interaction representation. This leads to

$$\begin{aligned}\hat{\rho}(t) &= K e^{-i\gamma_I \sigma B_0 \hat{I}_x t} \hat{I}_x(0+) e^{i\gamma_I \sigma B_0 \hat{I}_x t} \\ &= K \left[\hat{I}_x \cos \gamma_I \sigma B_0 t + \hat{I}_y \sin \gamma_I \sigma B_0 t \right].\end{aligned}\tag{1.27a}$$

where 0+ denotes the start of the evolution after the pulse. The time dependent expectation value of the magnetization in the interaction representation describes a precession at the chemical shift frequency, $\gamma_I \sigma B_0$, about the z-axis. If our detector were fixed in a frame which precesses at the Larmor frequency (*i.e* fixed in the famous rotating frame), an a.c. signal at the chemical shift frequency would be observed. The signal would eventually decay away. The Fourier transform of this signal would be a single line at the chemical shift frequency. This result could have been deduced rather simply from the energies of the two eigenstates. However, for many situations, such as MASS, the tedious approach of calculating the time evolution of the density matrix will be necessary. The basic description of the time evolution of the density matrix after a "90° flip", which has just been described, is the basis for much of signal calculations discussed below and in the following chapters.

V. Tensors, randomness and spectra of powders

A. Calculation of static powder spectra

Having calculated the isotropic chemical shift spectrum of an ensemble of spins, we now

consider the general problem of calculating the spectrum of a polycrystalline sample of spins which undergo an anisotropic chemical shift interaction. First, some comments about the tensorial nature of spin interaction are in order. The Hamiltonians described in the previous section are all expressed in a tensor form. In each case, the tensors were represented by a 3 by 3 matrix. The value of the matrix elements of these tensors depend on the frame of reference. The reference frame in which a tensor can be expressed as a diagonal matrix is called the principal axis system (PAS), and the three non-zero matrix elements are referred to as the principal values. In general, the PAS of a tensor describing a spin interaction will not coincide with the laboratory/magnet (LAB) reference frame, which is the frame in which the quantum mechanical operators are expressed. Therefore, when the energy of a spin is calculated using the Hamiltonian, the appropriate tensor must be expressed in terms of the LAB frame. Since the tensor takes its simplest form in the PAS, it is often desirable to express the Hamiltonian in term of the three principal values. This involves the transformation of the tensor from its PAS frame into the LAB frame. As can be seen in Appendix B, the machinery to accomplish this task is well laid out.

In general the chemical shift interaction is anisotropic. In this case, the Hamiltonian is give by Eq. 1.20. It is assumed that the large static field, B_0 , is along the z-axis. Eq. 1.20 reduces to

$$\hat{H}_c = \gamma_I B_0 (\sigma_{xx} \hat{I}_x + \sigma_{xy} \hat{I}_y + \sigma_{zx} \hat{I}_z) \quad 1.28$$

where σ_{ij} are the elements of the shift tensor. The $\hat{I}_x$ and $\hat{I}_y$ terms do not commute with the Zeeman Hamiltonian and can be safely ignored. These terms do not, to first order, alter the energies of the two Zeeman states. The transition energy between the two states is

$$\omega = \gamma_I B_0 (1 - \sigma_{zz}) \quad 1.29$$

It is necessary to determine how the tensor expressed in PAS contributes to σ_{zz} . The pictorial representation of the problem is shown in Figure 1.2 . At this point the rotational properties of the shift tensor (or any other second rank tensor) are exploited, namely that

$$\mathcal{Q}_{LAB} = R \mathcal{Q}_{PAS} R^{-1} \quad 1.30$$

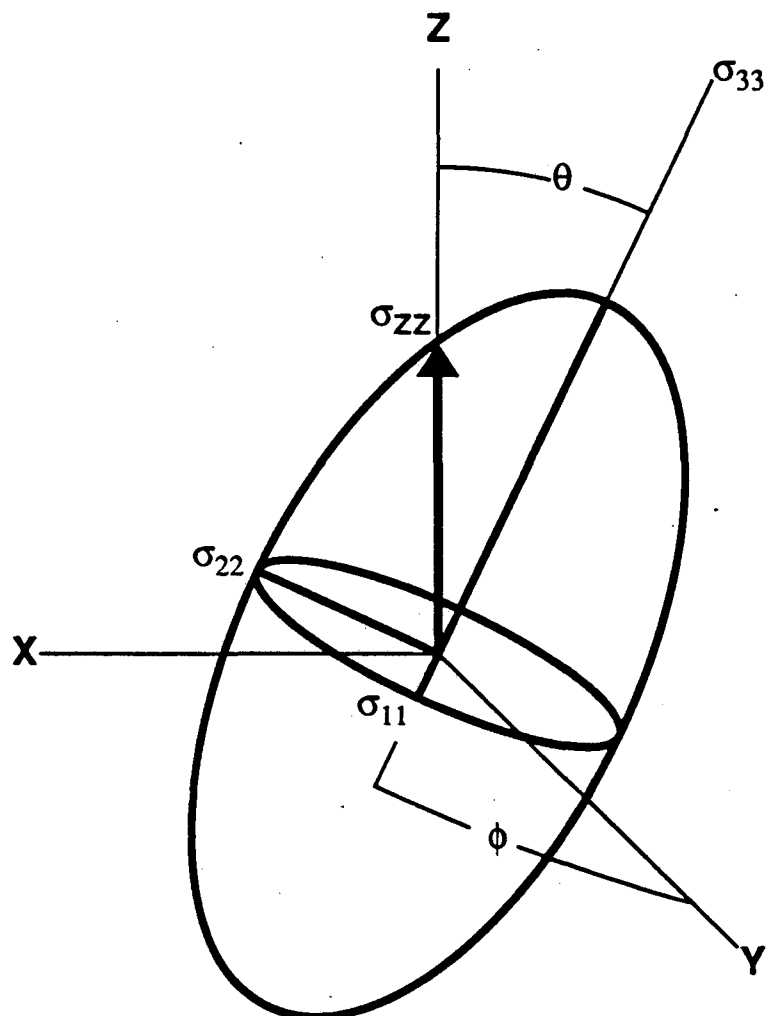


Figure 1.2. The orientation of the PAS of a chemical shift tensor with respect to the LAB frame. The magnitude of the chemical shift interaction along z-axis of the LAB frame is given by σ_{zz} . It is determined by the three principal values of the shift tensor, σ_{11} , σ_{22} and σ_{33} , and the two angles, ϕ and θ , which specify the orientation of the tensor.

where R is the Eulerian rotation matrix, a composite of three simple rotations (Edmond, 1974). The following observations are made: σ_{PAS} is diagonal; R is a symmetric matrix and σ_{zz} is the only component which is needed. Using these observations one can readily reduce Eq. 1.30 to

$$\sigma_{zz} = \sigma_{11}R_{31}^2 + \sigma_{22}R_{32}^2 + \sigma_{33}R_{33}^2 \quad 1.31$$

Substituting the values from the elements of the rotation matrix, which may be found in various texts (Cromwell, 1984) yields

$$\sigma_{zz} = \sigma_{11} \cos^2 \phi \sin^2 \theta + \sigma_{22} \sin^2 \phi \sin^2 \theta + \sigma_{33} \cos^2 \theta \quad 1.32$$

An alternate and more useful form of Eq. 1.29 is (see Appendix B)

$$\sigma_{zz} = \eta \delta \cos 2\phi \sin^2 \theta + \delta (1 - 3 \cos^2 \theta) \quad 1.32a$$

where σ_i is the isotropic value defined by

$$\sigma_i = \frac{1}{3} (\sigma_{11} + \sigma_{22} + \sigma_{33}) \quad 1.32b$$

and η and δ are known respectively as the asymmetry parameter and chemical shift anisotropy.

They are defined as

$$\delta = (\sigma_{33} - \sigma_i) \quad 1.32c$$

and

$$\eta = \frac{(\sigma_{11} - \sigma_{22})}{\delta} \quad 1.32c$$

For the special case where the chemical shift interaction is axially symmetric (*i.e.* $\sigma_{11} = \sigma_{22}$ or $\eta = 0$), eq. 1.32a becomes

$$\sigma_{zz} = \delta (1 - 3 \cos^2 \theta) \quad 1.32d$$

When Eq. 1.32 is substituted into Eq. 1.29, the result yields the transition energy as a function of the orientation of PAS relative to the LAB frame. As an example, consider the axially symmetric case (eq. 1.32c) where, without loss of generality, σ_i is assumed to be zero. In this case, the orientation of the σ_{33} element need only be considered. Although all possible values θ are equally probable, all values of σ_{zz} are not. It is clear that the probability of finding σ_{33}

oriented parallel to the field axis (the z-axis) with $\sigma_{zz} = -2\delta$ is infinitesimally small compared to the orthogonal direction, where $\sigma_{zz} = \delta$. In fact, the probability of a given frequency as a function of θ scales as $\sin \theta$. In general, a powder spectrum is not a single sharp line rather it is characterized by a distribution of intensities covering a finite frequency range. The method used to derive an equation for the intensity as a function of frequency was first determined by Bloembergen and Rowland (Bloembergen and Rowland, 1953, 1955). We briefly outline the pertinent parts of their discussion. Let the intensity of the spectrum at frequency, ω , be $I(\omega)$ and the probability of finding the orientation of the tensor between the interval from Ω to $\Omega + d\Omega$ be $P(\Omega)$ then

$$I(\omega)d\omega = P(\Omega)d\Omega \quad 1.33$$

As the noted above, all orientations of the PAS with respect to the LAB frame are equal probable or $P(\omega) = 1/4\pi$. For the axial symmetric case, Eq. 1.33 becomes

$$I(\omega) = \frac{\sin \theta}{4\pi} \frac{d\theta}{d\omega} \quad 1.34$$

where it is recognized that the number of orientations for a given θ scales as $\sin \theta$. Substitution of Eqs. 1.32d and 1.29 into Eq. 1.34 followed by differentiation yields

$$I(\omega) = \frac{1}{2} (\omega_{33} - \omega_{22})^{-\frac{1}{2}} (\omega - \omega_{22})^{-\frac{1}{2}} \quad 1.35a$$

For general case, the problem is a bit more complicated. The corresponding formulas were derived in the original papers by Bloembergen and Rowland (Bloembergen and Rowland, 1953, 1955). In general, the intensity of a chemical shift powder spectrum at a frequency, ω is given by

$$I(\omega) = \pi^{-1} (\omega - \omega_{11})^{-\frac{1}{2}} (\omega_{33} - \omega_{22})^{-\frac{1}{2}} K(m) \quad 1.35b$$

where

$$m = \frac{(\omega_{22} - \omega_{11})(\omega_{33} - \omega)}{(\omega_{33} - \omega_{22})(\omega - \omega_{11})}$$

for $\omega_{33} \geq \omega > \omega_{22}$ and

$$I(\omega) = \pi^{-1} (\omega_{33} - \omega)^{-\frac{1}{2}} (\omega_{22} - \omega_{11})^{-\frac{1}{2}} K(m)$$

where

$$m = \frac{(\omega - \omega_{11})(\omega_{33} - \omega_{22})}{(\omega_{33} - \omega)(\omega_{22} - \omega_{11})}$$

for $\omega_{22} > \omega \geq \omega_{11}$. $K(m)$ is the complete elliptic of the first kind, the values and polynomial approximation for which can be found in standard mathematical tables (Milne–Thomson, 1970). Figure 1.3 shows the calculated spectra based on Eqs. 1.35a and 1.35b. All three of these equations possess a logarithmic discontinuity at ω_{22} which makes these expressions somewhat unrealistic, since this implies an infinite number of spins. In order to make the calculated powder spectrum a bit more realistic, the spectrum is convolved with a Gaussian function. The spectrum at each point is replaced by a Gaussian function and then summed. The width of the Gaussian function is chosen to make the spectrum aesthetically pleasing. Figure 1.4 is comparison of the gaussian broadened calculated spectrum with an experimental powder spectrum. This figure demonstrates that Eq. 1.35 works reasonably well, especially with some Gaussian convolution.

B. Calculation of Bloch Decay spectra for powdered samples

An alternative method for calculating a powder spectrum is to calculate the Bloch decay, also known as the free induction decay (FID). As discussed earlier, the Bloch decay is just the transient time response of the spins to a rf-pulse. The FID of a polycrystalline sample is obtained by rewriting Eq. 1.27a, so that the density matrix takes into account the anisotropy. This yields

$$\hat{\rho}(t) = \hat{I}_x \cos \gamma_I B_0 \sigma_{zz} t + \hat{I}_y \sin \gamma_I B_0 \sigma_{zz} t. \quad 1.36$$

where σ_{zz} is given by Eq. 1.32a and 1.32d. This expression is then integrated over the three Euler angles (*i.e.* all possible orientations of the PAS with respect to LAB frame) to obtain the Bloch decay. Fourier transformation yields the powder spectrum. As is shown in Appendix D, the problem of discontinuities is not encountered using this method. However, increased computation time and complexity are the price. Figure 1.5 is a comparison of the powder spectrum obtained using this method which one obtained by applying Eq. 1.35a. The integrated area of the spectrum is a natural consequence of the time-domain calculation unlike the results obtained using Eq 1.35. This is because the numerical integration over the orientations by

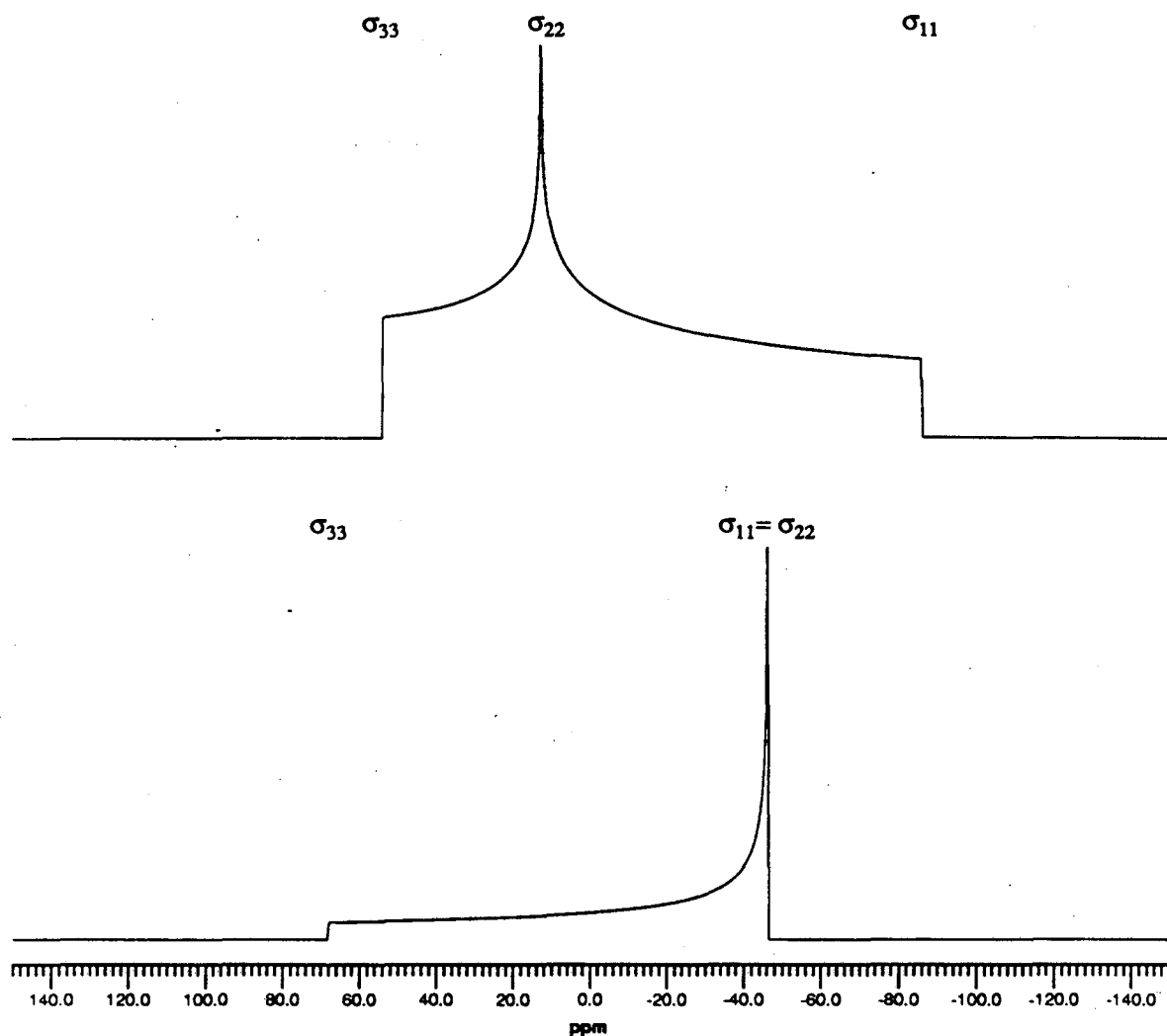


Figure 1.3. Calculated powder spectra based on standard formulae derived by Bloembergen and Rowland: the axial case (*lower*) and the general asymmetric case (*upper*). The convention $\sigma_{33} \geq \sigma_{22} \geq \sigma_{11}$ is used. In both cases, a logarithmic discontinuity exists at σ_{22} .

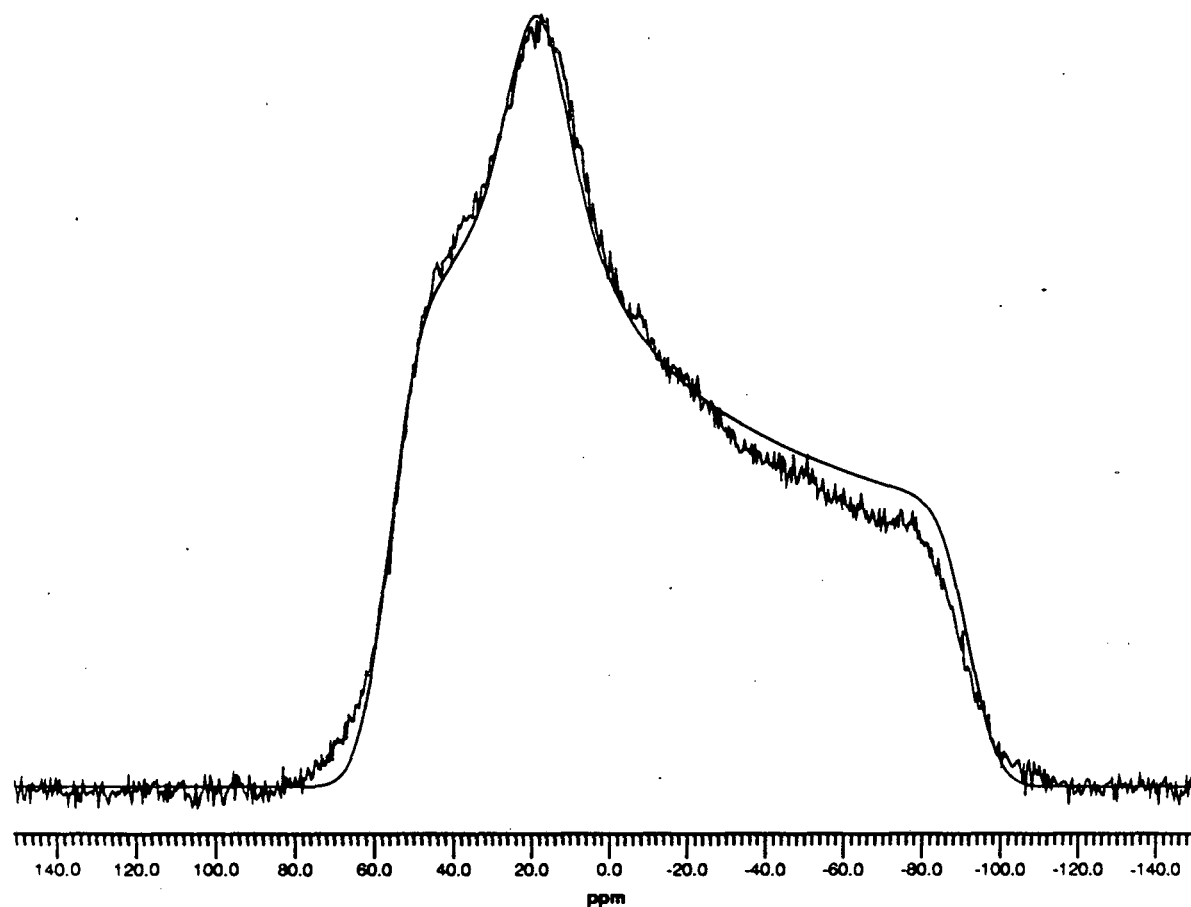


Figure 1.4. A comparison of the experimental ^{31}P spectrum of sodium dihydrogen phosphate with the calculated one. The calculated spectrum is based on the Bloembergen–Rowland formula and was Gaussian broadened to obtain a good fit. The chemical shift parameters needed for the calculation were determined by a technique which is described in Chapter 3.

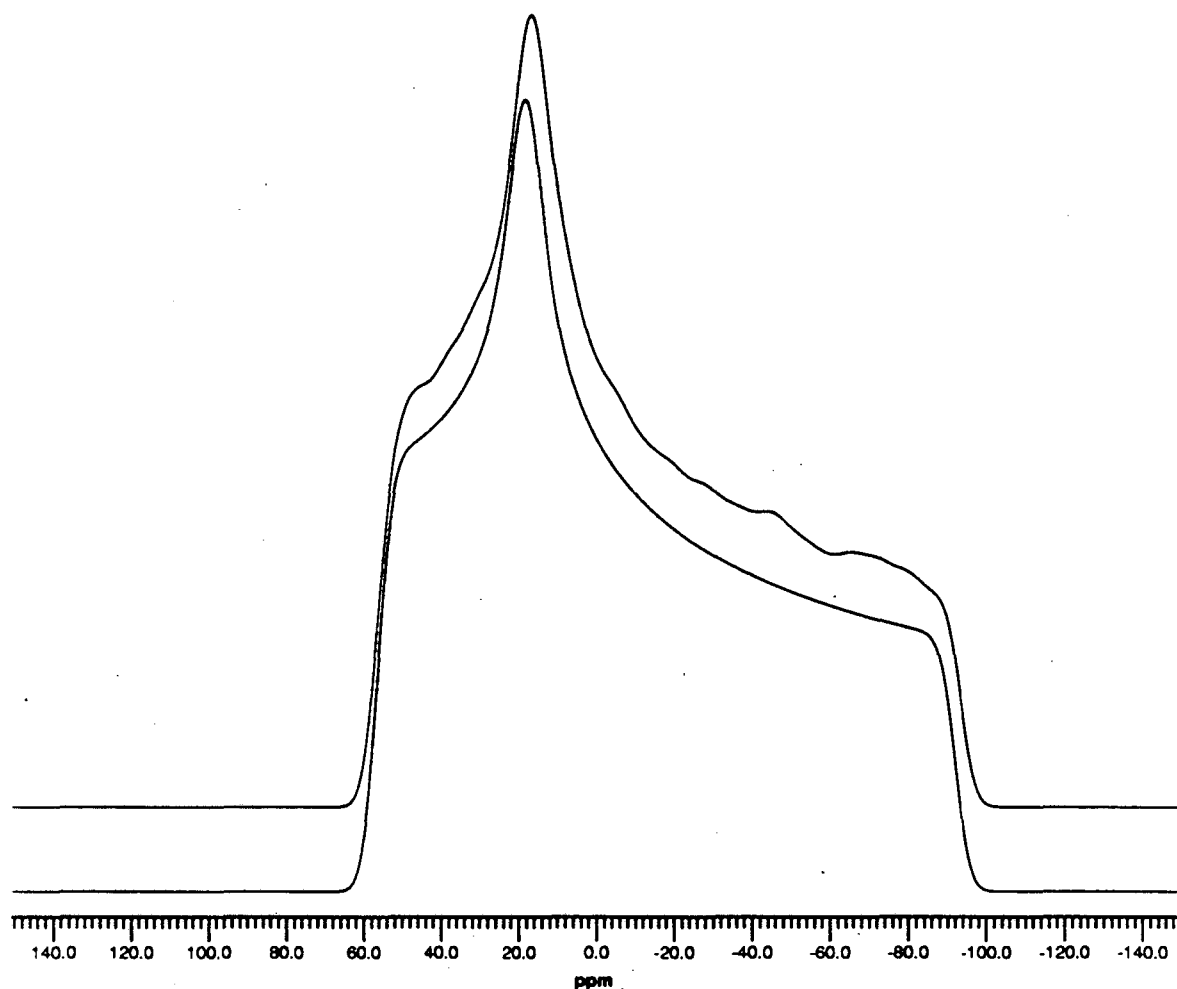


Figure 1.5. A comparison of the powder spectrum determined by Fourier transform of the calculated Bloch decay (*upper trace*) with the one calculated based on the Bloembergen-Rowland formula (*lower trace*). Both have been Gaussian broadened to an equivalent extent. The “noisiness” of the Bloch decay spectrum is a consequence of the numerical integration over Euler angles which is discussed in Appendix D.

its very nature considers only a finite number of orientations, or spins, and consequently the integrated area must be finite; hence, the spectrum cannot possess an infinite discontinuity.

A situation which occurs frequently is that the orientation of the PAS relative to the LAB frame becomes time dependent. This occurs when the nuclei are in motion, as would be the case for molecules in a solution. If the rotational component of the motion is isotropic, then for the axially symmetric case, the σ_{33} element of a single spin will sample all possible orientation. An average over all orientations is required for the angular expression. This yields

$$\langle 1 - 3 \cos^2 \theta \rangle_{ave} = \frac{1}{\pi} \int_0^\pi (1 - 3 \cos^2 \theta \sin \theta) d\theta = 0.$$

This means that the anisotropy of the interaction vanishes and one is left with a single sharp line, as in the simple isotropic case discussed previously. A similar argument follows for the asymmetric case. The rate of reorientation needed to achieve this averaging can be estimated by using the time-energy uncertainty principle (Cohen-Tannoudji, 1977)

$$\Delta\omega\Delta t \geq 1 \quad 1.37$$

The inverse of the rate of reorientation must be equal to or larger than $\Delta\omega = \sigma_{33} - \sigma_{11}$ in order for the anisotropy to collapse. The faster the rate of reorientation, the more effective the averaging. The rotational orientation times for biological molecules in water scales approximately as 1 ns/kDalton, which indicates that for a small molecule, such as ATP, the ^{31}P chemical anisotropy of which is on the order of 23 kHz in a 6.3 kG applied field, should be completely averaged.

C. Calculation of MASS Bloch decay spectra

Having considered the effect of random rotation on the chemical shift anisotropy, we now turn to the case where the sample rotates about a fixed axis or tumbles anisotropically. Again for simplicity the axially symmetric chemical shift interaction is considered. The resonance frequency is given by eqs. 1.29 and 1.35b. As the sample is spun, the σ_{33} element will execute a trajectory which is periodic having the same frequency as the rotation rate. Moreover, this tensor element can be decomposed into two components one of which is parallel to the spinning axis and the other which orthogonal. Figure 1.6 depicts this situation. The former is time invariant

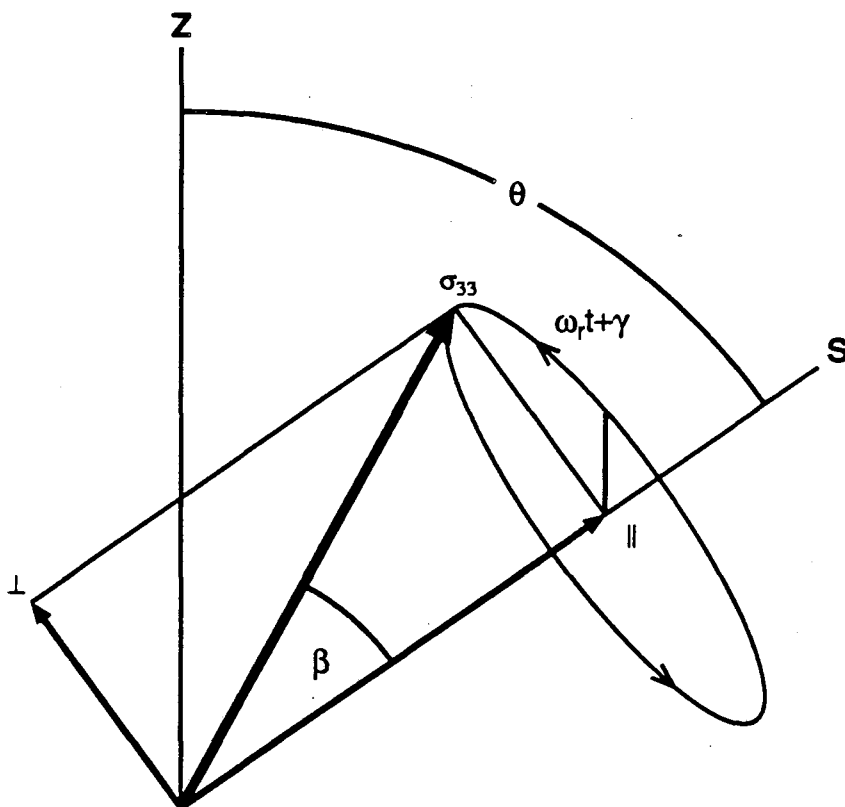


Figure 1.6. The orientation of the chemical shift element σ_{33} with respect to the MAS and LAB frames. For axially symmetric cases, the σ_{xx} is dependent only the σ_{33} element which can be decomposed into parallel and perpendicular components relative to the axis of rotation, labelled S. The parallel component contribution vanishes when $\theta = \theta_{magic}$ and the perpendicular has a vanishing average. A third angle, α , is needed in asymmetric cases. This angle relates the orientation of the σ_{22} and σ_{11} in the plane orthogonal to the rotation axis (S).

and the latter periodic. At some very high spinning rate, the periodic component will average to zero, leaving only the parallel component. If the axis of rotation is at $\theta_{magic} = \arctan \frac{1}{\sqrt{3}}$ with respect to field axis, Eq. 1.35b vanishes and only the isotropic part of the chemical shift is left. Again, it is expected that the spinning speed must be comparable to the breadth of the powder pattern in order to have any averaging effect. The powder patterns for ^{31}P are about 20 kHz broad at 63 kG. Although mechanically possible, sample spinning at such speeds is not routine. Currently speeds of 3 to 8 kHz can be easily achieved. Under these conditions, effects of sample spinning need to be analyzed in detail. Appendix B provides the details of the calculations and the results are presented in the following discussion. As it will turn out, very high spinning speeds may not be always desirable, especially when information about anisotropy of the chemical shift interaction is desirable.

In order to calculate the response of the chemical shift interaction under MASS, one must not only take into account the orientation of the PAS with respect to the LAB frame, but also the fact that this orientation becomes time dependent under MASS. This is achieved by first considering the orientation of the PAS with respect to the rotor frame (MAS) and then the time dependence of the MAS frame with respect to LAB frame using successive Euler transformations. This is depicted in Figure 1.6. The details of this calculation are covered in Appendix B and we start with the results of this derivation. For $\theta = \theta_{magic}$, the MASS chemical shift Hamiltonian (eq. B.1) may be expressed in the interaction representation as

$$H = \omega_0 \sigma_i \hat{I}_z + \omega_0 \hat{I}_z [C_1 \cos(\omega_r t + \gamma) - S_1 \sin(\omega_r t + \gamma) + C_2 \cos(2\omega_r t + 2\gamma) - S_2 \sin(2\omega_r t + 2\gamma)] \quad B.1a$$

where

$$\omega_0 = \gamma_I B_0$$

$$C_1 = \sin \theta \cos \theta \sin \beta \cos \beta [\cos 2\alpha (\sigma_{11} - \sigma_{22}) - 3(\sigma_{33} - \sigma_i)]$$

$$S_1 = \sin \theta \cos \theta \sin \beta \sin 2\alpha (\sigma_{11} - \sigma_{22})$$

$$C_2 = \sin^2 \theta \left\{ \frac{3}{4} \sin^2 \beta (\sigma_{33} - \sigma_i) + \frac{1}{4} (1 + \cos^2 \beta) \cos 2\alpha (\sigma_{11} - \sigma_{22}) \right\}$$

$$S_2 = \frac{1}{2} \sin^2 \theta \cos \beta \sin 2\alpha (\sigma_{11} - \sigma_{22}).$$

At this point, the time evolution of the density matrix after the initial "90°" pulse can be calculated. Since in both the static and the MASS cases the Hamiltonians are proportional to

$\hat{I}_x$, the MASS density matrix should have the same form as Eq. 1.27a except the frequency of precession is determined by Eq. B.1a.

$$\rho(t) = \hat{I}_x \cos \Omega t + \hat{I}_y \sin \Omega t \quad 1.38$$

where

$$\Omega = \omega_0 + \omega_0 [C_1 \cos(\omega_r t + \gamma) - S_1 \sin(\omega_r t + \gamma) + C_2 \cos(2\omega_r t + 2\gamma) - S_2 \sin(2\omega_r t + 2\gamma)]$$

The precession frequency is not only orientation dependent, but is also explicitly time dependent. The expectation value of the magnet moment (*i.e.* the signal) can be calculated from Eqs. 1.16 and 1.38. First, for convenience, let us note that the complex magnetic moment operator, $\hat{M}_c(t)$, is proportional to $\hat{I}_+$ and a multiplicative constant has been omitted for simplicity. The expectation value of the complex magnetization, as a function time, can be determined from Eq. 1.16 and is equal to

$$\langle \hat{M}_c(t) \rangle = \frac{1}{8\pi} \int_0^{2\pi} \int_0^\pi \int_0^{2\pi} \exp \left(i \int_0^t \Omega(t') dt' \right) \sin \beta d\alpha d\beta d\gamma \quad 1.39$$

where a "powder" integration has been included to account for all possible orientation of the PAS. The time integration may be carried out to give

$$\exp \left\{ i \int_0^t \Omega(t') dt' \right\} = \exp \{ i [\Phi_0(\alpha\beta\gamma, t) + \Phi_1(\alpha\beta\gamma, t)] \}$$

where

$$\begin{aligned} \Phi_0(\alpha\beta\gamma, t) &= \omega_0 \sigma_i t \\ \Phi_1(\alpha\beta\gamma, t) &= \frac{\omega_0}{\omega_r} [S_1 (\cos(\omega_r t + \gamma) - \cos \gamma) + C_1 (\sin(\omega_r t + \gamma) - \sin \gamma) \\ &\quad + \frac{1}{2} S_2 (\cos(2\omega_r t + 2\gamma) - \cos 2\gamma) + \frac{1}{2} C_2 (\sin(2\omega_r t + 2\gamma) - \sin 2\gamma)] \end{aligned} \quad 1.40$$

If the chemical shift parameters are known, then the time-domain response (*i.e.* Eq 1.39 along with 1.40) may be calculated, the Fourier transform of which is the MASS spectrum. The problem reduces to a computational one of carrying out the integrations, the details of which are covered in Appendix D. Figure 1.7 is an example of the Fourier transform of such a calculation.

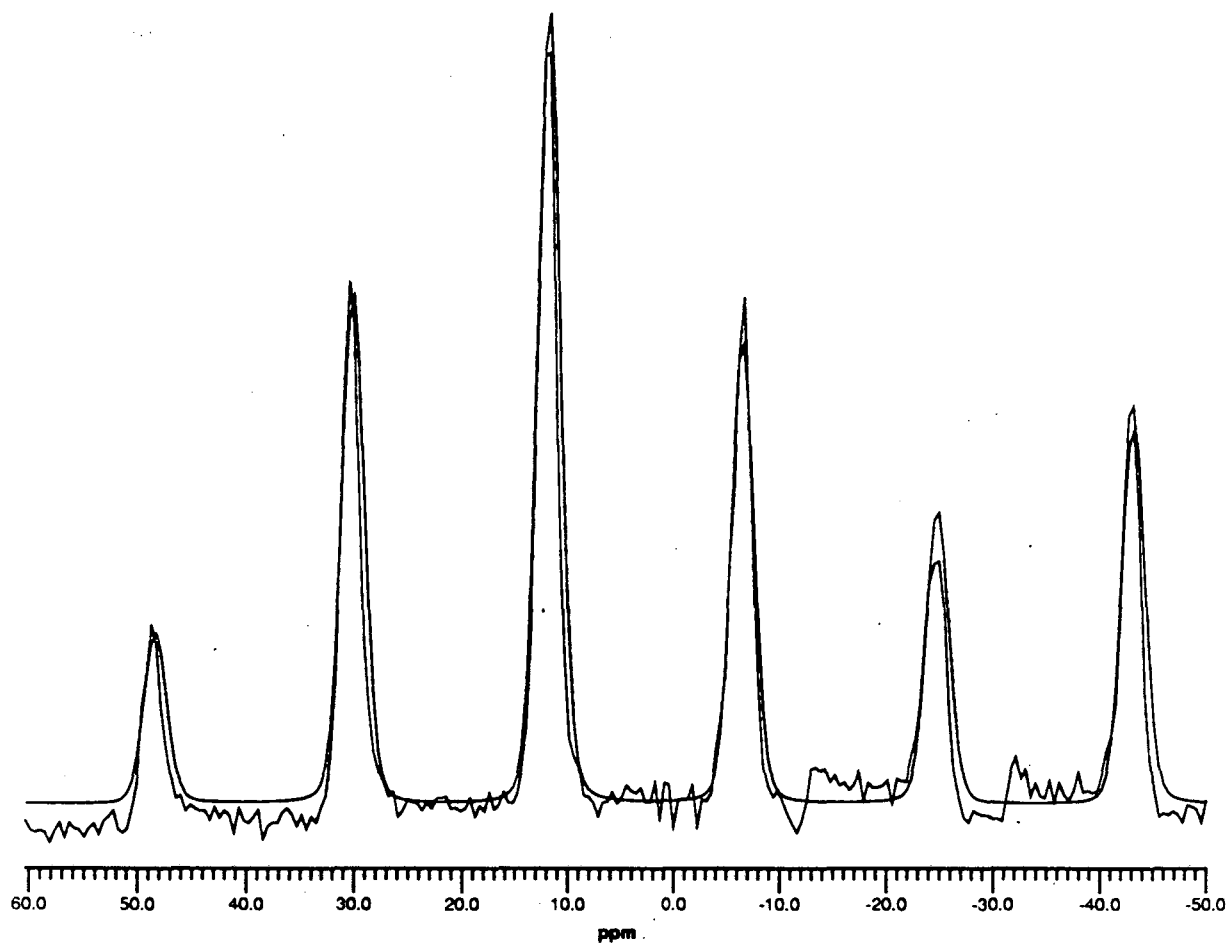


Figure 1.7. Comparison of the experimental ^{31}P MASS spectrum of sodium dihydrogen phosphate with the calculated. The later was determined from the Fourier transform of the Bloch decay calculated by integrating Eq. 1.40. The chemical shift parameters were determined by a technique which is described in Chapter 3. The sample was spun at 2.0 kHz.

Several qualitative features of this figure may be deduced without resorting to the complete calculation. First, there is an explicit time-dependent part of the Hamiltonian which may be divided into two parts: one which is dependent on the rotor speed and the other which is dependent on twice the rotor speed. These time-dependences make the Hamiltonian periodic. As a consequence, the expectation value of the magnetic moment also becomes periodic and the magnetization re-focuses (obtains its initial value and phase) at the end of each rotor cycle. That is, the density matrix at this point in time is equal to its initial value. The trajectory in the x - y plane shown in Figure 1.8 clearly demonstrates this point. The isotropic part of the chemical shift Hamiltonian is time-independent, but induces, through the evolution operator, a precession of the magnetization. The trajectory of the magnetization in this example may be thought of as two processes. The first is identical to the previous example where only the explicitly time-dependent part of the Hamiltonian was considered. The second is the precessional motion induced by the isotropic term. Hence, the position in the x - y plane at which the magnetization re-focuses becomes time dependent and precesses at a rate determined by the isotropic chemical shift frequency. This effect can be seen in Figure 1.9 where all the parameters have been kept the same as in the previous example except for an isotropic chemical shift of 800 Hz. Since, there are two periodic variations, a modulation effect is to be expected. The Fourier Modulation (Convolution) theorem would predict a frequency spectrum composed of a series of signals identical to each other, spaced at the modulation frequency (*i.e.* sample spinning frequency) and centered at the fundamental (*i.e.* isotopic chemical shift). A consequence of these observations is that when the sample speed is varied only the position of the centerband will remain unchanged while the sidebands reflect the change in speed. Figure 1.10 demonstrates this observation. This is of practical importance, since in general, it is not possible to identify which of the sidebands is the centerband. However, by recording the spectrum at two spinning frequencies, the centerband may be unambiguously identified. The main features of the MASS spectrum can be qualitatively understood from quantum mechanics and Fourier analysis.

At very fast spinning speeds, $\frac{\omega_a}{\omega_r}$ approaches zero and hence only the centerband at the isotropic chemical shift remains. It can also be shown that at very slow speeds the static

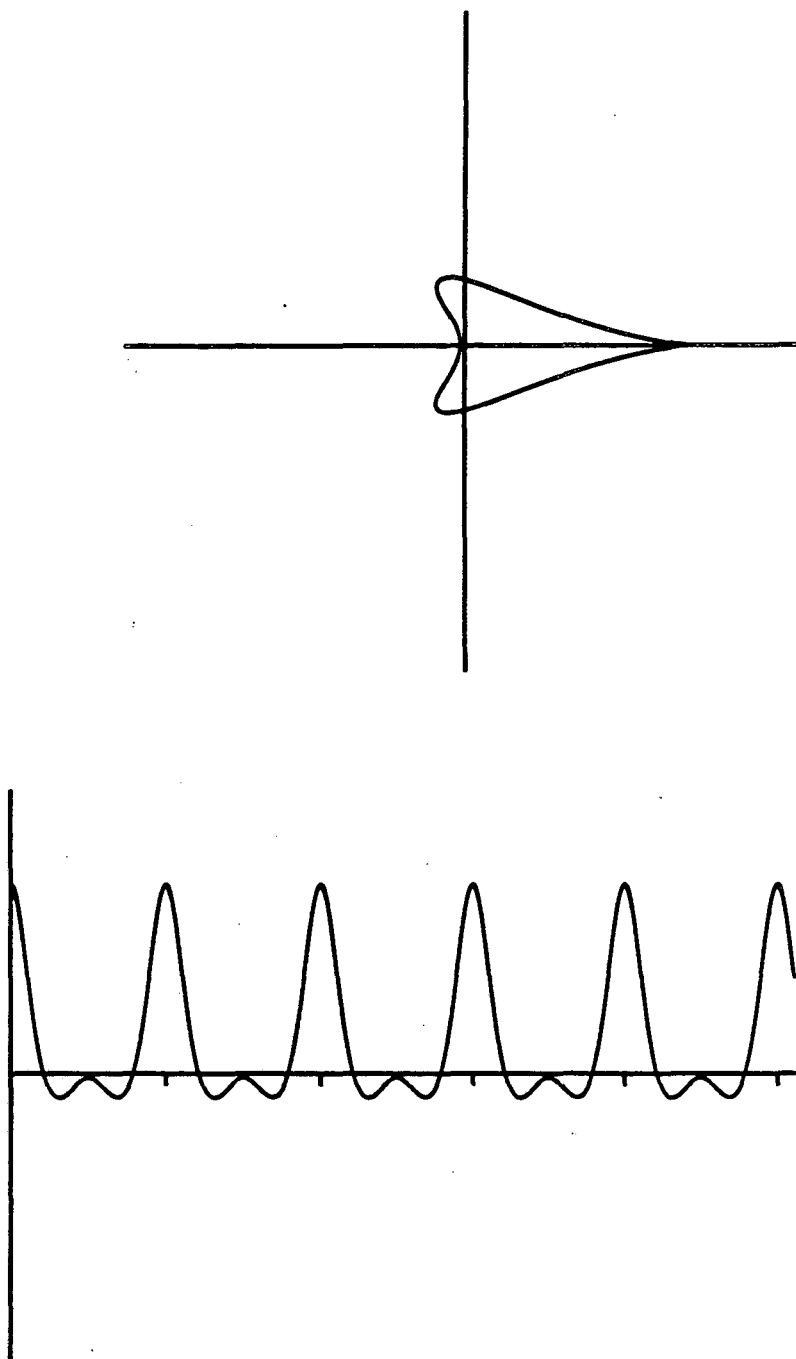


Figure 1.8. The calculated rotating frame complex magnetization trajectory in the x - y plane (*upper*) and the x magnetization as a function of time (*lower*) for the case where the isotropic shift is zero. The magnetization returns to the initial value and phase at the end of each rotor period. The calculation was carried out for 5 rotor periods using the following parameters: $\delta = 7.5\text{kHz}$, $\eta = 0$, $\sigma_1 = 0$, $\nu_r = 2.0\text{kHz}$.

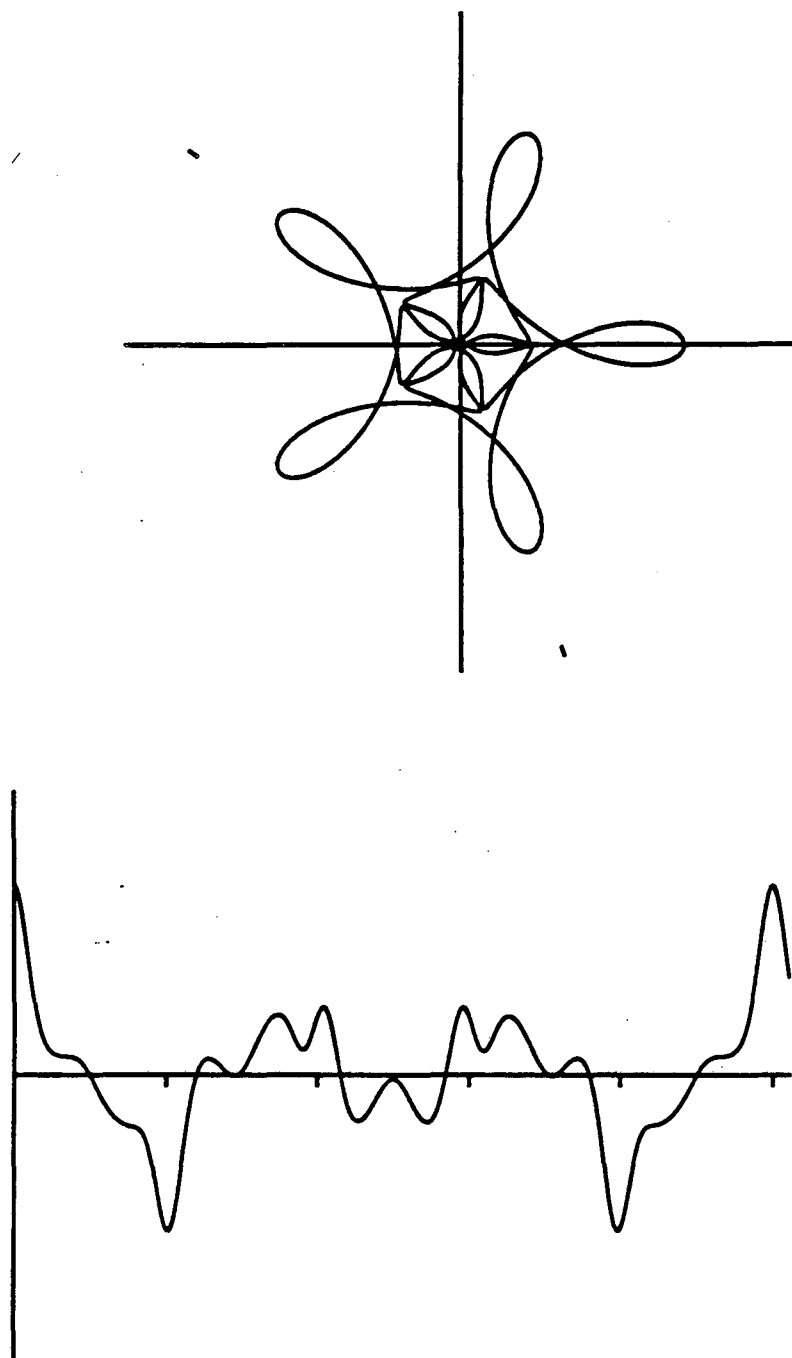


Figure 1.9. The calculated rotating frame complex magnetization trajectory in the x - y plane (*upper*) and the x magnetization as a function of time (*lower*) for the case where the isotropic shift is 800 Hz. The magnetization returns to initial value at the end of each rotor period; however, the phase at which the magnetization re-focuses precesses at the isotropic chemical shift frequency. After five rotor cycles the magnetization has return to its initial value and phase. The two small tic marks on the upper diagram mark the first two rotor periods. The calculation was carried out for 5 rotor periods using the following parameters: $\delta = 7.5\text{kHz}$, $\eta = 0$, $\sigma_1 = 0.8\text{kHz}$, $\nu_r = 2.0\text{kHz}$.

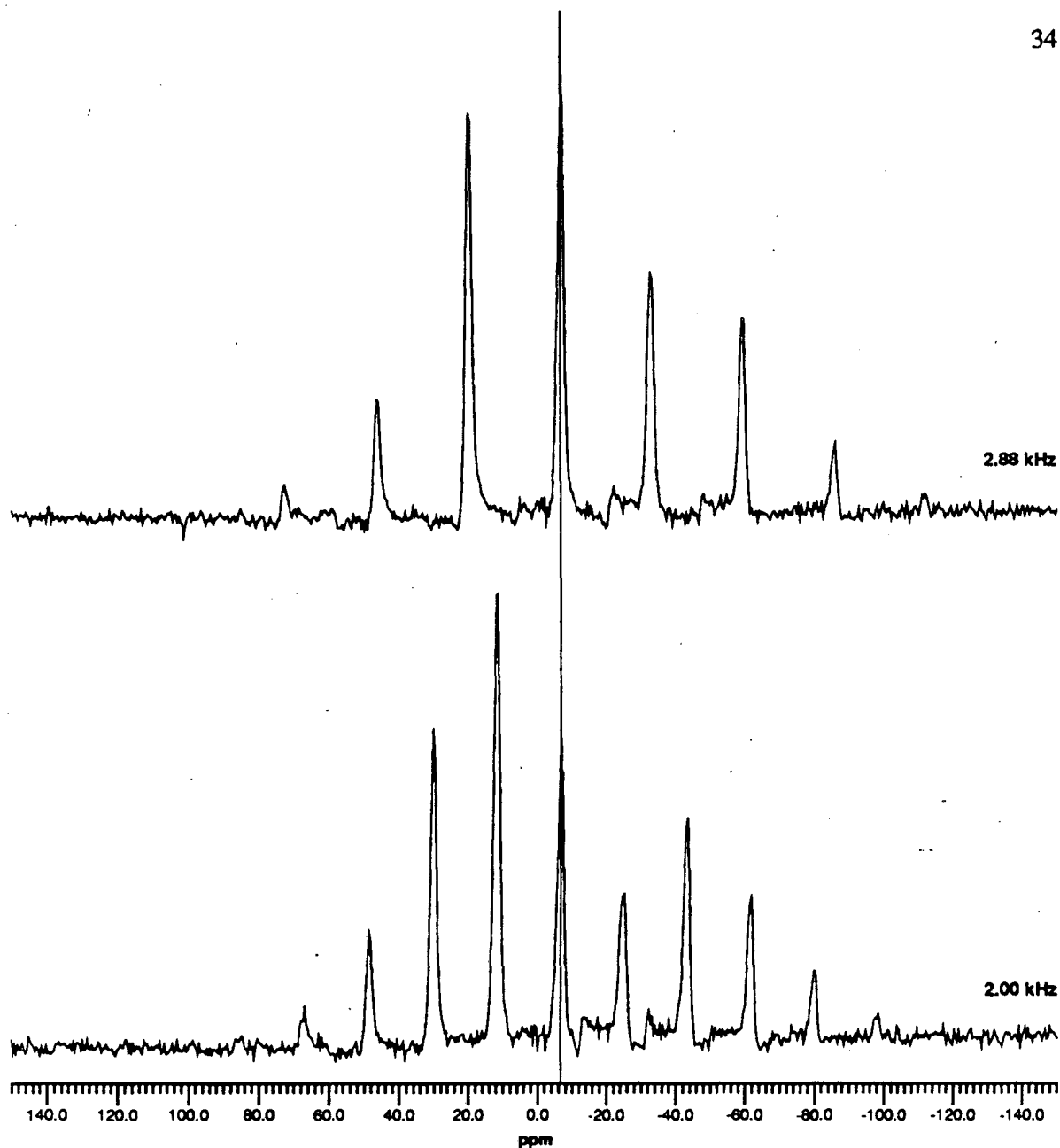


Figure 1.10. The ^{31}P MASS spectrum of sodium dihydrogen phosphate at two spinning speeds. The frequency of the centerband is invariant to sample spinning speed (as shown by the long line which marks the centerband frequency) and is equal to the isotropic chemical shift. The sidebands are positioned at integer multiples of the spinning frequency.

powder spectrum is obtained. In the intermediate cases, it is expected that the intensities of the spinning sidebands are a complex function of the spinning speed and chemical shift tensor parameters and, as stated above, one must resort to integrating Eq. 1.39. However, this assumes that the chemical parameters shift are known. A more useful approach would be one in which the knowledge of the sideband intensities and the spinning speed, both readily measurable quantities, could be used to determine the principal values of chemical shift tensor. Mariq and Waugh (Mariq and Waugh, 1979) showed that this could indeed be done. Another more useful method was developed by Hertzfeld and Burger (Hertzfeld and Burger, 1980). Appendix C describes this method in detail. The situation where this reverse mapping from intensities to shift parameters is of great practical importance is in case of overlapping powder patterns arising from nuclei in several distinct environments. An example is calcium dihydrogen phosphate, shown in Figure 1.11 . It is not possible from the powder spectrum to determine how many different ^{31}P nuclei contribute to the total spectrum. Application of MASS resolves the overlapping powder pattern into sets of sidebands corresponding to each nucleus. From the sideband intensities, the chemical shift parameters are deduced based on the method of Hertzfeld and Burger (Hertzfeld and Burger, 1980). The calculated total spectrum, as well as its constituents, are shown in Figures 1.11 and 1.12 . The calculated total spectrum is good agreement with the experimental powder pattern. Other examples are given throughout the following chapters.

At this point it is worthwhile to reiterate several points of practical importance about the effect of MASS. First a tremendous gain in resolution is achieved as is demonstrated in Figures 1.11. MASS transforms a broad static powder spectrum, in which the spectral intensity is distributed over a finite frequency range, into a series of equally spaced narrow lines. The intensities and positions of these lines contain information about both the anisotropic and isotropic components of the chemical shift. This is of particular importance for biological samples. In such samples, it is anticipated that a large number static powder patterns will overlap and information about the chemical shift anisotropy of the individual components will be obscured. Furthermore, a MASS spectrum should have a much greater signal-to-noise ratio than that of a static powder spectrum recorded under the same conditions. In the latter case the

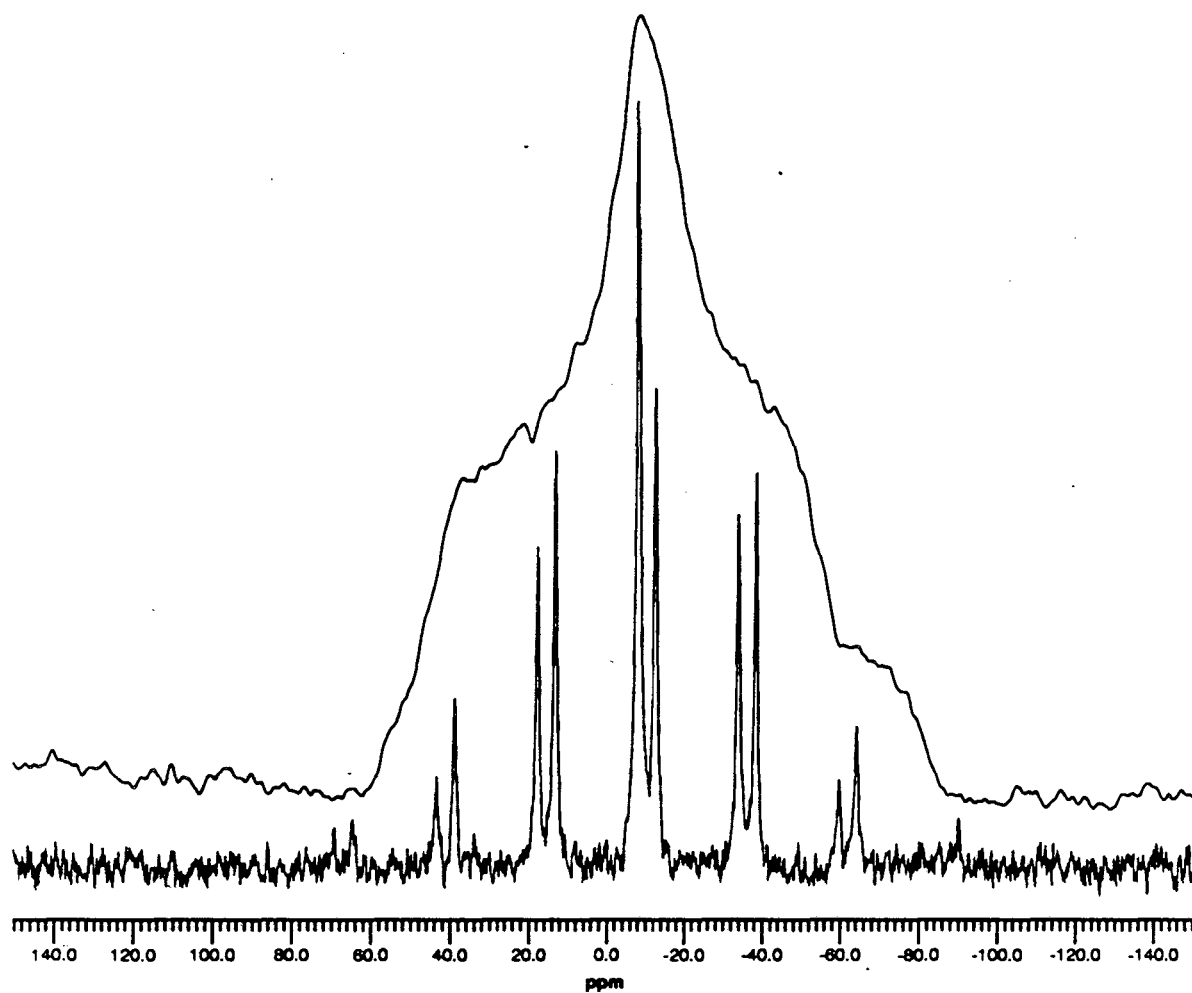


Figure 1.11. The ^{31}P static powder (*upper trace*) and MASS (*lower trace*) spectrum of calcium phosphate monohydrate. It is not possible to deduce from the static powder spectrum that there are two nonequivalent phosphate groups in the crystalline phase. MASS clearly resolves the two phosphate groups. It is possible to reconstruct the powder pattern from the sideband intensities as demonstrated in the next figure. Different number of acquisitions were averaged for each spectrum.

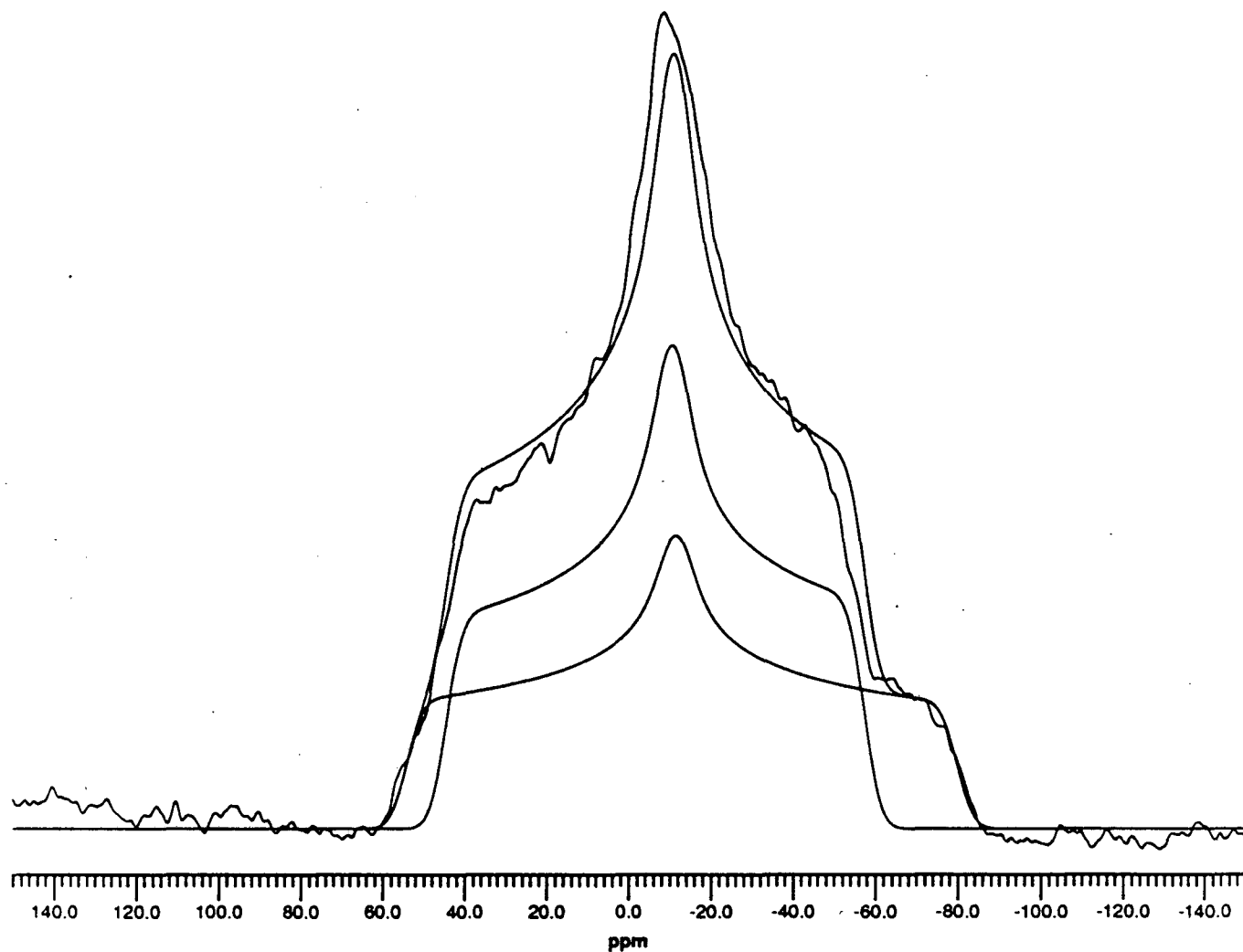


Figure 1.12. The ^{31}P experimental and calculated powder spectrum of Calcium Phosphate Monohydrate. The experimental and calculated spectrum are in good agreement. The calculated spectrum is based on the analysis of sideband intensities of the MASS spectrum shown in Figure 10. The method is described in Chapter 3. The contribution by each of the nonequivalent phosphate groups is also shown. It is clear that one of the groups has a larger anisotropy than the other.

integrated intensity is distributed over a finite range while in the MASS case the same intensity is concentrated in set of narrow lines. This observation is clearly demonstrated in Figure 1.13 where the MASS spectrum is the result of a single transient and the static powder is the average of 192 transients. The same enhancement in signal-to-noise and resolution is obtainable from solution NMR spectra. This assumes that the molecule of interest is both soluble and is sufficiently small so that the chemical shift anisotropy can be averaged. However, this last condition precludes obtaining information about anisotropic components of the interaction from solution spectra.

D. Calculation of Dipolar MASS Spectra

Finally, the effect of MASS on the dipolar interaction between like spins is considered where the chemical shift interaction is zero or very weak by comparison. This is not a completely realistic case. The closest example of such a case is a pair of protons of a methylene group where the chemical shift interaction is only a factor of 5 times smaller than the dipolar interaction. As pointed out in Appendix B, the treatment of the dipole interaction is very similar to that of the axially symmetric chemical shift interaction. The difference comes in the spin operators. As discussed in an earlier section, the occurrence of a dipolar interaction between two like spins results in a three level system. The two possible transitions are dependent on the orientation of the internuclear vector with respect to the magnetic field axis. The energies of these transitions differ by a constant, $3\gamma_I^2/2r^3$ and are given by

$$\omega(\theta) = \pm \frac{3\gamma_I^2}{2r^3} (1 - 3\cos^2\theta) . \quad 1.41$$

The powder spectrum may be calculated in the same manner as the chemical shift case. Eq. 1.41 is functionally the same as Eq. 1.32b which describes the axially symmetric chemical shift interaction given. From this observation, it would be expected that the powder pattern consists of two identical axially symmetric patterns which are symmetrically disposed about the isotropic frequency. This is shown in Figure 1.14. The powder pattern is known as a "Pake spectrum". The analysis of the of the MASS effects on dipolar interaction does not differ significantly from that of the chemical shift interaction. Figure 1.14 shows spectra calculated at the different speeds. Like the chemical shift case, the dipolar MASS spectra exhibit a spin

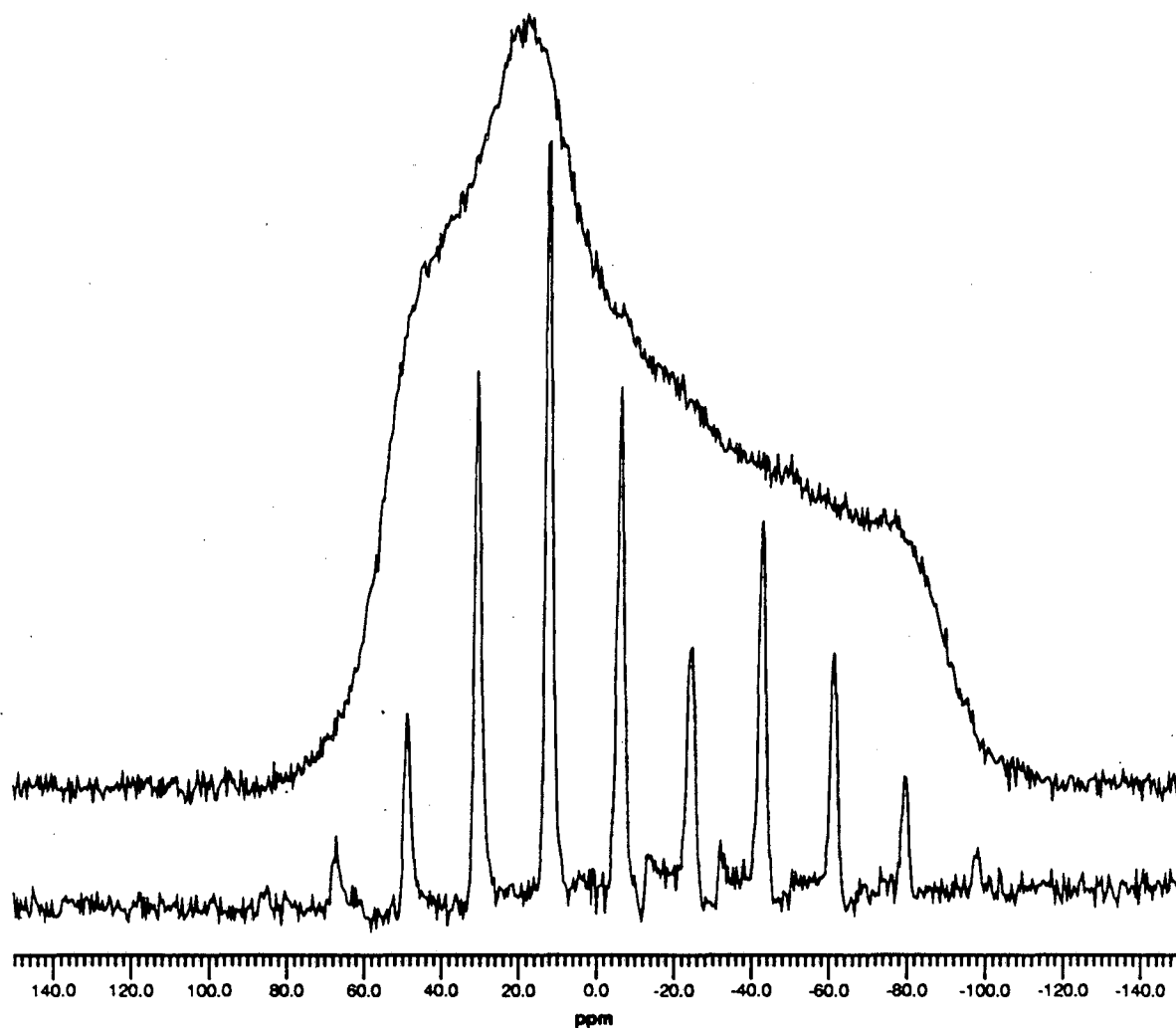


Figure 1.13. Comparison of the signal-to-noise between the static and MASS ^{31}P spectra of polycrystalline sodium dihydrogen phosphate taken under the same conditions. The powder spectrum is the result of 192 acquisitions and the MASS spectrum only one. The signal-to-noise ratio is approximately the same in both spectra.

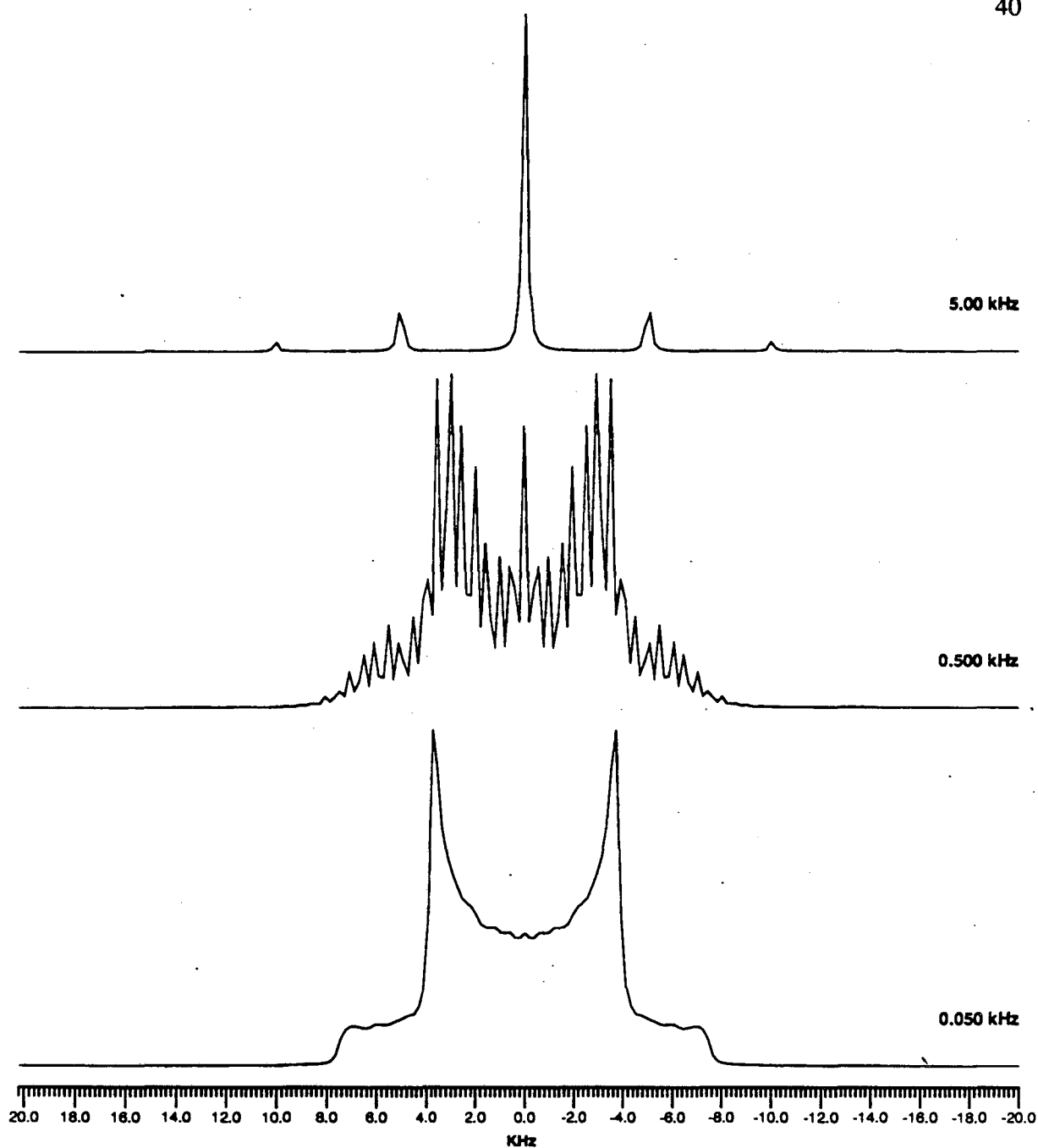


Figure 1.14. The calculated dipolar MASS spectrum of a isolated pair of $I=1/2$ spins at three different spinning speeds. The dipolar coupling constant was assumed to be 5.0 kHz.

rate invariant centerband and a set of sidebands positioned at rotor frequency.

Mariq and Waugh (Mariq and Waugh, 1979) have noted that when both chemical shift and dipolar interactions are present the problem is somewhat complicated by the fact that the chemical shift and dipolar parts of the Hamiltonian do not commute, which in turn leads to mixing of states and modification of energies. The transition energies under this situation are proportional to $D\Delta\sigma/\omega_r$, where D is the dipole coupling and $\Delta\sigma$ is the instantaneous chemical shift difference between the two spins. In addition, because of this homogeneous behavior resulting from the failure of the two interactions to commute, the structure of the individual sidebands will have four singularities (Mariq and Waugh, 1979). This is due to fact that the energy of transitions results from the product of two second order tensors. Since the breadth of each sideband scales inversely with spinning, at relatively high spinning speeds the effect vanishes and at low speeds other broadening mechanisms from insufficient averaging will obscure it. Finally, in the case of many like spins mutually coupled to each other, as would be the case of protons in a rigid solid organic sample, it is expected that MASS will have little effect until it is on the order of the total linewidth. This is because the spins through the dipole-dipole interaction (the "flip-flop" terms) will tend to average any perturbations over the total linewidth.

The topic of the following two chapters deals with the measurement of the ^{31}P chemical tensors of various phosphates and phosphate esters. The principal values of the shift tensors of these compounds were determined using the method of Hertzfeld and Burger (Hertzfeld and Burger, 1980). By measuring the tensor values and assuming an orientation with respect to the molecular structure (*i.e.* bonds), it was possible to infer information about the bonding and structure of these phosphates. The last chapter on the application of MASS discusses its application to the study of tissue. The samples were lyophilized, therefore, the motions which could interfere with the averaging effect of MASS were largely avoided (Waugh, 1979). It was expected that all of the ^{31}P nuclei could be observed under MASS conditions and, unlike in solution, the detectability of phosphorus bearing molecules was not limited to those which were relatively small and quickly reorienting. Hence, it was possible for the first time to obtain more "complete" spectra of these heterogeneous tissue samples than those observed under normal

solution conditions. Furthermore, the ^1H spectra of these tissue samples exhibited unusually narrow resonances when the samples were spun at the magical angle and are also discussed in the chapter on MASS and tissue.

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Chapter 2

The ^{31}P NMR Chemical Shift Tensors of ATP Complexes

Chapter 2. The ^{31}P NMR Chemical Shift Tensors of ATP Complexes

In this chapter, we discuss the measurement of the ^{31}P NMR chemical shift tensors for three complexes of adenosine triphosphate (ATP): sodium ATP trihydrate, bis(2-pyridyl)amine magnesium ATP and bis(2-pyridyl)amine calcium ATP.^{2,3} ATP plays a central role in energy metabolism. The pH and counterion dependence of the solution NMR spectra of ATP are not clearly understood. We undertook these measurements in the solid state with the aim of gaining a better understanding of these effects. Magic angle sample spinning (MASS) was employed to resolve the resonances of the three phosphate groups of the tripolyphosphate chain. The chemical shift tensors were determined using a two step procedure. First, the spinning side bands for each of the three resonances were computer fitted to obtain accurate relative intensities and then analyzed using programs based on the method of Hertzfeld and Berger.^{1,4} The high degree of spectral resolution allowed direct measurement of the isotropic shifts from the MASS spectra. Figures 2.1 and 2.2 are MASS spectra typical of those used in this study. Bloch decay and cross polarization experiments, both with proton decoupling, were used in obtaining the measurements. Typical linewidths were 200 Hz. Table 2.1 is a tabulation of the principal values of the chemical shift tensor for the three ATP complexes, as well as other shift values determined in our laboratory and from the literature. We use the convention $\sigma_{33} > \sigma_{22} > \sigma_{11}$ with positive denoting downfield shifts. In order to confirm our results, we calculated the powder spectrum for each of the ATP complexes using the chemical tensor values obtained from the sideband analysis. These calculated spectra were compared to non-spinning powder patterns obtained experimentally.⁵ Figures 2.3 and 2.4 show the success which we have had in recovering the powder patterns from sideband intensities. The magnesium complex exhibited an excellent fit. By contrast, the sodium complex showed only moderate agreement. This discrepancy was probably due to the fact that two sets of sidebands were not included in calculating the powder pattern. One set, most likely from impurities, appears at the downfield extreme of the spectrum and the other is a set of low intensity sidebands which shoulder the alpha resonance. This latter set may be due to either the heterogeneity in crystalline environments or impurities. Kennard⁶ has noted that obtaining crystallographic quality crystals is a very difficult and largely unfruitful

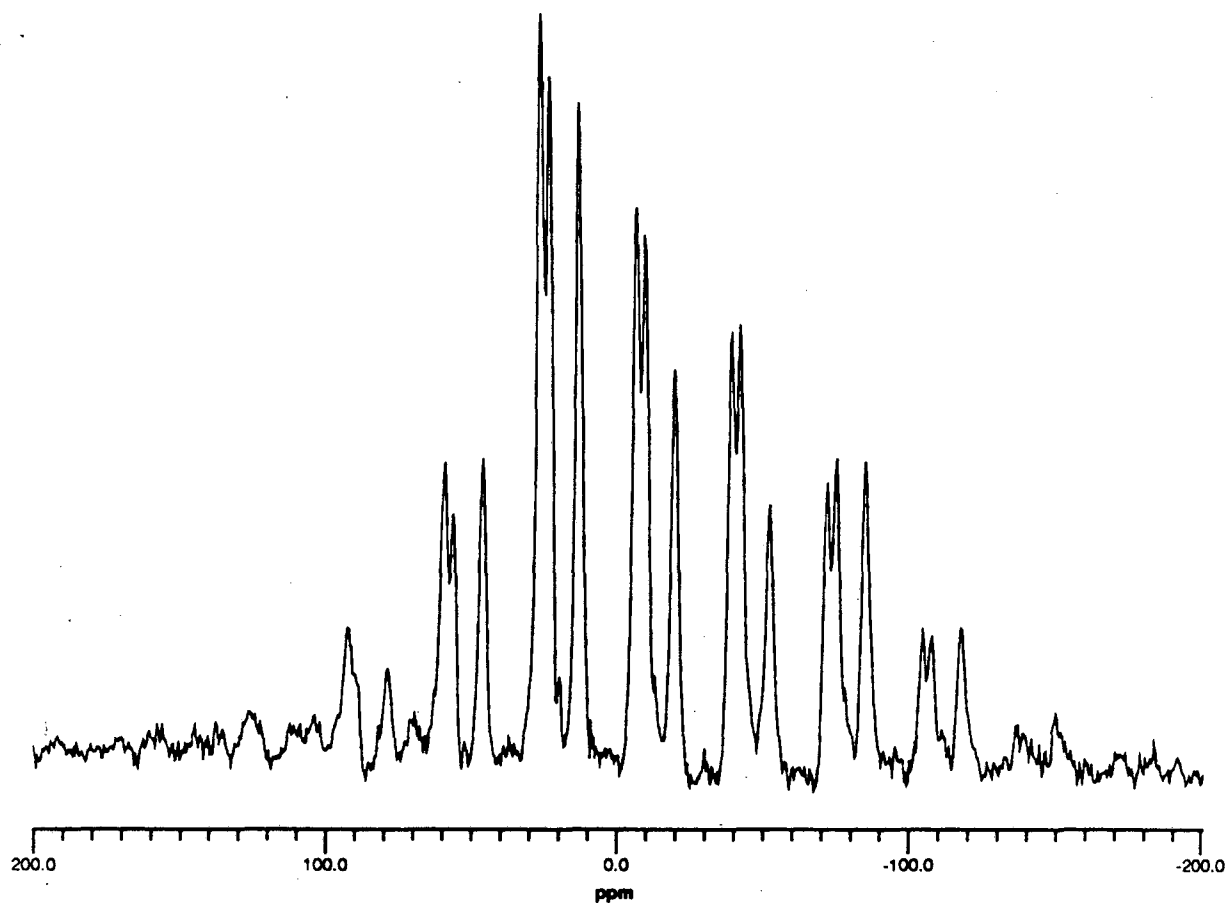


Figure 2.1. MASS spectrum of sodium ATP trihydrate, recorded using a proton decoupled one pulse experiment. The sample was spun at 3.2 kHz. Chemical shifts are relative to 85% phosphoric acid. The proton decoupling field was 45 kHz.

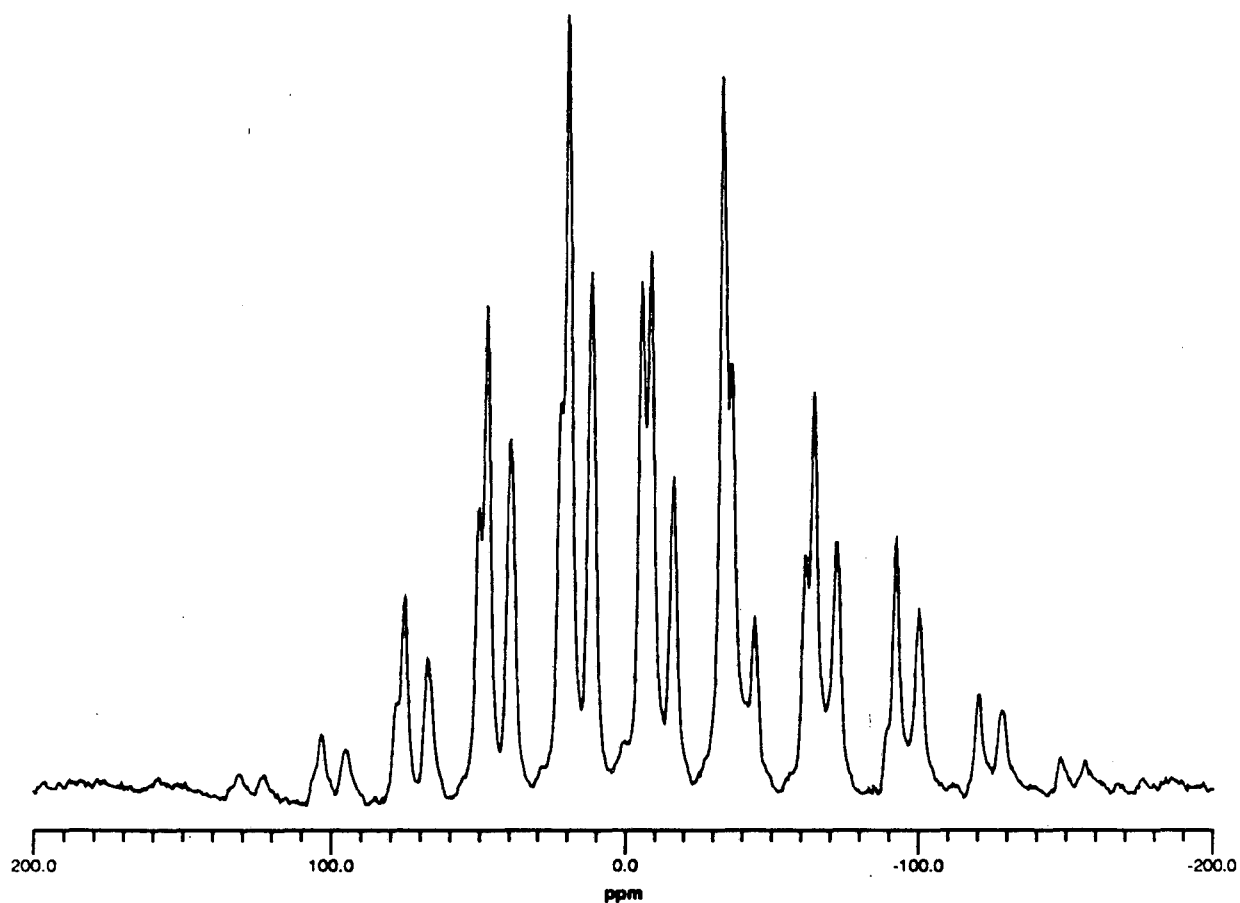


Figure 2.2. CPMAS spectrum of magnesium ATP-BPA, recorded using a Hartmann-Hahn matched cross polarization experiment. The sample was spun at 2.8 kHz. Chemical shifts are relative to 85% phosphoric acid. The proton decoupling field was 45 kHz.

Table 2.1. ^{31}P chemical shift tensor element values. Numbers in parentheses are used to distinguish different resonances arising from inequivalent nuclei and greek letters denote different positions on the same molecule. Chemical shifts were referenced to a 85% H_3PO_4 external standard.

COMPOUND		σ_{33}	σ_{22}	σ_{11}	σ_{iso}	$\sigma_{solution}^\dagger$
Barium diethylphosphate ¹²		75.9	17.5	-109.8	-5.5	
Phosphorylethanolamine ^{11,12}		68.6	-12.0	-67.2	-3.5	
Dilaurylphosphatidylethanolamine ¹²		84	23	-100	2.3	
Calcium Pyrophosphate						
	(1)	75	-39	-69	-11.0	
	(2)	64	-24	-66	- 8.0	
Sodium tripolyphosphate ¹³						
	α	107	-24	-69	4.6	
	β	80	20	-117	- 5.6	
Sodium ATP trihydrate						
	α	80.6	9.5	-120.0	-10.0	-10.38
	β	70.9	11.2	-142.2	-20.0	-21.15
	γ	78.6	10.1	-109.4	- 7.0	-7.17
Calcium ATP – DPA						
	α	80.4	11.8	-122.7	-10.2	
	β	72.2	12.9	-140.0	-18.2	
	γ	78.5	-25.4	-75.8	- 5.7	
Magnesium ATP – DPA						
	α	85.9	11.7	-125.5	- 9.3	-9.92
	β	73.7	14.2	-138.6	-16.9	-18.46
	γ	84.1	-23.9	-79.7	- 6.5	-4.82

[†] Data are from the work of Gadian.⁸ Chemical shifts are those measured at 7.0 pH.

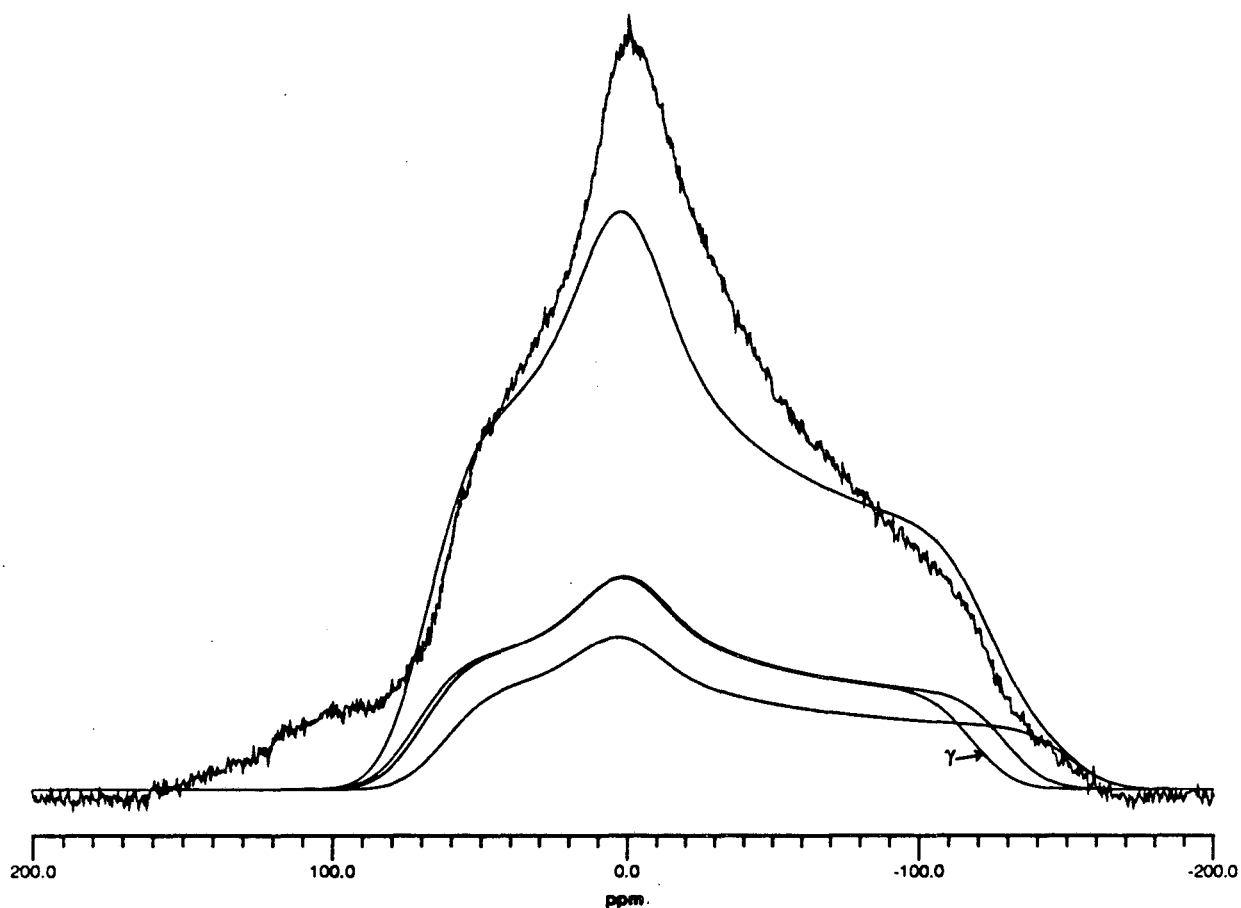


Figure 2.3. Comparison of the experimental powder pattern of the sodium ATP complex with the powder spectrum calculated from shift tensor elements listed in Table 1. The spectrum was recorded using a proton decoupled one pulse experiment. The individual components of the calculated powder pattern are also shown. The proton decoupling field was 45 kHz.

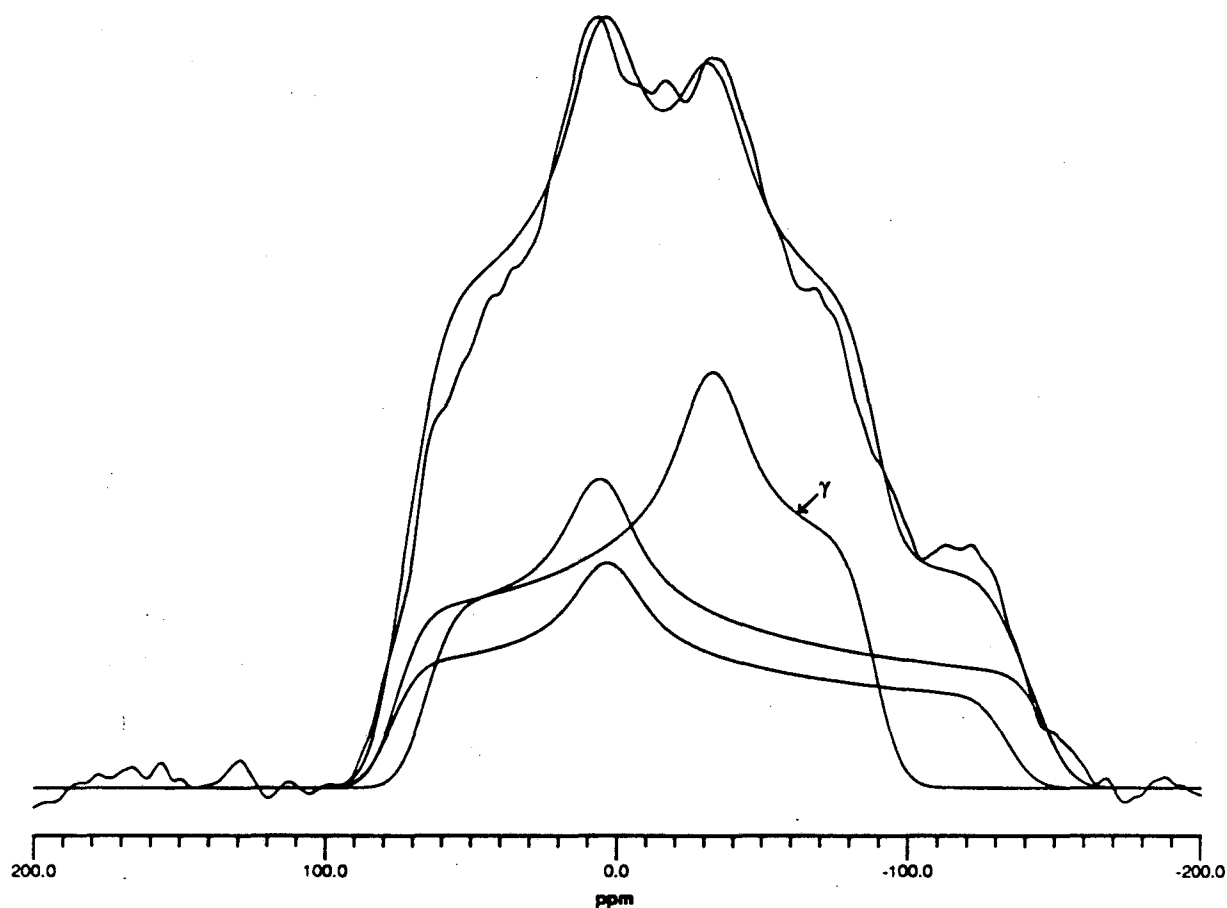


Figure 2.4. Comparison of the experimental powder pattern of the magnesium ATP complex with the spectrum calculated from shift tensor elements listed in Table 1. The powder pattern was taken using a chemical shift echo experiment.⁵ The individual components of the calculated powder pattern are also shown. The proton decoupling field was 45 kHz.

activity accounting for our unsuccessful attempts at recrystallization. We were confident of the sideband analysis of the sodium ATP complex since the characteristic discontinuities of the calculated pattern are well matched to those of the experimental patterns.

It would have been desirable to carry out the comparisons of the various ATP complexes without the use of the bis(2-pyridyl)amine, BPA, as a co-precipitant. However, attempts to obtain MASS spectra of commercially available magnesium ATP were unsuccessful. The spectra exhibited a single broad resonance, presumably due to the lack of crystalline homogeneity or the outright amorphous nature of the powder. By contrast, the BPA complexes were easy to prepare and the spectra were those expected with good resolution and free of impurities. Crystallographic studies have shown that the BPA does not interact with the phosphate chain.³ Based on this observation, we expected that the chemical shift tensors would not be affected by the presence of the co-precipitant. Of peripheral interest to us was the suggestion of some investigators that some dephosphorylation reaction intermediates may involve both aromatic residue-base interaction and the divalent metal binding for which the calcium and magnesium ATP-BPA could be a model.⁷ These two observations provide more than adequate reasons for studying these ATP-BPA complexes.

The isotropic chemical shifts, σ_{iso} , of the complexes of ATP in the solid state to first order resemble those observed in solution, as shown in Table 2.1. The data also reflect the observations made in solution that divalent metals induce shifts of the beta and gamma resonances relative to the alpha which is far less affected by the presence of the metal.⁸ In the case of the alpha position, the metal dependent shifts of the tensor elements seem to cancel to yield isotropic values which are similar. For the beta groups there is a small shift downfield of all three tensor elements of the divalent complexes relative to the sodium complex and the isotropic values of the magnesium and calcium complexes reflect a shift in that direction. This trend is in agreement with the capacity of the metal to polarize the ATP anion (*i.e.* $Mg^{+2} \geq Ca^{+2} \geq Na^{+}$). A similar dependence has recently been observed for simple phosphates by Turner and co-workers.⁹

By far the most interesting are the gamma phosphate resonances. The behavior of these resonances demonstrates the enhanced information obtained by analyzing the complete tensor in preference to isotropic chemical shifts. Although, the isotropic shifts of the gamma phosphate

group do not change drastically, the powder patterns undergo very large alterations. To better quantitate these differences, we used an alternate set of three parameters to describe the powder patterns, the isotropic shift, the chemical shift anisotropy (δ) and asymmetry parameter (η).¹⁰ These three quantities completely define a given chemical shift tensor. Table 2.2 lists the three values for the three ATP complexes. The sign of the shift anisotropy of the gamma position of sodium ATP is opposite to that of either the magnesium or calcium complex. Because of this, the terminal phosphate group of the sodium complex phosphate group has a powder pattern more reminiscent of a diester rather than monoester. This can be seen in Figure 2.3 where the gamma resonance of sodium ATP is barely distinguishable from that of the alpha position, a phosphate diester group. By contrast, for the magnesium complex (see Figure 2.4) the powder pattern for the gamma is clearly distinct from that of alpha and beta positions. In addition, as will be shown in Chapter 3, the σ_{22} value of 10.1 ppm is indicative of a singly charged phosphate group. We believe these observations lead to the conclusion that the gamma sodium ATP phosphate group is protonated. In contrast to this, the shift tensor values of the terminal phosphate groups of the magnesium and calcium complexes are similar to those of the monoester and we conclude that these sites are unprotonated. This is in disagreement with Cini and co-workers who proposed on the basis of potentiometric titration studies and reaction conditions that the gamma site was protonated.³

Table 2.2. Chemical shift tensor parameters of the three ATP complexes expressed in terms of σ_{iso} , δ and η .[†]

COMPOUND		δ	η	σ_{iso}
Sodium ATP trihydrate				
	α	-110	0.65	-10.0
	β	-122	0.49	-20.0
	γ	-102	0.67	-7.0
Calcium ATP – DPA				
	α	-113	0.63	-10.2
	β	-121	0.49	-18.2
	γ	84	0.53	-5.7
Magnesium ATP – DPA				
	α	-116	0.63	-9.3
	β	-121	0.49	-16.9
	γ	91	0.62	-6.5

[†] ordering conventions and definitions for δ and η are those of Haeberlen¹⁰:

$$|\sigma_{zz} - \sigma_{iso}| \geq |\sigma_{xx} - \sigma_{iso}| \geq |\sigma_{yy} - \sigma_{iso}|$$

$$\sigma_{iso} = \frac{1}{3}(\sigma_{zz} + \sigma_{xx} + \sigma_{yy})$$

$$\delta = \sigma_{zz} - \sigma_{iso}$$

and

$$\eta = \sigma_{yy} - \sigma_{xx} / \delta.$$

References and Notes

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- (2) The sodium ATP was purchased from Sigma and used without further purification. The magnesium and calcium ATP were prepared using the procedure of Cini.³
- (3) Cini, R.; Burla, M. C.; Nunzi, A; Polidori, G. P.; Zanazzi P. F. *J. Chem. Soc. Dalton Trans.*, **1984** , 2467-2476.
- (4) Each MASS spectrum was fitted using the program NTCCAP with various admixtures of lorentzian-gaussian lineshapes to obtain the best fit.
- (5) Rance M.; Byrd R. A. *J. Mag. Res.*, **1983** , 52, 221-240. Fully phased cycled chemical shift echo spectra were taken in order to obtain high quality powder patterns. Additional powder spectra were taken in same manner as the MASS spectra except without sample rotation.
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Chapter 3
Study of ^{31}P NMR Chemical Shift Tensors and
Their Correlation to Molecular Structure

Chapter 3. Study of ^{31}P NMR Chemical Shift Tensors and Their Correlation to Molecular Structure

I. Introduction

^{31}P NMR has become a very useful tool for studying cellular metabolism *in vivo*. The chemical shift interaction in phosphates and phosphate esters which are of interest in such studies often exhibit large pH and counterion dependences. For example, the presence of magnesium ions induces large differential shifts of all three ATP resonances (Gadian, 1979). Although the factors which control the ^{31}P chemical shift interaction have been identified (Lechter and Van Wazer, 1967), the specific interactions which bring about these dependences are not well understood. To gain a better understanding of the ^{31}P chemical shift interaction, we have undertaken a magic angle sample spinning (MASS) study of a variety of phosphate-group containing molecules. The chemical shift tensors of a number of phosphates and phosphate esters were measured. The down- and upfield extremes of the tensors, the σ_{33} and σ_{11} elements respectively, were found to vary with bond length and bond angles. The variation in bond lengths of tetrahedrally coordinated phosphorus atoms have been shown by others to be function of d-p π -molecular orbital interactions of the ligand and phosphorus atoms (Cruickshank, 1961). Therefore, we have established a direct link between variations of the chemical shift tensor and phosphorus-oxygen bonding. Deviations from these correlations were also found. Some of these were attributed to metal counterion effects which have recently been characterized by Turner (Turner, 1986).

II. Background

Based on approximate quantum mechanical calculations, Lechter and Van Wazer (Lechter and Van Wazer, 1967) have identified three predominant factors which determined the changes in ^{31}P isotropic chemical shifts and expressed their effect with the equation

$$\Delta\delta = a\Delta n_{\pi} + b\Delta\chi - c\Delta\Theta_{\sigma} \quad 3.1$$

where a,b,c are constants and n_{π} is the π -bond order, χ the electronegativity of the substituent, Θ_{σ} is the σ -bond angle and finally δ is the chemical shift. The relative importance of each

of the three contributions has been under some debate (Gorenstein, 1984,1985). Recent data indicate that the O-P-O bond angles play a predominant role in determining differences in the isotropic chemical shifts (Blackburn,1971, Gorenstein, 1984,1985, Prado, 1979,Dustrata, 1981).

Most of the experimental and theoretical studies have been limited to the isotropic part of the chemical shift interaction, while only a few have exploited its tensorial nature. Dustrata and coworkers (Dustrata, 1981) studied the cyclic thioxo-phosphonates and phosphane oxides, sulfides and selenides. A linear relationship between the asymmetry parameter (for definition see Chapter 1.) and the intracyclic O-P-O bond angles of the cyclic thioxo-phosphonates was found. Grimmer (Grimmer, 1978) observed a linear relationship between the chemical shift anisotropy, $\Delta\sigma = \sigma_{||} - \sigma_{\perp}$, and the average P-O bond length of various compounds of the form POX_3 (X=F, Cl, Br and methyl) all of which are axially symmetric. More recently, Turner and co-workers (Turner, 1986) have determined empirical correlations between the chemical shifts of orthophosphates with the charge and the cationic radius of the counterion. They also found correlations between the chemical shift anisotropy and average deviation of the O-P-O bond angle from the tetrahedral value.

A direct comparison of structural parameters, such as bond lengths and angles, and the ^{31}P chemical shift tensors has not yet been reported. It is known that the degree of π -bonding can be inferred from bond lengths and changes in σ -bonding from bond angles. Cruickshank (Cruickshank, 1961) has shown that P-O bond lengths vary linearly with the d-p π -bond order. Figure 3.1 shows the two P-O d-p π -bonds. Deviations from this behavior were attributed to σ -bond effects, although this observation has been under debate (Huheey, 1978). Cruickshank's observations along with those of Lechter and Van Wazer (Lechter and Van Wazer, 1967) provide a direct means for relating changes in chemical shifts to variations in structure and P-O bonding. More importantly, if both the structure and the chemical shift tensor were known, it would be possible to deduce the bonding effects of a particular P-O bond on the chemical shift interaction without limiting the analysis to the effect on the isotropic chemical shift value which is an average over all directions and, hence, over all P-O bonds. Based on these observations, we have carried out an analysis of P-O bonding effects on the ^{31}P chemical shift interactions in the solid state.

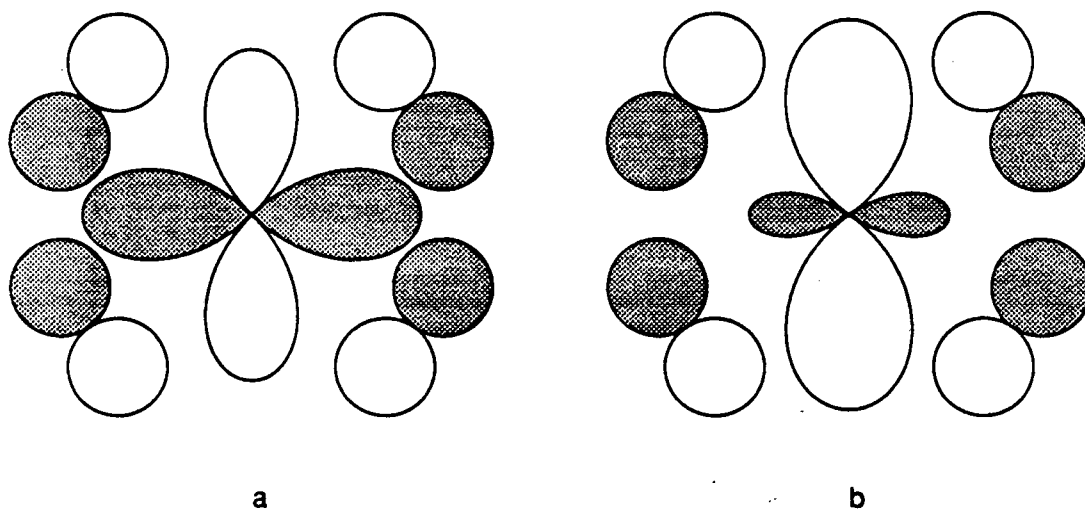


Figure 3.1. The two d-p π bonding orbitals: (a) is the overlap of the $P(3d_{x^2-y^2})$ with 4 $O(2p)$ and (b) is the overlap of the $P(3d_{xz})$ with 4 $O(2p)$.

Our approach was to measure and compile from the literature ^{31}P shift tensors of a variety of phosphates and phosphate esters. Comparisons between shift tensors and structures were facilitated by existing x-ray crystallographic data for most of the compounds. A major problem confronting an analysis of this type is the severe overlap of several powder patterns arising from the presence of several distinct phosphorus nuclei. For the simple case of a single ^{31}P nuclei, the principal elements of the chemical shift tensor can be measured directly from the spectrum. However when several powder patterns overlap, it is in general not possible to determine the individual chemical shift tensor elements. This is clearly demonstrated in the powder patterns for the ATP complexes discussed in the previous chapter. The individual contribution from each phosphorus nucleus of the triphosphate chain cannot be clearly discerned. This problem can be overcome by the application of magic angle sample spinning (MASS). MASS experiments allow the determination of both the isotropic chemical shifts and the complete shift tensor. The two common features present in all MASS spectra are the centerband and the spinning side-bands. The frequency of the centerband corresponds to the isotropic shift and is invariant to sample spinning rate (Marciq and Waugh, 1979). The side-bands appear at integer multiples of the spinning rate about the centerband. The relative intensities of the centerband and its spinning side bands contain information from which the chemical shift tensor values can be determined. The detailed theoretical treatment of the recovery of the chemical shift tensor from MASS spectra has been given by Marciq and Waugh (Marciq and Waugh, 1979) who have used a moment analysis and by Hertzfeld and Berger (Hertzfeld and Berger, 1980) who have numerically calculated the theoretical spinning side-band intensities.

In order to completely specify the chemical shift tensor, its orientation, in addition to the three principal values, must also be established. From single crystal studies of Kohler and co-workers (Kohler, 1976) and Hertzfeld and co-workers (Hertzfeld, 1978) it can be shown that the orientation of the ^{31}P chemical shift tensors of phosphates and phosphate esters are relatively constant and, more important, the principal axes of the tensors are orientated roughly along the P-O bonding directions. In a MASS study of sodium tripolyphosphate, Burlinson and co-workers (Burlinson, 1986) deduced the tensor orientations to be very similar to those cited above. The approximate orientation of the shift tensors which have been studied is shown

in Figure 3.2 . This orientation has been rationalized by Kohler and co-workers based on the anticipated electron density around the phosphorus nuclei. We have assumed that this is the approximate tensor orientation of all of the phosphates and phosphate esters which are discussed in this paper.

III. Experimental

A. Materials

NaATP was purchased from Sigma and used without further purification. Mg- and CaATP were prepared using the method of Cini, *et al* (Cini, 1984). Equimolar quantities of the appropriate metal sulfate, 2,2'-dipyridylamine, and NaATP were mixed in 150 mL of 30% ethanol. The precipitate was washed with 30% ethanol followed by cold water. Sodium dihydrogen pyrophosphate was made using a standard synthesis procedure (Bell, 1950). Sodium pyrophosphate anhydrate was made by dehydrating the decahydrate at 130°C to constant weight, which was equal to the theoretical weight for complete dehydration. The other simple phosphates were either used directly or washed with cold anhydrous ethanol to remove phosphoric acid contaminant.

B. Instrumentation

All spectra were recorded on a spectrometer of local design (Shih, 1979)(109.298 MHz for ^{31}P) using a Doty MASS probe. Ninety degree pulses were typically 5 μs for both protons and phosphorus. Cross polarized MASS (CP-MASS) spectra were taken under Hartmann-Hahn matched condition with a flip-back pulse at the end of each acquisition (Stejskal and Schaefer, 1975) which took advantage of short proton T_1 's. Various cross polarization times were used (0.5-2.0 ms). For simple Bloch decay experiments with proton decoupling, long repetition times were necessary due to long ^{31}P T_1 's, approximately 24 s for NaATP. Sample spinning rates were typically 2.0 to 4.0 kHz. The rotor speeds were determined by an home-built optical tachometer fitted to the bottom of the stator.

Static powder patterns were obtained in the same manner as MASS spectra without sample rotation. In addition, fully phased cycled chemical shift echo spectra were recorded to obtain accurate powder patterns (Rance and Byrd, 1983).

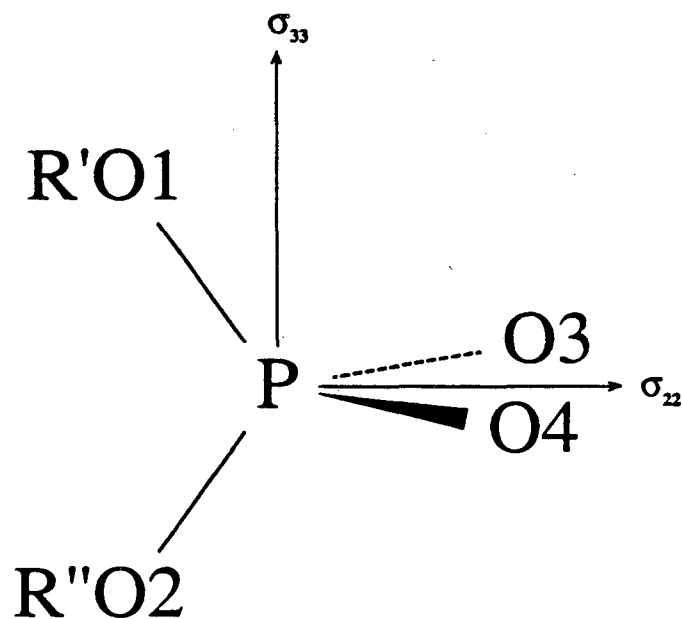


Figure 3.2. Orientation of the chemical shift tensor with respect to the molecular frame. The σ_{11} element is perpendicular to the elements shown and extends out towards the viewer. $R'=P,C,H$ and $R''=P,C,H$.

C. Recovery of chemical shift tensors

The general procedure for recovering chemical shift tensor elements was to match the experimental spinning side-band intensities with those calculated by Hertzfeld and Berger (Hertzfeld and Berger, 1980) using a non-linear least squares analysis. First, the MASS sideband intensities were extracted using NTCCAP, a spectrum simulation program, with admixture of lorentzian-gaussian lineshape. This method was especially useful for those spectra which were not completely resolved as was the case for all ATP complexes studied. Ratios of intensities, I_n/I_m , were calculated, where I_m was normally taken to be the most intense peak. These ratios were then compared to a theoretically calculated set which were tabulated by Hertzfeld and Berger as a function of a pair of parameters, μ and ρ , defined by,

$$\mu = \frac{(\gamma H_0)(\sigma_{33} - \sigma_{11})}{\omega_r}, \quad 3.2$$

$$\rho = \frac{(\sigma_{11} + \sigma_{33} - 2\sigma_{22})}{(\sigma_{33} - \sigma_{11})}. \quad 3.3$$

The actual computation was initiated using a coarse minimum search program to determine the approximate μ and ρ values for which there was a minimum total residual between the observed and theoretical I_n/I_m ratios of all the measured side-band intensities. These approximate values were then used as initial guesses for subsequent analysis. A nonlinear least squares analysis program using the IMSL subroutine ZXSSQ (IMSL, 1984) was used to determine the final values for μ and ρ . From μ , ρ , the rotor speed and the isotropic chemical shift a set of the tensor elements was then determined. It is important to note that the isotropic shift is measured directly from the MASS spectrum, the frequency of the centerband being equal to the isotropic shift (Maricq and Waugh, 1979). Since the frequency of the centerband is invariant to sample spinning rate, it was sufficient to obtain spectra at two different spinning rates to identify the centerband. The frequency of the centerband was measured with respect to a 85% H_3PO_4 standard. The frequency of the standard was measured under conditions identical to that of the solid sample before and after each experiment.

Analysis using both NTCCAP fitted and raw intensity data for the beta MgATP resonance, which is well resolved, indicated no difference in resulting shift tensor component values. This

comparison was also made on several other well resolved resonances with good agreement as was discussed in the previous chapter.

IV. Results and Discussion

The chemical shift tensor elements of a variety of phosphates and phosphate esters are tabulated in Table 3.1. Included in this table are results obtained in our laboratory and those published elsewhere (Kohler, 1976; Hertzfeld, 1978; Rothwell, 1980). The isotropic chemical shifts measured for the solids were not equal to those measured in solution. The variation in the isotropic shifts of the sodium, calcium and magnesium hydrogen phosphates probably reflects the strength of the acidic hydrogen bond to the phosphate group and counterion dependence (see below and Turner, 1986). The upfield shift associated with the protonation is not manifest in our MASS data (Gadian, 1979). Although, the solid state isotropic data do not agree in detail with those measured in solution, the tensor elements which contribute to determining these average values do form a consistent set of data with respect to the details of their molecular structure. This is the subject of the following observations.

Figure 3.3 relates the σ_{33} element, the extreme downfield element, to the maximum P-O bond length. A strong linear trend is observed. From Figure 3.2, it can be seen that the P-O-R bond and σ_{33} element roughly lie in the same direction. In general, this bond will be the longest (see Table 3.2). Cruickshank (Cruickshank, 1961) showed that the variations in bond lengths in phosphates and their esters result from reduction in or elimination of the capacity of one or more of the oxygens to contribute to d-p π -bonding. This occurs when one or more of the oxygen p orbitals, which contribute to the two d-p π -bonding molecular orbitals, must also accommodate a bond resulting from protonation (R=H) or esterification (R=C,P). This weakens the π -bonding interaction. The degree to which the bond is elongated is then a measure of how effectively the R group reduces the π -bonding capabilities of the oxygen. Furthermore, according to Cruickshank (Cruickshank, 1961), this effect should be linear. The consequence of these observations is that the P-OR bond will in general be the longest P-O bond of the phosphoester. The fact that a linear trend is observed in Figure 3.3 indicates that that degree of d-p π -bonding plays a central role in determining the magnitude of the σ_{33} tensor element.

It should be noted that this correlation encompasses a wide variety of phosphates. It

Table 3.1. ^{31}P chemical shift tensor element values. Numbers in parentheses are used to distinguish different resonances arising from inequivalent nuclei and greek letters denote different positions on the same molecule. Chemical shifts are referenced against 85% H_3PO_4 with positive denoting downfield. (References are given at the end of this chapter.)

COMPOUND		σ_{33}	σ_{22}	σ_{11}	σ_{iso}
Urea - phosphoric acid complex ¹		26.6	2.5	-44.6	- 5.2
Magnesium phosphate octahydrate		42.5	-27.2	-64.7	-16.5
Sodium hydrogen phosphate dodecahydrate		55.4	-35.5	-69.8	-16.6
Magnesium hydrogen phosphate trihydrate (newberyite)		48.0	-28.0	-67.8	-15.9
Calcium hydrogen phosphate dihydrate (brushite) ²		70.0	-12.0	-53.0	-1.0
Calcium dihydrogen phosphate monohydrate ²					
	(1)	49.0	1.0	-48.8	0.7
	(2)	59.0	7.0	-66.0	0.0
Barium diethylphosphate ¹		75.9	17.5	-109.8	-5.5
Sodium GMP Heptahydrate		70.2	-15.2	-37.9	5.7
Phosphorylethanolamine ^{1,3}		68.6	-12.0	-67.2	-3.5
Dilaurylphosphatidylethanolamine ¹		84	23	-100	2.3
Calcium Pyrophosphate					
	(1)	75	-39	-69	-11.0
	(2)	64	-24	-66	- 8.0
Sodium pyrophosphate anhydrate					
	(1)	87	-33	-46	2.7
	(2)	81	-27	-50	1.3
Sodium pyrophosphate dodecahydrate					
	(1)	76	-32	-49	-1.7
	(2)	78	-26	-58	-2.0
Sodium dihydrogen pyrophosphate anhydrate		65	-24	-80	-13.0
Sodium tripolyphosphate ⁴					
	α	107	-24	-69	4.6
	β	80	20	-117	- 5.6
Sodium ATP trihydrate					
	α	80.6	9.5	-120.0	-10.0
	β	70.9	11.2	-142.2	-20.0
	γ	78.6	10.1	-109.4	- 7.0
Calcium ATP - DPA					
	α	80.4	11.8	-122.7	-10.2
	β	72.2	12.9	-140.0	-18.2
	γ	78.5	-25.4	-75.8	- 5.7
Magnesium ATP - DPA					
	α	85.9	11.7	-125.5	- 9.3
	β	73.7	14.2	-138.6	-16.9
	γ	84.1	-23.9	-79.7	- 6.5

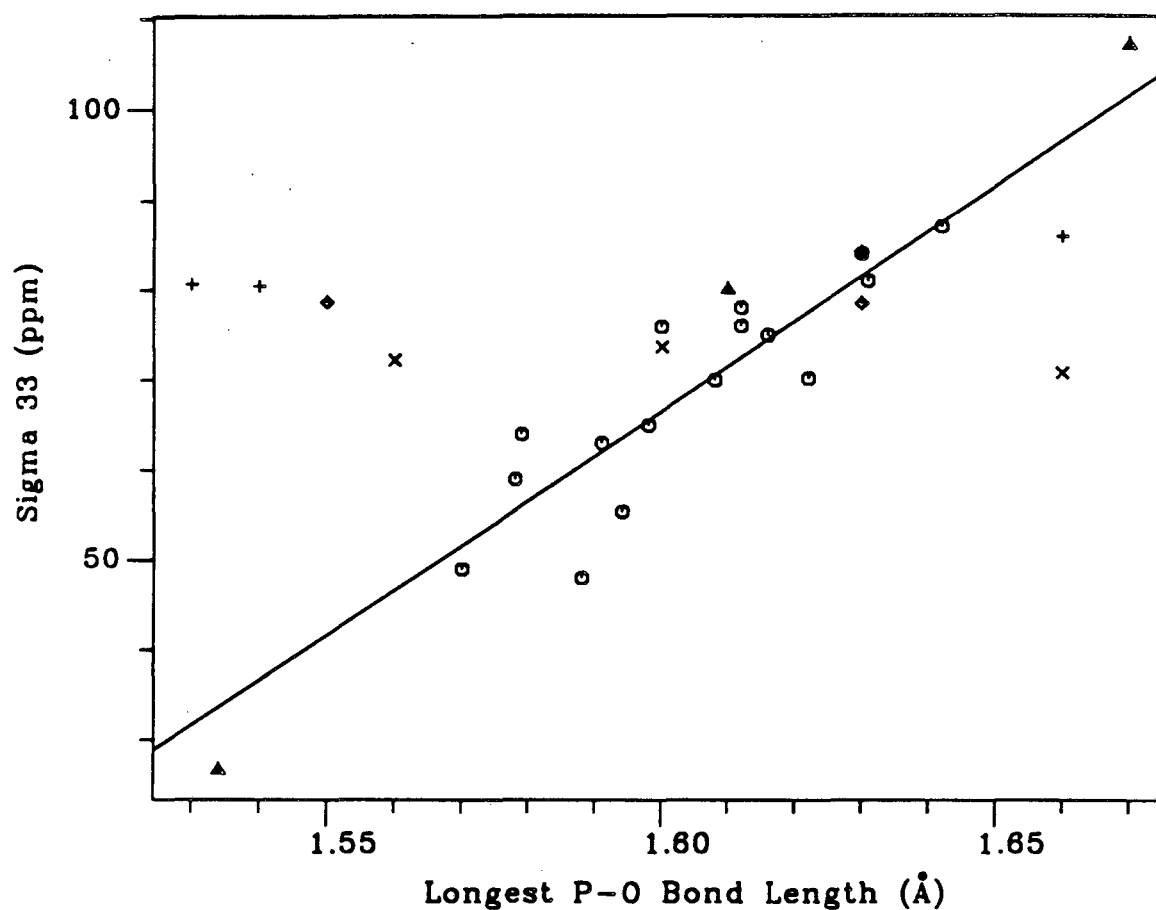


Figure 3.3. σ_{33} plotted as a function of the longest P-O bond length. The Δ symbols represent the urea-phosphoric acid complex and the sodium tripolyphosphate. The + symbols represent the alpha position, the $\times$ the beta positions and the $\diamond$ the gamma positions of the ATP complexes. The $\circ$ symbols represent the remaining compounds listed in Table 1. The best-fit line was determined using only the $\circ$ points. The equation for the best-fit line is $\sigma_{33} = 499\text{\AA}^{-1}\text{ppm} \cdot r - 731\text{ppm}$ where r is the bondlength.

Table 3.2. Bond lengths and bond angles used to determine the correlations shown in Figures 4,5,6 and 7. (References are given at the end of this chapter).

COMPOUND		P-O Bond Lengths		Bond Angles	
		Maximum	Minimum	O=P=O	ROPOR'
Urea - phosphoric acid complex ¹		1.534	1.508	111.9	110.8
Sodium hydrogen phosphate dodecahydrate ²		1.594	1.510	—	—
Magnesium hydrogen phosphate trihydrate ³ (newberyite)		1.588	1.500	—	—
Calcium hydrogen phosphate dihydrate ⁴ (brushite)		1.608	1.511	112.7	108.0
Calcium dihydrogen phosphate monohydrate ⁵	(1)	1.570	1.496	115.3	107.8
	(2)	1.578	1.505	119.7	107.7
Barium diethylphosphate ⁶		1.60	1.52	121.6	103.5
Sodium GMP heptahydrate ⁷		1.622	1.511	—	—
Phosphorylethanolamine ⁶		1.591	1.493	117.4	106.2
Dilaurylphosphatidylethanolamine ⁶		1.63	1.43	119	101
Calcium Pyrophosphate ⁸	(1)	1.616	1.505	114.8	105.3
	(2)	1.579	1.493	113.4	106.4
Sodium pyrophosphate anhydrate ⁹	(1)	1.642	1.512	—	—
	(2)	1.513	1.631	—	—
Sodium pyrophosphate dodecahydrate ¹⁰		1.612	1.503	—	—
Sodium dihydrogen pyrophosphate anhydrate ¹¹		1.598	1.492	117.7	104.5
Sodium tripolyphosphate ¹²	α	1.61	1.49	—	—
	β	1.67	1.50	116	99
Sodium ATP trihydrate ¹³	α	1.53	1.51	122	101
	β	1.66	1.52	122	98
	γ	1.55	1.46	—	97
Calcium ATP - DPA ¹⁴	α	1.54	1.54	116	101
	β	1.56	1.51	117	108
	γ	1.63	1.46	—	104
Magnesium ATP - DPA ¹⁴	α	1.66	1.51	117	101
	β	1.60	1.45	121	104
	γ	1.63	1.49	—	100

accommodates the sodium tripolyphosphate σ_{33} tensor values recently determined by Burlinson and co-workers (Burlinson, 1986). The longest P-O bond length for this compound is 1.670 Å which is, also, the longest P-O bond encountered in our study. The urea-phosphoric acid complex, the shift tensor of which was determined by Hertzfeld and co-workers (Hertzfeld, 1978), also lies very close to the best fit line. However for this compound, we took into account the presence of an anomalously short hydrogen bond, as determined by neutron diffraction (Sands, 1972), to one of the oxygens in σ_{11} - σ_{22} plane. Similar to protonation, such a strong hydrogen bond would necessarily influence the P-O d-p π bond (Cruickshank, 1961). Hertzfeld and co-workers (Hertzfeld, 1978) found that the orientation of the σ_{33} element for this complex was significantly different to that found for barium diethylphosphate. This difference results in the σ_{33} element lying further away from the longest bond than in other phosphates. To account for these observations, We averaged the bond length along this direction with the maximum bond length, to obtain a value of 1.534 Å, which yielded a better agreement with the σ_{33} versus maximum bond length correlation.

As it can be seen in Figure 3.3, the σ_{33} values of several phosphates deviate from the best fit line. They are the alpha and beta positions of the calcium ATP complex and all three positions of the sodium ATP complex. In contrast, the values of this tensor element for the magnesium ATP complex and sodium tripolyphosphate lie close to the line. The difference between these two groups of tripolyphosphates was somewhat perplexing at first. However, we observed that these phosphates form a separate group which was characterized by the independence of their σ_{33} tensor element on the maximum bond length. This indicated that some other factor effectively negated d-p π bonding contribution. A likely candidate would be the σ -bond angle contribution, discussed below.

Since bond length variations were not expected to be isolated along one direction, we examined the variation of the σ_{11} tensor element, that is, the upfield extreme of a powder pattern, with P-O bond lengths. As discussed above, the ability of oxygens, O1 and O2 in Figure 3.2, to contribute to d-p π -bonding is diminished when they are involved in esters bonds. This in turn increases the d-p π -bond orders in the non-ester oxygens, O3 and O4 in Figure 3.2. Therefore, the P-O3 and P-O4 bonds should be the shortest bonds. For all of

the compounds listed in Table 3.2, this is the case. The variation in bond distances reflects the degree of d-p π -bonding along these bonds which are in the σ_{11} - σ_{22} plane. Therefore, the shortest bond length of each compound was compared to its σ_{11} value. The comparison is shown in Figure 3.4. The large scatter in this correlation, we believe, is due primarily to the small range of bond lengths which nature has provided. Again because the overall trend is roughly linear, it can be concluded that the chemical shift interaction is predominately affected by the degree of d-p π -bonding.

The phosphate diesters exhibit the main exceptions to the observed correlation. On the one hand, monoesters, in particular the gamma ATP group of the calcium and magnesium complexes, lie close to the best-fit line. The gamma sodium ATP group behaves effectively as a diester because it is most likely protonated (see Chapter 2). The deviations were the largest for the beta positions of the ATP complexes. These formed a group the σ_{11} elements of which were seemingly independent of bond length. It is interesting to note that Cruickshank (Cruickshank, 1961) predicted the beta position P-O bond length of sodium tripolyphosphate based on d-p π -bond order to be about 1.45 Å, the same value predicted by us on the basis of the best-fit line in Figure 3.4. Cruickshank argued that since the bond angle of 99° for the tripolyphosphate is midway between 109.5° and 90.0°, which correspond to the unperturbed sp^3 and sp^3d^2 bond angles respectively, the σ bond contribution must be significantly different due to a difference in hybridization.

In order to test this postulate, we plotted both the σ_{33} and σ_{11} values against the appropriate bond angle (see Figure 3.1). We selected bond angles and tensor elements which were clearly related to each other either through single crystal NMR studies or by the uniqueness of the bond angle relative to the predicted tensor element orientation. For the σ_{33} case the R'O-P-OR'' bond angles (R'=P,C,H and R''=P,C,H) were used. In cases where single crystal NMR data were available, the bond angle formed by O-P-O roughly orthogonal to the plane formed by σ_{22} and σ_{11} was used. Figure 3.5 relates the magnitude of σ_{33} to the appropriate bond angles. A reasonably linear behavior was observed. The σ_{33} values of the three alpha ATP complexes were found to be close to the best-fit line, while the beta positions of the sodium and calcium complexes, as well as the beta sodium tripolyphosphates, seems to be relatively

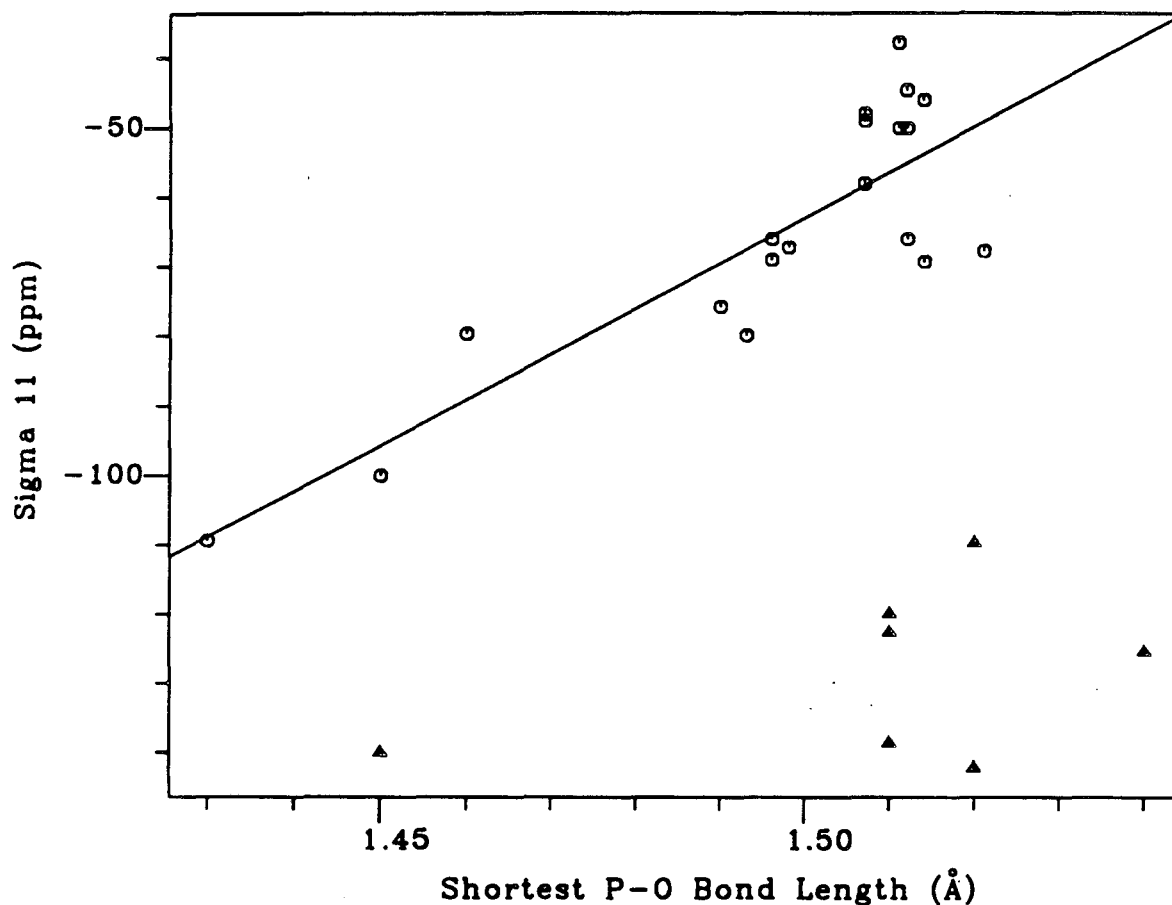


Figure 3.4. σ_{11} plotted as a function of the shortest P-O bond length. The Δ symbols represent barium diethylphosphate, dilaurylphosphatidylethanolamine, and the beta position of the sodium tripolyphosphate. The $+$ symbols represent the alpha positions, the $\times$ symbols the beta positions and the $\diamond$ the gamma positions of the ATP complexes. The $\circ$ symbols represent the remaining compounds listed in Table 1. The best-fit line was determined using only $\circ$ points. The equation for that line is $\sigma_{11} = 1260\text{\AA}^{-1}\text{ppm} \cdot r - 1950\text{ppm}$ where r is the bondlength.

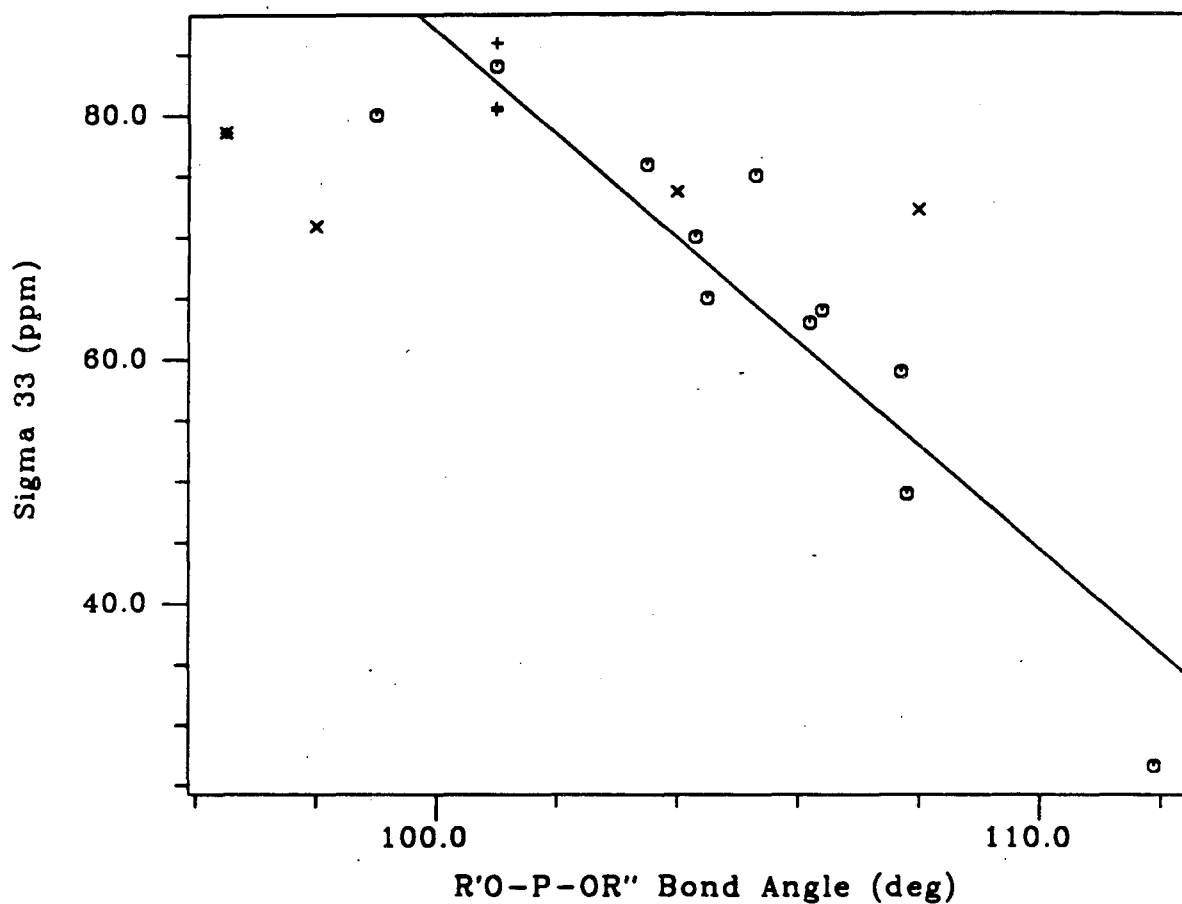


Figure 3.5. σ_{33} plotted as a function of the R'OPOR'' bond angle. The + symbols represent the alpha position and the x symbol represent the beta positions at the ATP complexes. The star symbol * represents the average of the POPOH angle of the γ position of the NaATP complex. The o are the remaining listed in Table 2. The best-fit line was determined using only the o points and the equation for this line is $\sigma_{33} = -4.24\text{deg}^{-1}\text{ppm}\Theta + 511\text{ppm}$ where Θ is the bond angle.

independent of bond angle. In these cases, the π contribution appears to be negated by the σ -bond angle contribution in the same sense as in Eq. 3.1 where the two effects oppose each other. Continuing this line of reasoning, we believe that the alpha resonances are dominated by the σ -bond angle effect. These two classes are unique in their bonding. One class consist of diesters of the type CO-P-OP and the other PO-P-OP. The effect we see may in part be due to some properties of the R' P-OP bond for which we have not accounted. Finally, we correlated the σ_{11} elements to the bond angles formed by the two non-ester bonds, a set of angles orthogonal to those just discussed in the case of the σ_{33} relationship. This is shown in Figure 3.6 . A linear behavior was again observed, albeit a very weak one. We see, as in the bond length correlation (Figure 3.4), that the alpha and beta ATP values form separate unrelated groups. As in the σ_{33} -ester bond angle correlation, we propose that this is due to the mutual cancellation of the π -bonding and σ -bond angle effects.

We have conspicuously not mentioned two subjects in this discussion. The first is the σ_{22} element and the other is the role of the counterion, a topic which we introduced in the context of the ATP complexes. The σ_{22} element does not correlate well with any bond lengths. However, σ_{22} seems to be sensitive to the formal charge of the phosphate group. From Table 3.1, it can be seen that the diesters and the doubly protonated simple phosphates form a group significantly downfield shifted in the region 1 to 25 ppm. The monoesters fall in a region from -12 to -40 ppm. We believe that the effect of counterions must be relatively small since the rationale given for the above correlations are to first order independent of their presence. The counterion effect has recently been addressed by Turner and co-workers (Turner, 1986) who found that the isotropic chemical shift is dependent on the electronegativity of the the cation. This effect is reflected in the data shown in Table 3.1. Comparing the calcium to the sodium pyrophosphate, we observe that all three elements of the tensor shift upfield. This is consistent with a greater degree of electron-withdraw by the divalent cation and with the electronegativity term of Eq. 3.1. The counterion would enhance the electronegativity of the oxygen to which it is bound. An alternative possibility is that the counterion imposes small changes in σ -bonding of the phosphate moiety. This effect is implied in the above discussion and may explain the variations of the alpha and beta position in the three ATP complexes. In general, a small counterion effect

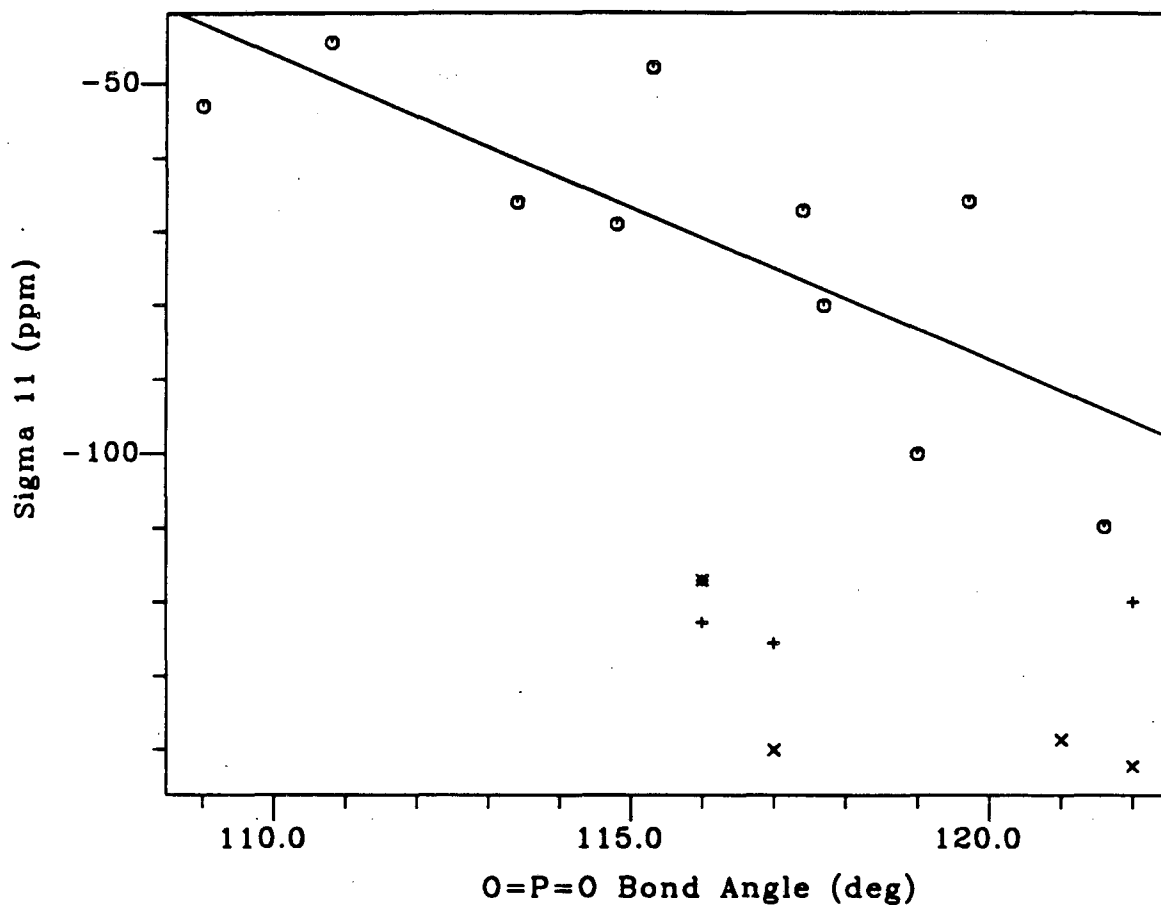


Figure 3.6. σ_{11} plotted as a function of the O=P=O bond angle. The + symbols represent the alpha position and the x represent the beta positions at the ATP complexes. The * symbol represents the β position of the sodium tripolyphosphate. The o are the remaining compounds listed Table 2. The best-fit line was determined using only the o points. The equation for this line is $\sigma_{11} = -4.11\text{deg}^{-1}\text{ppm}\Theta + 406\text{ppm}$ where Θ is the bond angle

may explain the scatter from the best-fit lines observed in all of the correlations discussed above.

V. Conclusion

We have studied a wide variety of phosphate bearing compounds in the solid phase using ^{31}P -magic angle sample spinning. Although the dataset is limited due to naturally small variation in structure, there seems to be a good correlation between bond length and the two discontinuities which characterize the extrema of the chemical shift powder pattern, most significantly the linear relationship between the σ_{33} element and the maximum bond length, and the σ_{11} element and the minimum bond length. We have found that the related bond angles also correlate to the tensor elements. The bond length relationships reflect the perturbing influences of protonation or esterification on the d-p π bonding between the phosphorous and oxygen atoms which result in the total spread of the powder pattern increasing with formation of bonds to the phosphate oxygens. These observations are consistent with those of Cruickshank, who related bond distance to π bonding order, and are in qualitative agreement with isotropic theory of Van Wazer and Lechter. They also indicate that Eq. 3.1, which is linearly dependent on π bond order, electronegativity and σ bond, is valid for the σ_{33} and σ_{11} elements of phosphates and phosphate esters. The σ_{22} component does not correlate well with any structural variations. However, it seems to be sensitive to the overall charge of the phosphate group. For the molecules that we have studied, we conclude that the major contribution to the chemical shift tensor interaction arises from the perturbation of the d-p π -bonding.

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Chapter 4

A Study of Rat Tissue using Magic Angle Sample Spinning

Chapter 4. A Study of Rat Tissue using Magic Angle Sample Spinning

I. Introduction

It has long been recognized that *in vivo* ^{31}P NMR spectra are incomplete. Evans (Evans, 1979) noted that only 25 to 30% of the total cell phosphorus is observed. This portion was found to correspond to the acid-soluble fraction of the total phosphorus pool. The remaining fraction of up to 60% was distributed among relatively large macromolecules, such as DNA, RNA, and phospholipids. Table 4.1 is a compilation of the cell phosphorus distribution in three types of tissue from rats. The majority of the phosphorus bearing molecules in these tissues, with the exception of skeletal muscle, are large and their rotational correlation times are expected to be long. This would render these molecules unobservable under normal *in vivo* NMR conditions. In essence, the rotational correlation time acts as a natural filter for ^{31}P NMR spectra. The small acid-soluble phosphates, such as ATP, play an important role in cellular metabolism and their observability, both in terms of resolution and intensity, without interference from the dominant population of phosphorus containing macromolecules is one of the reasons why *in vivo* ^{31}P NMR spectroscopy has been so successfully exploited. In contrast to this situation, ^1H NMR has suffered from the fact that the spectra of complex biological systems are often obscured by a large water resonance and are dominated by a multiplicity of large nuclear dipole-dipole interactions which yield broad featureless lines. In both cases, the barrier to obtaining complete and meaningful spectra is the presence of large inhomogeneous broadening interactions.

In the case of ^{31}P NMR, chemical shift anisotropy and heteronuclear phosphorus-proton dipolar interactions dominate the spectrum while for the ^1H case, the homonuclear dipolar interaction is the leading inhomogeneous broadening mechanism. Interactions with paramagnetic metals probably also contribute some role in line broadening (Cohen, 1962). Although, this latter mechanism is not readily manipulable, chemical shift and dipolar interactions are. A straightforward method for achieving some control over these interactions is magic angle sample spinning (MASS). A resonance broadened by an anisotropic inhomogeneous interaction is replaced by a much narrower set of signals, one at the isotropic value of that interaction along with a series of spinning sidebands positioned at integer multiples of the spinning frequency centered about the isotropic frequency (see Chapter 1). At spinning speeds comparable to

Table 4.1. The chemical shifts and relative quantities of various ^{31}P bearing compounds in three rat organs. Quantities are given as percent of total phosphorus and chemical shifts are in ppm from 85% phosphoric acid.

COMPOUND	Chemical shift	Muscle ⁵	Kidney	Liver	References
Nucleic Acid P		7	22	25	1,2
DNA	0.3	2	6	5	1,2,3
RNA		3	14	20	1,2,3
Lipid P		13	39	37	1,2,3
Phosphatidyl choline	~-0.7	8	13	19	4
Phosphatidyl ethanolamine	~-0.5	3	11	9	4
Others		2	15	10	4
Acid soluble P		68	28	28	1,2
α ATP+NAD(H)	-10.5	17	8	8	6
β ATP	-18.5	9	4	4	6
γ ATP	- 4.8	9	4	4	6
phosphocreatine	- 3.0	12	+	1	6
phosphodiester	0.0	+	4	1	6
inorganic phosphate	2.3	11	2	4	6
sugar phosphate	3.7,4.7	10	5	6	6
Protein P		11	10	10	1,2

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- 5.) acid soluble data for this category was taken from heart experiments reported in reference 6.
- 6.) Koretsky A. (1985), Doctoral Thesis, University of California, Berkeley.

the strength of the interaction, the intensities of these sidebands contain complete information about the magnitude of the anisotropy of the interaction (Maricq,1979). In order for MASS to have significant averaging effects, the spinning frequency must be comparable to or larger than the broadening interaction. For the phosphorus bearing molecules of biological interest, this condition is easily met and has been demonstrated in numerous studies (for example see Haberkorn, 1978). In the proton case, at reasonable spinning rates, MASS would have little effect in averaging the large homonuclear dipolar interactions, unless these interactions were in part motionally averaged at the molecular level.

In the following sections, we shall report on the application of both the ^1H and ^{31}P MASS NMR techniques to the study of lyophilized rat tissues: thoracic skeletal muscle, liver and kidney. Cholli and co-workers (Cholli,1986) have studied frog oocytes using ^{31}P MASS and have demonstrated that this technique can be applied to studying biological systems. However, we felt that this technique could be improved significantly by combining the cross polarization technique with sample rotation as has been done by us (see previous chapters) and others (Hertzfeld,1978; Rothwell,1980) on simpler systems. In addition, as demonstrated in earlier chapters, we have found that the principal values of the chemical shift tensor of a particular ^{31}P nucleus can be used to limit the possible identity of the molecule to which the phosphorus atom is bound. The sideband analysis technique described by Hertzfeld and Berger (Hertzfeld and Berger, 1980) provided a means of unambiguously determining the chemical shift tensor elements for each of the observed species. The usefulness of this method has been demonstrated in the previous chapters and several other studies (Hertzfeld and Berger, 1980, and Burlinson,1986). These studies also provided a source of data for model compounds used in this present work.

Recently, Bloom and co-workers (Bloom,1986) have been able to separate and characterize the various components of the proton spectra of phospholipid bilayer model membranes and rat mammary adenocarcinoma cells. They elicited the narrow components by application of the CPMG pulse sequence and the dipolar broadened components by the a Jeener-Broekaert echo sequence (Jeener, 1967). An alternative method for studying the narrow and partially narrowed proton resonances would be MASS. The spinning rate would effectively discriminate between

these and the very broad resonances which would remain essentially unaffected (Waugh, 1979).

By applying magic angle sample spinning and subsequent analysis, we have observed the "complete" ^{31}P NMR spectrum or, more correctly, a much greater percentage of the cell phosphorus than detected in *in vivo* experiments. Furthermore, from the proton MASS spectra and ^{31}P spectra, we have deduced information about the mobility and the state of hydration of the various proton and phosphorus pools.

II. Experimental

A. Instrumentation and tissue preparation

All spectra were obtained on a home-built 270 MHz spectrometer equipped with a Nicolet 1180 computer and Doty MASS probe. Most samples were spun at rates of 1 kHz to 5 kHz. The nature of the samples seemed to facilitate both packing and spinning. Proton spectra were obtained by the normal one pulse experiment. Proton pre-saturation experiments will be described elsewhere (see Appendix A). The phosphorus experiments were obtained with both proton decoupled one pulse method and the cross polarization (CP) method under Hartmann-Hahn matched and mismatched conditions. The effect of decoupling on linewidth was tested and indicated that a minimum could be readily reached.

The rat tissues were excised while the animal was under anesthesia and the tissue samples were immediately immersed in liquid nitrogen to preserve as many high energy metabolites as possible. The tissue was stored under liquid nitrogen until further processing. The samples were ground under liquid nitrogen and then lyophilized for three to four days to a constant weight. The appearance of the proton spectra depended slightly on exposure to moisture, but these changes were reversible on further drying.

B. Data Analysis

In order to obtain accurate intensities from the MASS tissue spectra from which chemical shift parameters could be extracted, it was necessary to fit each of the resonances, as well as their corresponding sidebands, to a set of Gaussian-Lorentzian lines. This was accomplished using the interactive program NTCCAP (Nicolet, 1978). Every attempt was made to keep the number of lines used to a minimum. The fact that sidebands are essentially duplicates of the centerband spaced at the rotor frequency (see Chapter 1) was used to constrain the number

and position of the lines used in the fitting. For each tissue type, the fitting procedure was repeated at least twice, at two different spinning speeds. In addition, at least two different samples (from different rats) were used. The intensities obtained in this way were expressed as ratios, I_n/I_m , where in general I_m was the most intense sideband. These ratios were compared to those calculated by Hertzfeld and Berger (Hertzfeld and Berger, 1980). At the heart of the program used in this analysis was the IMSL routine ZSSQ, a non-linear minimization subroutine based on the Levenberg-Marquardt algorithm which was used to minimize the sum of squares the differences between the experimental and calculated intensity ratios. The minimization procedure was essentially a non-linear two parameter fit of the variables, μ and ρ , defined by Hertzfeld and Berger (Hertzfeld and Berger, 1980 and Chapter 1 and Appendix C of this thesis) as

$$\mu = \frac{\gamma B_0 (\sigma_{33} - \sigma_{11})}{\omega_r}$$

and

$$\rho = \frac{(\sigma_{11} + \sigma_{33} - 2\sigma_{22})}{(\sigma_{33} - \sigma_{11})}.$$

The isotropic chemical shift values for each of the observed species were obtained directly from the fits of the spectra. The principal elements of the chemical shift tensor of a particular ^{31}P resonance were calculated from the values of μ , ρ and the isotropic chemical shift. The principal elements of the chemical shift tensor reported here are averages of the various trials. For some resonances, no consistent set of chemical shift parameters could be obtained and are not reported for this reason (see below).

The general procedure, outlined above, was tested on well resolved lines for which the tensor values were previously measured and the results obtained were consistent with reported values (see previous chapter and chapter 1). Further verification was obtained in two ways. The chemical shift tensor elements obtained from the analysis were used to calculate the powder spectra from standard formulae (see Chapter 1). The powder spectrum for each constituent were scaled, added together and then compared to experimental powder spectra. The intensity of each pattern was adjusted until the best fit was obtained. Further discussion of these comparisons is given in the next section. The second method involved the calculation of time domain

response (*i.e.* the free induction decay or FID) corresponding to the MASS spectrum. Again, these calculations were based on the chemical shift parameters obtained using the Hertzfeld and Berger method. The FID's of each component were individually scaled to reflect their relative contributions, added and the sum Fourier transformed. This method had the advantage that the relative intensities of the resonances had physical meaning (see Appendix E). In all cases where such quantum mechanical calculations were carried out good agreement between the calculated and experimental spectra were obtained (see below). This was considered sufficient proof that the principal chemical shift tensor values were accurate. To reiterate, it was possible to calculate either the powder or the MASS spectrum with good agreement with experimental results using the chemical shift parameters obtained by first fitting the experimental data and then analyzing the resulting intensities using the method of Hertzfeld and Berger.

III. Results

A. ^1H MASS NMR spectra of lyophilized tissue

The ^1H NMR spectra of lyophilized tissue samples of rat skeletal muscle, liver and kidney consisted of a broad featureless resonance with a linewidth of about 1 kHz. The lineshape was characteristic of a super-lorentzian (Bloom, 1977). Figure 4.1 is a proton spectrum of rat muscle. An inversion recovery experiment carried out on a sample of muscle tissue indicated that several overlapping resonances could be discriminated based on longitudinal relaxation. Two time points of the relaxation data set are shown in Figure 4.2. The T_1 of the major component was 0.42 s at 25°C. This major component had a linewidth at half height of 230 Hz. When the samples were spun at the magic angle further narrowing was achieved. The typical linewidth of the major components was about 40 Hz. Figures 4.3, 4.4 and 4.5 are the ^1H MASS NMR spectra of the lyophilized rat muscle, kidney and liver. Spectral resolution was approximately constant for samples of the same tissue type. In general, the highest resolution was observed in muscle tissue. MASS inversion recovery experiments on the kidney and muscle samples indicated overlapping lines with individual linewidths of roughly 12 Hz. This is shown in Figure 4.6. MASS spin lattice relaxation data for several of the MASS resonances are summarized in Table 4.2. The proton chemical shift assignments implicit in Table 4.2 are discussed in the following section. In all kidney and liver samples, a very broad resonance was

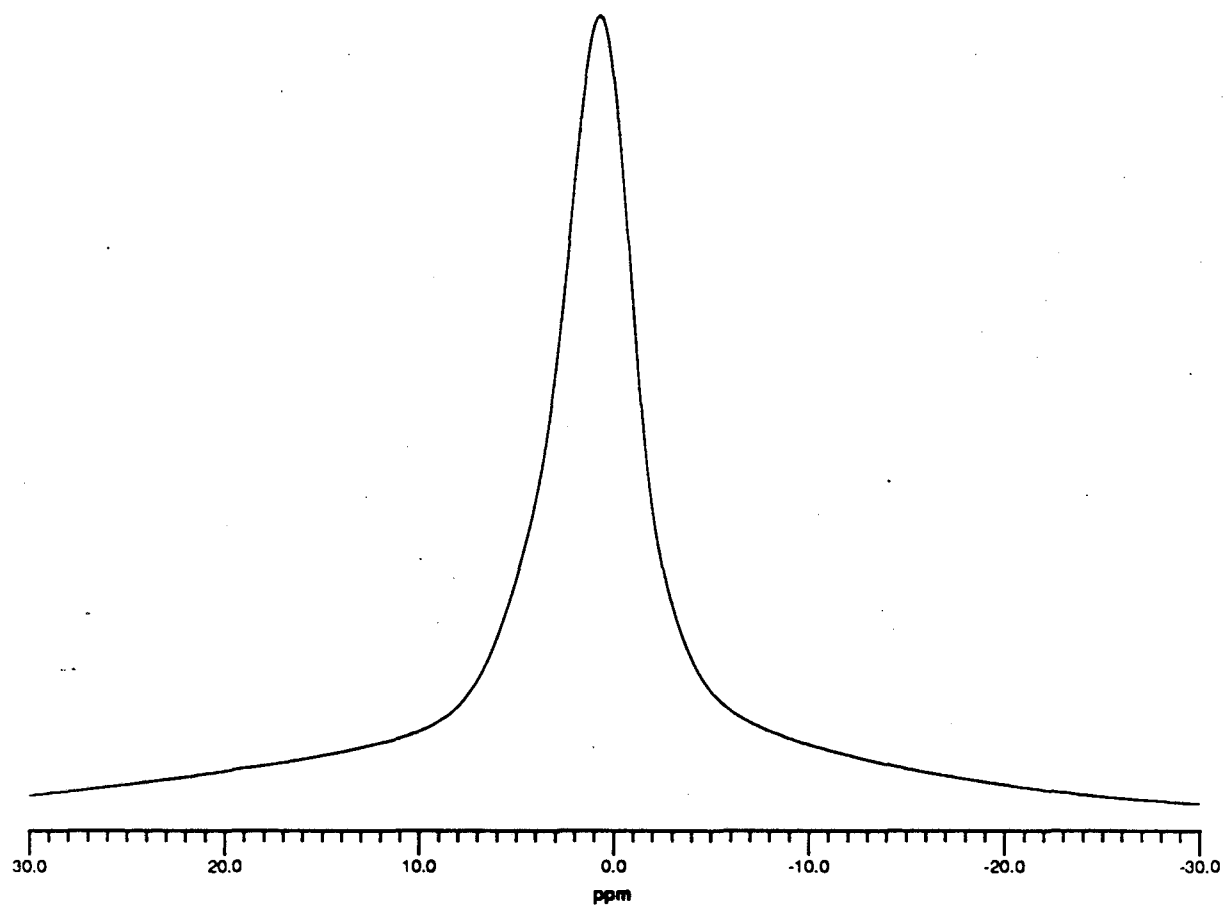


Figure 4.1. The static ^1H spectrum of lyophilized rat skeletal muscle. The line is characteristic of a super-lorentzian.

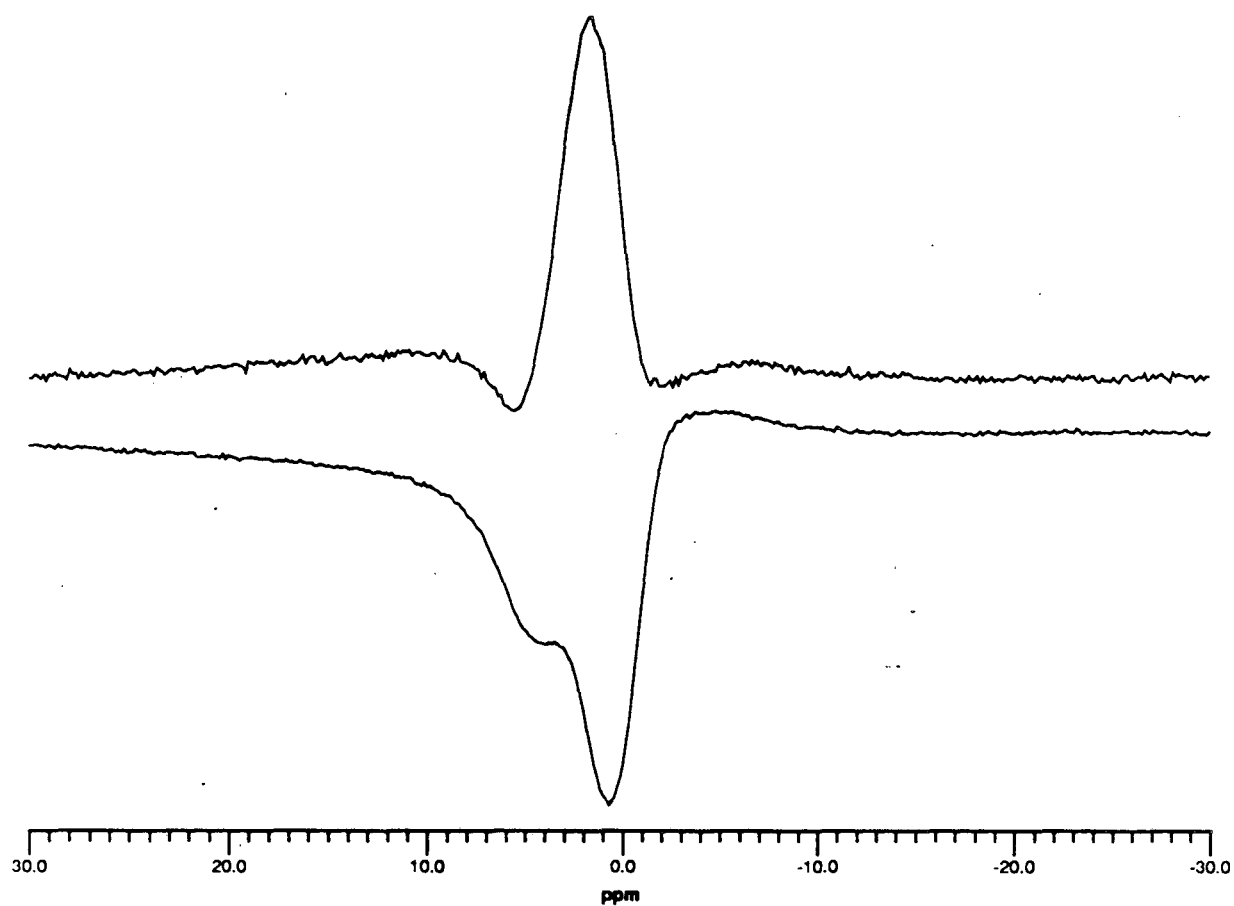


Figure 4.2. Two partially relaxed static ^1H spectrum of lypophilized rat skeletal muscle. The spectra were taken as part of an inversion–recovery spin lattice relaxation (T_1) experiment. The lower spectrum was taken 250 ms after the inverting pulse and the upper after 300 ms. Several components are clearly visible.

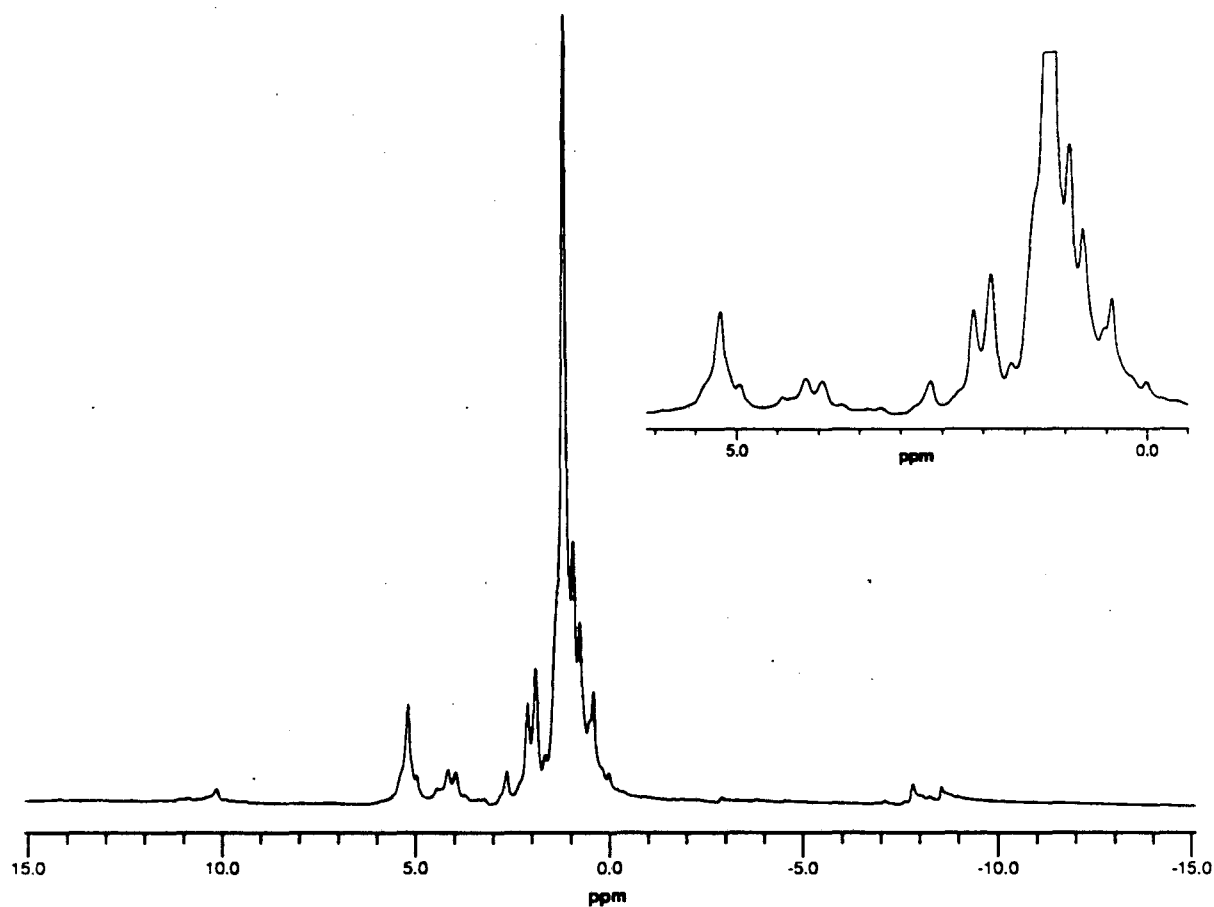


Figure 4.3. The ^1H MASS spectrum of lyophilized rat muscle. The sample was spun at 2.5 kHz. The inset is an expansion of the isotropic region.

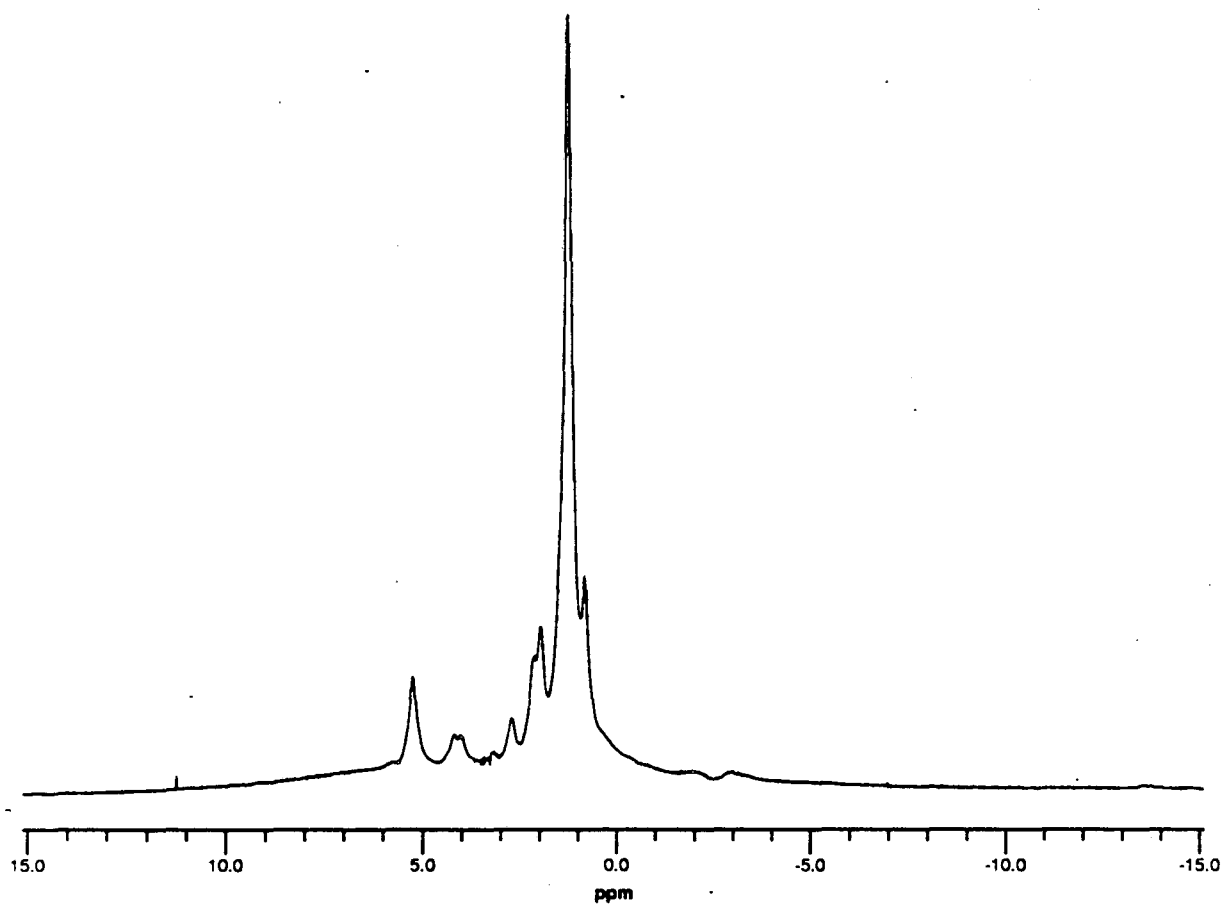


Figure 4.4. The ^1H MASS spectrum of lyophilized rat kidney. The sample was spun at 5.3 kHz. The spinning sidebands are beyond the plotted region.

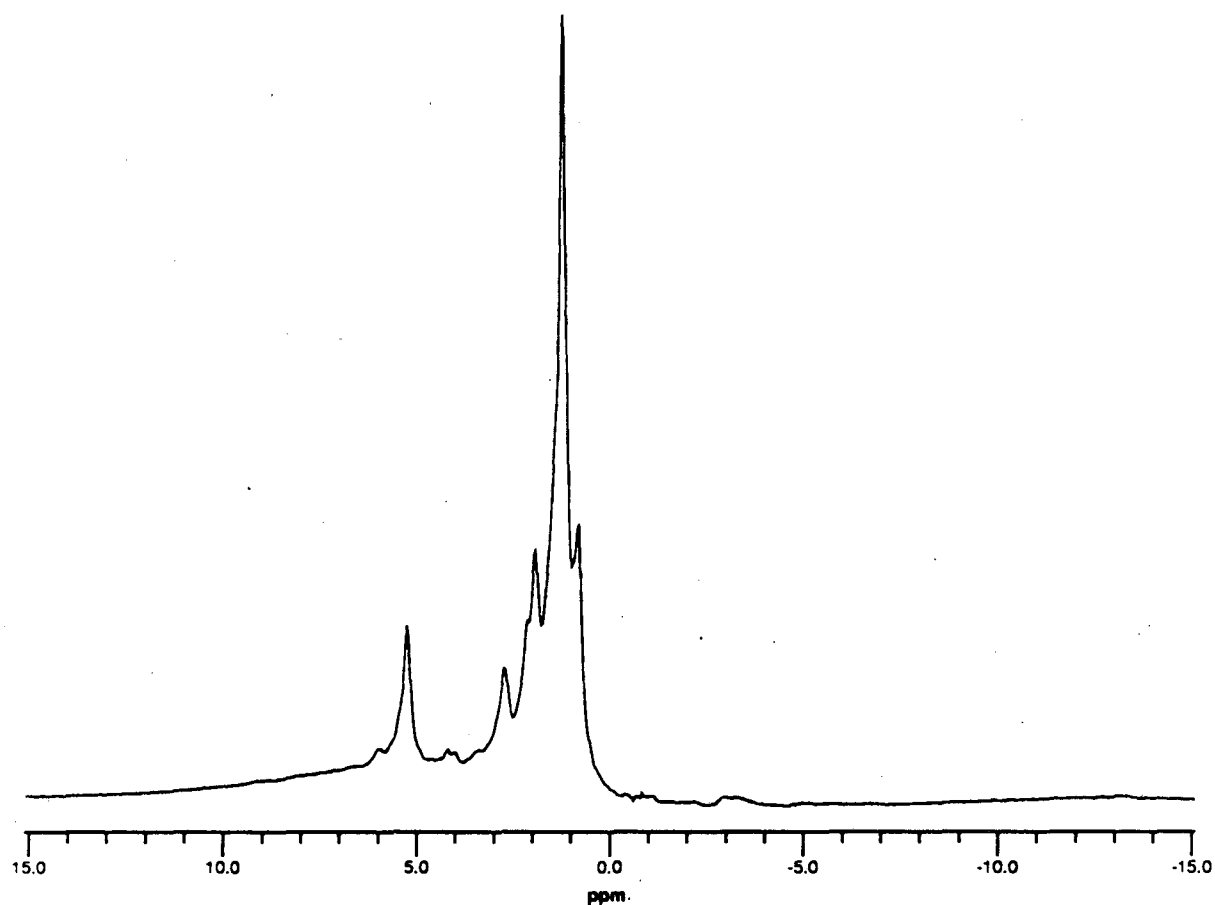


Figure 4.5. The ^1H MASS spectrum of lyophilized rat liver. The sample was spun at 4.9 kHz. The spinning sidebands are beyond the plotted region.

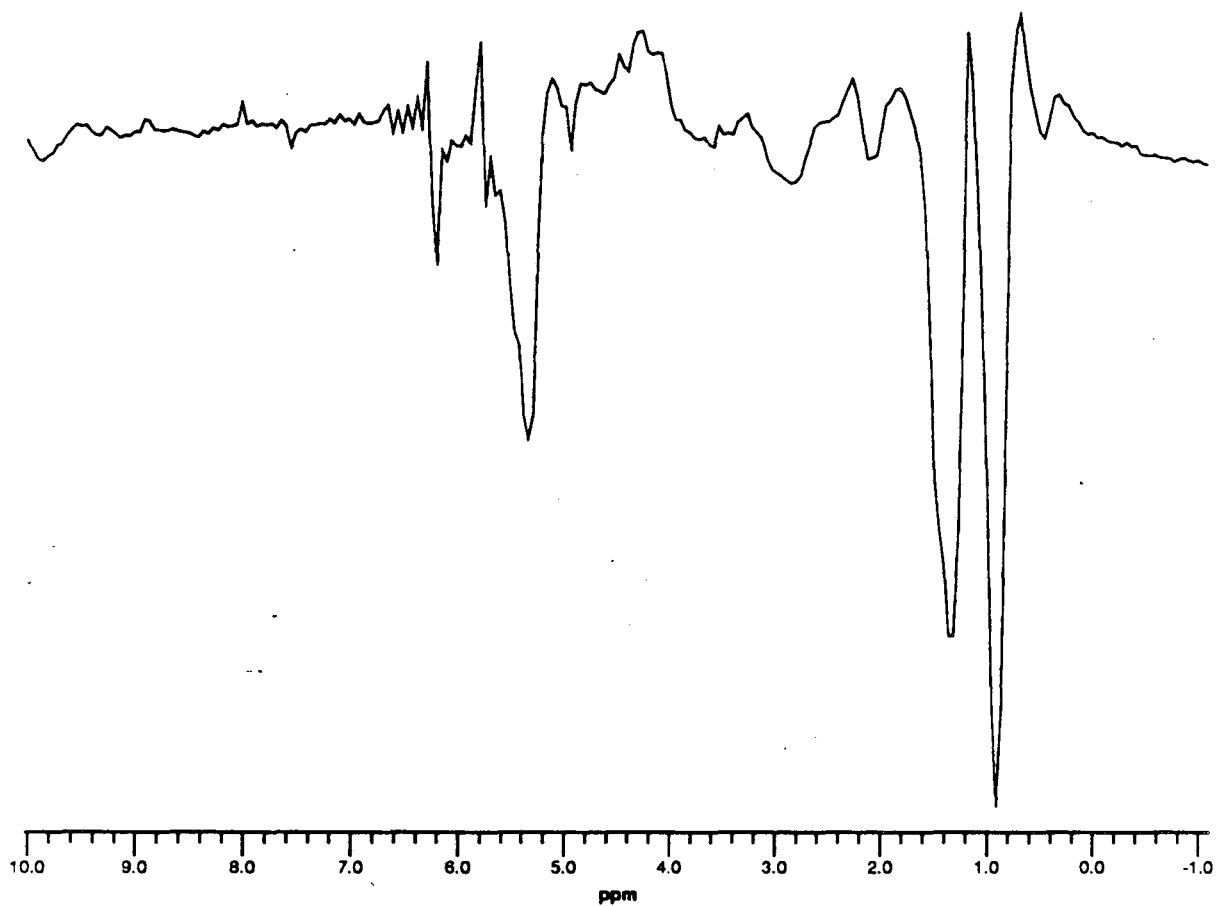


Figure 4.6. The ^1H spectrum of lyophilized rat kidney. The spectrum is a result of an inversion recovery experiment where the time between the inverting and 90° pulse was 300 ms. The linewidths in this spectrum are about 12 Hz (compare with Fig. 4).

Table 4.2. The spin lattice relaxation times for several types of protons in rat muscle and kidney. Times are given in seconds. Measurements made at 22°C using the inversion recovery method while magic angle spinning.

Proton Type	Egg lecithin ¹	Muscle ²	Kidney ³
-CH ₂ -	0.47	0.44	0.46
H-C _{,p} ²	0.54	0.68	0.50
-CH ₂ -C _{,p} ²	0.41	0.40	0.41
-CH ₂ -CO ₂	0.34	0.31	0.31

1. Horwitz, A. L., *et al* (1972) *Proc. Nat. Sci.*, 69, 590-593. Results were determined at 40°C
2. Based on zero crossing measurements.
3. Based on full inversion recovery datasets

observed and the spectral resolution in these tissues was not as high as in the muscle samples. We found that the broad component could be effectively removed by saturation of the ± 2 order sidebands. The results of these experiments are reported elsewhere (see Appendix A). The ^1H MASS spectra of the three tissues exhibited several common features. All three were dominated by a sharp resonance at about 1.2 ppm. This chemical shift corresponded to the center of the broad 1 kHz feature observed in static samples. The next most intense features were a pair of lines at about 2.0 ppm, followed by another pair at 4.0 ppm. A common downfield feature at about 5.2 ppm was also observed.

Two variable temperature studies were undertaken on the muscle samples. The results of the variable temperature T_1 studies on static muscle samples are summarized in the Arrhenius plot shown in Figure 4.7. A minimum in the spin lattice relaxation was observed at 0°C. This corresponded to a rotational correlation time of roughly 0.5 ns (Seelig, 1981). The proton MASS muscle spectrum as a function of temperature is shown in Figure 4.8. The linewidth data are summarized in Figure 4.9. All of the resonances broaden with decrease in temperature. Although the magnetic field was shimmed at each temperature, without a linewidth standard it was difficult to assess how much of the broadening was due to inhomogeneity of the field. Differential broadening was the best indication that the observed temperature effect was not entirely artifactual.

B. ^{31}P MASS NMR spectra of lyophilized tissue

For each of the tissue types, the ^{31}P static spectra exhibited two components. The relative proportions of the two were dependent on the tissue type. In the case of the liver and kidney the broader component constituted about 65% of the total area, while in muscle this component was approximately 85% (see Table 4.6 for further details). The linewidth of the narrow component was approximately 700 Hz while the broad component was about 13.0 kHz for the muscle and about 1.5 kHz for the kidney and liver. The Bloch decay spectrum of a static muscle sample with proton decoupling is shown in Figure 4.10. The proton cross polarized spectrum of the same muscle sample is shown in Figure 4.11. The cross polarized spectrum extends from about 100 ppm to -120 ppm. Unlike the Bloch decay spectrum, this spectrum is more reminiscent of the typical case of overlapping powder patterns. In either case, the spectra provided minimal

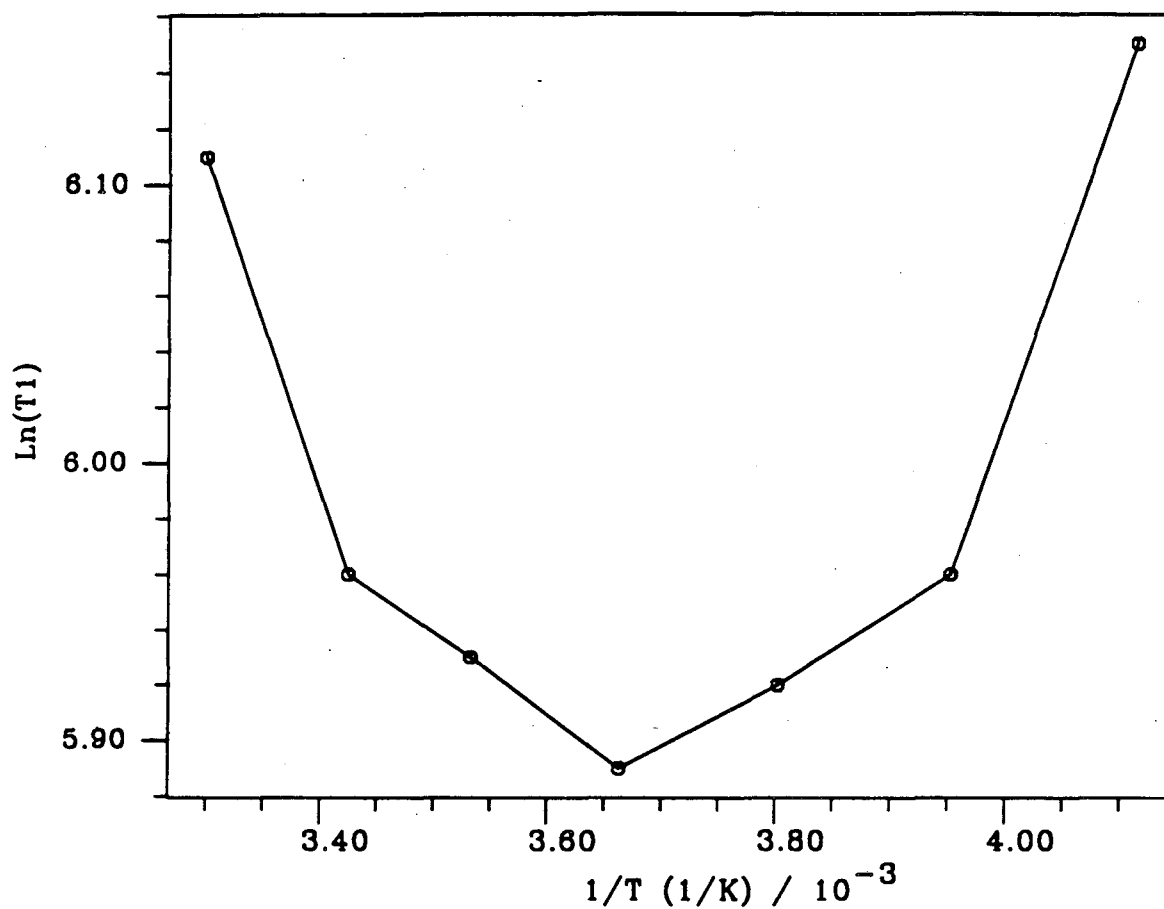


Figure 4.7. The temperature dependence of the spin lattice relaxation time (T_1) of the major component found in the static ^1H spectrum of rat skeletal muscle. The minimum corresponds to a correlation time of 0.5 ns.

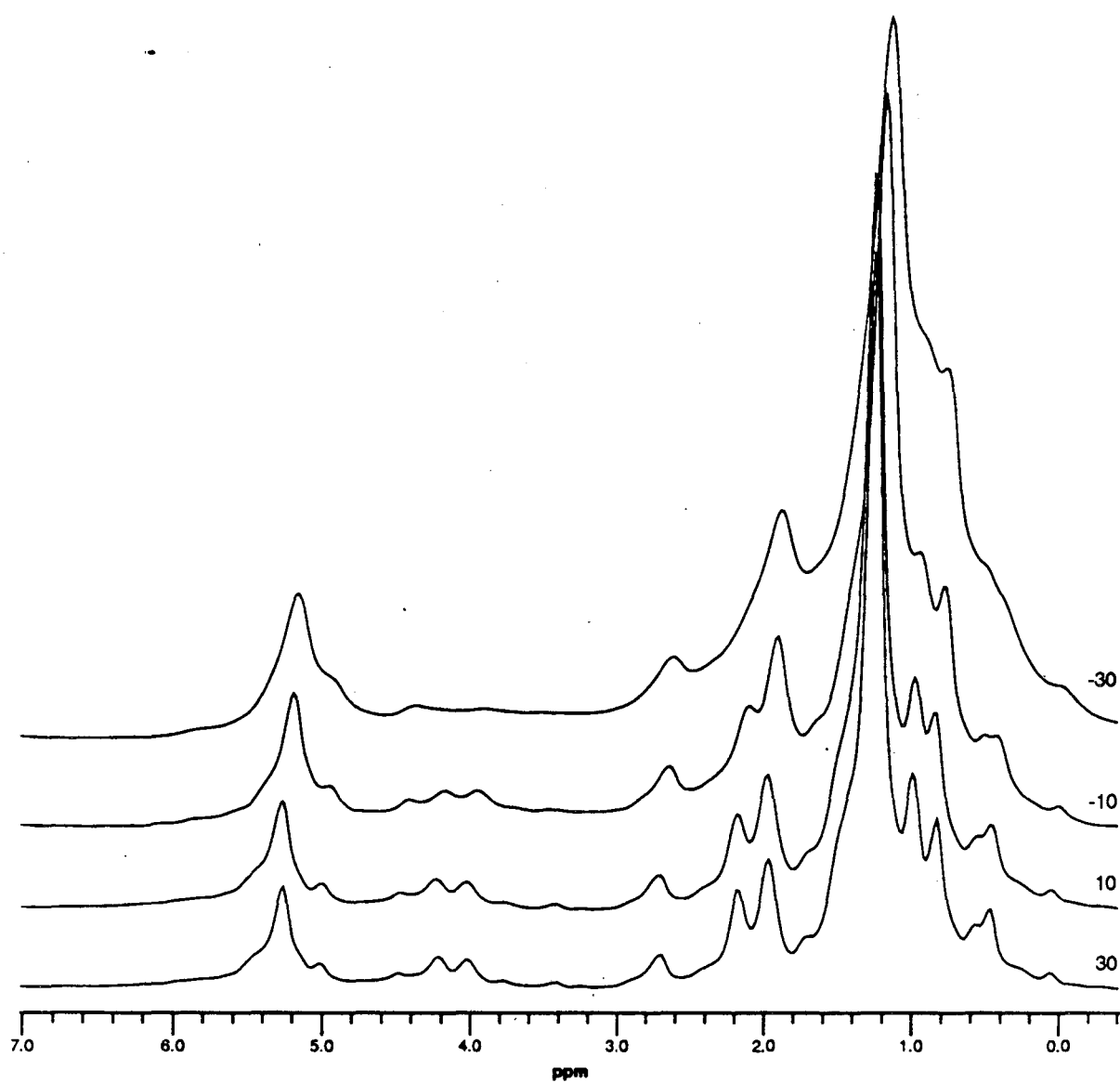


Figure 4.8. The temperature dependence of the isotropic ^1H MASS resonances in rat skeletal muscle. The linewidths of some of the resonances are summarized in Figure 9.

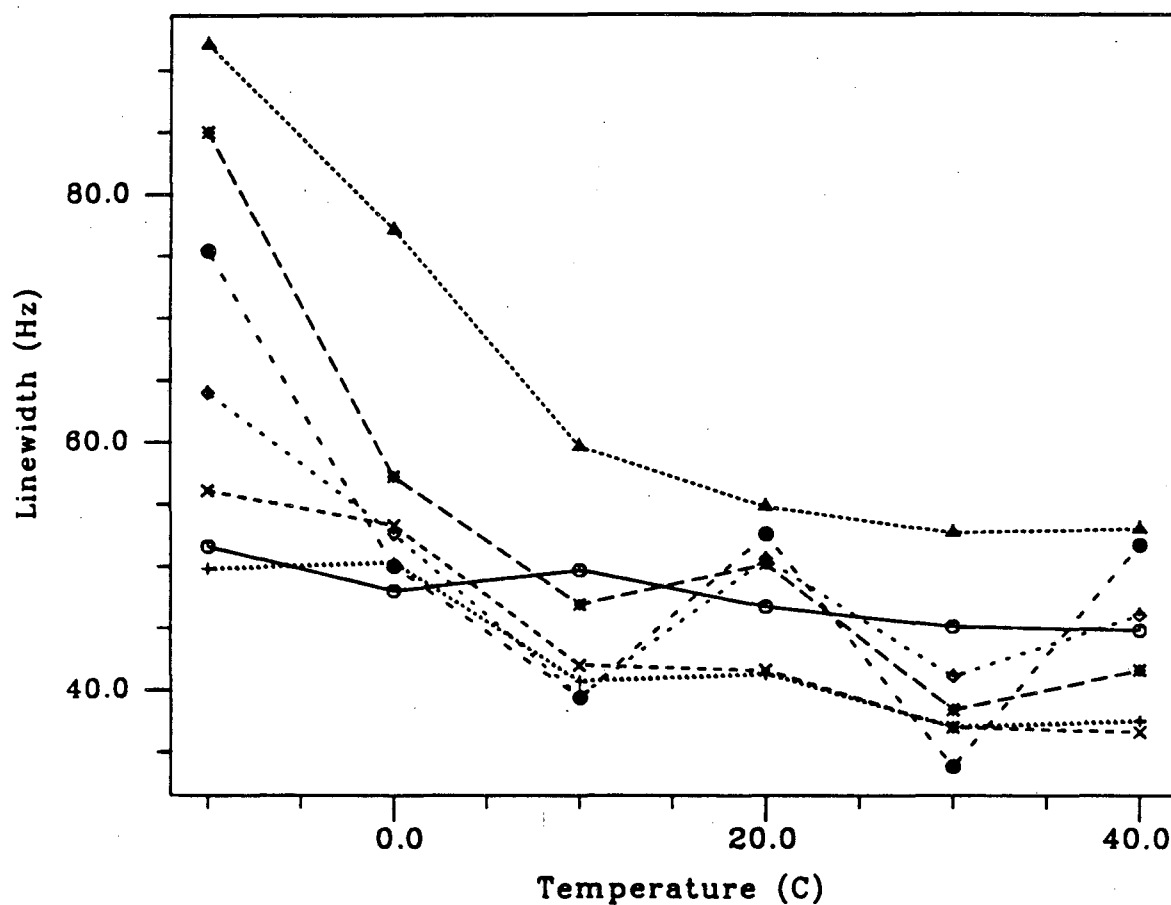


Figure 4.9. The temperature dependence of the isotropic ^1H MASS resonances in rat skeletal muscle. The $\circ$ symbols represent the resonance at 0.82 ppm, $\times$ at 1.24 ppm, $\diamond$ at 1.96 ppm, $*$ at 2.16 ppm, $\bullet$ at 2.70 ppm, Δ at 4.00 ppm and $+$ at 1.24 ppm.

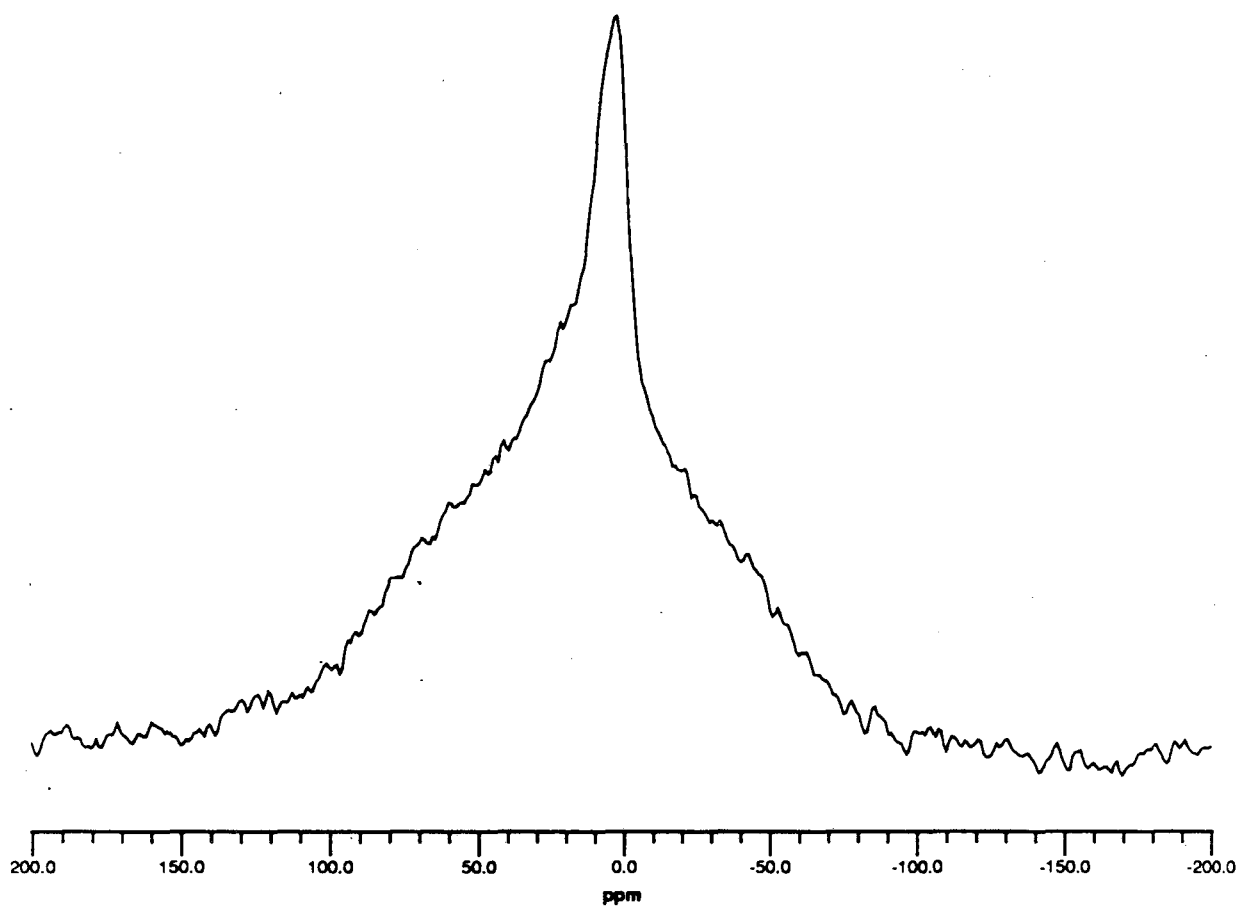


Figure 4.10. The static ^{31}P Bloch decay spectrum of lyophilized rat skeletal muscle. The spectrum was taken with proton decoupling. The the ratio of narrow to broad component is aproximately 15:85. The narrow component has a linewidth of 1.4 kHz and the broad, 12 kHz.

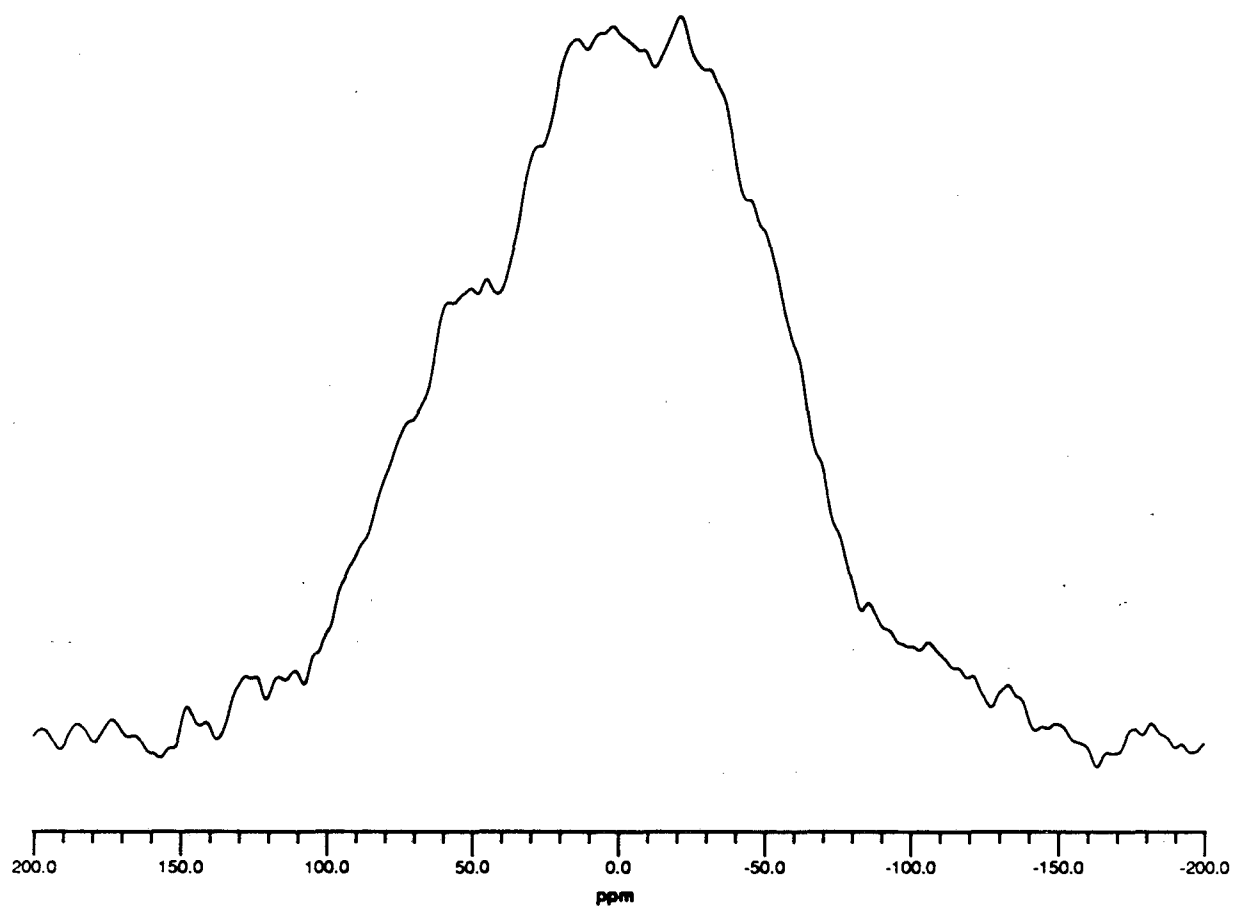


Figure 4.11. The ^{31}P cross polarized (CP) spectrum of lyophilized rat skeletal muscle. The cross polarization time was 1.5 ms under Hartmann-Hahn matched conditions.

information. Figure 4.12 is a spectrum taken under conditions identical to Figure 4.11 with magic angle sample spinning. As expected, sample rotation did afford greater resolution. However, the centerband region, 10 to -20 ppm, was dominated by the narrow component which was observed in the Bloch decay spectrum. This feature obscured the centerband region of the spectra.

Figures 4.13, 4.14 and 4.15 are ^{31}P CP-MASS spectra of rat muscle, kidney and liver. As in the case of MASS Bloch decay spectrum with proton decoupling, we were immediately struck by the relatively broad lines. Typically, the centerband and sidebands were in excess of 700 Hz. Typical linewidths of about 150 Hz were observed for inorganic samples (see Chapter 3). Despite the broadness of the spectra, several features could be clearly discerned in the CP-MASS spectra. Resonances at about -1.3 and -9.6 were observed in all three tissue types. In addition, a feature at approximately 17.0 ppm was observed in muscle and kidney samples. As can be seen in Figures 4.13 to 4.15, the resonance at -1.3 ppm clearly dominated the spectra. Furthermore, on closer examination, it was observed that this feature possessed a shoulder centered at about 3.6. This can be seen most clearly in the expansion of the isotropic region of the skeletal muscle spectrum (see Figure 4.13). These observations are summarized in Table 4.3.

C. Calculation of ^{31}P chemical shift tensor elements from sideband intensities

For each type of tissue, using the procedure outlined in a previous section, we analysed the sideband intensities to determine the chemical shift tensor elements of each component. These data are summarized in Table 4.3. Not surprisingly, we found that there was a large uncertainty in the shift tensor element determination. By this, we mean that the standard deviation for a particular shift tensor element determined from several measurements was relatively large. Typically, the uncertainties encountered for inorganic phosphates were on the order of five times smaller, about 2 to 3 ppm. Therefore, the results for the rat tissue given in Table 4.3 should be viewed with some caution. This is especially true for the 3.6 ppm component which is common to all three tissues. In the case of the kidney, we were unable to obtain a consistent set of tensor element for this component. The anisotropy (*i.e.* $\sigma_{33} - \sigma_{11}$) of this component in muscle and liver samples was 73 ppm and 99 ppm, respectively. At routine spinning speeds of 4.5 to 5.2 kHz,

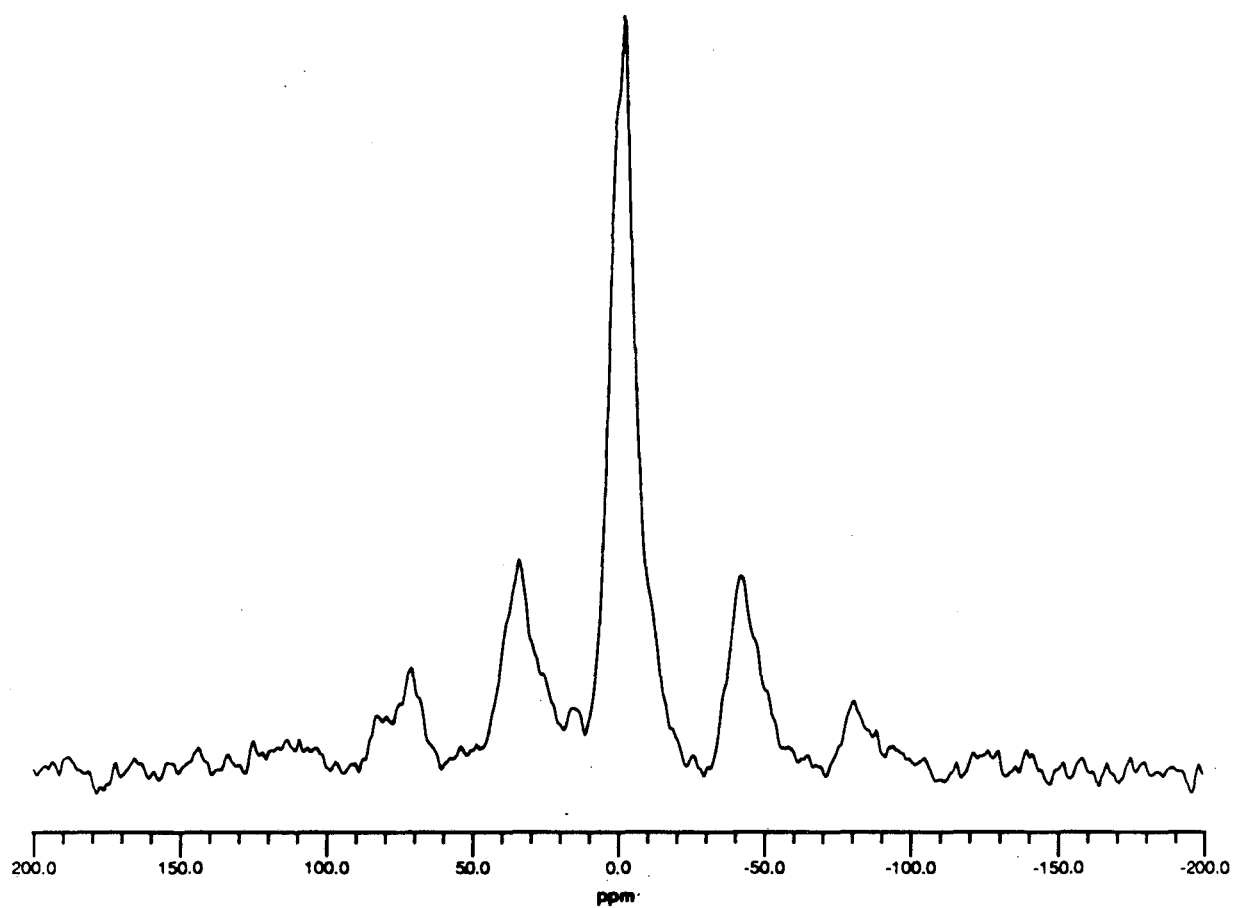


Figure 4.12. The ^{31}P MASS Bloch decay spectrum of lyophilized rat skeletal muscle. The spectrum was taken under identical conditions as Figure 10, except with sample spinning at 4.4 kHz.

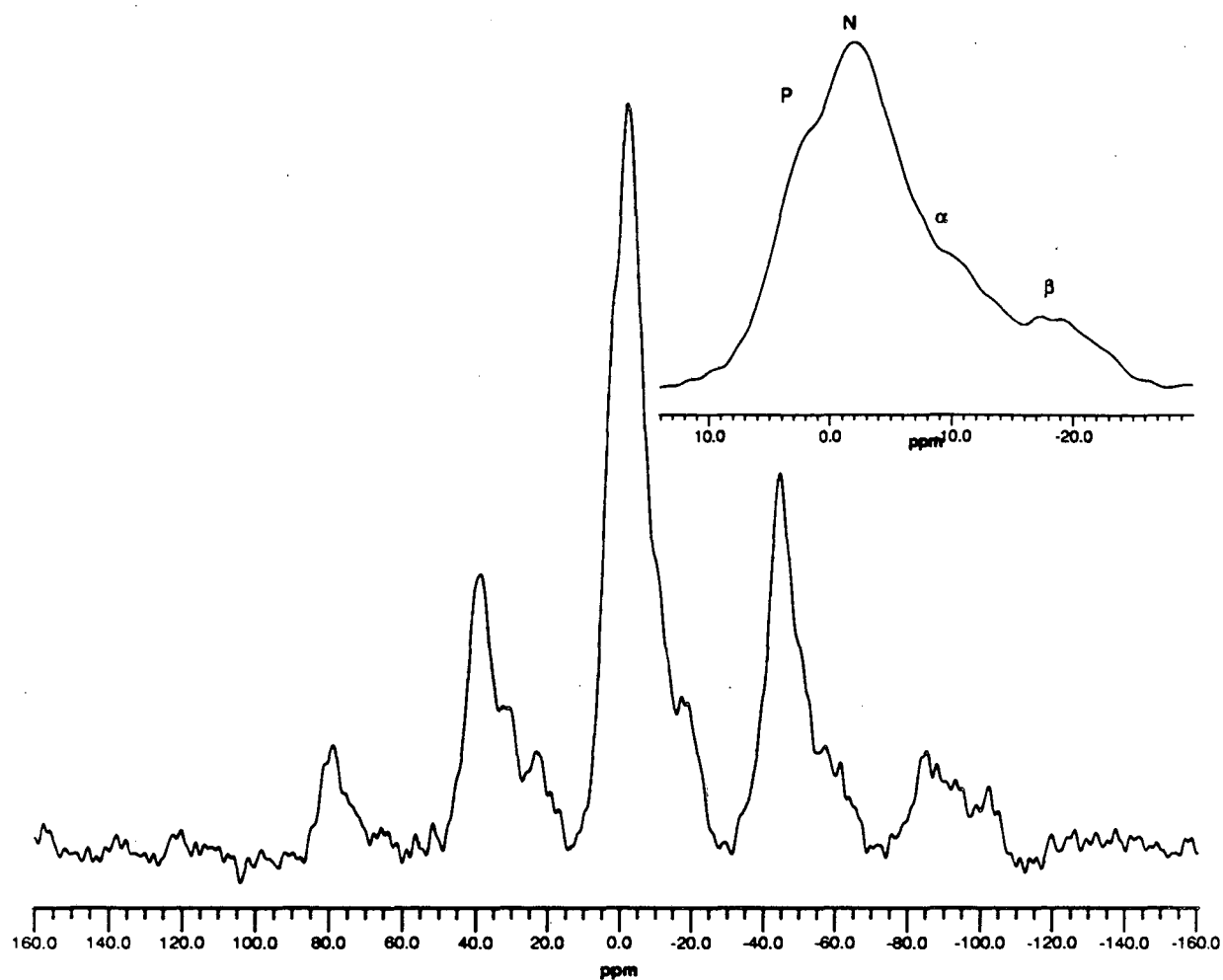


Figure 4.13. The ^{31}P CP-MASS spectrum of lyophilized rat skeletal muscle. The sample was spun at 4.5 kHz. The inset shows the isotropic region of the MASS spectrum. The phospholipid component is denoted, P, the DNA-RNA, N, and the ATP resonances as α , which includes NAD and β . The spectrum was obtained under Hartmann-Hahn matched conditions with a cross polarization time 2 ms.

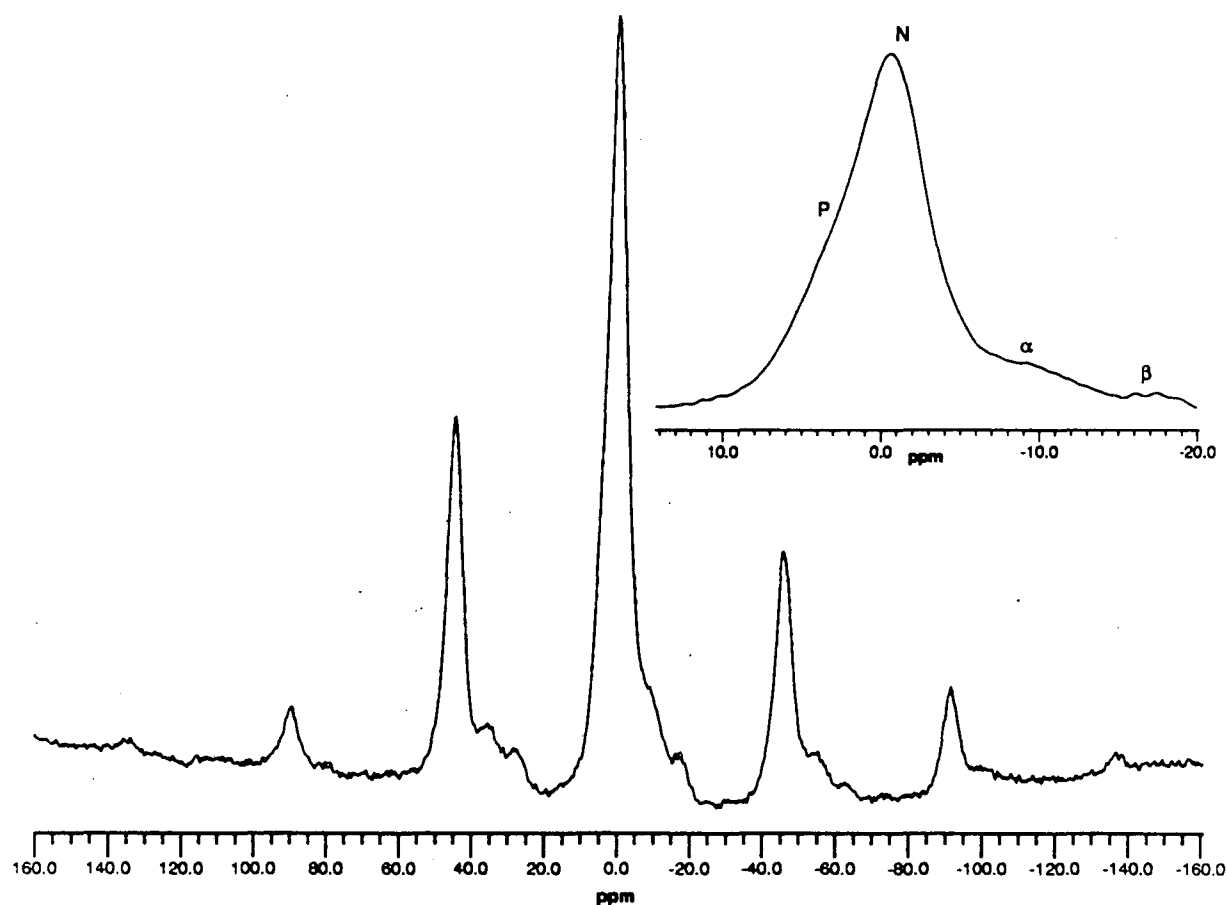


Figure 4.14. The ^{31}P CP-MASS spectrum of lyophilized rat kidney. The sample was spun at 4.9 kHz. The inset shows the isotropic region of the MASS spectrum. The phospholipid component is denoted, P, the DNA-RNA, N, and the ATP resonances as α , which includes NAD, and β . The spectrum was obtained under Hartmann-Hahn matched conditions with a cross polarization time 2 ms.

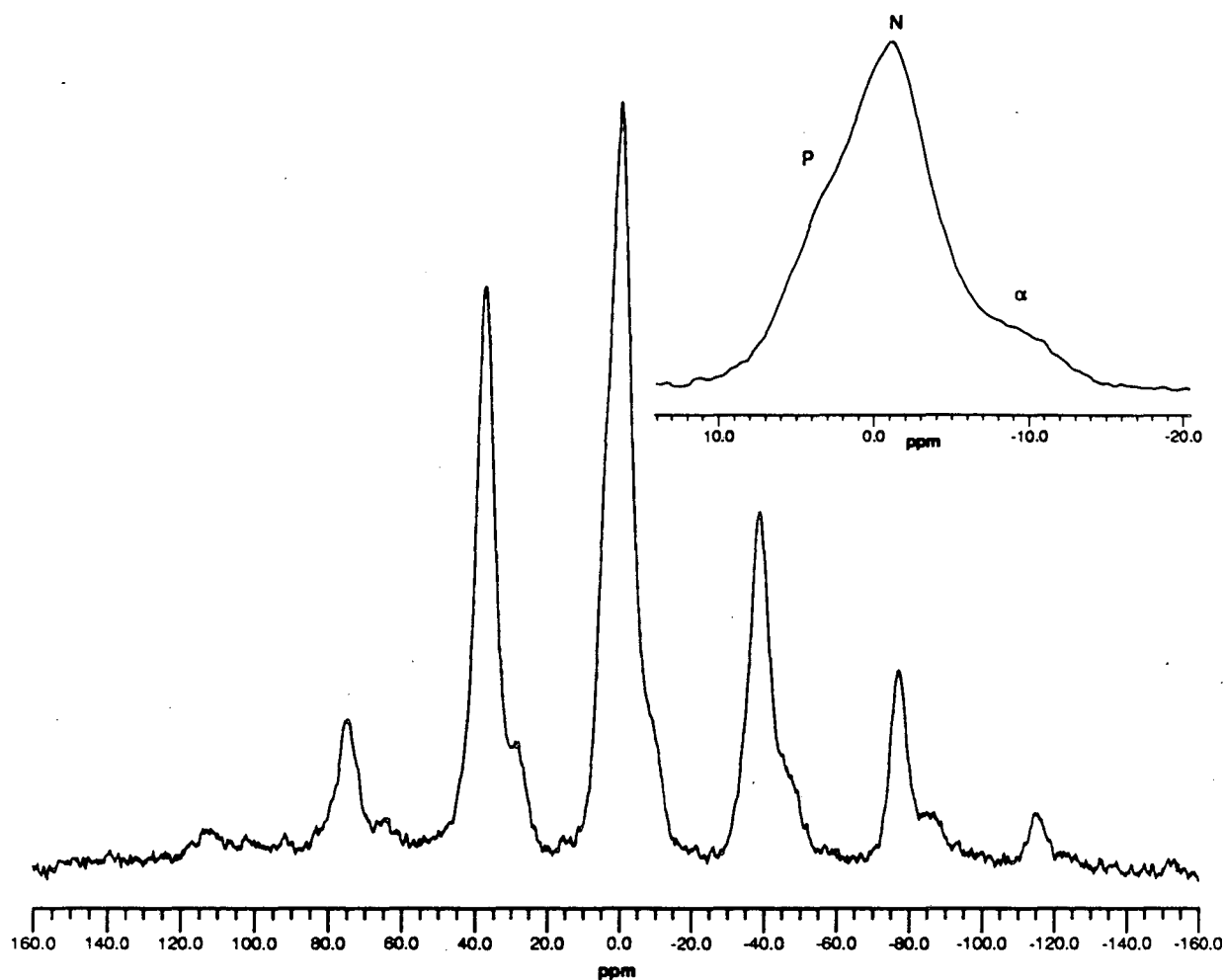


Figure 4.15. The ^{31}P CP-MASS spectrum of lyophilized rat liver. The sample was spun at 4.1 kHz. The inset shows the isotropic region of the MASS spectrum. The phospholipid component is denoted, P, the DNA-RNA, N, and the NAD resonance as α . The spectrum was obtained under Hartmann-Hahn matched conditions with a cross polarization time 1 ms.

Table 4.3. ^{31}P chemical shift tensor element values of lyophilized rat organs and various phosphate esters. Chemical shifts are referenced to 85% H_3PO_4 with positive denoting downfield. For the rat data, typical standard deviations were about ± 10 ppm for the shift tensor elements and ± 0.4 ppm for the isotropic shifts. For the rest of the data, of those determined by us, the standard deviation was about ± 3 ppm for the shift tensor elements and ± 0.4 ppm for the isotropic shifts. (References are given at the end of this chapter.)

COMPOUND	σ_{33}	σ_{22}	σ_{11}	σ_{iso}
Rat				
Skeletal Muscle	49	-14	-24	3.6
Kidney [†]	—	—	—	3.8
Liver	64	-16	-35	4.3
Skeletal Muscle	88	-10	-82	-1.6
Kidney	73	18	-96	-1.3
Liver	78	23	-105	-1.3
Skeletal Muscle	77	10	-115	-9.6
Kidney	76	6	-111	-9.6
Liver	88	2	-118	-9.8
Skeletal Muscle	78	8	-121	-16.8
Kidney	41	40	-136	-17.6
DNA ¹	85	25	-109	0.3
DNA ^{1a}	78	17	-97	-0.6
Phosphorylethanolamine ^{2,3}	68.6	-12.0	-67.2	-3.5
Dilaurylphosphatidylethanolamine ²	84	23	-100	2.3
Sodium tripolyphosphate ⁴				
α	107	-24	-69	4.6
β	80	20	-117	-5.6
Sodium ATP trihydrate				
α	80.6	9.5	-120.0	-10.0
β	70.9	11.2	-142.2	-20.0
γ	78.6	10.1	-109.4	-7.0
Calcium ATP – DPA				
α	80.4	11.8	-122.7	-10.2
β	72.2	12.9	-140.0	-18.2
γ	78.5	-25.4	-75.8	-5.7
Magnesium ATP – DPA				
α	85.9	11.7	-125.5	-9.3
β	73.7	14.2	-138.6	-16.9
γ	84.1	-23.9	-79.7	-6.5
Sodium GMP Heptahydrate	70.2	-15.2	-37.9	5.7

[†] No consistent set of shift tensor elements could be determined. The anisotropy of the tensor is most likely about 100 ppm.

the sideband intensities were very low and accurate fittings were not possible. The combined effects of having only a few sideband intensities compounded by inaccurate measurements of intensities resulted in even greater uncertainties for these components. Although slower spinning speeds could have been used to obtain more sidebands, in practice the loss of resolution at lower speeds was not acceptable. We report these values because their magnitudes do have qualitative value in the discussion to follow and as we shall discuss are probably close to the correct values. A similar situation also holds true for the muscle and kidney component at - 16.8 and - 18.4 respectively. In these cases, due to the relatively low intensities, it was difficult to obtain a consistent set of shift tensor elements. It is important to note that the isotropic shifts were determined independently of the tensor elements and were solely determined by how well we could fit the centerband region of the spectra. They were much more reproducible and, therefore, these results were considered more reliable. In Table 4.4, we include chemical shift tensors which have been determined by us (see Chapter 3) and others (Hertzfeld, 1978; Burlinson, 1986; Mai, 1983).

D. Linewidths of ^{31}P resonances

The relatively broad lines were a source of concern. Cholli and co-workers (Cholli, 1985) have also noted poor resolution in their study of lyophilized frog oocytes. VanderHart and co-workers (VanderHart, 1981) have analyzed the sources of linebroadening in ^{13}C MASS NMR. Of those tabulated by them, we eliminated insufficient proton decoupling field and off resonance effects as possible causes of line broadening based on studies in which the linewidth of the centerband was measured as a function of decoupling power and proton frequency offset (see previous section). The effect of missetting of the magic angle was difficult to determine. The angle was adjusted for minimum linewidth. However, the minimum was ambiguous due to the persistence of broad and overlapping lines. The linewidths were modestly dependent on spinning speed with smaller linewidth achieved with higher spinning speed. Samples were routinely spun from 2.5 to 5.0 kHz. The stability of the spinning speed was determined by examination of the optical tachometer output using an oscilloscope and frequency counter and found to be stable to better than 5 Hz. Given these observations and the relatively narrow lines observed for inorganic samples under the same instrument conditions, it did not seem

reasonable that these above mentioned factors were responsible for the broad lines. Hence, we concluded that the predominant sources of linebroadening were not instrument dependent and were inherent to the samples.

IV. DISCUSSION

A. Interpretation and assignment of ^1H MASS spectra

The high spectral resolution of the ^1H MASS NMR spectra of lyophilized rat tissue was somewhat surprising. The dipolar interaction for a rigid pair of methylene protons is about 16 kHz. The observed linewidths for the static tissue samples were about one order of magnitude smaller. The MASS linewidth were another two orders of magnitude smaller. These observations were attributed to the partial motional averaging of the dominant homonuclear dipolar interactions as well as the small proton chemical shift anisotropy. Further averaging was achieved through MASS. Since the homonuclear dipolar interaction has a vanishing average, the frequencies of the centerbands in the proton spectra are the isotropic or average values of the chemical shift interaction for various types of proton in analogy to proton spectra of liquids and solution.

If we assume that these chemical shifts resemble those measured in solution, the following assignments can be made for the major features observed in all three tissues. For the sake of explicitness, we refer to the muscle proton spectrum (Figure 4.3) which exhibited the highest resolution. Peaks in the 0.0 to 2.0 ppm region, which includes the dominant feature at 1.2 ppm, were assigned to methyl and methylene groups where the adjacent atom was a C_{sp^3} . The resonances between 1.7 to 2.4 ppm most likely arose from CH-X groups where X is more electronegative than C_{sp^3} with the pair of peaks at 2.1 ppm assigned to $\text{CH}_2\text{-C}_{sp^2}$ protons. The 4.0 ppm features were most likely methylene groups adjacent to a phosphate group. The peak at 5.2 ppm was assigned to olefinic protons. These assignments along with the corresponding relative integrated areas are summarized in Table 4.4. These data are consistent with those expected for a proton spectrum of phospholipids.

Chemical analysis data (West and Todd, 1961) show that lipids and proteins constitute the two largest classes of compounds in lyophilized mammalian muscle, approximately 20% and 80% respectively. This indicated that a large fraction of the tissue protons was unobserved. The absence of proton intensity in the 6.0–7.5 ppm region from aromatic protons and the presence of

Table 4.4. The relative integrated areas of the rat muscle proton spectrum compared to the calculated areas based on phospholipid distribution given in Table 4.1.

Proton Type(s)	Integration region	Measured Area	Estimated Area
H-C _{sp²}	4.7-5.8	100	100
-CH ₂ -O	3.5-4.7	66	150
-N(CH ₃) ₃	3.0-3.5	11	200
-CH ₂ -CH ₂ -N	2.4-3.0	38	120
-CH ₂ -C _{sp²}	1.7-2.4	177	200
-CH ₂ -, -CH ₃	-0.5-1.7	1000	1800

a relatively large olefinic signal indicated that only the lipids were observed. The relative intensities, especially the dominance of the methylene/methyl signals, are also strong evidence that no proteinous protons are observed. The lipid content of most mammalian tissue is about 50% phospholipids (White, 1973). Furthermore, more than 60% of the phospholipid pool is comprised of phosphatidylcholine and phosphatidylethanolamine. In light of these data, we believe that the ^1H MASS NMR spectra of the three types of rat tissue are those of the phospholipid pool in the tissues and in particular of phosphatidylcholine and phosphatidylethanolamine. It is interesting to note that the $\text{N}(\text{CH}_3)_3$ resonances of the phosphatidylcholine which is expected to resonate at 3.3 ppm is greatly diminished.

The contention that the proton spectra of the rat tissues is that of phospholipids is supported by earlier works on the solution proton spectra of sarcoplasmic reticulum membranes (Davis and Inesi, 1971; Robinson, 1972). The solution spectra of membrane fragments and sonicated vesicles closely resemble, both in chemical shift and relative intensities, the ^1H MASS spectra of the three types of tissue (see Figure 4.3). There are two further supporting lines of evidence from other MASS data. We found that commercially available crude soy bean phosphatidylcholine preparations also exhibited narrow resonance. Figure 4.16 is the comparison of this sample and that of rat kidney. The soy bean phosphatidylcholine spectrum contains essentially all of the major features. Another example which supports our contention comes from the work of Eckman (Eckman, 1982), who obtained a ^1H MASS spectrum of 95% deuterated lauric acid which resembles those obtained in this study. He made the important observation that the chemical shifts measured from the solid may not be identical to those in solution. The differences between solid state and solution isotropic chemical shifts were as large as 2.7 ppm. He was unable to give an explanation for these differences. Regardless of their origin, these differences serve to point out that comparisons of proton chemical shifts between solid state and solution should be made with caution. However, the fact that all of the major features in the rat tissue spectra have nearly identical chemical shifts to those in the solution spectra of the sarcoplasmic reticulum membranes (Davis and Inesi, 1971) encourages us to believe that we can make direct chemical shift correlations between those observed for the lyophilized rat tissues and solution data of membranes.

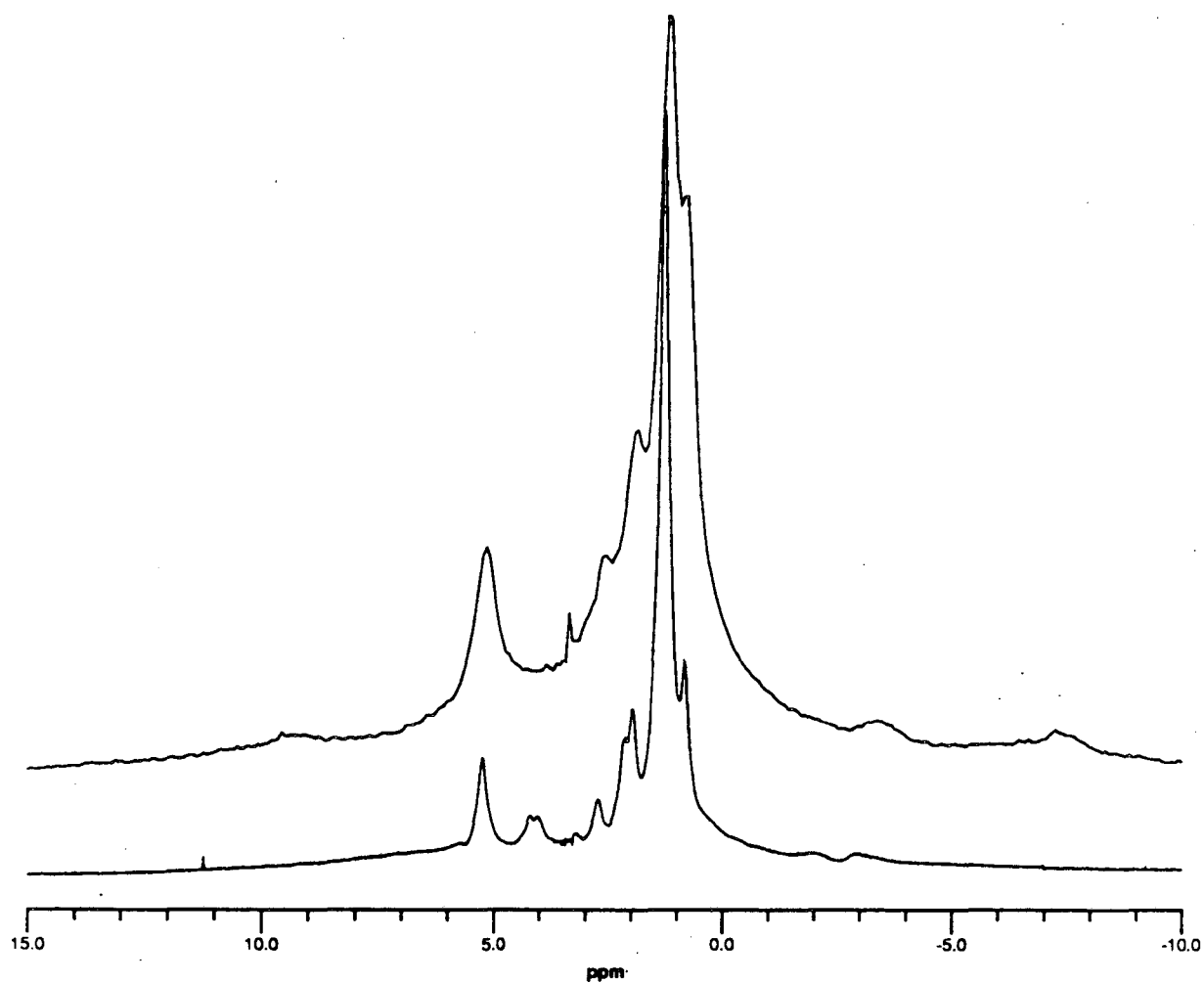


Figure 4.16. A comparison of the ^1H MASS spectrum of lyophilized rat kidney and soy phosphatidylcholine. The kidney spectrum is identical to Figure 4. The kidney sample was spun at 5.3 kHz and soy, at 2.3 kHz.

One prominent difference between the muscle ^1H MASS spectrum and those of the kidney and liver is the presence of a large very broad baseline feature. Chemical composition data for liver and muscle (West and Todd; 1961) indicate no significant differences which could account for the broad feature. One possible source is bound water. It was clear that not all of the water was removed (see below) and if the extent of dehydration were tissue dependent, this could lead to a large and potentially broad water signal.

B. Interpretation and assignment of ^{31}P MASS spectra

We turn to the ^{31}P CP-MASS NMR spectra of the three types of rat tissue. From previous work by us (see Chapter 2) and others (Kohler, 1976; Hertzfeld, 1978; Burlinson, 1986; Mai, 1983), the isotropic chemical shifts and the shift tensor elements of almost all of the phosphorous bearing molecules listed in Table 4.1 are known or could be estimated from model compounds. Based on these data, we were able to assign the various ^{31}P resonances. The upfield most resonance observed in both muscle and kidney, at -16.8 and -18.4 respectively, are from the β -ATP phosphate group. This assignment was supported by the isotropic shift which was similar to those of the polycrystalline magnesium and calcium ATP-DPA. From previous work on these and other phosphate compounds (see previous chapters), it was known that the σ_{11} tensor element of the β position of a tripolyphosphate chain is characterized by a large upfield value ranging from -117 ppm to -142 ppm. The σ_{33} element for this type of phosphate group ranges from 70 ppm to 80 ppm. The σ_{22} reflects the formal charge of a phosphate group with singly charged group ranging from 1 to 25 ppm. The -16.8 ppm muscle component is consistent with these observations. The corresponding kidney component was not in complete agreement. The low intensities of the sidebands may have contributed to a large error, although a relatively consistent set of tensor values was obtained from several different spectra. However, the isotropic chemical shift was characteristic of the β position of ATP.

The -9.6 ppm feature common to all three tissues were assigned to the CO-P-OP group. Based upon quantities shown in Table 4.1, this feature most likely corresponds to the α ATP and NAD phosphate groups. The shift tensor elements measured from the tissue samples were entirely consistent with model compounds. Likewise, the common -1.5 ppm component was assigned to the CO-P-OC group which could potentially correspond to DNA, RNA and phos-

pholipids. Again the shift tensor elements agreed nicely with previous works on phospholipids (Kohler, 1977; Hertzfeld, 1978) and DNA (Mai, 1983). Based on these works, we anticipated that the anisotropy and asymmetry parameters of the shift tensors for these compounds would vary with environment and mobility. This has been documented for DNA by Mai (Mai, 1983) and for phospholipids by Griffin (Griffin, 1976). Based on these works and other observations, we believe that the -1.5 ppm component arises from DNA and RNA. The reason for this will be discussed in the following section. Furthermore, the 3.8 ppm component is most likely indicative of a mobile phospholipid group, the shift tensor of which is nearly axial and of diminished anisotropy. The large narrow contribution observed in the proton decoupled static and MASS Bloch decay experiments (see Figure 4.7a and c) were probably due to a different pool of phospholipids which had undergone further motional narrowing (see below).

C. The calculation of the MASS spectra of rat tissue

The above assignments are highly dependent on how well we were able to fit the experimental spectra. In light of the fact that most of the spectral features overlapped greatly, an independent verification was desirable. As discussed in the previous section, this was achieved in two ways. The first was to calculate a powder spectrum using the principal values of the chemical shift tensors listed in Table 4.4 and standard formulae for the powder patterns (Mehring, 1983). Figure 4.17 is such a comparison. In this case, the intensities of the constituent powder patterns were varied until the best match to the experimental powder pattern was achieved. For this particular case, this procedure yielded good agreement. Unfortunately, quantification of powder patterns intensities calculated in this manner is not straightforward. This arises from the fact that standard powder formulae exhibit a logarithmic discontinuity at σ_{22} . The second method of verification was a direct quantum mechanical calculation of the MASS spectra based upon shift tensor elements. Although this method was computationally more complex than the first, it afforded a straightforward means for quantifying the relative amounts of each component (see Appendix D). The calculated spectra obtained in this way were in good agreement with the experimental MASS spectra. Figures 4.18 and 4.19 show the results of such calculations for muscle and kidney tissues along with the experimental MASS spectra. For clarity, it should be noted that relative intensities of the centerbands need not correspond to relative

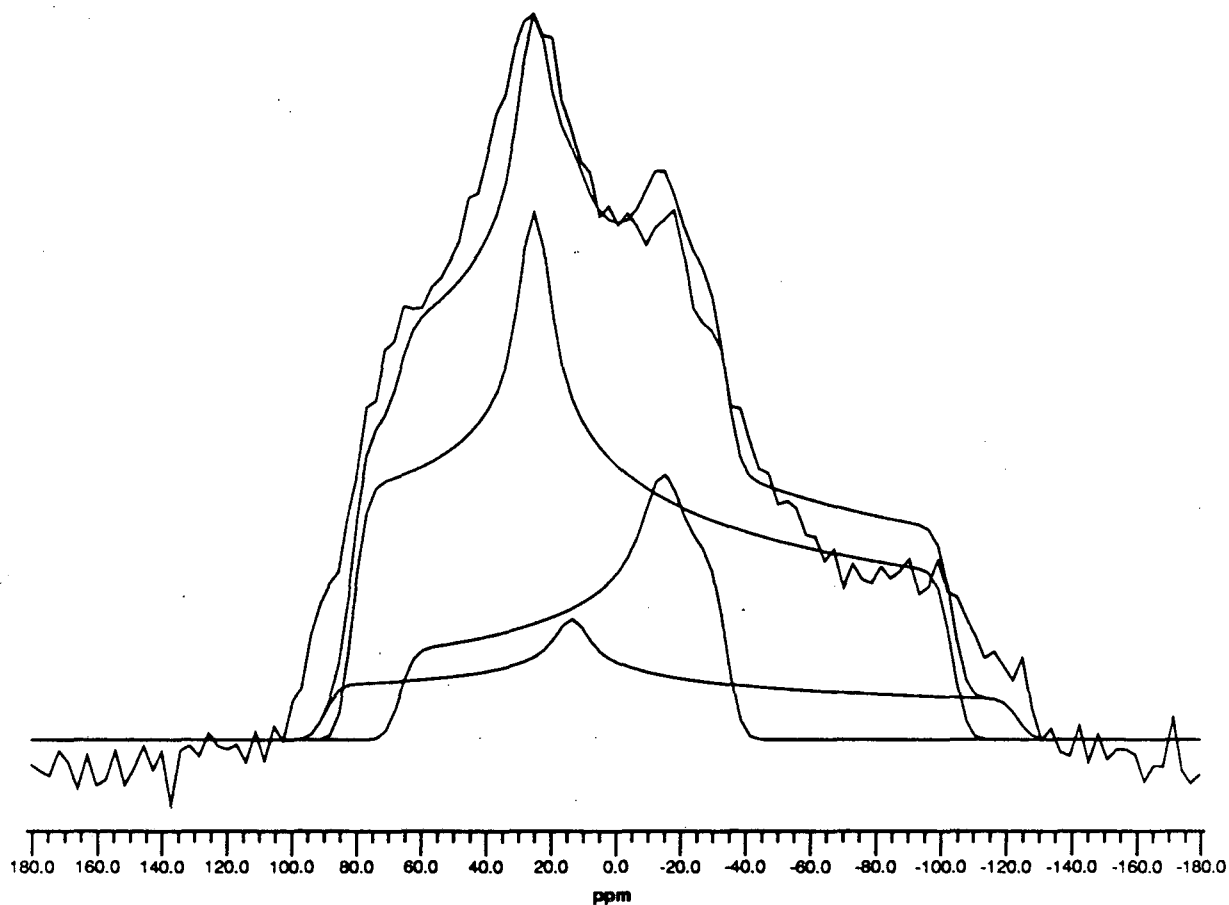


Figure 4.17. The experimental powder spectrum of lyophilized rat liver compared to the calculated powder spectrum based on the chemical shift parameters shown in Table 4. The powder spectra for the three components used to generate the total are also shown. The intensity of each component was adjusted until the best fit of the total to the experimental spectrum was achieved.

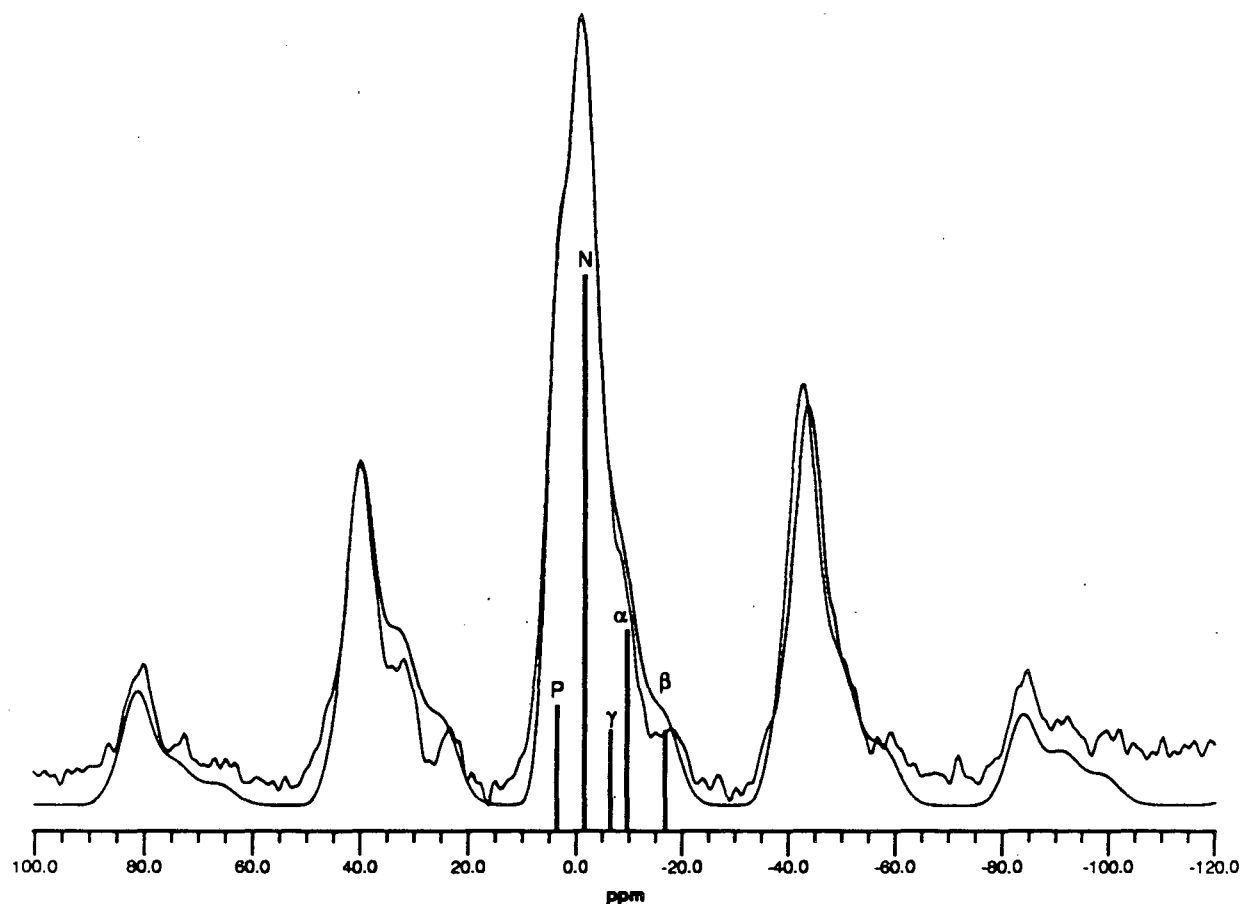


Figure 4.18. The experimental MASS spectrum of lyophilized rat skeletal muscle compared to the calculated MASS spectrum based on the chemical shift parameters shown in Table 4. The chemical shift parameters used for the phospholipid (P), DNA and RNA (N), α and β ATP components are those determined from muscle tissue and the parameters for the γ ATP component come from the magnesium ATP. The latter was included to obtain a better overall fit to the experimental spectrum. The heights of the bars denote the relative scaling factors for each of the components used in calculating the spectrum and are tabulated in Table 6. The positions of the bars are the isotropic chemical shifts of the corresponding components.

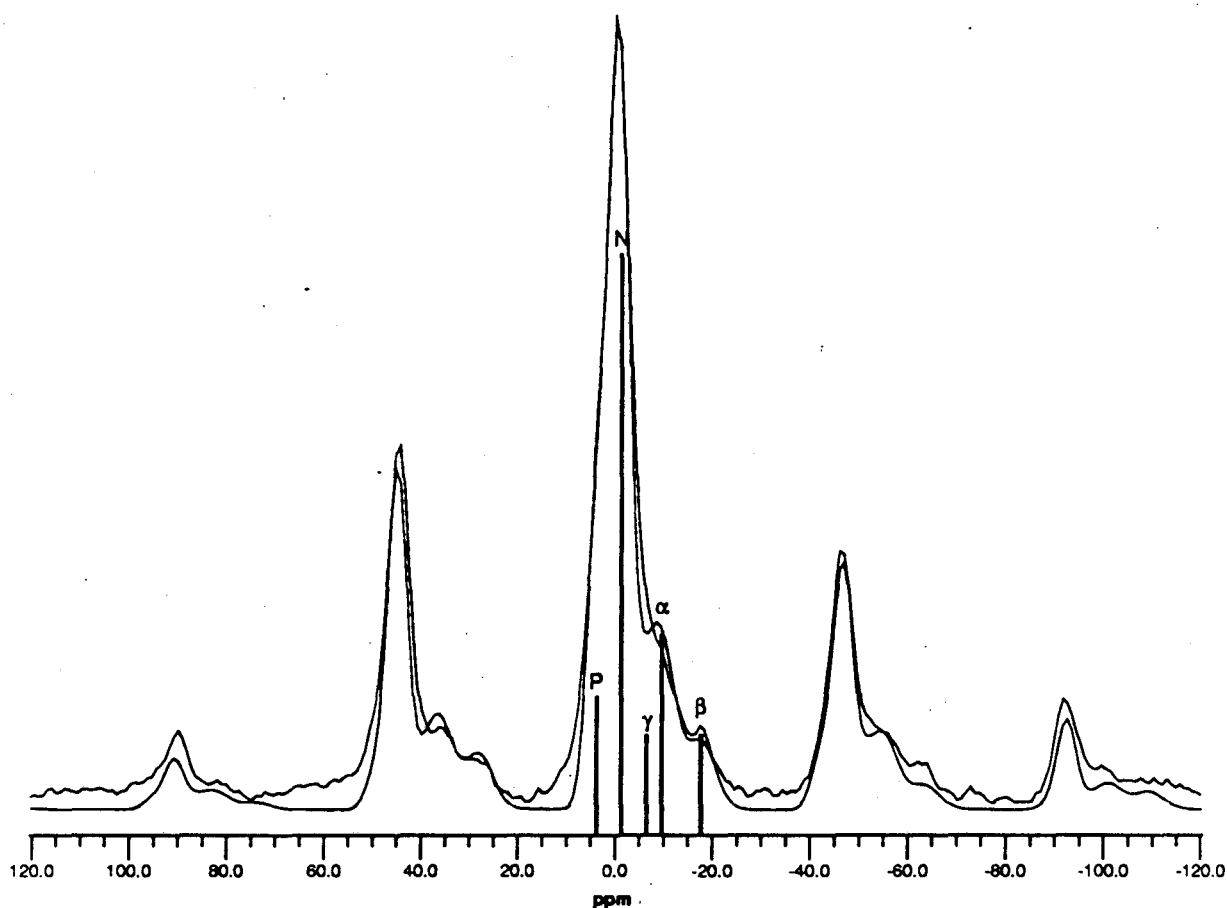


Figure 4.19. The experimental MASS spectrum of lyophilized rat kidney compared to the calculated MASS spectrum based on the chemical shift parameters shown in Table 4. The chemical shift parameters used for the DNA and RNA (N), α and β ATP components are those determined from kidney tissue and the parameters for the γ ATP component come from magnesium ATP. Isotropic chemical shift value of the phospholipid (P) component come directly from the fit of the spectrum and rest of the shift parameters are those of corresponding component in skeletal muscle. The muscle parameters were used since no consistent set of shift parameters could be obtained. The γ ATP was included to obtain a better overall fit to the experimental spectrum. The heights of the bars denote the relative scaling factors for each of the components used in calculating the spectrum and are tabulated in Table 6. The positions of the bars are the corresponding isotropic chemical shifts.

populations of the various components. The bars shown in Figures 4.18 and 4.19 reflect the relative contributions to the total population and not centerband intensities. We found that better agreement between calculated and experimental spectra could be obtained by including a contribution corresponding to a γ -ATP component. The necessary shift parameters needed to calculate the spectrum were obtained from our analysis of polycrystalline magnesium ATP (see Chapters 1 and 2). The presence of a β -ATP resonance indicated that the inclusion of this γ -component was indeed reasonable. For the kidney spectrum, we found a much better fit could be obtained by including the downfield near axial component observed for the liver and muscle. We used the shift tensor element obtained for the muscle in order to calculate the spectrum. As noted above, although the sidebands for this component in the kidney spectra were observed, no consistent set of tensor elements could be obtained. The fact that reasonable agreement exists between calculated and experimental spectra indicates that the chemical shift tensor elements tabulated in Table 4.1 for the various components observed in the ^{31}P CP-MASS spectra are reasonable.

Based on the calculated spectra, it was possible to estimate the relative distribution of cell phosphorus. The relative quantities of the mobile and immobile fractions were estimated from the relative areas of the narrow component in comparison to the broad component in the non-spinning spectra. The difference in linewidth of the narrow component in non-decoupled and decoupled experiments was not appreciable. From this we deduced that the second moment of the heteronuclear dipole coupling is small and, therefore, the cross polarization efficiency should be low (Mehring, 1983). Consequently, the CP-MASS spectra were expected to solely reflect the immobile fraction of the total cell phosphorus. This was supported by the observation that decoupled Bloch decay MASS spectra (Figure 4.10) contained a large centerband contribution which possessed no sideband intensity. From the comparison of the experimental CP-MASS spectra with the calculated spectra, it can be seen clearly that all of the sideband and centerband intensities can be accounted for without recourse to inclusion of additional centerband intensity (as distinguished from inclusion of both center and sideband intensities as in the case of the γ -ATP contribution). Therefore, the relative scaling factors used in the calculation of the theoretical spectra (*i.e.* relative bar heights in Figures 4.18 and 4.19) should reflect the relative

distribution of the various phosphorus bearing molecules which were assigned above. The results are tabulated in Table 4.5. In the case of kidney data, if it is assumed that all of the CO-P-OC fraction arises from DNA and RNA and the axial and mobile fractions from phospholipids, the relative quantities agree surprisingly well with that tabulated in Table 4.1. In the case of muscle tissue, a similar simple interpretation of the distribution as given in Table 4.5 cannot be made. This may be due to fact that the bonding categories used in Table 4.5 certainly overlap and, in addition, O-P-N bonds found in phosphocreatine, which is a significant contribution to the total phosphorus pool in muscle, are not accounted for in Table 4.5. The only remedy to this problem would be much higher spectral resolution. Nonetheless, the phosphorus distribution obtained in this study does reflect the fact that the cell phosphorus distribution is significantly different in the muscle and kidney, as shown by larger amount of phospholipids in the kidney in comparison to the muscle. The data also show that for the two tissue types the relative amount of NAD is that expected from *in vivo* NMR results. They also suggest that the relative ATP content is higher in muscle than in kidney. However, because of the potentially large errors, this result is, at best, tentative.

In passing, we note that cross polarization rates (T_{IS}) and rotating frame relaxation rates ($T_{1\rho}$) for the major CO-P-OC component in the tissue samples are similar to those measured for the three resonances of polycrystalline magnesium ATP. The cross polarization times were about 300 μ s and the relaxation rate 3.0 ms at a ^1H decoupling field of 35 kHz. At cross polarization times of 1 to 2 ms, the relative ratio of the phospholipid to the CO-P-OC component appeared to be constant. Therefore, the relative intensities were believed to accurately reflect the relative populations. The errors estimates given in Table 4.5 are primarily arise from how well the calculated spectra could be fitted to experimental and the precision with which the static spectra could be partitioned between narrow and broad components.

D. Recalculation of MASS spectra based on model data

The agreement between calculated and experimental data led us to recalculate the MASS spectra based on the chemical shift tensor data for the model compounds (*i.e.* DNA-RNA, NAD, ATP and phospholipids) rather than on the data obtained directly from the tissue samples. In these calculations we retain the near axially symmetric component because no model for this

Table 4.5. The chemical shifts and relative quantities of various ^{31}P bearing compounds in rat muscle and kidney as determined in this study. Quantities are given as percent of total phosphorus and chemical shifts are in ppm, from 85% phosphoric acid.

COMPOUND	Chemical shift	Muscle	Kidney
Mobile fraction¹	-3.0-3.0	15	35
Immobile fraction¹		85	65
Axial	3.6	9	7
CO-P-OC	-1.5	44	29
CO-P-OP	-9.6	15	10
PO-P-OP	-17.5	8	5
PO-P-O ²		8	5

1. the error in these data are about $\pm 10\%$.
2. assumed based on presences of PO-P-OP component.

component could be clearly identified. NAD was assumed to have shift parameters similar to α -ATP. Furthermore, we assumed that all of CO-P-OC components arose entirely from DNA since no RNA data were available. The anisotropy parameter (*i.e.* $\sigma_{33} - \sigma_{11}$) of the DNA is known to be sensitive to state of the hydration (Mai, 1983). The shift tensor elements for the DNA were chosen based on the comparison of the anisotropies of the CO-P-OC component of the tissue samples with those tabulated by Mai and coworkers (Mai, 1983) for DNA in various states of hydration. The DNA chemical shift tensor values which best agreed with tissue data corresponded to hydration states of 9, 11 and 13 D₂O per nucleotide. The average of these values was used in subsequent calculations. The relative quantities tabulated in Table 4.5 were used to scale the various components. Figure 4.20 and 4.21 show the comparison between experimental MASS spectra of muscle and kidney and those calculated based on model data described above. In the case of the muscle, the agreement was modest with the largest error coming from the DNA component. This may reflect the fact that this component may not entirely arise from DNA. As pointed out earlier there is good reason to believe that other types of molecules may contribute to this component. The model-based calculated MASS spectra of the kidney was in good agreement with the experimental spectrum. These calculations further support the assignments and interpretation of the MASS spectra of tissue which we presented.

E. Motion in Phospholipids

Finally, we turn to a common feature of both ¹H and ³¹P spectra, that of molecular motion. As pointed out earlier, the fact that a relatively high resolution MASS spectra of lipids can be obtained from the tissue samples indicated the presence of anisotropic motion. Without such motion it would be expected that the proton homonuclear dipolar interactions would strongly dominate the spectrum to the extent that modest spinning speeds (2 to 5 kHz) MASS would not be effective. In addition, if isotropic motions were responsible for the averaging, we would expect that MASS would likewise not be effective. This effect has been discussed by Waugh (Waugh, 1979). He also noted that in order for MASS to have an effect in cases where dipolar interaction is significant the spinning speed must be on the order of or greater than the linewidth, since the "flip-flop" terms would tend to average any effect of slower spinning rates. There were further indications that varying degrees of motion were present in the lyophilized tissue

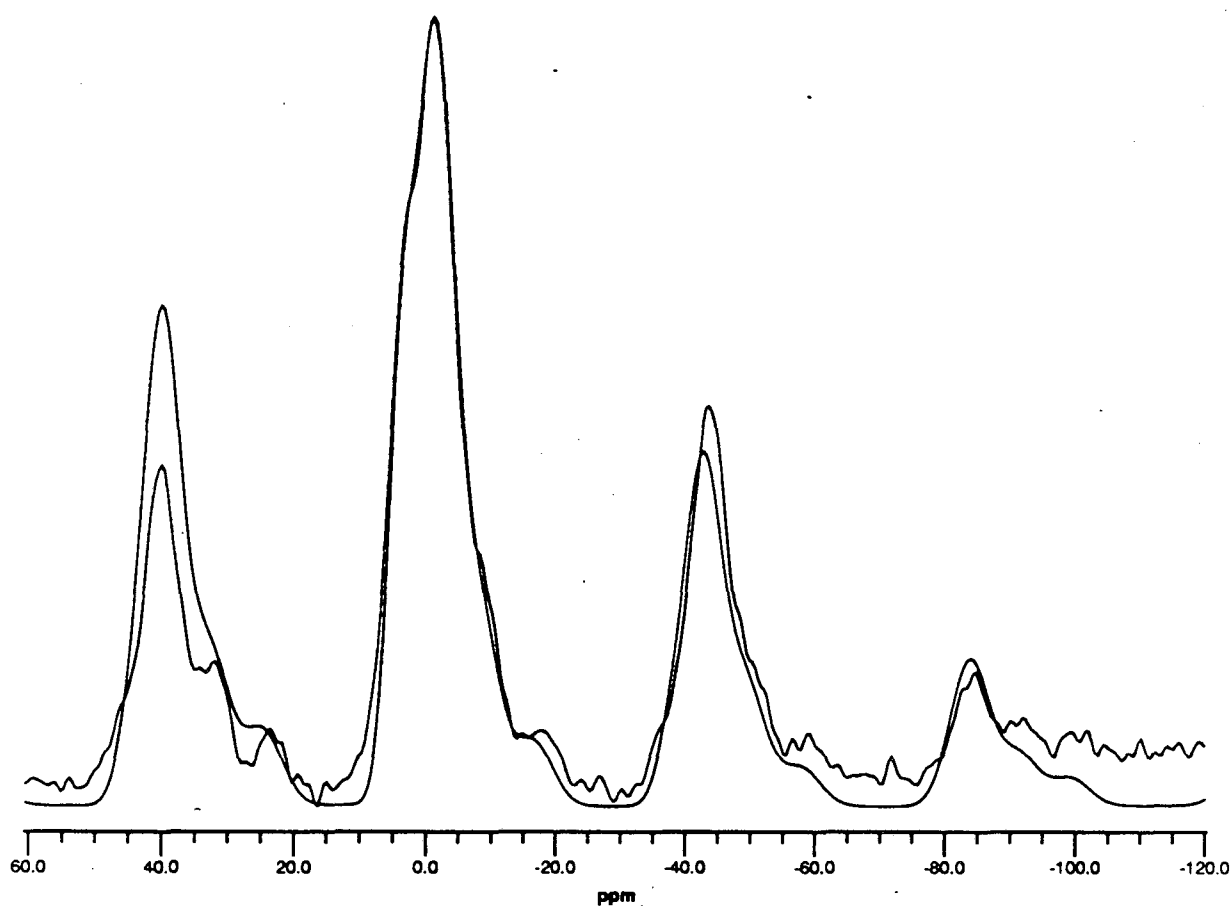


Figure 4.20. The experimental MASS spectrum of lyophilized rat skeletal muscle compared to the calculated MASS spectrum based on the chemical shift tensor parameters from models. The calculated spectrum was determined in the same manner as Figure 18. The same relative contributions were used. The chemical shift tensor values were from model (*i.e.* DNA with 11 D₂O per nucleotide, magnesium ATP with the α ATP position also used to model NAD). The phospholipid component was retained.

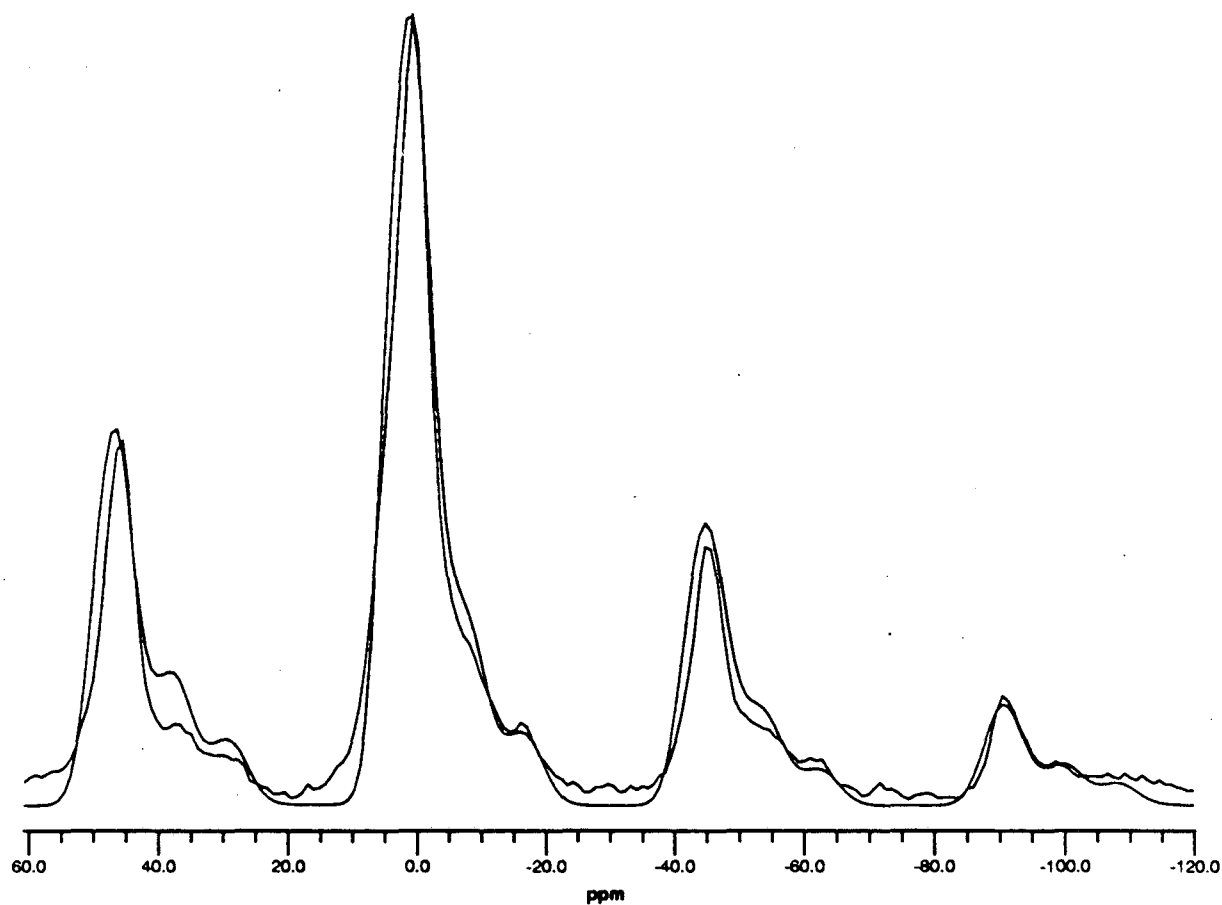


Figure 4.21. The experimental MASS spectrum of lyophilized rat kidney compared to the calculated MASS spectrum based on the chemical shift tensor parameters from models. The calculated spectrum was determined in the same manner as Figure 19. The same relative contributions were used. The chemical shift tensor values were from model (*i.e.* DNA with 11 D₂O per nucleotide, magnesium ATP with the α ATP position also used to model NAD). The phospholipid component from the muscle sample was also used.

samples. The ^1H spin lattice relaxation of the dominant feature in the muscle spectrum time reached a minimum at about 0°C . This corresponds to a correlation time of roughly 0.5 ns at about 273°K . This correlation time is comparable to those measured by Seelig and coworkers (Seelig, 1981) for deuterium labelled 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC). They measured the correlation time for the bilayer to be 0.17 ns and for the reconstituted sarcoplasmic reticulum membrane vesicles, 0.21 ns. The increase in correlation time was attributed to presence of proteins. The MASS T_1 measured at 293°K for various features of both the muscle and kidney spectra are shown in Table 4.2. Based on proton assignments made above, these relaxation times are comparable to those measured by Horwitz and coworkers (Horwitz, 1972) for sonicated lecithin dispersions. The presence of differential proton relaxation was attributed to the fact that spin diffusion was not a contributing mechanism and that the relaxation most likely occurs through modulation of the homonuclear dipolar interaction. A similar situation probably exists in the lyophilized tissue where spin diffusion is in part suppressed by MASS and molecular motion. The variable temperature MASS T_1 experiments have not yet been carried out. However, the increase in linewidth with decrease in temperature may indicate that the effectiveness of motional averaging of dipolar interactions has been diminished at lower temperatures. By combining observations from both ^1H and ^{31}P spectra, it was possible to reach some conclusions about the dynamics of the head group. The presence of the narrow component in ^{31}P Bloch decay experiments and near axial component in CP-MASS experiments indicate that these phosphate groups are undergoing motions. The near axial pattern arises from averaging about a single axis of rotation. This motional averaging process is well understood (Griffin, 1976; Seelig, 1978; Campbell, 1979). From the work of Campbell (Campbell, 1979) on dipalmitoylphosphatidylcholine, it is possible to estimate the rotational reorientation time based on the chemical shift anisotropy. Based on that study, the reorientation time of the near axial component was estimated to be roughly $2\ \mu\text{s}$. The narrow component most likely arises from head groups for which the axis of rotation wobbles and causes further averaging of the shift tensor but is insufficient to obtain either the isotropic linewidth or chemical shift. We found that this signal was the most variable from sample to sample of a given tissue type. This was attributed to the final state of hydration of the samples. It has been shown by Griffin (Griffin,

1976) that the degree of hydration plays an important role in the dynamics of the head group. In that study, the rigid lattice chemical shift tensor was observed for completely dehydrated dipalmitoyllecithin. Upon successive degrees of hydration the anisotropy both decreased and underwent sign reversal. The near axial pattern which we have observed is of the opposite sign and much narrower than that of the model phospholipid data. Since we made no rigorous attempt to completely dehydrate our samples we expect the state of hydration to vary and lead to the differences observed for the most mobile phosphates. The motion observed for the head group however was not sufficient to render the $N(CH_3)_3$ of the choline head group observable. The expected integrated intensity of these protons should be comparable to those protons bound to unsaturated carbons (see Table 4.5). The isotropic chemical shift for these proton is 3.3 ppm. This region in all three tissues is devoid of any large narrow signal. The absence of signal from these protons at 25° has been noted by Davis and Inesi (Davis and Inesi, 1971) in their study of sarcoplasmic reticulum. In this system, the N-methyl proton signals were only observed upon increase in temperature. This was attributed to increased mobility of the choline group. As pointed out earlier, increase in temperature does induce some amount of line narrowing. However, unlike the study of Davis and Inesi, even at 313°K, the feature at 3.4 ppm has not appreciable changed from 283°K. Based on trimethylammonia studies, the expected linewidth of the N-methyl protons in the presence of rotation about the carbon C_3 axis is about 30 to 40 kHz and if the rotation about the C_γ -N bond is taken into account, the linewidth reduces to 18 kHz (Haigh, 1968). As discussed above, the motionally narrowed ^{31}P chemical shift tensor of the phospholipids indicates that the head groups reorient with correlation times of approximately 2 μs . Reorientation rates of this magnitude would be sufficient to average the residual proton dipolar couplings in the choline groups to the point where the methyl protons would be observable under MASS conditions. Apparently, the motions of the N-methyl groups are even more restricted. One reason for this may be that the choline group may not be very flexible and rotation about the C_γ -N bond maybe hindered (Buldt and Wohlgemuth, 1981). It is interesting to note that the soy phosphatidylcholine does have a sharp signal a 3.3 ppm at room temperatures (see Figure 4.16). The significance of this observation needs to be investigated. The final picture is one where the motions of the phospholipids are severely restrained at the

head group and progressively less restrict along the fatty acid chains.

V. Conclusion

By applying ^{31}P MASS NMR to lyophilized tissue samples from rat muscle, liver and kidney, we have been able to detect a large fraction of the total cell phosphorus population. In particular, DNA, RNA and phospholipids were observed. The identities of these species, as well as the resonances from ATP and NAD, were based on not only the isotropic shift but also by the principal values of the chemical shift tensors. These tensor parameters were determined from analyses of sideband intensities. They were found to be in good agreement with model compounds. Furthermore, from these data it was possible to deduce that the phospholipid headgroups in the nominally lyophilized samples did possess waters of hydration and were undergoing motion. The DNA in the tissue was also hydrated with about 11 waters per nucleotide. By comparing the calculated MASS spectra with experimental CP-MASS spectra, it was possible to quantitate the relative cell phosphorus populations. These relative quantities seem to be in good agreement with chemical analysis for at least the kidney samples. The liver sample did not possess any β -ATP resonance and we concluded from this that the ATP was depleted from this sample. Based on comparisons to model data, all of the ATP appears to be complexed to divalent metals such as magnesium and calcium.

In addition to these findings, we observed high resolution ^1H spectra for the three tissue types. We believe these spectra are of phospholipids, in particular phosphatidylcholine and phosphatidylethanolamine. This conclusion was based on the absence of aromatic resonance characteristic of proteins and the presence of intense methyl/methylene signals. The relative integrated areas also were roughly those expected for phospholipids. The fact that relatively high resolution spectra could be obtained by MASS indicated the presence of anisotropic motion. Diminished signal intensity from the N-methyl protons of the choline headgroup indicated that headgroups were not as mobile as fatty acid chain protons. The ^{31}P signals associated with the headgroups appeared to be narrowed indicating that they too were not immobile.

The set of experiments described indicate that solid state NMR techniques can be very useful in the study of tissue. Although lyophilized tissue samples were used in this study, we foresee only moderate logistical complications in applying these techniques to frozen tissue

and we will shortly undertake these experiments. The results presented also indicate that other biological problems may benefit from ^1H and ^{31}P MASS NMR.

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References and Notes for Table 4.3

1. Mai, M. T.; Wemmer, D. E.; Jardetzky O., *J. Am. Chem. Soc.*, **105**, 7149 (1983).
- 1a. Second DNA entries are the average of 9,11 and 13 D₂O per nucleotide. These were chosen based on comparable anisotropies to those observed in tissue. Data are from reference 1.
2. Hertzfeld, J.; Griffin R. G.; Haberkorn, R. A. , *Biochemistry*, **17**, 2711 (1978).
3. Kohler, S. J.; Ellett, J. D.; Klein M. P., *J. Chem. Phys.*, **50**, 4451 (1976).
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Chapter 5

A Study of Glycolysis in Erythrocytes

Chapter 5. A Study of Glycolysis in Erythrocytes

I. Introduction

Much of the recent developments in biological NMR has centered primarily around four nuclei: ^{31}P , ^{13}C , ^1H and ^2H . To a lesser extent, ^{15}N , ^{23}Na and ^{39}K have also been used. A nucleus which has not been extensively utilized and, yet, provides a great deal of promise is tritium. ^3H NMR has become an invaluable analytical tool for determining the extent and specificity of tritium labelling procedures (Bloxsidge and Elvidge, 1983). However, the applications of ^3H NMR to biological problems have been limited and, to date, *in vivo* applications have not been explored.

There are several factors which make tritium an attractive alternative to hydrogen and deuterium as NMR spin probes. Like the proton, the tritium spin is $1/2$ and consequently is not complicated by quadrupole interactions (and the resulting residual broadening) as would be the case for deuterons. Its magnetogyric ratio is greater than that of the proton by approximately 7%. This has several consequences. First, since the sensitivity is between linearly to quadratically dependent on the magnetogyric ratio, a triton should be approximately 12 and 18 times more sensitive than ^2H and ^{13}C nuclei, respectively. Second, the chemical shift interaction is, to first order, identical to that of the proton when expressed on a relative part per million (ppm) basis (Bloxsidge, 1979). Second order isotope effects have been shown to exist and to provide information about the multiplicity of labels (Bloxsidge, 1979). J-couplings are, also, scaled by ratio of magnetogyric ratios for the proton and triton, that is proton-triton couplings are about 7% greater than the analogous proton-proton couplings and the triton-triton couplings are about 14% larger. Aside from the advantages afforded by its nuclear spin properties, tritium has the obvious advantage common to most isotopic probes: signals from only the probe and subsequent products resulting from chemical reactions involving the probe are observed. In general this makes the *in vivo* monitoring of reactions and analysis of spectra easier. The analysis is also facilitated by the fact that the tritium spectra will be nearly identical to the proton spectra without the presence of large background signals such as water.

The major disadvantage in using tritium labelled probes is the radioactivity. The discussion,

at this point, is limited to those concerns related to the implementing NMR experiments. Tritium is a soft 18 keV beta emitter and the glass walls of an NMR sample tube are much more than adequate for protection against radiation of this energy. Nonetheless, the disintegration of a tritium will result in the formation of reactive radicals which are the products of the initial decay, subsequent collisions and further radical chemistry. The effects of the chemistry involved in these reactions on NMR observations are minimal and are only a problem in very long term experiments and/or when the samples are not fresh (Bloxsidge and Elvidge, 1983). From the health physical point of view, as long as the sample is contained, the radioactivity and associated chemistry pose no real problems or hazards to the spectroscopist. The major precautions are then one of contamination and minimization of exposure. This is especially important in the context of biological applications where the labelled compounds are most likely non-volatile and biologically active. There are several well tested procedures to accomplish acceptable safety standards. The review by Bloxsidge and Elvidge (Bloxsidge and Elvidge, 1983) outlines the measure they have taken. Below, we discuss our simple approach which has worked with good success. We found that the bulk of the safety concerns from the spectroscopist point of view can be addressed by the use of commercially available teflon inserts for the standard NMR sample tubes. They afford more than sufficient protection against spillage and contamination. We have purposefully shattered sample tubes and found that the insert remains undamaged. The only drawback in these inserts is the loss of resolution which, in turn, also affects signal-to-noise, a valuable commodity in tritium experiments. However, considering both the health of spectroscopist and spectrometer this compromise cannot be avoided.

Our first efforts towards applying ^3H NMR was the study of glycolysis in red blood cells. The glycolytic pathway in erythrocytes is well characterized *in vitro* and mathematical models exist for the processes which control the glycolytic pathway (Rapoport, 1976). Figure 5.1 schematically shows the substrates and enzymes involved in the pathway. *In vivo* glycolysis in *E. coli* has been extensively studied by Ugurbil (Ugurbil, 1979) using ^{13}C NMR. Brindle (Brindle, 1979) has studied the transport of alanine and lactate across the cell membrane in human erythrocytes using ^1H NMR. This wealth of information provided us with an excellent starting point for investigating the potentials of ^3H NMR. An immediate consequence of studying

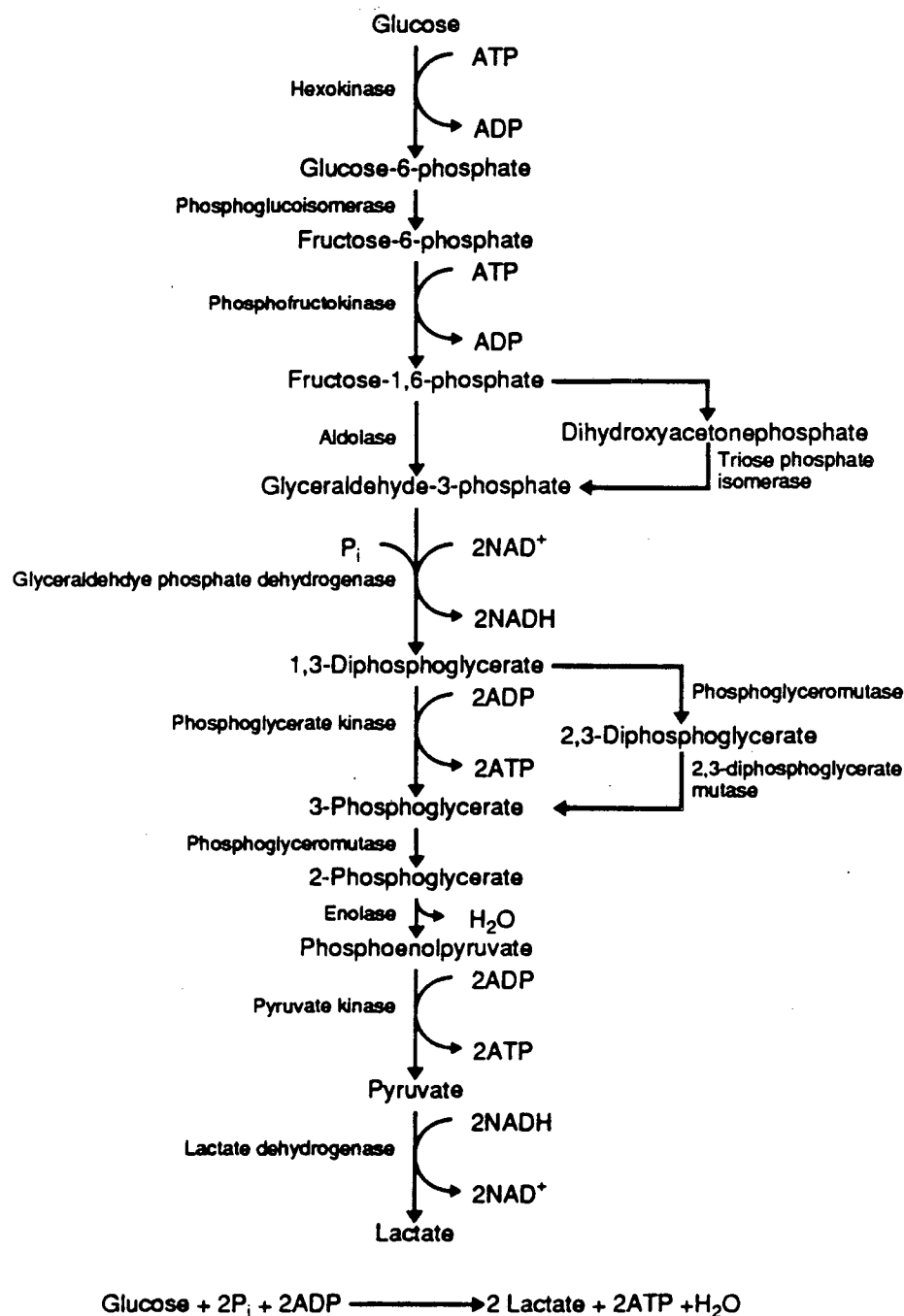


Figure 5.1. The glycolytic pathway. The structures of the intermediates are shown in Figure 4.

this reaction pathway was the unique opportunity to trace the fate of protons/tritons and, in particular, the observation of the production of exchangeable tritons, a feat which cannot be achieved in the case of the protons in the presence of 100 M water proton spins. It was also anticipated that kinetics information obtained under various conditions could be used to test the existing regulatory models.

II. Experimental

The first glycolysis experiments were carried out on a home-built 270 MHz, proton frequency, spectrometer. Because of the closeness of the resonant frequencies, particular care was taken to filter both the tritium and proton decoupling channels. This was accomplished by bandpass filters at the output of the broadband observe transmitter amplifier. The proton transmitter was a tuned amplifier. Further filtering of both transmitter and receiver sides was achieved with low loss bandpass filters on both the observe and decoupling channels. Although the NMR signal is not normally filtered before preamplification, in this particular case, it was found that higher signal-to-noise could be achieved. Broadband proton decoupling was achieved by gating the proton irradiation frequency with a noise generator (Ernst, 1966). All subsequent glycolysis experiments were carried out on a IBM/Bruker NR 300 configured in much the same manner as the 270 MHz instrument. In all experiments, the chemical shifts were determined relative to the proton water signal at 4.65 ppm at 37°C and the lactate methyl resonance at 1.32 ppm with respect to TMS. Individual free induction decays (FID's) were collected at a fast rate with 100 ms delay between acquisitions and small tip angles in order to efficiently maximize signal-to-noise (Becker, 1980). Intensities from spectra taken under these conditions cannot, in general, be used quantitatively except when the various nuclear spin populations have similar longitudinal relaxation (*i.e.* T_1). In the following sections, it will be shown that the T_1 's are sufficiently similar so that relative intensities are indicative of relative spin population.

Tritiated glucose was prepared by the National Tritium Labelling Facility. The glucose was labelled in C-1 position. The specific activity was 6.8 Ci per mmol of glucose. The glucose was dissolved in 0.9% saline solution to a total glucose, labelled and unlabelled, concentration of 0.28 M. The ^3H NMR spectrum of the tritiated $[1-^3\text{H}]$ glucose used in these experiments is shown in Figure 5.2. The two major signals are those of the anomers of glucose. At 3.6 ppm

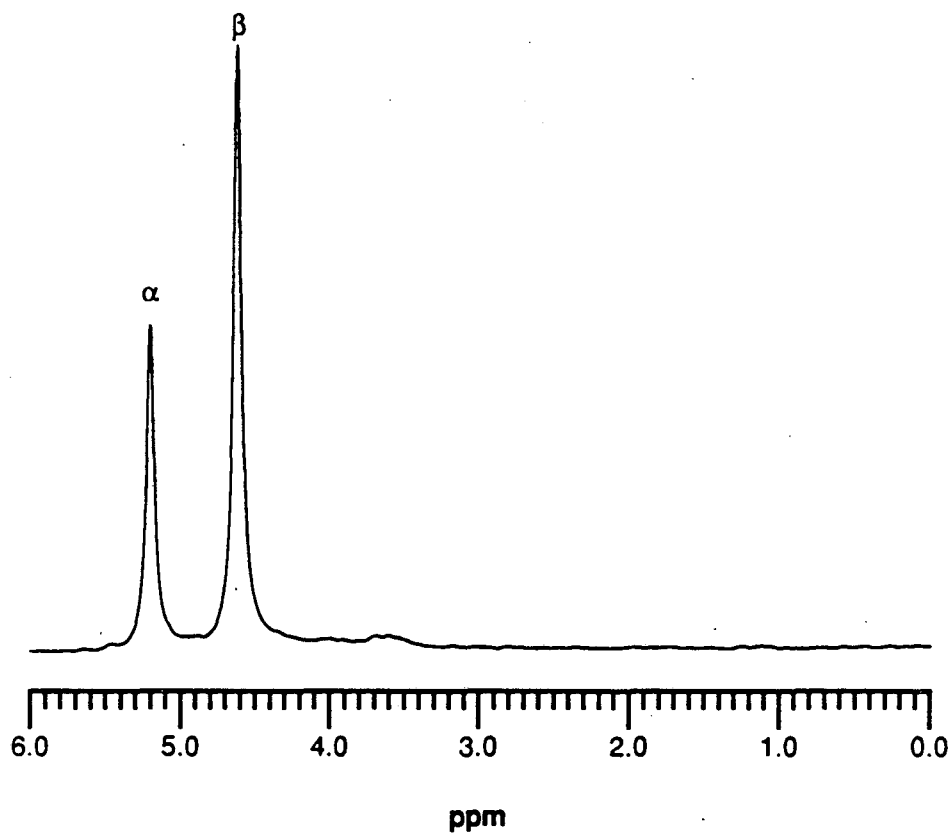


Figure 5.2. The ^3H spectrum of 1- ^3H -glucose. The two resonances arise from the two anomeric forms which exist in a 60:40 ratio (see Figure 4 for structures).

there is a small amount of contaminant.

The blood was prepared by Mr. Paul Carson and his procedure is summarized here for clarity and completeness. In the experiments involving human erythrocytes, the isotonic glucose solution without buffer was simply mixed with packed red blood cells. Typical blood sample contained 200 μL of the glucose solution and 200 μL of packed blood cells. These experiments were carried out at 22°C. In the rat blood experiments the procedure was more refined. The heparinized (2 units/ mL of whole blood) whole blood samples from anesthetized rat were packed by centrifugation and washed several times with pH 8.0 phosphate buffer and repacked. On the final wash the blood sample was treated with penicillin (0.12 mg/ml of packed cells) and streptomycin (0.14 mg/ml of packed cells). Typical preparation for the blood contained: 50 μL of the isotonic glucose solution, 50 μL of 8.0 pH phosphate buffer, 70 μL of deuterium oxide, for NMR locking purposes and 170 μL of packed red blood cells. These experiments were carried out at 37°C.

III. Results

The initial experiments were carried out on packed human blood cells. The results of these are summarized in Figure 5.3 . These spectra are representative of several trials. The length of time for total conversion was approximately 66 hrs. The anaerobic conversion of glucose to lactate is clearly demonstrated by the appearance and growth of two new signals, one at 1.32 ppm from the tritium label of the lactate methyl group and the other at 4.9 ppm from exchangeable tritons (denoted HOT). The spectra taken 4 hours after mixing of the glucose and blood sample exhibits the two anomeric forms of glucose (see Figure 5.3). The α anomer is downfield at 5.19 ppm and the β upfield at 4.61. The observed intensity ratio of 40:60 is in good agreement with the literature values (Maple and Allerhand, 1987, Angyal, 1984). The spectrum at 46.6 hours indicated the presence of an intermediates at resonating 3.2 to 3.8 ppm. This chemical shift range corresponded to tritons adjacent to a phosphate group. This observation was consistent with the fact that starting with the first phosphorylated intermediate, glucose-6-phosphate, of the glycolytic pathway continuing to the 3-phosphoglycerate, the triton is expected to be adjacent to a phosphate group (see Figure 5.4). After 66 hours, all of the glucose has been consumed and only the signals from lactate and HOT are observed.

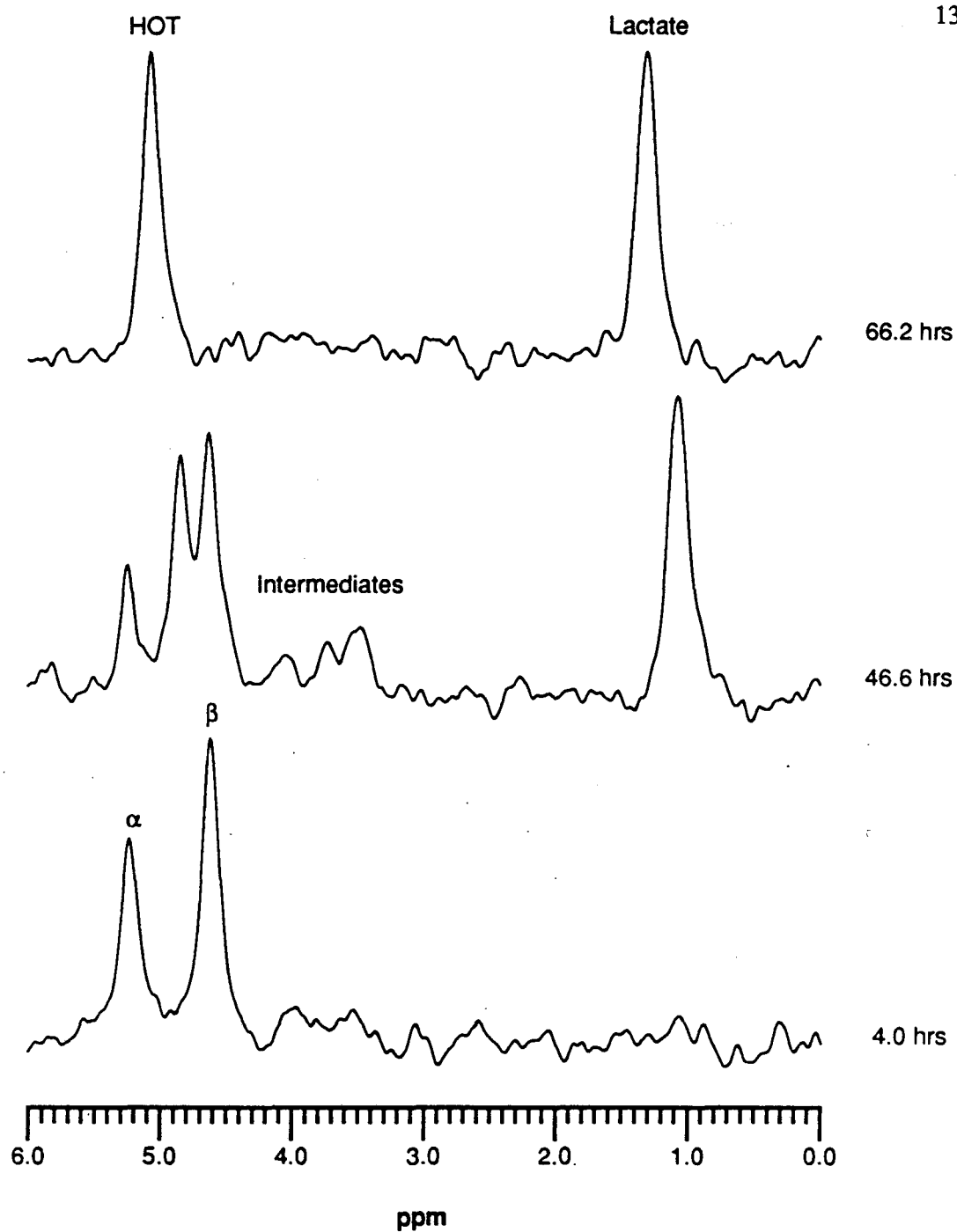


Figure 5.3. ^3H NMR spectra of glucose and glycolytic intermediates in packed human erythrocytes. Spectra were recorded at three different times following the addition of labelled glucose to the packed cells (see text for details).

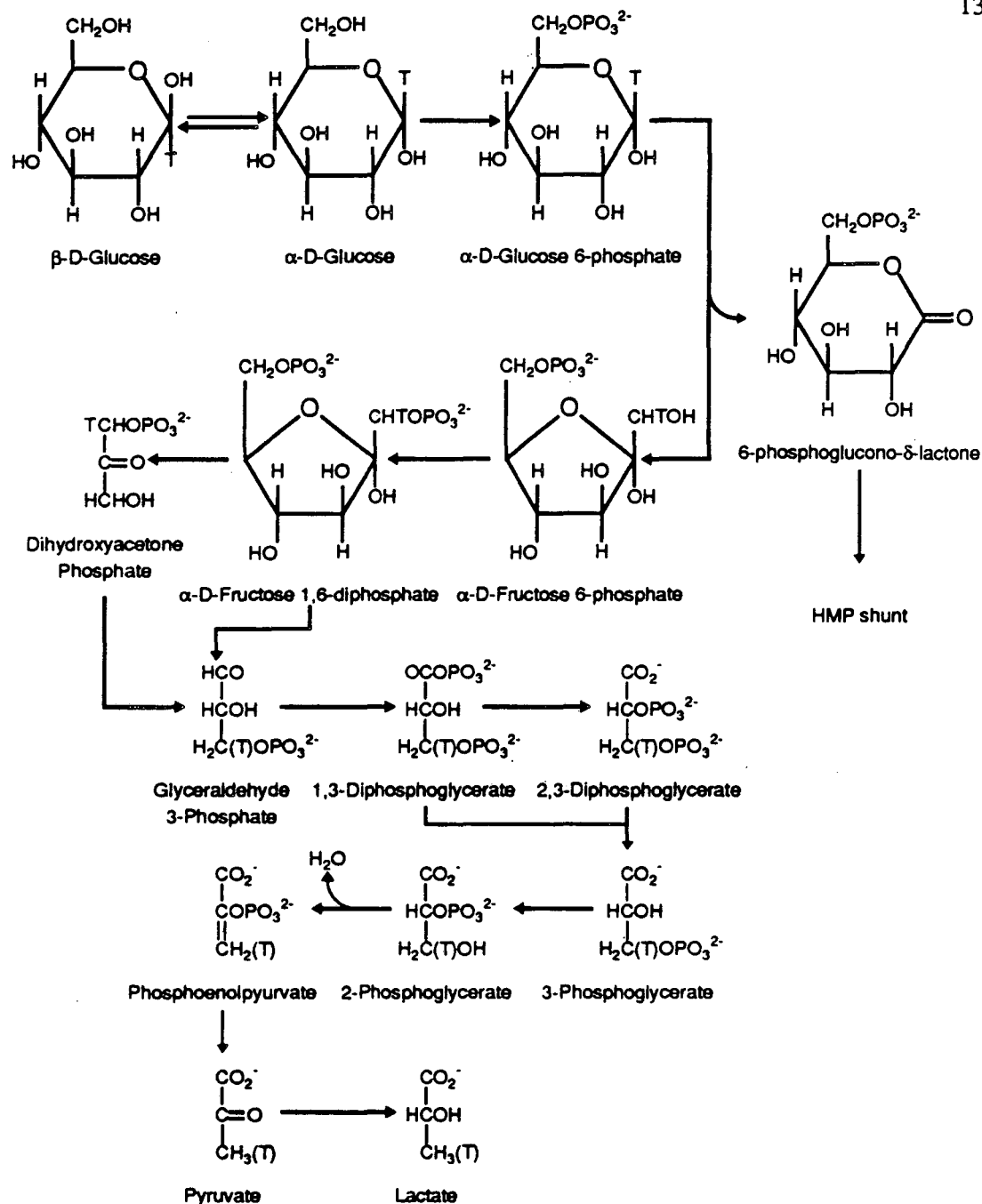


Figure 5.4. The structure of the glycolytic intermediates. Only one of the anomeric forms of each of the cyclic phosphorylated intermediates are shown. However, both do exist. The enzymes involved in the reactions are shown in Figure 1. The tritium label is lost when the labelled glucose-6-phosphate is diverted into the HMP shunt.

It is well known that increasing the pH and/or the concentration of inorganic phosphate elevates the concentration of all intermediates, (Minakawa and Yoshikawa, 1966). Furthermore, it has also been found that rat erythrocytes are capable of higher glycolytic rates than human blood cells (Kim, 1983). The availability of rat blood cells was an added advantage. Based on these observations, the more recent experiments were carried out on rat erythrocytes at higher pH (pH 8.0) and high inorganic phosphate concentration (30 mM). Furthermore, the packed blood samples were buffered at pH 8.0 and antibiotics were added (see above). As expected, the rate of reaction was much faster under these conditions. In Figure 5.5, the time course of glycolysis in packed rat erythrocytes is shown (for clarity this experiment is denoted as experiment I in future discussions). At 4.0 ppm, an intense resonance belonging to an intermediate was observed. Unfortunately at 37°C, the HOT resonance was overlapped with the β -glucose resonance at 4.6 ppm. Figure 5.6 shows the sum of spectra taken over the duration of the experiment. An additional set of peaks from 4.4 to 3.8 ppm are clearly discernable. These were due to impurities found in the original glucose solution (see Figure 5.2). During the course of experiment I, a spectrum was obtained under fully relaxed (*i.e.* intensity accurate) conditions. The difference between this spectrum and a subsequent spectrum taken under optimum signal-to-noise conditions indicated that, within the signal-to-noise, the intensities were identical and that the differences were most likely due to ongoing concentration changes due to glycolysis. In another experiment (denoted experiment II), nominally run at the same temperature, the water resonance was partially resolved as shown in Figure 5.7. We attributed this to the actual sample temperature being 2 to 3 degrees cooler than in experiment I caused by an error in the sample temperature control unit. The chemical shift of water is known to be highly temperature dependent and the slightly cooler temperatures resulted in the shift of 0.08 ppm of the HOT peak (Wemmer, 1987). During experiment II, the separation between the HOT and β -glucose peaks remained constant indicating that the temperature was constant over the course of the experiment. This experiment also differed in that 30% v/v more cells were used. The rate of glucose consumption for this experiment increased roughly 30%, indicating a linear dependence of the overall rate on amount of packed cells used in the experiment.

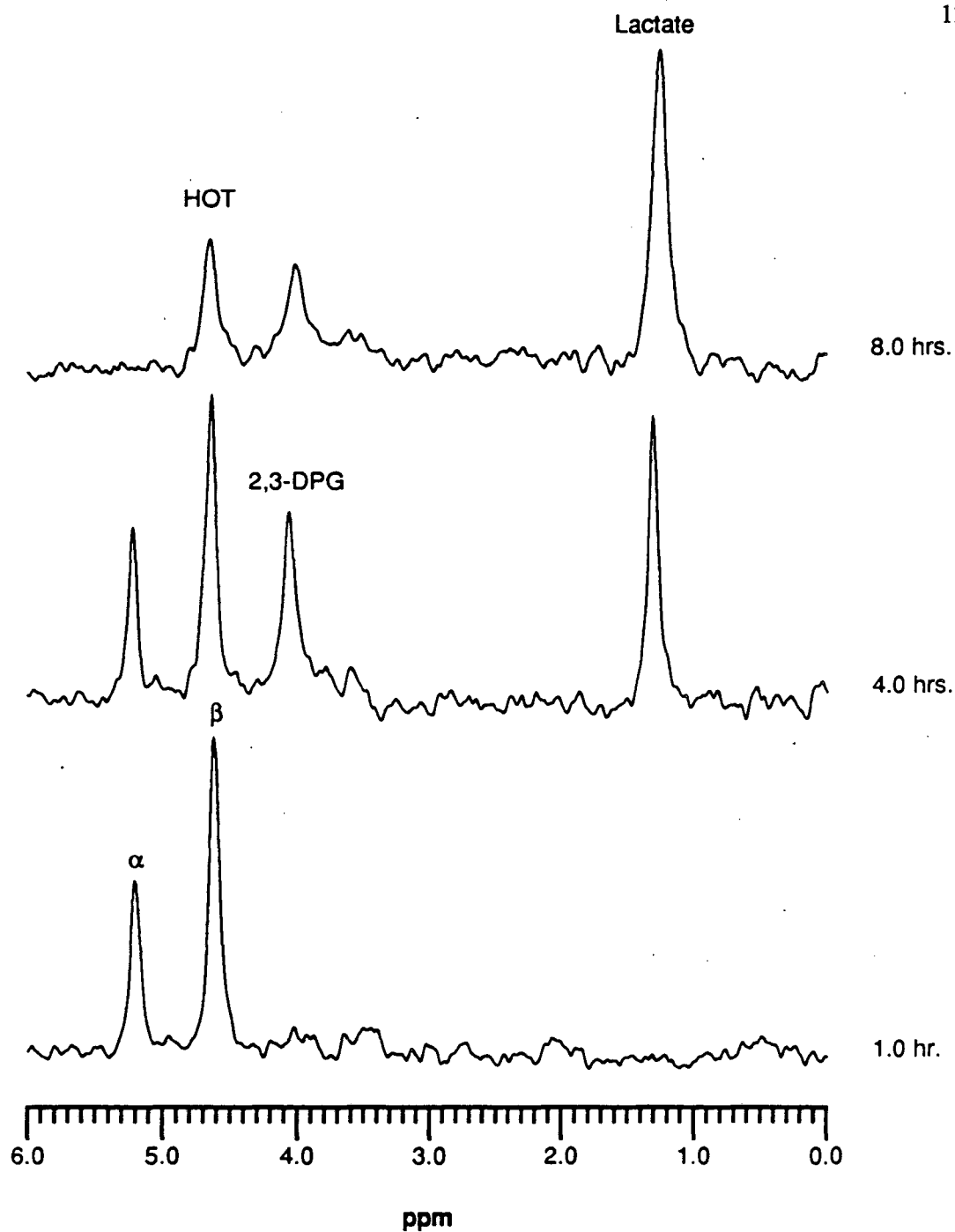


Figure 5.5. ^3H NMR spectra of glucose and glycolytic intermediates in packed rat erythrocytes. Spectra were recorded at three different times following the addition of labelled glucose to the packed cells. The production of the glycolytic intermediate 2,3-diphosphoglycerate was observed (see text for details).

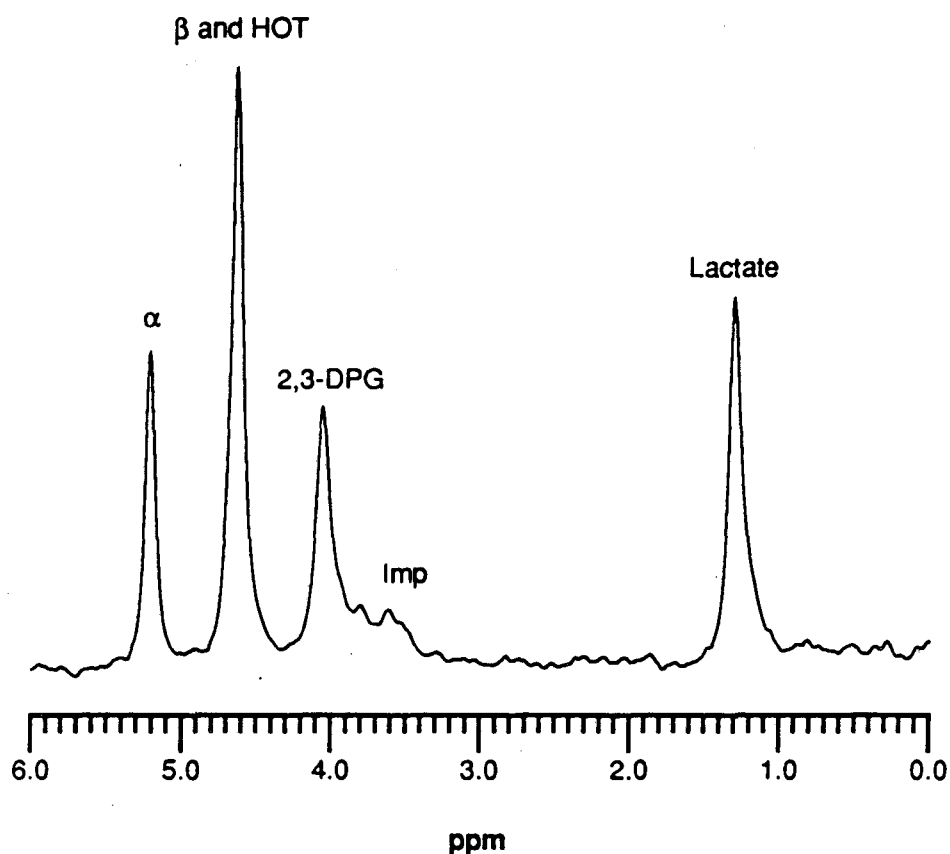


Figure 5.6. ^3H NMR spectrum of glucose and glycolytic intermediates in packed rat erythrocytes. This spectrum is the sum of individual spectra taken over a two day interval, three of which are shown in Figure 5. The resonance at 4.6 ppm is a superposition of the β -glucose and HOT. The low intensity resonances centered about 3.6 ppm are impurities which were present in the original solution containing the labelled glucose.

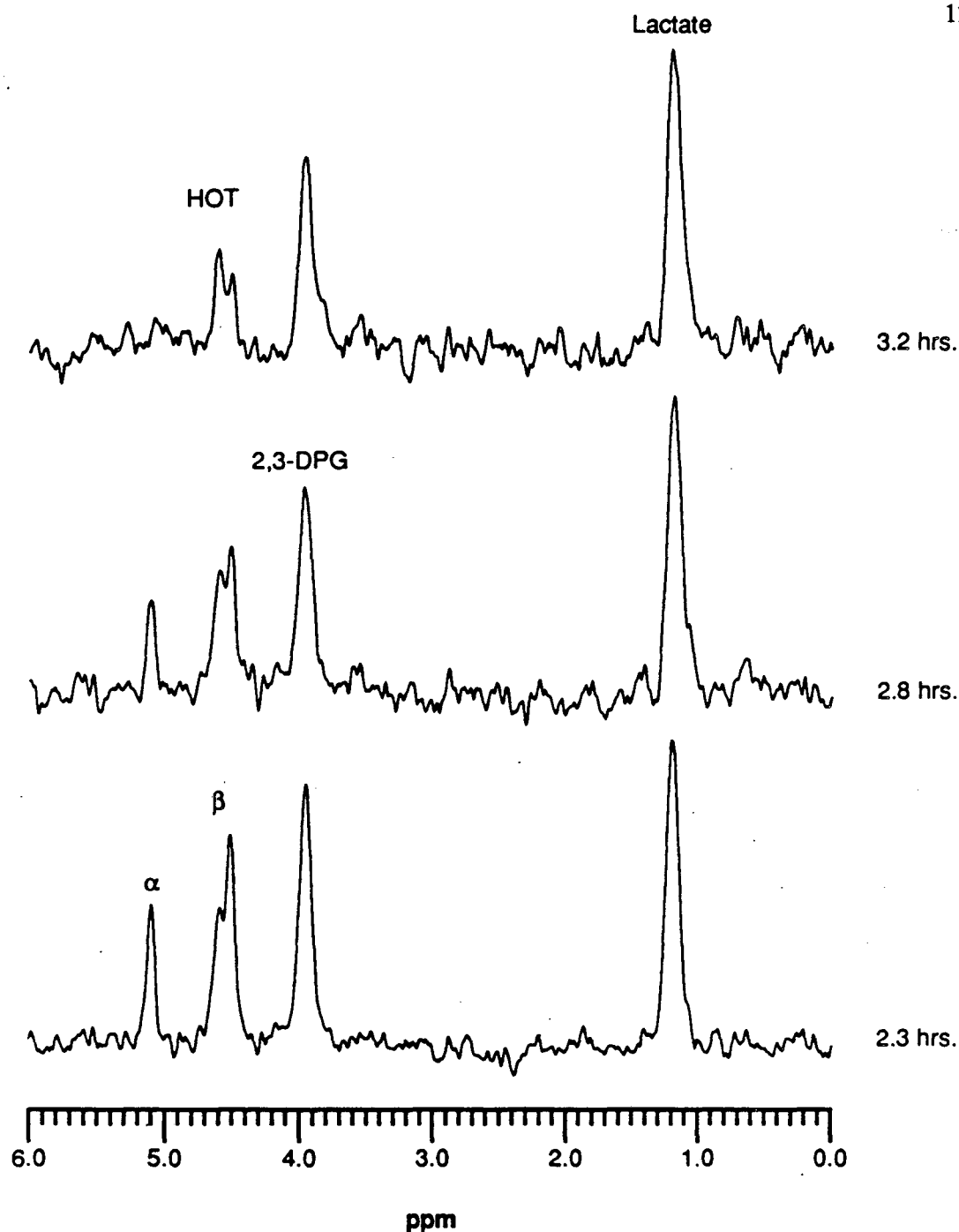


Figure 5.7. ^3H NMR spectra of glucose and glycolytic intermediates in packed rat erythrocytes. Spectra were recorded at three different times following the addition of labelled glucose to the packed cells. These spectra differ from those of Figure 5 in that the sample temperature was 2 to 3°C lower and 30% v/v more cells were used which accounted for the overall increase in the reaction rate.

The relative integrated intensities of the glucose, lactate and intermediate resonance from experiment I were plotted as a function of time. These plots are shown in Figures 5.8 , 5.9 and 5.10 . Relative integrated intensities were chosen to reduce effects of instrument instabilities and it was assumed that all of the tritium was observed, that is, no significant population was broadened to the point of being undetectable. The time dependent variations in concentrations of these species reflect the roles of each in the glycolytic pathway. The resonance at 4.05 ppm behaved like a transient intermediate increasing at the beginning at one rate and later decreasing at another. The lactate signal at 1.32 ppm was sigmoidal. The two glucose signals behaved roughly exponentially. Based upon these observations, the time course data were fitted to a set of simple rate equations (see Table 5.1 for the forms of the equations). The purpose of this was to obtain approximate rates of reaction. These rate equations were descriptive rather than based on any mechanistic considerations. There was clearly not enough data to make a rigorous determination of kinetic parameters (*e.g.* concentration dependences for Michaelis–Menton analysis). Nonetheless, the fits to the simple rate equations were reasonable. Only the initial 4 hours of the β glucose signal at 4.61 ppm were fitted since it was known from experiment II that the HOT signal, which increased with time, was unresolved from the resonance of this anomer of glucose. Table 5.1 summarizes all of the kinetic data.

IV. Discussion

A. Rates and anomeric preference

Although the analysis of kinetics were based on a rather simplistic model, they point out the basic features of the time dependences of the various tritiated compounds (*i.e.* glucose, lactate and the intermediate). As noted above the forms of the time dependences are those indicative of the roles in the enzymatic pathway of initial substrate, intermediate and end product. The kinetic parameters derived from the simple models are reasonable. The initial concentration of glucose, as determined by the least squares analysis of the intermediate and lactate data, are in rough agreement with the known initial concentration of glucose. The initial rate of glucose consumption expressed in terms of volume of packed cells used is 2.9 and 3.1 $\mu\text{mol/mL}$ of packed cells/h⁻¹ for the β and α anomer of glucose, respectively. These rates can be compared to the reported activity for hexokinase of 1.5–2.5 $\mu\text{mol/mL}$ of packed cells/h⁻¹ (Bruns, 1958).

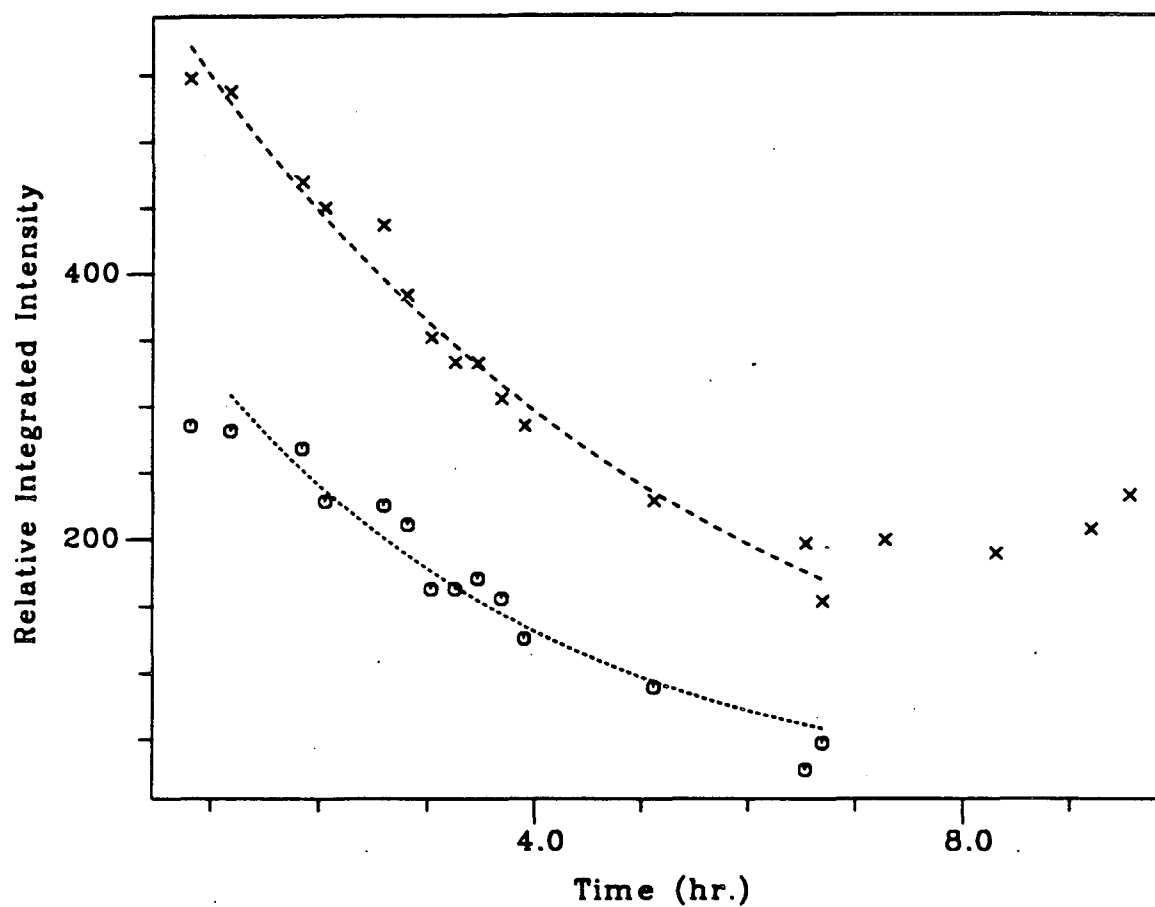


Figure 5.8. Intensities of the ^3H resonances of the α and β anomers of glucose as a function of time. The $\times$ symbols represent the β anomer and $\circ$, the α . The lines represent the non-linear least squares fit of the initial intensities. The numerical results as well as the equation used in the analysis are summarized in Table 1. The β anomer intensities level off due the contribution from HOT.

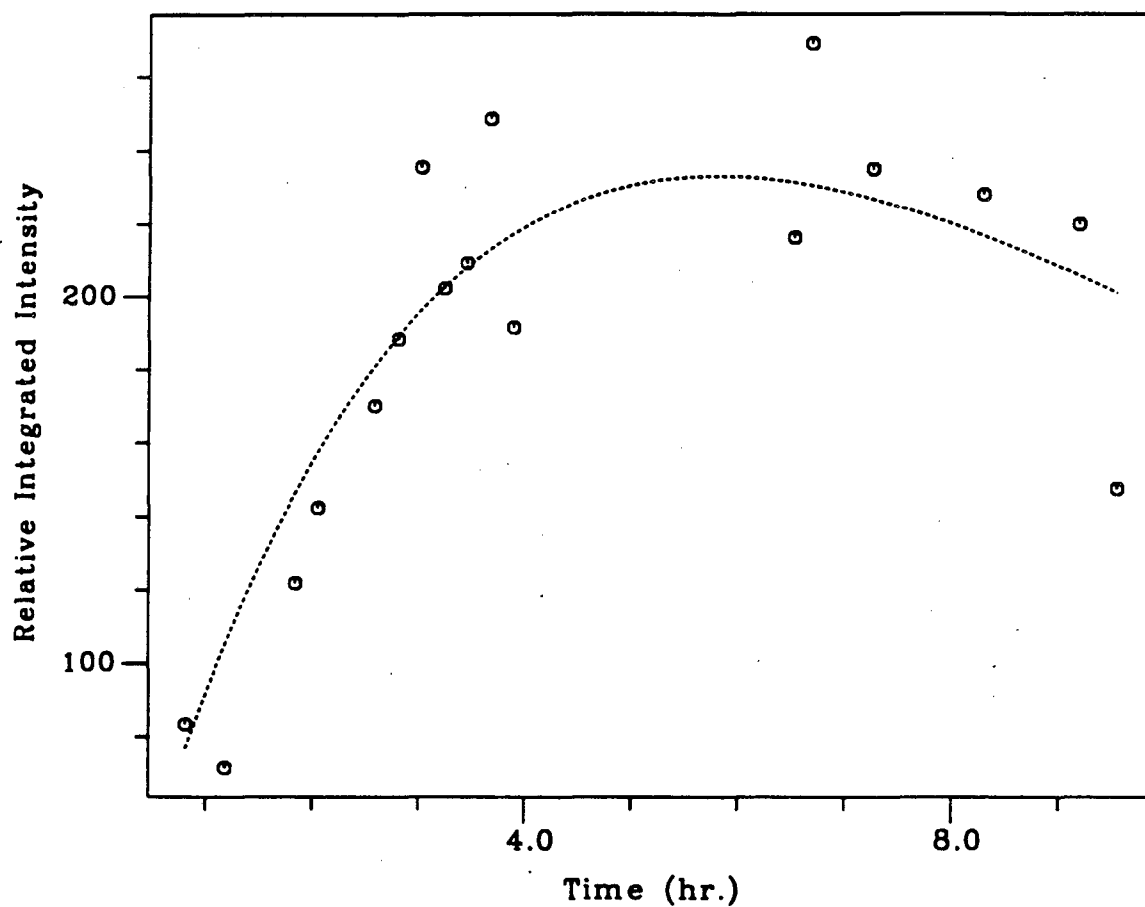


Figure 5.9. Intensity of the ^3H resonance of the intermediate, identified as 2,3-diphosphoglycerate, as a function of time. The line represents the non-linear least squares fit of the intensities. The numerical results as well as the equation used in the analysis are summarized in Table 1.

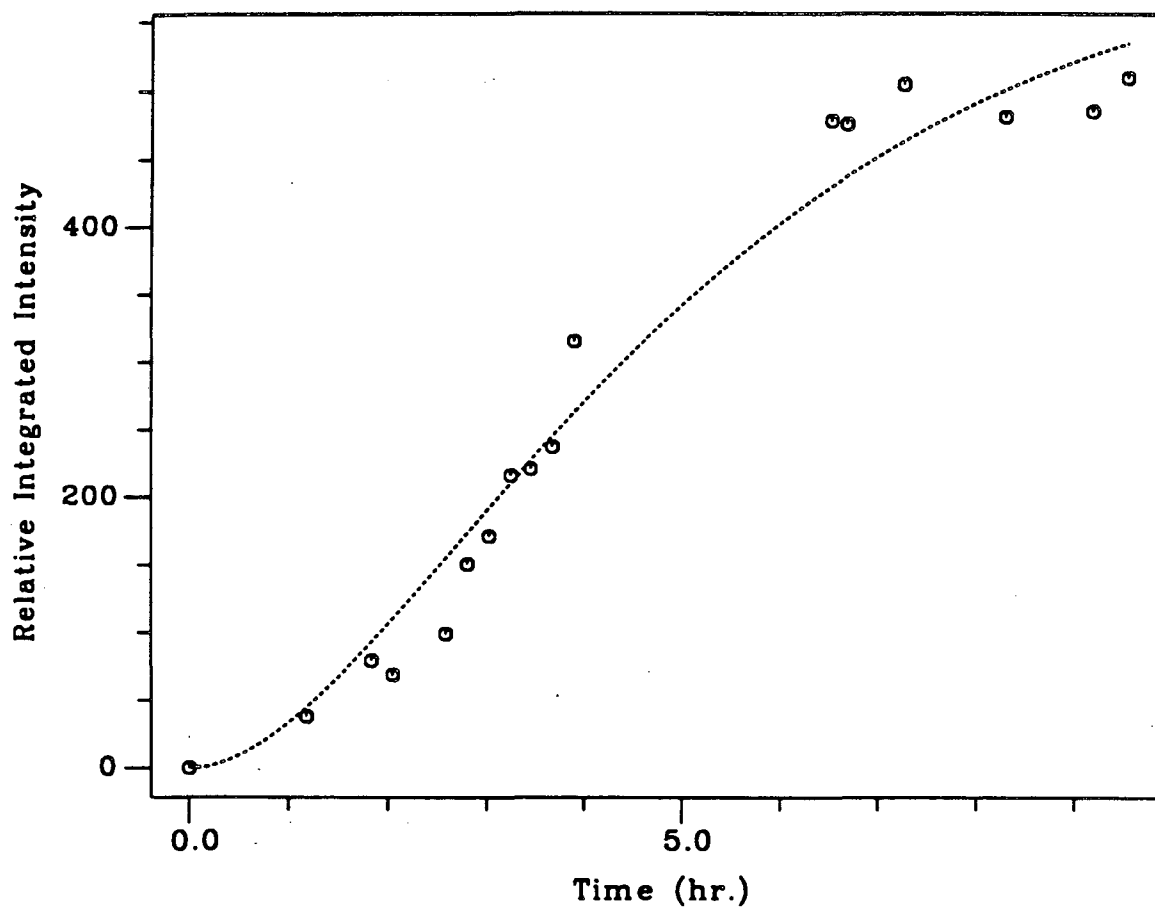
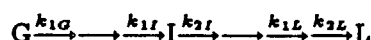


Figure 5.10. Intensity of the ^3H resonance of lactate as a function of time. The line represents the non-linear least squares fit of the intensities. The numerical results as well as the equation used in the analysis are summarized in Table 1.

Table 5.1. Kinetic parameters of rat erythrocyte glycolysis as determined by ^3H NMR. The parameters are the results of a non-linear least squares fit of data shown in Figures 8, 9 and 10 using simple rate laws described below.¹ The error in kinetic parameters are estimated to be $\pm 15\%$.

Compound	Chemical Shifts (ppm)	Kinetic Parameters ¹		
		$[\text{G}]_0(\text{mM})$	$k_1(\text{h}^{-1})$	$k_2(\text{h}^{-1})$
Glucose (α) ^{2,3,4}	5.21	4.1	0.303	—
Glucose (β) ^{2,3,4}	4.63	6.4	0.209	—
Intermediate	4.07	5.9	0.173	0.171
Lactate	1.32	5.7	0.373	0.375

1. The kinetic parameters result from a non-linear least squares fit of the data to the rate laws of the reaction



where G is glucose, I the intermediate and L lactate. The relevant equations are

$$\begin{aligned} [\text{G}] &= [\text{G}]_0 e^{-k_{1G}t} \\ [\text{I}] &= \frac{k_{1I}[\text{G}]_0}{k_{2I} - k_{1I}} \left(e^{-k_{1I}t} - e^{-k_{2I}t} \right) \\ [\text{L}] &= [\text{G}]_0 \left(1 - \frac{k_{2L}}{k_{2L} - k_{1L}} e^{-k_{1L}t} + \frac{k_{1L}}{k_{2L} - k_{1L}} e^{-k_{2L}t} \right) \end{aligned}$$

where $[\text{G}]_0$ is the initial concentration of glucose. $[\text{G}]_0$ was determined from the fit and the initial total tritium concentration of 9.4 mM.

2. The ratio of the α to β anomer at 37°C is 39.4:60.2 (Maple and Allerhand, 1987). The ratio as determined by the kinetic fits is 39:61.
3. The anomeric conversion rate is approximately 0.379 h^{-1} (Pigman, 1957).
4. The initial glucose consumption rates are 2.9 and $3.1 \mu\text{mol/mL}$ of packed cells/hr. The activity of hexokinase is 1.5–2.5 $\mu\text{mol/mL}$ of packed cells/hr (Burns, 1958).

In rat erythrocytes, the phosphorylation of glucose occurs solely through hexokinase. K_m in erythrocyte homogenates has been measured to be 0.06 mM (Malaisse, 1985). It is also known that glucose is both actively and passively (*i.e.* by diffusion) transported into the red cells (Kim, 1983). The total concentration of glucose in the experiments discussed here was about 282 mM. These observations indicated that the hexokinase was most likely saturated. Therefore, if it assumed that glucose transport in the cells and glucose-hexokinase binding is much faster than anomeric conversion, then the 42% difference between the two anomer consumption rates, as shown in Table 5.1, must be due to the anomeric specificity of the enzyme. This difference can be compared to the specificity measured for hexokinase in erythrocyte homogenates which is 60% at 8°C (Malaisse, 1985). The difference in the specificities probably reflects the difference in temperatures.

B. Identity of the intermediate

Under the conditions of high phosphate concentration and high pH, it is known that human erythrocytes build-up large amounts of fructose-1,6-diphosphate, dihydroxyacetone phosphate and 2,3-diphosphoglycerate. It has been reported that, at a phosphate concentration of 30 mM at pH 8, the human erythrocytes accumulated 1.540, 1.920 and 4.600 $\mu\text{mole/ml}$ of packed cells, of fructose-1,6-diphosphate, dihydroxyacetone phosphate and 2,3-diphosphoglycerate, respectively, after 1 hour of incubation (Table 5.2, Minakami and Yoshikawa, 1966). The concentration of lactate under these conditions was reported to be 2.500 $\mu\text{mole/ml}$ of packed cells with all other intermediates being at least an order of magnitude less concentrated (Minakami and Yoshikawa, 1966). The dependence on temperature, pH and phosphate concentration, as well as, the large build-up of 2,3-diphosphoglycerate is known to occur in most mammals (Agar and Broad, 1983). Based on integrations of the ^3H spectra obtained from rat erythrocytes in experiment I, under conditions similar to those used by Minakami and Yoshikawa (Minakami and Yoshikawa, 1966), after 2.6 hours, the concentration of the 4.07 ppm intermediate was estimated to be 4.1 $\mu\text{mole/ml}$ of packed cells and lactate, 2.4 $\mu\text{mole/ml}$ of packed cells (Table 5.3). The fact that only one intermediate was observed and that the relative concentration of this intermediate was very high strongly suggests that it was 2,3-diphosphoglycerate.

In order to clarify the situation, the 500 MHz ^1H NMR spectra of the three most abundant

Table 5.2. The concentrations of several glycolytic intermediates as determined by chemical assay.¹ The chemical shift data are from the ¹H NMR spectra of the isolated compounds (see Figure 11) and are of the C-1 position, the carbon to which the triton is bound.

Compound	Chemical Shift (ppm)	Concentration (μ mol/mL of packed cells)
Fructose-1,6-diphosphate	4.16	1.54
Dihydroxyacetone phosphate	4.43	1.92
2,3-Diphosphoglycerate	3.95	4.60
3-Phosphoglyceraldehyde	—	0.26
3-Phosphoglycerate	—	0.10
Lactate	1.32	2.50

1. From: Minakawa and Yoshikawa, 1966. Conditions: human erythrocytes, pH 8.0 and $P_i=30$ mM, 37°C, 1 hr. incubation time.

Table 5.3. The estimated concentrations of several glycolytic intermediates as determined by ^3H NMR after 2.6 hours.¹ Data are from Figures 8, 9 and 10 and are based on the initial tritiated glucose concentration of $24.4\ \mu\text{mol/mL}$ of packed cells.

Compound	Chemical Shift (ppm)	Concentration ($\mu\text{mol/mL}$ of packed cells)
α -glucose	5.21	5.5
β -glucose and HOT	4.63	10.6
Intermediate	4.07	4.1
Lactate	1.32	2.4

1. Conditions: rat erythrocytes, pH 8.0 and $P_i=30\ \text{mM}$, 37°C , 2.6 hr. incubation time.

glycolytic intermediates were obtained. The data are summarized in Table 5.3. The spectra of dihydroxyacetone phosphate (Figure 5.11) exhibited twice the number of peaks anticipated. These were attributed to the ketone (I) and ketal (II) tautomers.



The ^1H NMR data, as well as, the structures and carbon numbering schemes are summarized in Figure 5.12. Based on the work of Reynolds (Reynolds, 1971), the doublet at 4.43 ppm was identified as the proton bound to the first carbon (denoted C-1) of the ketone. For the ketal, the C-1 proton was found to resonate at 3.65 ppm. At 37°C, the ratio of the ketone to ketal forms was 62:38 which is in agreement with the results obtained by Reynolds (Reynolds, 1971) in solution. The equilibrium concentrations of these two forms in blood should not differ from those measured in solution. Based on the concentration of dihydroxyacetone phosphate measured by Minakami and Yoshikawa (Minakami and Yoshikawa, 1966), the estimated concentration of the ketal form in human erythrocytes is about 0.73 $\mu\text{mole/ml}$ of packed cells, a concentration which should have been detectable (this can be compared to the concentration of lactate which was detected after 1.2 hrs of 0.9 $\mu\text{mole/ml}$ of packed cells). This observation had two consequences: first, neither dihydroxyacetone phosphate (I) nor its ketal tautomer (II) was the intermediate which gave rise to the ^3H signal at 4.07 ppm and second, for glycolysis in rat erythrocytes, the level of dihydroxyacetone phosphate must have been less than that found in human erythrocytes.

The proton spectrum of the fructose-1,6-diphosphate was complex with the total spectrum spread over only 0.5 ppm (see Figure 5.11). The two most intense resonances at 3.93 and 4.16 ppm were assigned to the C-6 and C-1 protons, respectively (see Figure 5.12 for carbon numbering scheme). Furthermore, these resonances were probably from the major β anomer. These assignments were based on the following observations. First, as can be seen from Figure 5.12, the C-1 and C-6 positions of fructose-1,6-diphosphate have two equivalent protons; therefore, the signal arising from these protons are expected to be the most intense. The C-1

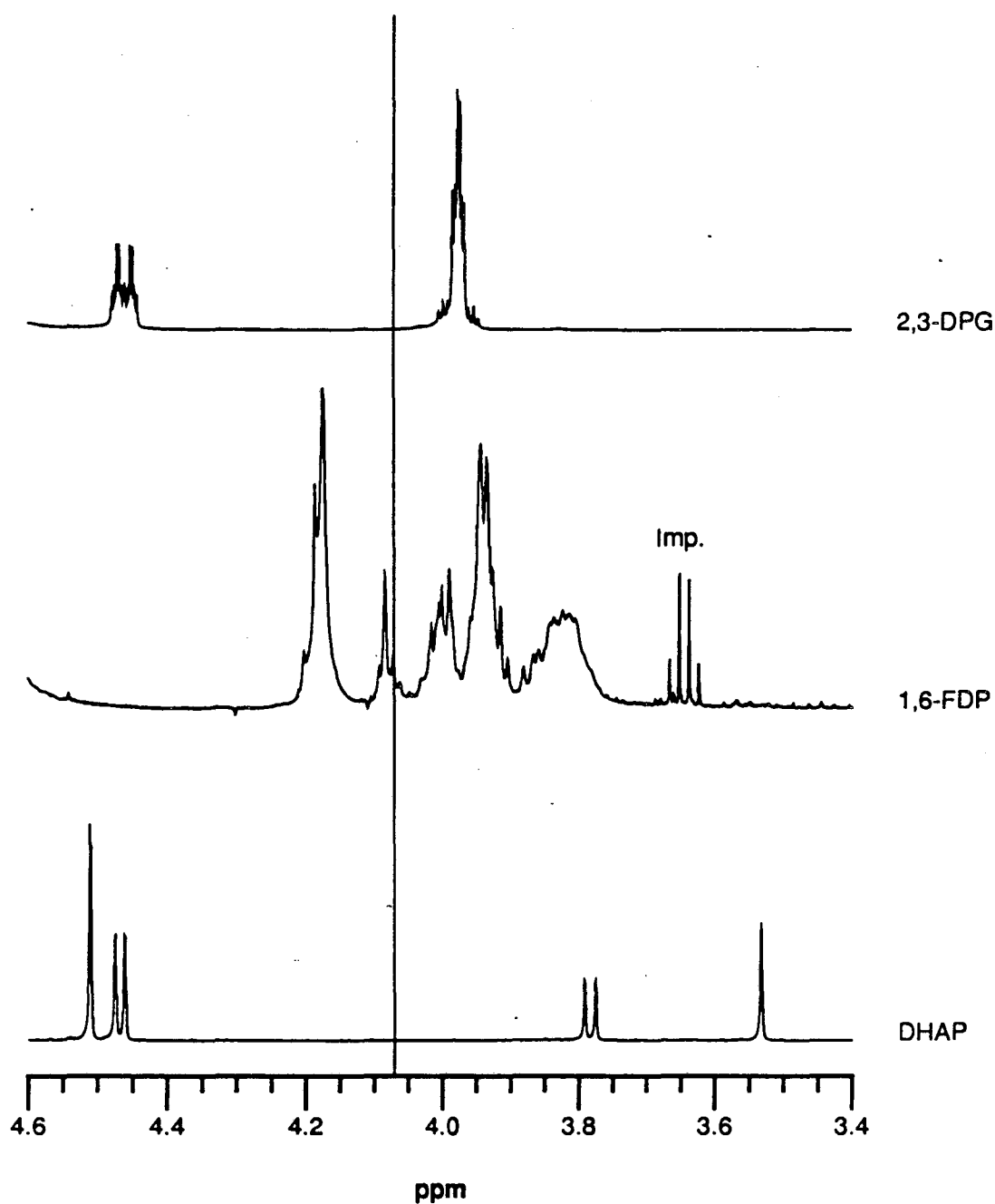
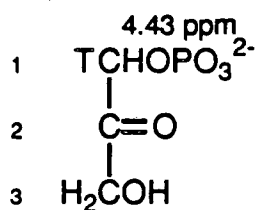
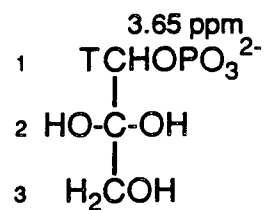


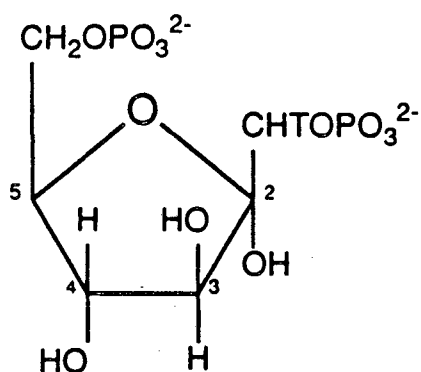
Figure 5.11. ^1H NMR spectra of three glycolytic intermediates: dihydroxyacetone phosphate (DHAP), fructose-1,6-diphosphate (1,6-FDP) and 2,3-diphosphoglycerate (2,3-DPG). The vertical line represents the ^3H chemical shift of the intermediate, identified as 2,3-diphosphoglycerate (see text for details). The fructose-1,6-diphosphate contained a contaminant denoted "Imp."



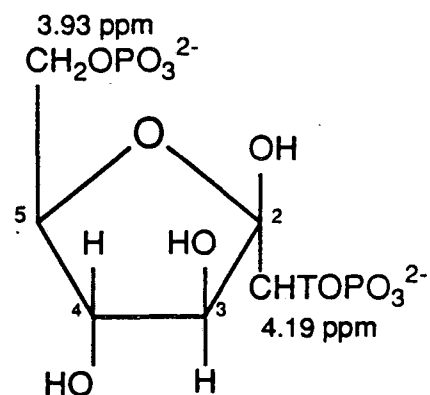
Dihydroxyacetone
Phosphate
(Ketone Form)



Dihydroxyacetone
Phosphate
(Ketal Form)



α -D-Fructose 1,6-diphosphate



β -D-Fructose 1,6-diphosphate

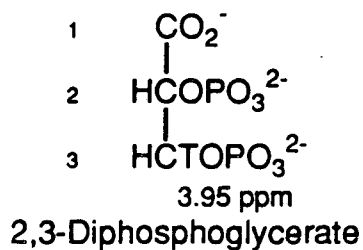


Figure 5.12. The structure of the three most abundant glycolytic intermediates and the ^1H NMR signal assignments. The corresponding spectra are shown in Figure 11. The small numbers, without units, denote the carbon numbering scheme used in the text.

protons are expected to be more downfield than the C-6 protons because of the electron-withdrawing OH group on the adjacent carbon (C-2). The anomeric ratio of the β to α forms of fructose-1,6-diphosphate was determined by Koerner and coworkers (Koerner, 1980) to be 77:23. Therefore, the β resonances should be more intense. The minor anomeric form could not be clearly identified. The resonance at 4.08 ppm is mostly likely one of the two more intense resonances of this anomer. Finally, the 2,3-diphosphoglycerate spectrum exhibited resonances centered at 3.95 and 4.44 ppm (Figure 5.11). Based on integrated intensities the 3.95 ppm upfield signal was assigned to the C-3 protons (see Figure 5.12).

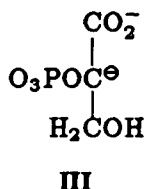
Based on the ^1H NMR observations, it was not possible to assign the signal from the intermediate at 4.07 ppm to either of the two known intermediates, fructose-1,6-diphosphate and 2,3-diphosphoglycerate. Although, the α -fructose-1,6-diphosphate resonance at 4.08 ppm was very close, this would have meant that this anomer was the dominant form in blood. This did not seem likely. Several factors complicate the identification of the 4.07 ppm signal. The first was the tritium isotope effect. For tritons bound to sp^3 carbons, which is the case in fructose-1,6-diphosphate and 2,3-diphosphoglycerate, the tritium isotope effect is known to be as large as 0.1 ppm and on the average 0.03 ppm with the triton resonating upfield from the calculate shift based on the proton chemical shift (Al-Rawi, 1974). The difference between the chemical shift of the observed intermediate in the tritium spectrum (position of the vertical line in Figure 5.11) and the 4.16 ppm resonance of fructose-1,6-diphosphate is about 0.08 ppm. Although this difference is larger than average isotope shift, it is certainly not unreasonable. The second source of complication is that 2,3-diphosphoglycerate is known to bind to deoxyhemoglobin (Marshall, 1977). This interaction causes a magnetic susceptibility shift of the ^{31}P resonance of 2,3-diphosphoglycerate in intact blood cells of about 0.08 to 0.16 ppm downfield at pH 7.2 (Marshall, 1977). This susceptibility induced shift should be about the same size in ppm units for the protons and tritons. These observations tend to suggest that it is the 2,3-diphosphoglycerate which is observed in the ^3H spectrum. The ^1H NMR spectra of these intermediates do not provide much information about the glycolytic intermediate which gives rise to the 4.07 ppm ^3H NMR signal. Given the results of biochemical analysis discussed above, it seems more likely that the intermediate is 2,3-diphosphoglycerate. If this is the case,

given the relatively broad lines, small contributions from the fructose-1,6-diphosphate could be obscured by the 2,3-diphosphoglycerate. A more interesting possibility is that the 4.07 ppm ^3H signal does come from fructose-1,6-diphosphate. This would mean that rat erythrocytes did not build-up 2,3-diphosphoglycerate. This implies that hemoglobin in rats may be different from other mammals. Hemoglobin from cats and sheep are known not to respond to 2,3-diphosphoglycerate and in these animals much lower levels of 2,3-diphosphoglycerate are found compared to most mammals (for a biochemical review see Isaacks and Harkness, 1983). It is clear that further NMR studies need be to carried out. The effects of 2,3-diphosphoglycerate binding to rat hemoglobin under varying states of oxygenation need to be studied in more detail in order to determine the susceptibility induced changes in tritium chemicals shifts in the intact erythrocytes.

C. Water and significance of water-lactate ratio

The hexose monophosphate (HMP) shunt provides one unequivocal source of exchangeable tritium. As can be seen in Figure 5.4, this pathway diverts glucose-6-phosphate from the glycolytic pathway. The first reaction of this pathway oxidizes glucose-6-phosphate to 6-phosphoglucono- δ -lactone with concomitant reduction of NADP. This reaction causes the immediate loss of the C-1 position tritium label. Therefore, the relative amount of exchangeable tritium must minimally reflect the amount of glucose which is diverted to the HMP pathway. Depending on the conditions, about 2 to 11% of total glucose goes through this pathway (Tsubio and Fukunaga, 1965; Murphy, 1960). The ratio of the water to the lactate is about 30:70 in the rat blood samples. This clearly indicates that a considerable amount of tritium is released at some other point in the glycolytic pathway. Simon and coworker have carried HOT exchange studies for a variety of isolated enzymatic reactions (Simon, 1968). Of those reported, the glucose-6-phosphate to fructose-6-phosphate catalyzed by phosphoglucisomerase and the conversion of phosphoenolpyruvate to pyruvate by pyruvate kinase are potential candidates for release of the tritium label, as well as the enolase catalyzed elimination reaction of 2-phosphoglycerate to phosphoenolpyruvate (see Figures 5.1 and 5.4). It is assumed that although other reactions allow exchange of protons (tritons) of the intermediates with the medium, these three in particular involve the labilization of the tritium label. Based on the mechanistic studies by Stubbe

and Westhead (Stubbe and Westhead, 1980) and Dinovo and Boyer (Dinovo and Boyer, 1971), it seemed unlikely that the tritium label on the 3-position of 2-phosphoglycerate would be lost to the medium. The mechanism involves hydrogen abstraction by the enzyme to form a carbanion (III) intermediate which proceeds to product by loss of the hydroxyl group.



There is no reason to believe that the label would scramble under these circumstances. It is known from the work of Topper (Topper, 1957) that in the presence of phosphoglucose isomerase the C-1 position label of the glucose-6-phosphate is not exchanged with the media. However, in the presence of both the phosphoglucose and phosphomannose isomerases exchange does occur at 26°C and pH 6.8. The original work by Gottschalk (Gottschalk, 1947) indicated that the phosphomannose undergoes thermal inactivation between 28°C and 30°C which suggest that the loss of labels under the influence of this enzyme at 37°C may be small in the rat erythrocytes. Finally, the pyruvate kinase reaction is known to exchange the C-1 proton. Robinson and Rose (Robinson and Rose, 1972) have studied the loss of the label from the phosphoenolpyruvate during conversion to pyruvate (last two reactions of Figure 5.4). At pH 8.9, 18% of the tritium was lost to the medium. High pH was cited as one of the factors which enhanced detritiation. In the presence of Mg^{+2} ions, the ratio of rate of label loss to rate of conversion to pyruvate was almost a factor of 9 times greater at pH 8.8 than 7.3. If the isolated enzymatic reactions are indicative of what happens in the cells, then the total detritiation due to the HMP shunt combined with the pyruvate kinase reaction, which ranges from 18 to 30%, can account for all of the exchangeable tritons which were observed.

V. Conclusion

One of the purposes for these experiments was to show that ^3H NMR can be a valuable complement to the use of other nuclei in the non-invasive application of NMR to biological problems. Clearly, the present application demonstrates this point. Several interesting features of erythrocyte glycolysis were observed. We were able to observe the *in vivo* glucose anomeric

specificity of the enzyme pathway. Study of isolated enzymes using ^3H NMR could lead to a more detail knowledge about the role of anomeric specificity in glycolysis. We were able to measure a build-up of an intermediate the identity of which is still unresolved. For the first time, a direct measure of the amount of exchangeable proton/triton produced in glycolysis has been carried out and these measurements are consistent with studies made on isolated enzymes. Present experiments indicate that relatively subtle effects such as oxygenation levels may be investigated by ^3H NMR. In principle, measurements of this type could be made in a number of different biological systems.

Of course, much of the success in these and other experiments are strongly dependent on the availability of tritiated probe molecules and safe and effective means of carrying out the spectroscopic investigation. The question of availability of specifically tritiated probes may be somewhat problematic. These chemical problems were largely solved by the National Tritium Labelling Facility, the speciality of which is the labelling of probe molecules. The safety and instrumental aspects of NMR applications has been clearly demonstrated by the group at Surrey (Bloxsidge and Elvidge, 1983) and by us. We have adopted a simple protective teflon insert which works well, although with loss of resolution. This problem needs to be addressed further and it is anticipated that the situation can be improved without too much loss of convenience or ease-of-use which the teflon inserts afford. Safety considerations aside, the experiments just described above demonstrate that ^3H NMR can be effectively and safely used in application to biological problems.

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Appendices

Appendix A. Saturation of MASS sidebands

Appendix B. Derivation of MASS Hamiltonian

Appendix C. The Intensity of MASS sidebands

Appendix D. Powder Averaging – A Random Approach

Appendix A. Saturation of MASS sidebands

The motivation for this study came from the observation that the broad component in the kidney ^1H MASS spectra had a significant ± 2 sideband contribution while the sharp resonances contributed very little at these sidebands; hence, based on rather simplistic considerations, it seemed possible to selectively saturate the broader component by irradiating its second order sideband before the acquisition of the spectrum. The rationale was that a given spin packet contributed intensity to several sidebands during a rotor cycle. Saturation of a sideband over many rotor cycles would saturate all the spin packets which contributed intensity to that sideband and, furthermore, partially or completely saturate all the other sidebands to which the saturated spin packets contributed intensity. This situation is somewhat analogous to that encountered in a saturation transfer experiment.

We started these experiments by first examining the ^1H spectra of muscle tissue which had no broad component. Low power, approximately 5 to 50 mw, was applied to the sample using a directional coupler. We irradiated both of the first order sidebands first individually and then simultaneously. This latter experiment was carried out by modulating the irradiation frequency (*i.e.* carrier frequency) by the tachometer output signal using a double balanced RF mixer. The tachometer output signal was a square wave, the frequency of which was equal to the sample spinning frequency. The tachometer signal was band-pass filtered about the rotor frequency to obtain a single frequency sinusoidal waveform. The RF carrier and tachometer signals were mixed to obtain the modulated saturation signal, the two frequency components of which were the sum and the difference of the carrier and tachometer frequencies. The manufacturer's specification for the isolation (*i.e.* suppression of the carrier at the output) of the double balanced mixer, an HP-10514A, was greater than 40 db. This insured that none of the centerband (carrier frequency) region was irradiated. This method also insured equal irradiation of the both sidebands. Figure A.1 shows the effect a single frequency saturation experiment on a stationary sample. It was a classic demonstration of a hole-burning experiment of an inhomogeneously broadened line. Figures A.2 and A.3 show the non-irradiated control, the single and double frequency irradiation of a spinning muscle sample. Only one of the pair of first order sidebands was saturated in the single frequency irradiation experiments. This

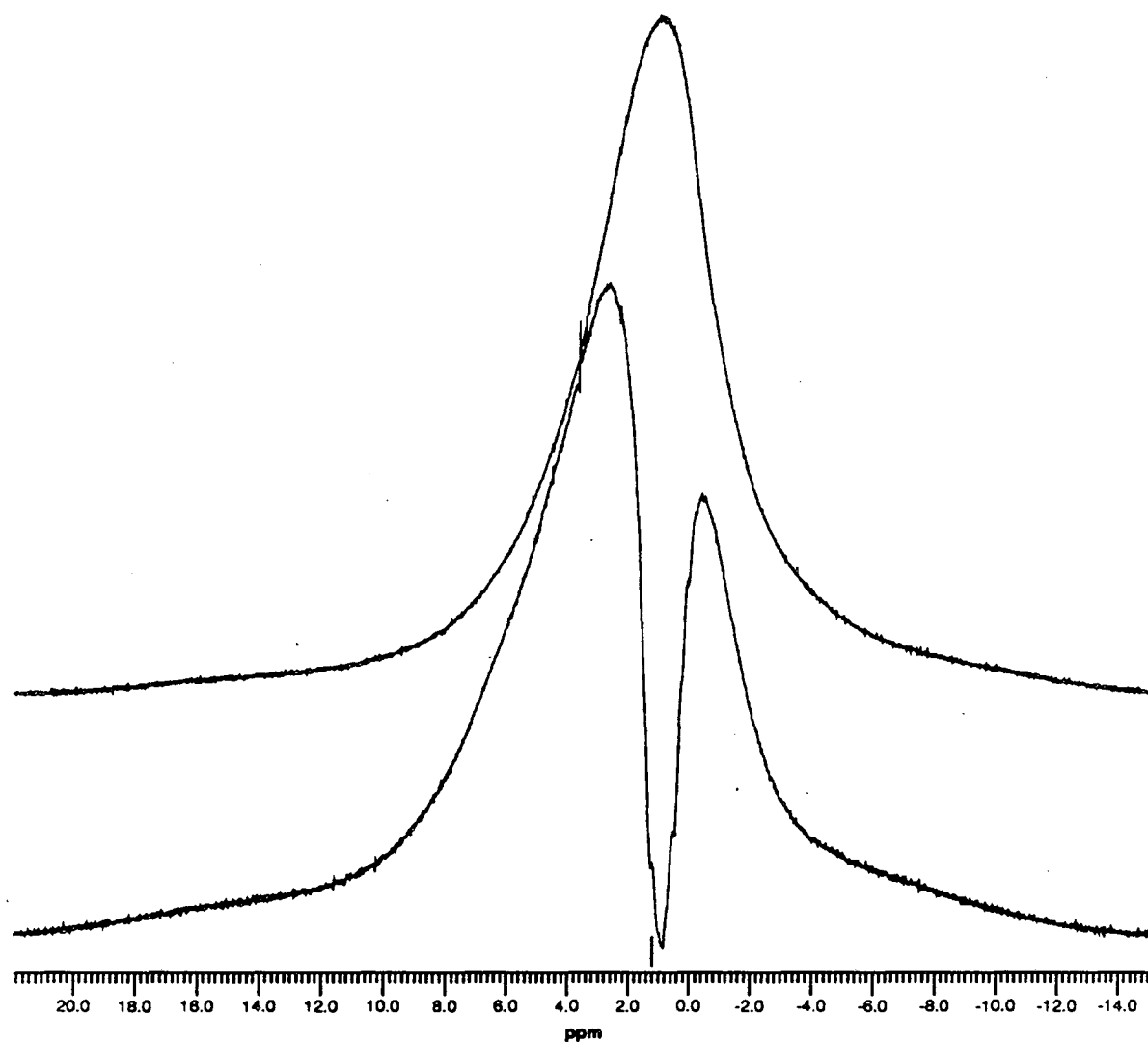


Figure A.1. The ^1H NMR spectra showing the effect of saturating a part of the static spectrum of lyophilized rat muscle. The top spectrum is the control where no saturating power was applied and the bottom one shows the effect of saturating a part of the spectrum at a frequency indicated by the small tick mark which is above the scale. The spectrum is a classic demonstration of a hole-burning experiment on an inhomogeneously broaden resonance.

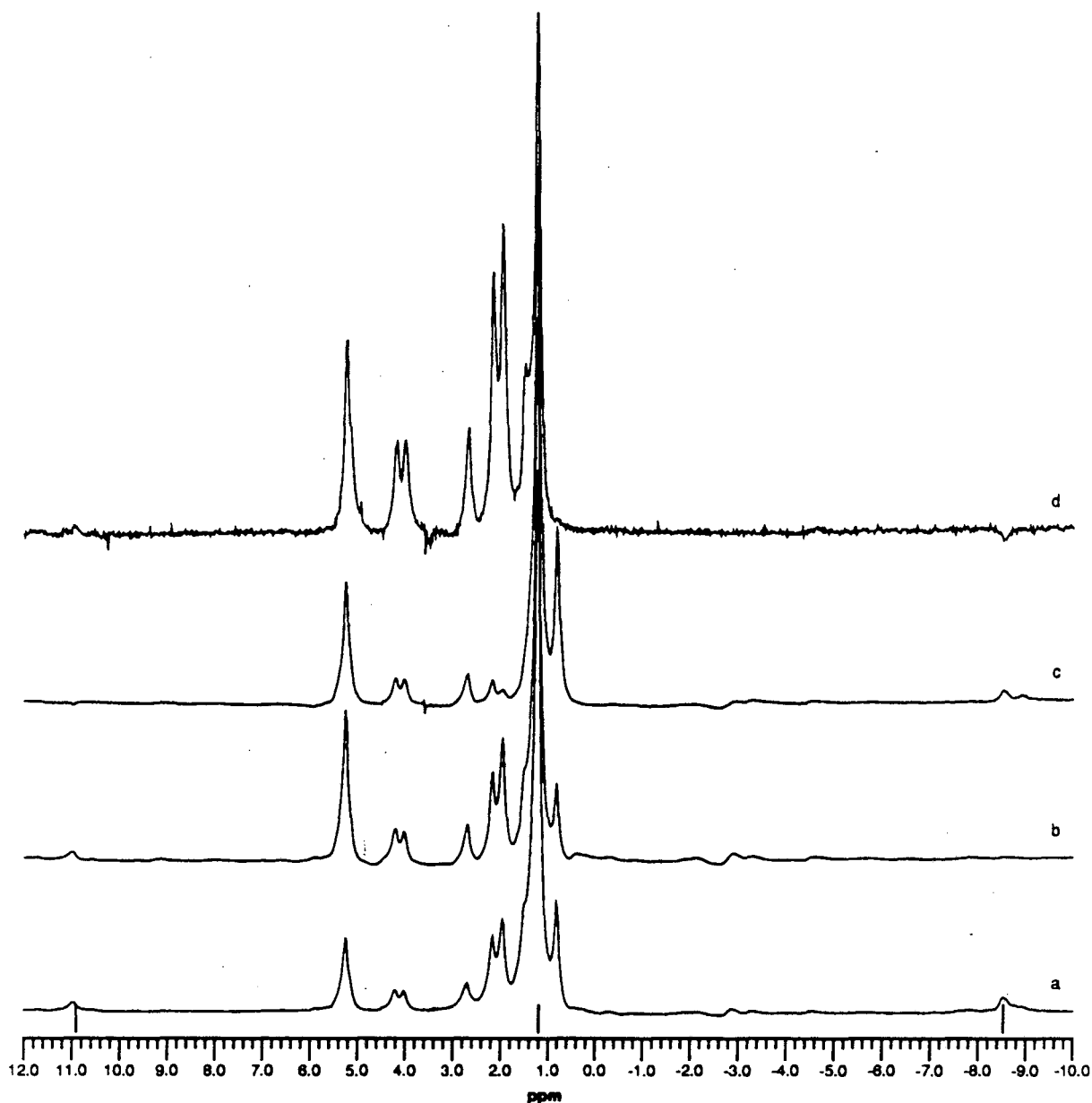


Figure A.2. The ^1H NMR spectra showing the effects of saturating the first order sidebands of a MASS spectrum of lyophilized rat muscle. Spectrum (a) is the control where no saturating power was applied; (b) shows the effect of saturating the upfield sideband; (c) the downfield sideband and (d) both sidebands simultaneously. The two small tic marks at 10.9 and -8.5 ppm indicate the frequencies at which the saturating power was applied. The tic mark at 0.2 ppm is the position of the corresponding isotropic signal, the sidebands of which were saturated. This is also the frequency of the carrier signal arising from the modulation scheme used in irradiating both sidebands simultaneously. The vertical scaling is different in each spectra.

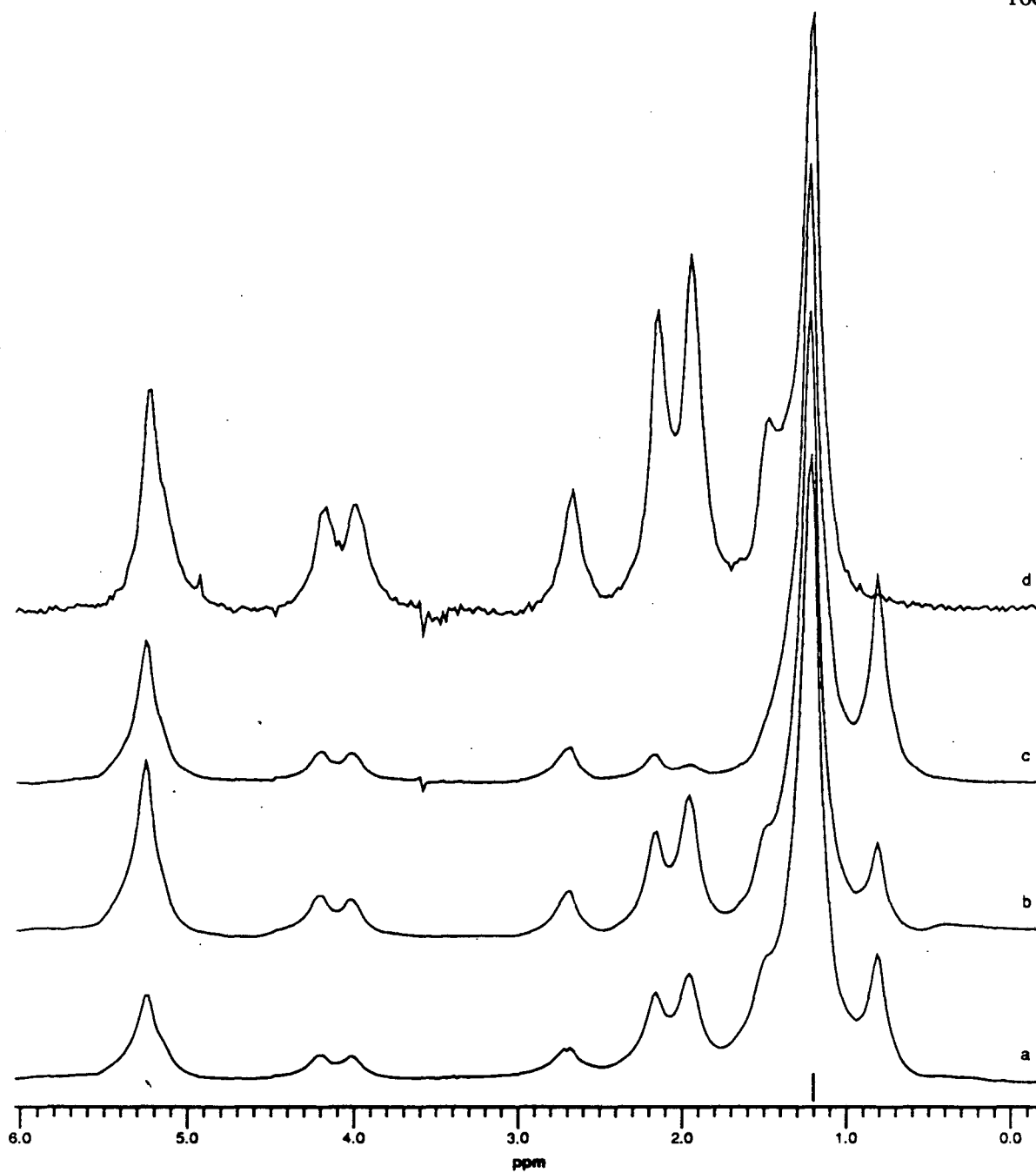


Figure A.3. The isotropic regions of the spectra shown in Figure A.2. The resonance at -0.8 ppm is lost when both of the first order sidebands are saturated. The relative intensities of the isotropic resonances vary in a complex manner which is not yet understood. The order of the spectra is the same as in Figure A.2.

attributed to the fact that only one of the two allowed transitions of the dipolar system was saturated. When both first order sidebands were irradiated, both sidebands became saturated with noticeable loss of intensity at the centerband (see Figure A.2). The peak at 0.9 ppm is lost during the double frequency saturation. The loss of centerband intensity was attributed to two causes: first, all spin packets contribute some amount of intensity to the centerband; therefore, partial saturation of the centerband is to be expected. Second, a finite amount of power at the carrier frequency centered is also expected from imperfect modulation. All frequency components of the irradiation will have a finite frequency coverage, as evidenced by the saturation of the signal at 0.9 ppm when the modulated saturation signal centered at 1.2 ppm. Lower saturation powers were not obtainable due to the non-linearity of the transmitter amplifier of the spectrometer. The relative intensities of all the other signals also diminished. This could not be readily accounted for and has yet to be investigated. These variations in intensities may be indicative of spin diffusion processes.

We applied this technique to a kidney sample with the expectation that the broad component observed in the spectra of this tissue could be preferentially saturated leaving the narrow components behind. The second order sidebands for these narrow components were much less than 1% of the corresponding centerband intensities. Figure A.4 shows the control and saturation experiments in which the second order sidebands were irradiated. The broad feature in the spectrum was suppressed. However, there was a significant loss of intensity for all resonances especially near the carrier frequency at which the modulated saturating power was applied. The greatest relative intensity loss was at the carrier frequency. Some of the intensity loss was certainly due to the presence of finite second order sideband intensity of the narrow resonances. Figure A.5 is the vertical expansion of Figure A.3 and it clearly shows the complete saturation of the broad feature.

These observations on the saturation of MASS sidebands which were to be due to dipolar broadening led us to repeat the same experiment on ^{31}P MASS sidebands which result from chemical shift anisotropy. The compound we chose was monocalcium phosphate monohydrate. This compound has two distinct phosphate groups in the solid state. From previous studies by Rothwell (Rothwell, 1980) and us, the chemical shift tensors were known to be different (see

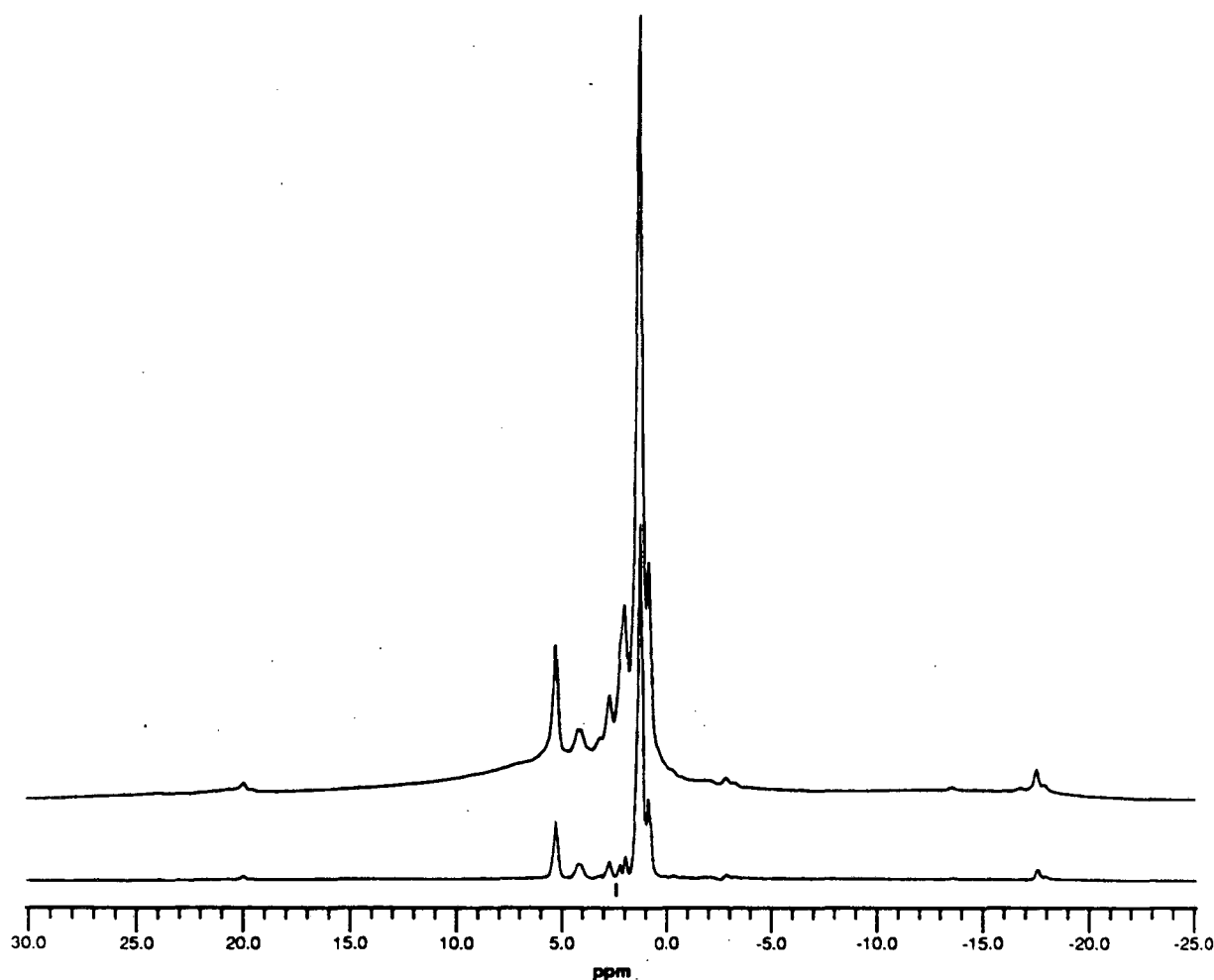


Figure A.4. The ^1H NMR spectra showing the effect of saturating both of the second order sidebands of the broad component in spectrum of a lyophilized rat kidney. The top spectrum is the control where no saturating power was applied and the bottom one shows the effect of saturating the sidebands. The small tic mark which is above the scale indicates the frequency of the carrier signal arising from the modulation scheme used in irradiating both sidebands simultaneously. The resonances near to this carrier frequency have lost more relative intensity than those which are further away (*e.g.* compare signals at 5.5 ppm and 2.0 ppm). The spectra have been plotted on a common intensity scale.

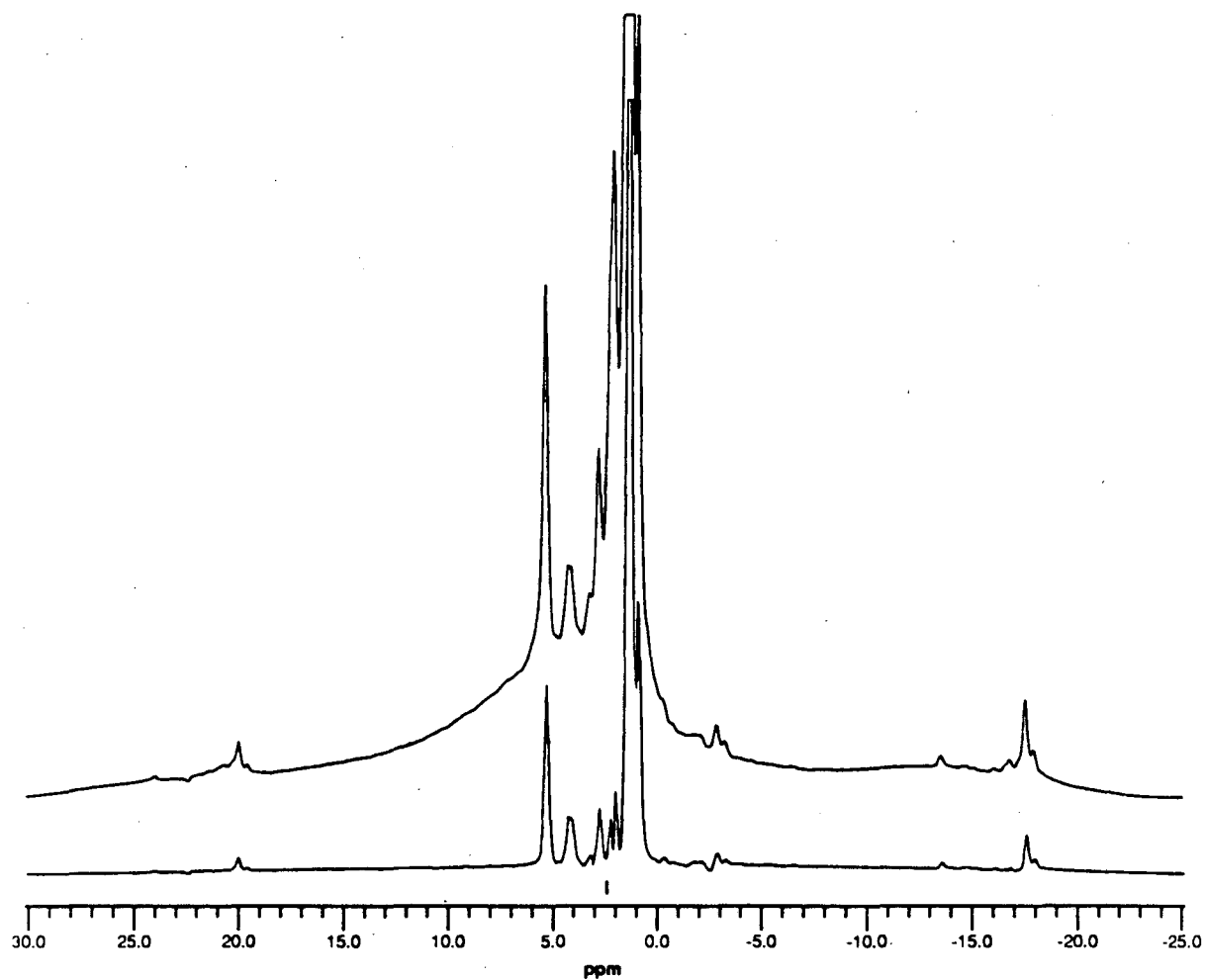


Figure A.5. A vertical expansion of Figure A.4. The saturation of the broad component can be clearly seen.

table in Chapter 3) with one phosphate group having a greater anisotropy of about 27 ppm; for clarity this group will be referred to as I and the other II. When the upfield second order sideband of I was irradiated (see Figure A.6), all signals of I became saturated. This was in contrast to the ^1H case, discussed above, where the downfield sidebands did not saturate when the upfield sideband was irradiated. This may be due to fact that the chemical shift case is a two-level system while the dipolar is a three-level one (see Figure 1 of Chapter 1). What was very surprising was the non-irradiated signals arising from II also lost intensity. A control experiment was carried out to verify that the lost of signals from II were not due to any off-resonance component of the saturating irradiation. A saturating signal was applied at a frequency, the offset of which was equal to the separation of the second order sidebands of I and II, but on the opposite side of the sideband of II (see Figure 6). We assumed that the off-resonance effects on II would be same in both cases. A small amount of intensity was lost. This was attributed to the off-resonance component of the irradiation signal. The large amount of intensity loss in the sidebands of II when the second order sideband of I was saturated was attributed to spin diffusion. When the second order sideband of II was irradiated, the sidebands of II were saturated with loss of intensity in the sidebands of I.

Maricq and Waugh (Maricq and Waugh, 1979) have pointed out that under magical angle spinning, the strength of the homonuclear dipole interaction in the presence of a chemical shift interaction scales as $\delta\sigma/\omega_r$, where $\delta\sigma$ is the instantaneous chemical shift difference. Based on the isotropic shift difference of about 73 Hz, a spinning speed of 2 kHz and a separation of 4.6 Å between the two phosphate groups (based on the x-ray structure, Dickens and Bowen, 1971), the residual dipolar coupling would be approximately 10 Hz. The instantaneous shift difference could be larger than the isotropic difference and the residual dipolar interaction could be larger. Nevertheless, residual amount of dipolar interaction must exist and provides the mechanism for spin diffusion which is observed. Further studies are in order.

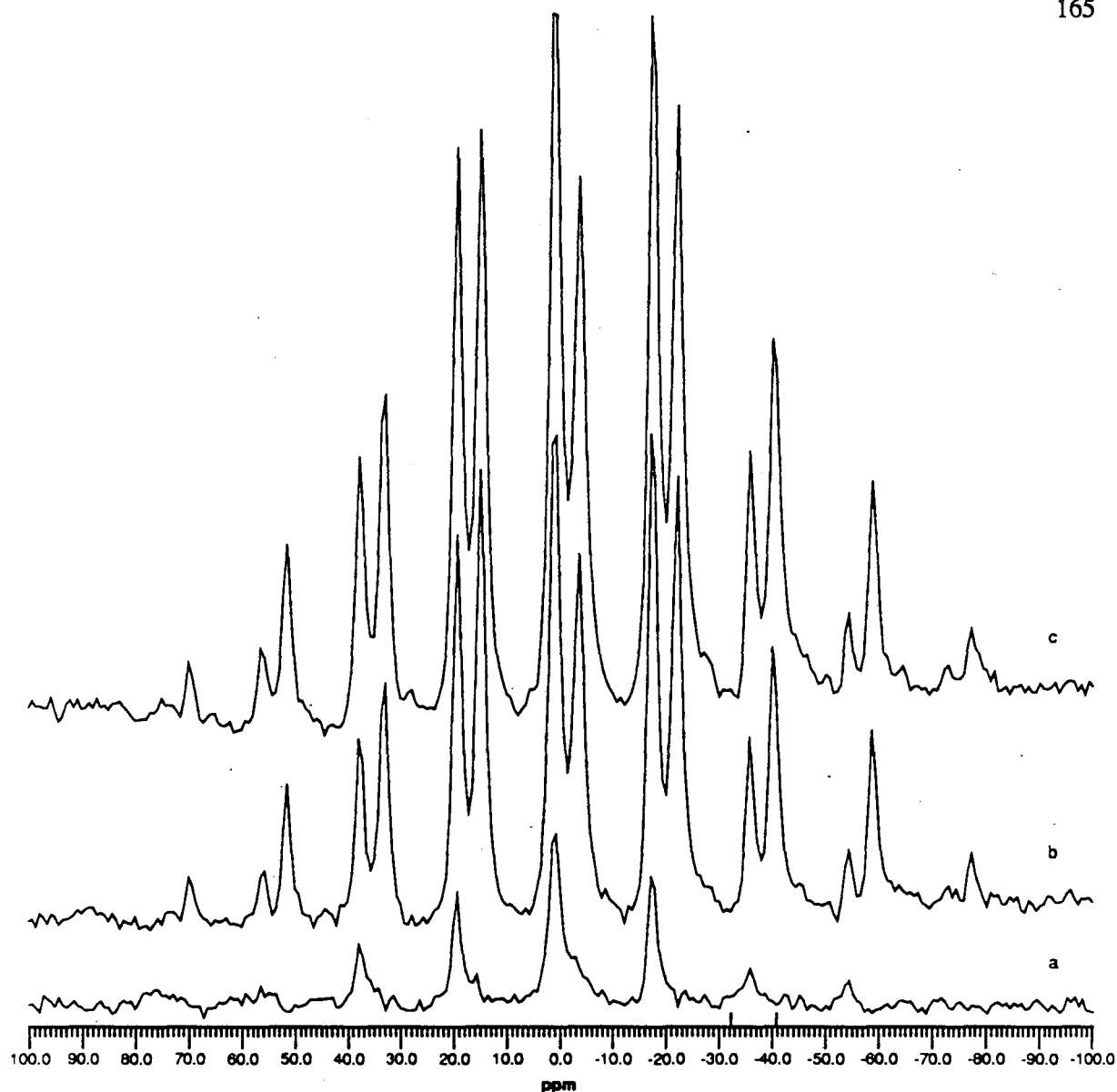


Figure A.6. The ^{31}P NMR spectrum of monocalcium phosphate monohydrate showing the effects of saturating the upfield third order sideband of the upfield component. Spectrum (a) shows the effect of saturating the third order sideband at -41.0 ppm, indicated by the tic mark. The upfield component (designated I in the text) has been entirely saturated and the downfield component (designated II in the text) has been partially saturated. Spectrum (b) shows the effect of applying the same saturating irradiation as in (a), except at -32.5 ppm, indicated by the upfield tic mark. The upfield third order sideband of II is equidistant to both irradiation frequencies. The effect of this irradiation is small compared to the on-resonance irradiation in (a). Spectrum (c) is the control where no saturating power was applied. The spectra have been plotted to a common intensity scale.

Appendix B. Derivation of MASS Hamiltonian

In general a spin Hamiltonian may be expressed as the tensor equation

$$H = \mathbf{X} \cdot \hat{\mathbf{A}} \cdot \mathbf{I} \quad B.1$$

where $\mathbf{I}$ is spin vector operator, $\hat{\mathbf{A}}$ is a symmetric second order Cartesian tensor relating the spacial dependence of the interaction. $\mathbf{X}$ can be a variety of things depending on the specific interaction: for example it may be the static magnetic field vector in the case chemical shift interaction or the spin vector operator of a second spin in the case of the dipolar interaction. The values of the components of the tensor $\hat{\mathbf{A}}$ are dependent upon the specific reference frame. The reference in which $\hat{\mathbf{A}}$ is diagonal is called the principal axis system (PAS). In general the PAS will not coincide with the laboratory/magnet (LAB) reference frame. However, the spin operators are normally expressed in laboratory/magnet reference frame since quantization is along the z-axis, the direct of the magnetic field. Therefore, the spacial part must be transformed from its PAS to the laboratory frame. This is essentially an Euler rotation problem. In order to accomplish this, the Cartesian tensor is reexpressed as a spherical tensor in which case Eq B.1 becomes

$$H = \sum_{k=0}^2 \sum_{q=-k}^k (-1)^q A_{kq} T_{k-q} \quad B.2$$

where A_{kq} are the spherical tensors of rank k due to the spacial or lattice variables and T_{kq} are the spin operators expressed in spherical tensor forms. The relationship between the spherical and cartesian spin operators are the following

$$\begin{aligned} T_{00}^{cs} &= -\sqrt{\frac{1}{3}} I_z B_0 & T_{00}^{dd} &= 0 \\ T_{10}^{cs} &= 0 & T_{10}^{dd} &= 0 \\ T_{1\pm 1}^{cs} &= -\frac{1}{2} I_{\pm} B_0 & T_{1\pm 1}^{dd} &= 0 \\ T_{20}^{cs} &= \sqrt{\frac{1}{3}} I_z B_0 & T_{20}^{dd} &= \sqrt{\frac{1}{6}} (I_z S_z - \mathbf{I} \cdot \mathbf{S}) \\ T_{2\pm 1}^{cs} &= \mp \frac{1}{2} I_{\pm} B_0 & T_{2\pm 1}^{dd} &= \mp I_z S_{\pm} + I_{\pm} S_z \\ T_{2\pm 2}^{cs} &= 0 & T_{2\pm 2}^{dd} &= \frac{1}{2} I_{\pm} S_{\pm} \end{aligned} \quad B.3$$

where T^{cs} refers to chemical shift operators and T^{dd} to dipole-dipole operators. For the lattice

tensor operators, the relationships are as follows

$$\begin{aligned}
 A_{00} &= -\sqrt{\frac{1}{3}}(A_{xx} + A_{yy} + A_{zz}) \\
 A_{10} &= -\frac{i}{\sqrt{2}}(A_{xy} + A_{yx}) \\
 A_{1\pm 1} &= -\frac{1}{2}[A_{xx} - A_{yy} \pm i(A_{xy} - A_{yx})] \\
 A_{20} &= \sqrt{\frac{1}{6}}[3A_{zz} - (A_{xx} + A_{yy} + A_{zz})] \\
 A_{2\pm 1} &= \mp \frac{1}{2}[A_{xx} + A_{yy} \pm i(A_{xy} + A_{yx})] \\
 A_{2\pm 2} &= \frac{1}{2}[A_{xx} - A_{yy} \pm i(A_{xy} - A_{yx})]
 \end{aligned}
 \quad
 \begin{aligned}
 A_{ij}^{ca} &= \gamma_I \sigma_{ij} \\
 A_{ij}^{dd} &= \frac{\gamma_I \gamma_S \hbar}{r^3} (\delta_{ij} - 3e_i e_j) \\
 i, j &= x, y, z
 \end{aligned}
 \quad B.4$$

where in, the case of A_{ij}^{dd} , δ_{ij} is the Kronecker delta function and e_i is the component of the unit internuclear vector connecting the two spins. This form of the expression can be found in the book by Mehring (Mehring, 1983). One way to visualize these Cartesian lattice tensors is as "energy" ellipsoids (see Chapter 1). In the PAS, the A_{ii} ($i = x, y, z$) are the axes of an ellipsoid with units of energy. For the dipolar case, this ellipsoid is axially symmetric and for the chemical shift interaction, the ellipsoid is asymmetric. In this geometric context, the problem becomes one of determining the contribution of each of these axes to a specified direction in laboratory frame: for example in the case of chemical shifts the magnetic field axis. The reason the Hamiltonian is expressed in this way is that these irreducible spherical tensor operators will rotationally transform in a relatively simple manner, similar to the spherical harmonics (Edmond, 1960). For situation such as the MASS problem two sets of transformations are needed and are schematically expressed in the following way

$$A''(PAS) \xrightarrow{R(a, \beta, \gamma)} A'(MAS) \xrightarrow{R(\phi, \theta, \psi)} A(LAB) \quad B.5$$

where $R(a, b, c)$ represent an Euler transformation about the angles (a, b, c) . For the transformation from the MAS frame to the LAB frame the following operation is carried out,

$$A_{kq} = R(\phi, \theta, \psi) A'_{kq} R^{-1}(\phi, \theta, \psi) \quad B.6$$

Figure 1.2 in Chapter 1 shows the relative orientations of the various angles. Equation B.5 can be reexpressed using Wigner rotation matrices to

$$A_{kq} = \sum_{p=-k}^k A'_{kp} D_{pq}^k(\phi, \theta, \psi) \quad B.7$$

where

$$D_{pq}^k(\phi, \theta, \psi) = \exp(-ip\phi) d_{pq}^k(\phi, \theta, \psi) \exp(-iq\psi). \quad B.9$$

The detailed derivations of these equations can be found in numerous textbooks (Baym, 1974, Cornwell, 1984). Each transformation shown in Eq. B.5 will involve a separate application of Eq. B.7, that is, each A_{kq} term must be expressed as a sum of A'_{kq} terms, each of which in turn must be expressed as a sum of A''_{kq} terms. Finally, each A_{kq} term, having been expanded into grand summation, is multiplied by the suitable spin tensor operator which results in the MASS Hamiltonian being in a useful form.

Before we continue with the detailed algebraic manipulation of this type of problem, several observations are made which will reduce the number of terms which are involved. All problems of interest are under high field conditions, that is the Zeeman term is dominant. Therefore, all operators which involve raising and/or lowering operators, like $I_{\pm}$, will be ignored since they are non-energy conserving. More precisely these operators do not commute with the Zeeman operator and do not to first order contribute to the spectrum. For the chemical shift case, Bloch-Siegert shifts are a consequence of these so-called 'non-secular terms'. A more immediate consequence is that all of terms except those involving T_{00} and T_{20} are dropped (see Eq. B.3). Finally, the A_{00} term is the scalar part of the particular interaction and it is invariant to rotational transformation. Because of this and the secular approximation, the only term that will be of interest is the $A_{20}T_{20}$ term for which the A_{20} must be expanded in term of the PAS as described above.

The A_{20} term results from two transformations which can be expressed in the same way as equation B.7

$$A_{20} = \sum_{q,p=-2}^2 A''_{2p} D_{pq}^{(2)}(\alpha\beta\gamma) D_{q0}^{(2)}(\phi\theta\psi) \quad B.10$$

In order to facilitate the algebraic manipulations, the Wigner rotations which will be needed are listed below

$$D_{00}^{(2)}(\alpha\beta\gamma) = \frac{1}{2}(3 \cos^2 \beta - 1)$$

$$\begin{aligned}
D_{10}^{(2)}(\alpha\beta\gamma) &= -\sqrt{\frac{3}{2}} \sin \beta \cos \beta e^{-i\alpha} & D_{01}^{(2)}(\alpha\beta\gamma) &= \sqrt{\frac{3}{2}} \sin \beta \cos \beta e^{-i\gamma} \\
D_{-10}^{(2)}(\alpha\beta\gamma) &= \sqrt{\frac{3}{2}} \sin \beta \cos \beta e^{i\alpha} & D_{0-1}^{(2)}(\alpha\beta\gamma) &= -\sqrt{\frac{3}{2}} \sin \beta \cos \beta e^{i\gamma} \\
D_{22}^{(2)}(\alpha\beta\gamma) &= \frac{1}{4} (1 + \cos \beta)^2 e^{-2i\alpha} e^{-2i\gamma} & D_{2-2}^{(2)}(\alpha\beta\gamma) &= \frac{1}{4} (1 - \cos \beta)^2 e^{-2i\alpha} e^{2i\gamma} \\
D_{-22}^{(2)}(\alpha\beta\gamma) &= \frac{1}{4} (1 - \cos \beta)^2 e^{2i\alpha} e^{-2i\gamma} & D_{-2-2}^{(2)}(\alpha\beta\gamma) &= \frac{1}{4} (1 + \cos \beta)^2 e^{2i\alpha} e^{2i\gamma} \\
D_{21}^{(2)}(\alpha\beta\gamma) &= -\frac{1}{2} \sin \beta (1 + \cos \beta) e^{-2i\alpha} e^{-i\gamma} & D_{2-1}^{(2)}(\alpha\beta\gamma) &= \frac{1}{2} \sin \beta (\cos \beta - 1) e^{-2i\alpha} e^{i\gamma} \\
D_{-21}^{(2)}(\alpha\beta\gamma) &= \frac{1}{2} \sin \beta (1 - \cos \beta) e^{2i\alpha} e^{-i\gamma} & D_{-2-1}^{(2)}(\alpha\beta\gamma) &= \frac{1}{2} \sin \beta (1 + \cos \beta) e^{2i\alpha} e^{i\gamma} \\
D_{20}^{(2)}(\alpha\beta\gamma) &= \sqrt{\frac{3}{8}} \sin^2 \beta e^{-2i\alpha} & D_{02}^{(2)}(\alpha\beta\gamma) &= \sqrt{\frac{3}{8}} \sin^2 \beta e^{-2i\gamma} \\
D_{-20}^{(2)}(\alpha\beta\gamma) &= \sqrt{\frac{3}{8}} \sin^2 \beta e^{2i\alpha} & D_{0-2}^{(2)}(\alpha\beta\gamma) &= \sqrt{\frac{3}{8}} \sin^2 \beta e^{2i\gamma}.
\end{aligned}$$

B.11

Examination of Eq.B.4 shows that $A''_{2,\pm 1}$ terms are zero because in the PAS the symmetric part of the tensor (A''_{2q}) is diagonal. Furthermore, as it will be shown later $A''_{2-2} = A''_{22}$. Under these constraints, equation B.7 can be expanded to yield

$$A_{20} = A''_{20} \sum_{q=-2}^2 D_{0q}^{(2)}(\alpha\beta\gamma) D_{q0}^{(2)}(\phi\theta\psi) + A''_{22} \sum_{q=-2}^2 \left(D_{-2q}^{(2)}(\alpha\beta\gamma) D_{q0}^{(2)}(\phi\theta\psi) + D_{2q}^{(2)}(\alpha\beta\gamma) D_{q0}^{(2)}(\phi\theta\psi) \right)$$

B.12

The second summation on the right-hand side of this equation can be reexpressed as

$$\begin{aligned}
\sum_{q=-2}^2 \left(D_{-2q}^{(2)}(\alpha\beta\gamma) D_{q0}^{(2)}(\phi\theta\psi) + D_{2q}^{(2)}(\alpha\beta\gamma) D_{q0}^{(2)}(\phi\theta\psi) \right) = \\
\sum_{q=-2}^2 \left(D_{-2q}^{(2)}(\alpha\beta\gamma) D_{q0}^{(2)}(\phi\theta\psi) + D_{2-q}^{(2)}(\alpha\beta\gamma) D_{-q0}^{(2)}(\phi\theta\psi) \right).
\end{aligned}$$

B.13

This regrouping is done to take advantage of the following equalities

$$\begin{aligned}
D_{10}^{(2)} &= -\overline{D_{-10}^{(2)}} & D_{20}^{(2)} &= \overline{D_{-20}^{(2)}} \\
D_{21}^{(2)} &= -\overline{D_{-2-1}^{(2)}} & D_{2-2}^{(2)} &= \overline{D_{-22}^{(2)}} \\
D_{2-1}^{(2)} &= -\overline{D_{-21}^{(2)}} & D_{22}^{(2)} &= -\overline{D_{-2-2}^{(2)}}.
\end{aligned}$$

Substituting these relationships in to Eq B.11 leads to

$$\begin{aligned}
\sum_{q=-2}^2 (D_{-2q}^{(2)}(\alpha\beta\gamma) D_{q0}^{(2)}(\phi\theta\psi) + D_{2-q}^{(2)}(\alpha\beta\gamma) D_{-q0}^{(2)}(\phi\theta\psi)) \\
= \sum_{q=-2}^2 (D_{-2q}^{(2)}(\alpha\beta\gamma) D_{q0}^{(2)}(\phi\theta\psi) + \overline{D_{-2q}^{(2)}(\alpha\beta\gamma) D_{q0}^{(2)}(\phi\theta\psi)}) \quad B.13a \\
= 2 \sum_{q=-2}^2 \operatorname{Re} (D_{-2q}^{(2)}(\alpha\beta\gamma) D_{q0}^{(2)}(\phi\theta\psi)). \quad B.13b
\end{aligned}$$

Now substituting eq B.10 into B.13a

$$\begin{aligned}
2 \sum_{q=-2}^2 \operatorname{Re} (D_{-2q}^{(2)}(\alpha\beta\gamma) D_{q0}^{(2)}(\phi\theta\psi)) &= \sqrt{\frac{3}{2}} \left(\frac{1}{4} (1 + \cos \beta)^2 \sin^2 \theta \cos(2\phi + 2\alpha + 2\gamma) \right. \\
&+ \sin \theta \cos \theta \sin \beta (1 + \cos \beta) \cos(\phi + 2\alpha + \gamma) + \frac{1}{2} (3 \cos^2 \theta - 1) \sin^2 \beta \cos 2\alpha \\
&- \sin \theta \cos \theta \sin \beta (1 - \cos \beta) \cos(-\phi + 2\alpha - \gamma) \\
&\left. + \frac{1}{4} \sin^2 \theta (1 - \cos \beta)^2 \cos(-2\phi + 2\alpha - 2\gamma) \right). \quad B.14
\end{aligned}$$

Expanding and recombining the trigonometric terms

$$\begin{aligned}
2 \sum_{q=-2}^2 \operatorname{Re} (D_{-2q}^{(2)}(\alpha\beta\gamma) D_{q0}^{(2)}(\phi\theta\psi)) &= \sqrt{\frac{3}{2}} \left(\frac{1}{2} (3 \cos^2 \theta - 1) \sin^2 \beta \cos 2\alpha \right. \\
&+ \frac{1}{2} \sin^2 \theta (1 + \cos^2 \beta) \cos 2\alpha \cos(2\phi + 2\gamma) - \sin^2 \theta \cos \beta \sin 2\alpha \sin(2\phi + 2\gamma) \\
&\left. + 2 \sin \theta \cos \theta \sin \beta \cos \beta \cos 2\alpha \cos(\phi + \gamma) - 2 \sin \theta \cos \theta \sin \beta \sin 2\alpha \sin(\phi + \gamma) \right). \quad B.15
\end{aligned}$$

In a similar manner, the first term of the right hand side of eq B.11 can be reduced to

$$\begin{aligned}
\sum_{q=-2}^2 D_{0q}^{(2)}(\alpha\beta\gamma) D_{q0}^{(2)}(\phi\theta\psi) &= \frac{1}{4} (3 \cos^2 \theta - 1) (3 \cos^2 \beta - 1) \\
&- 3 \sin \theta \cos \theta \sin \beta \cos \beta \cos(\phi + \gamma) + \frac{3}{4} \sin^2 \theta \sin^2 \beta \cos(2\phi + 2\gamma). \quad B.16
\end{aligned}$$

Finally, by combining eq B.15 and B.16 and replacing $\phi = \omega_r t$, eq B.9 may be expressed in the following way

$$\begin{aligned}
A_{20} &= \sum_{q,p=-2}^2 A_{2p}'' D_{pq}^{(2)}(\alpha\beta\gamma) D_{q0}^{(2)}(\phi\theta\psi) \\
&= \frac{1}{4} (3 \cos^2 \theta - 1) [(3 \cos^2 \beta - 1) A_{20}'' + 2 \sqrt{\frac{3}{2}} \sin^2 \beta \cos 2\alpha A_{22}''] \\
&+ \sin \theta \cos \theta \sin \beta \cos \beta [2 \sqrt{\frac{3}{2}} \cos 2\alpha A_{22}'' - 3 A_{20}''] \cos(\omega_r t + \gamma) \\
&- \sin \theta \cos \theta [2 \sqrt{\frac{3}{2}} \sin \beta \sin 2\alpha A_{22}''] \sin(\omega_r t + \gamma) \\
&+ \sin^2 \theta [\frac{3}{4} \sin^2 \beta A_{20}'' + \frac{1}{2} \sqrt{\frac{3}{2}} (1 + \cos^2 \beta) \cos 2\alpha A_{22}''] \cos(2\omega_r t + 2\gamma) \\
&- \sin^2 \theta [\frac{1}{2} \sqrt{\frac{3}{2}} (1 + \cos^2 \beta) \sin 2\alpha A_{22}''] \sin(2\omega_r t + 2\gamma). \quad B.17a
\end{aligned}$$

or

$$\begin{aligned}
A_{20} = & \frac{1}{4}(3 \cos^2 \theta - 1) [(3 \cos^2 \beta - 1) A_{20}'' + \sqrt{\frac{3}{2}} \sin^2 \beta \cos 2\alpha A_{22}''] \\
& + C_1 \cos(\omega_r t + \gamma) - S_1 \sin(\omega_r t + \gamma) + C_2 \cos(2\omega_r t + 2\gamma) \\
& - S_2 \sin(2\omega_r t + 2\gamma)
\end{aligned}$$

$$C_1 = \sin \theta \cos \theta \sin \beta \cos \beta [2\sqrt{\frac{3}{2}} \cos 2\alpha A_{22}'' - 3A_{20}''] \quad B.18b$$

$$S_1 = 2\sqrt{\frac{3}{2}} \sin \theta \cos \theta \sin \beta \sin 2\alpha A_{22}''$$

$$C_2 = \sin^2 \theta \left\{ \frac{3}{4} \sin^2 \beta A_{20}'' + \frac{1}{2} \sqrt{\frac{3}{2}} (1 + \cos^2 \beta) \cos 2\alpha A_{22}'' \right\}$$

$$S_2 = \sqrt{\frac{3}{2}} \sin^2 \theta \cos \beta \sin 2\alpha A_{20}''$$

Now, if $\theta = \theta_{magic}$, then the total Hamiltonian (Eq B.1) may be expressed in complete form

$$\begin{aligned}
H = & T_{00} A_{00} + T_{20} [C_1 \cos(\omega_r t + \gamma) - S_1 \sin(\omega_r t + \gamma) \\
& + C_2 \cos(2\omega_r t + 2\gamma) - S_2 \sin(2\omega_r t + 2\gamma)] \quad B.1a
\end{aligned}$$

Several things are immediately apparent. First, there is a time-independent part to this portion of the Hamiltonian. The time-dependent parts may be divided into two parts: one which is dependent on the rotor speed and the other which is dependent on twice the rotor speed. This time-dependent part makes the Hamiltonian periodic. Hence, the density matrix of the spin system will behave periodically, that is the density matrix will become identical to the initial density matrix ($t=0$) once every rotor period. This periodicity is the cause of the rotary echos observed in the time-domain signal.

Until this point, the specific type of spin interaction has not been specified and the treatment has been general except for the constraint that $A_{2\pm 2}$ terms are equal. In the chemical shift case, the asymmetric components, σ_{ij} where $i \neq j$, have been shown to contribute to second order energy shifts (Buckingham and Malm, 1971; Griffin, 1972). Therefore, for the purposes for this discussion the asymmetric parts are considered to be zero. In the case of the dipolar interaction, the internuclear vector lies along z direction of PAS and hence the x and y components are zero. By Eq B.4, all of the terms except for the A_{zz} should vanishes. Except for the operators the dipolar case is equivalent to the axially symmetric chemical shift case.

Appendix C. The intensity of MASS sidebands.

As described in Chapter 1, in order to calculate the time-domain response of the system after the application of a "90°" pulse, it is necessary to calculate the density matrix as a function of time. In addition, since the energy of a given spin packet will depend on the orientation of its principal axis system (PAS) with respect to the lab/magnet frame, the density matrix must also be integrated with respect to time (see Appendix D). The frequency-domain spectrum is then the Fourier Transform of the integrated time response. The spectrum, which consists of the centerband and a series of sideband separated by the rotor frequency, is expected to be a complex function of the three tensor elements and the spinning speed. Thus far, it has been established that if one has knowledge of the tensor parameters and the spin rate, the MASS spectrum may be calculated. However, a mapping from the spectrum back to the tensor parameters has not been derived. This is purpose of this Appendix.

We start with the MASS Hamiltonian derived in the previous appendix

$$H = \omega_0 \sigma_i \hat{I}_z + \omega_0 \hat{I}_z \left\{ \frac{1}{4} (3 \cos^2 \theta - 1) [(3 \cos^2 \beta - 1) (\sigma_{33} - \sigma_i) + \sin^2 \beta \cos 2\alpha (\sigma_{11} - \sigma_{22})] \right. \\ \left. + C_1 \cos(\omega_r t + \gamma) - S_1 \sin(\omega_r t + \gamma) + C_2 \cos(2\omega_r t + 2\gamma) - S_2 \sin(2\omega_r t + 2\gamma) \right\} \quad C.1$$

where

$$\omega_0 = \gamma_I B_0$$

$$C_1 = \sin \theta \cos \theta \sin \beta \cos \beta [\cos 2\alpha (\sigma_{11} - \sigma_{22}) - 3 (\sigma_{33} - \sigma_i)]$$

$$S_1 = \sin \theta \cos \theta \sin \beta \sin 2\alpha (\sigma_{11} - \sigma_{22})$$

$$C_2 = \sin^2 \theta \left\{ \frac{3}{4} \sin^2 \beta (\sigma_{33} - \sigma_i) + \frac{1}{4} (1 + \cos^2 \beta) \cos 2\alpha (\sigma_{11} - \sigma_{22}) \right\}$$

$$S_2 = \frac{1}{2} \sin^2 \theta \cos \beta \sin 2\alpha (\sigma_{11} - \sigma_{22}).$$

Without loss of generality, σ_i may be set to zero, since this is a relative quantity. Second, notice that, despite the time-dependence, the parts of the Hamiltonian commute amongst themselves at all times. This last observation makes it possible to separate the explicitly time-dependent, $\hat{H}_1$, and time-independent, $\hat{H}_0$, parts in the time evolution operator. Eq 1.5 is used along with the above Hamiltonian to yield

$$\hat{U}(t) = \exp \left(i \hat{H}_0 t + i \int_0^t \hat{H}_1(t') dt' \right) \quad C.2$$

where the Dyson time operator has been omitted and time ordering is implicit. Both parts of the Hamiltonian are proportional to $\hat{I}_z$, and the density matrix immediately following the "90°"

rf-pulse is proportional to $\hat{I}_x$. Using eq 1.18, in much the same way in which Eq. 1.33 was derived, it is possible to show that the signal (*i.e.* magnetization), expressed as a complex number, is

$$M(\alpha\beta\gamma, t) = \exp(i\Omega_0 t) \exp\left(i \int_0^t \Omega_1(t') dt'\right) \quad C.3$$

where

$$\Omega_0(\alpha\beta\gamma, t) = \omega_0 \sigma_i + \frac{1}{4} \omega_0 (3 \cos^2 \theta - 1) [(3 \cos^2 \beta - 1) (\sigma_{33} - \sigma_i) + \sin^2 \beta \cos 2\alpha (\sigma_{11} - \sigma_{22})]$$

and

$$\Omega_1(\alpha\beta\gamma, t) = \omega_0 [C_1 \cos(\omega_r t + \gamma) - S_1 \sin(\omega_r t + \gamma) + C_2 \cos(2\omega_r t + 2\gamma) - S_2 \sin(2\omega_r t + 2\gamma)].$$

Integrating the time variable yields

$$\begin{aligned} \Phi_0(\alpha\beta\gamma, t) &= \Omega_0 t \\ \Phi_1(\alpha\beta\gamma, t) &= \int_0^t \Omega_1(t') dt' \\ &= \frac{\omega_0}{\omega_r} [S_1 (\cos \gamma - \cos(\omega_r t + \gamma)) + C_1 (\sin(\omega_r t + \gamma) - \sin \gamma) \\ &\quad + \frac{1}{2} S_2 (\cos 2\gamma - \cos(2\omega_r t + 2\gamma)) + \frac{1}{2} C_2 (\sin(2\omega_r t + 2\gamma) - \sin 2\gamma)] \\ &= F(\gamma, 2\gamma) - F(\omega_r t + \gamma, 2\omega_r t + 2\gamma) \end{aligned} \quad C.4$$

where the last equality points out that $\Phi_1(\alpha\beta\gamma, t)$ can be written as a single function which is used twice with different arguments. An integration of eq C.3 over all orientations must be carried out in order to obtain the desired answer. At this point, the derivation of Mehring (Mehring, text) is closely followed. He starts with following identity

$$G(\gamma) = \frac{1}{2\pi} \int_0^{2\pi} \delta(\epsilon - \gamma) G(\epsilon) d\epsilon \quad C.5$$

which results from the property of a δ -function (Stalkgold, 1979). From distribution theory (Stalkgold, 1979) it can be shown that

$$\delta(\epsilon - \gamma) = \sum_{m=-\infty}^{+\infty} e^{im(\epsilon - \gamma)}. \quad C.6$$

The quantity $e^{i\Phi_1(\alpha\beta\gamma, t)}$ can be expanded using the last equality given in eq C.4 to yield

$$e^{i\Phi_1(\alpha\beta\gamma, t)} = e^{iF(\gamma, 2\gamma)} e^{-iF(\omega_r t + \gamma, 2\omega_r t + 2\gamma)}.$$

Substitution of this equation into eq C.5 gives

$$e^{i\Phi_1(\alpha\beta\gamma,t)} = \frac{1}{4\pi} \int_0^{2\pi} \delta(\epsilon - \gamma) e^{iF(\epsilon, 2\epsilon)} d\epsilon \int_0^{2\pi} \delta(\epsilon - \omega_r t - \gamma) e^{-iF(\epsilon, 2\epsilon)} d\epsilon$$

Furthermore, substituting the identity Eq. C.6 into the preceeding equation results in

$$e^{i\Phi_1(\alpha\beta\gamma,t)} = \sum_{m=-\infty}^{+\infty} \sum_{n=-\infty}^{+\infty} e^{i(m-n)} e^{i(m\omega_r t)} \int_0^{2\pi} e^{im\epsilon} e^{iF} d\epsilon \int_0^{2\pi} e^{-in\epsilon} e^{-iF} d\epsilon$$

where the arguments for F have been droppped. Integration over γ yields

$$\begin{aligned} e^{i\Phi_1(\alpha\beta\gamma,t)} &= \sum_{m=-\infty}^{+\infty} \sum_{n=-\infty}^{+\infty} \delta(m-n) e^{i(m\omega_r t)} \int_0^{2\pi} e^{im\epsilon} e^{iF} d\epsilon \int_0^{2\pi} e^{-in\epsilon} e^{-iF} d\epsilon \\ &= \sum_{m=-\infty}^{+\infty} e^{i(m\omega_r t)} \int_0^{2\pi} e^{im\epsilon} e^{iF} d\epsilon \int_0^{2\pi} e^{-im\epsilon} e^{-iF} d\epsilon \end{aligned}$$

At this point, we include the Φ_0 term and the Fourier transform of the product yields

$$\begin{aligned} \mathcal{F} [e^{i\Phi_1(\alpha\beta\gamma,t)} e^{i\Phi_0(\alpha\beta,t)}] &= e^{i\Phi_1(\alpha\beta\gamma,\omega)} * e^{i\Phi_1(\alpha\beta,\omega)} \\ &= \sum_{m=-\infty}^{+\infty} \delta(\omega - \Omega - m\omega_r) \int_0^{2\pi} e^{im\epsilon} e^{iF} d\epsilon \int_0^{2\pi} e^{-im\epsilon} e^{-iF} d\epsilon \end{aligned} \quad C.7$$

where the Fourier transform of the product is expressed as a convolution which is denoted by the $*$ symbol and the Φ_0 term is only dependent the two specified angles. If $\theta = \theta_{magic}$ then the Φ_0 term becomes independent of the orientation of the PAS with respect to the Lab/magnet frame and is only dependent on the isotropic chemical shift. The discussion is restricted to this case. The δ -function term results in a series of signals spaced at the rotor frequency about the isotropic chemical shift. The intensity of the M^{th} -sideband is

$$I_M = \left| \int_0^{2\pi} e^{im\epsilon} e^{iF} d\epsilon \right|^2 \quad C.9$$

and for clarity the function F is stated in again

$$F(\epsilon, 2\epsilon) = \frac{C_1}{\omega_r} \sin \epsilon + \frac{S_1}{\omega_r} \cos \epsilon + \frac{C_2}{2\omega_r} \sin 2\epsilon + \frac{S_2}{2\omega_r} \cos 2\epsilon.$$

If the magnetization, as expressed by Eqs. C.3 and C.7, is normalized to unity at $t = 0$, the right side of eq C.7 must also be unity which implies

$$\sum_{m=-\infty}^{+\infty} I_m = 1.$$

Furthermore, as $\omega_r \rightarrow \infty$, $e^{iF} \rightarrow 0$ and $I_0 \rightarrow 1$, that is the all of intensity will be in the centerband which was predicted in a previous appendix (see Appendix B). From Eq. C.1, it can be seen that the sideband intensities, as given by eq C.9, are dependent on essentially two variables (*i.e* assuming that the isotropic shift is zero) and Hertzfeld and Berger (Hertzfeld and Berger, 1980) used the following variables

$$\mu = \frac{\omega_0}{\omega_r} (\sigma_{33} - \sigma_{11}) \quad D.10a$$

and

$$\rho = \frac{\sigma_{11} + \sigma_{33} - 2\sigma_{22}}{\sigma_{33} - \sigma_{11}}. \quad D.10b$$

In order to connect these variables to those in eq C.1, Eqs D.10a and D.10b can be rewritten to give

$$\mu = \frac{\omega_0}{\omega_r} (\sigma_{11} - \sigma_{22})$$

and

$$\mu\rho = \frac{3\omega_0}{\omega_r} (\sigma_{33} - \sigma_i).$$

Using these variables, Hertzfeld and Berger (Hertzfeld and Berger, 1980) numerically integrated Eq. C.9, a Bessel function, as a function of μ and ρ . In addition, they also integrated the remaining angular variables, α and β in order to obtain the powder average. These intensities were reported in ratios of the form, I_N/I_0 for up to $N = \pm 5$. These are the data, in conjunction with a non-linear fitting computer routine, which were used to determine the principal values of the chemical shift tensor from the experimental MASS sideband intensities.

Appendix D. Powder Averaging – A Random Approach

In this appendix, we discuss a method for determining the powder averaged equation which describes the complex magnetization as function of time. The integral is of the form

$$\langle \hat{M}_c(t) \rangle = \frac{1}{8\pi} \int_0^{2\pi} \int_0^\pi \int_0^{2\pi} \exp \left(i \int_0^t \Omega(t') dt' \right) \sin \beta d\alpha d\beta d\gamma. \quad 1.39$$

The time integration may be carried out in a straight forward manner and usually can be solved analytically. This leaves the spacial integrations. In principle, one of the numerous quadrature techniques may be applied to determine the integral.

Before detailing the method used to integrate Eq. 1.39, we deviate a bit from the discussion and point out a useful consequence of integrating the time domain response. The integrated area of the frequency domain spectrum is directly proportional to the value of the time zero point, $\langle \hat{M}_c(0) \rangle$, of the time domain response or the free induction decay (*FID*). In a Bloch decay experiment, all of the spin magnetization starts with the same phase; hence, at this point in time the amplitude of the FID is simply directly proportional to the total number of spins and the integrated area of the frequency domain spectrum. This has two consequences. First, for a finite number of spins, the integrated area of the frequency spectrum cannot possess infinite discontinuities. This is contrasted to the Bloembergen–Rowland equations for static powder spectra which do possess logarithmic discontinuities. The absence of infinite discontinuities allows two different calculated powder spectra to be scaled with respect to each other. This is important when simulating spectra containing contributions from several inequivalent nuclei. The second consequence of the relationship of the time zero point and the integrated area of the frequency spectrum is that the relative error in determining this time point is equal to the relative error in the integrated area. This will have important consequences in characterizing the numerical integration of Eq. 1.39

The spacial integration of Eq. 1.39 results from the fact that one must take into account the fact that in a powder, the principal axis system (PAS) of given interaction can take on any random orientation with respect to the LAB/magnet frame; hence, since all orientations are equally probable and there are very large numbers of nuclei, one can replace the sum over all the orientations with an integral. Hertzfeld and Berger (Hertzfeld and Berger, 1980) used a

combination of Trapezoid and two point Gaussian methods to integrate similar expressions (see Appendix C.). They pointed out that this combination was adequate for accurately determining the integrals. We choose two different integration techniques: the Trapezoid rule and a simple Monte Carlo method. The Monte Carlo method, in particular, offered several attractive features which now will be discussed. More rigorous mathematical discussion of Monte Carlo theory is given in a review James (James, 1980).

The Monte Carlo estimate of the integral improves with each new randomly chosen orientation. Since the orientations are generated without bias and randomly, each additional orientation contributes equally as the rest of previously chosen points. In the case of the simple Trapezoid rule, one must calculate the contribution of new sets of orientations which are evenly distributed over the total integration region in order to improve the estimate of the integral. In the simple case this means dividing the interval of integration by half leading to a doubling of the points to be calculated before the integration can be improved without bias (James, 1980). In additions, the computer program necessary to iteratively generate the regular lattice of orientations needed for the trapezoid integration can be tedious and complex, especially in multiple dimensions. In contrast, a single random orientation generator is very compact and has been shown to be efficient (Marsaglia, 1971).

Error estimates of the Monte Carlo integration are easily calculated. The estimate of the variance is given by

$$\sigma^2 = \frac{N}{N-1} \left[\frac{1}{N} \sum F^2(\Omega_n, t) - \left(\frac{1}{N} \sum F(\Omega_n, t) \right)^2 \right]$$

where F is the value of the integrand for the orientation Ω_n at time, t . Again, the variance, in theory, may be calculated after the contribution of each new orientation has been added. For the trapezoid rule case, the variance can only be calculated after the contribution from a complete set of new lattice points have been calculate.

The biggest drawback to the Monte Carlo method is that for three dimensional problems it converges as $n^{-1/2}$ while the Trapezoid method converges as $n^{-2/3}$ where n is the number points calculated. Simple Gaussian methods converges even faster depending on the how many points are used. The Gaussian quadrature and adaptive techniques were not explored. We saw

no need to use these more complex techniques since both the Trapezoid and the Monte Carlo techniques were simple to implement and provided results which were sufficiently accurate. A typical trapezoid rule routine using 35,000 points was sufficient to achieve a standard deviation of 1% of the time zero point for an axially symmetric problem involved integration over only two angles. The performance of the Monte Carlo routine using the same number points was slightly better in execution and accuracy. The primary disadvantage of the Trapezoid routine was that it utilized a fixed lattice of orientations and could not be run iteratively to an arbitrary variance level. We were unable to construct an algorithm which could iteratively and efficiently determine new sets of orientations and remain competitive with the Monte Carlo method. Our attempts clearly do not preclude the existence of such an algorithm.

In order for the Monte Carlo integration technique to work correctly, random numbers must be generated. It is sufficient to state that adequate pseudo-random generators are available, as well as, tests which measure their degree of randomness. The method by which these random numbers were generated and tested is beyond the scope of thesis and discussion. Good discussions may be found in articles by James (James, 1980), Press (Press, 1986), Marsaglia (Marsaglia, 1984). Specifically, the random number generator *RAN2*, found in *Numerical Recipes*, was used in all calculations. Random points on a spherical surface, corresponding to two of the angles of integration, were determined using the method of Marsaglia (Marsaglia, 1971). Random points on a circle, corresponding to the remaining angle of integration, were determined by scaling a version of *RAN2* for 0 to 2π . Figure D.1 shows the first 4096 random points chosen on a spherical surface which have been projected onto a surface. This can be compared to the regular lattice used for the trapezoid methods. The lattice is shown in Figure D.2. Finally, the initial 4050 points chosen on a circle are shown in the last figure, Figure D.3. The typical MASS spectra calculated using the Monte Carlo method took 35,000 points to achieve a standard deviation in the time zero of 1% of the total amplitude. The CPU time for such a calculation was about 18.5 minutes on a VAX 11/785. The Fourier transform intensities of the sideband obtained in this way were well within 5% of the sideband intensities obtained by Hertzfeld and Berger (Hertzfeld and Berger, 1980).

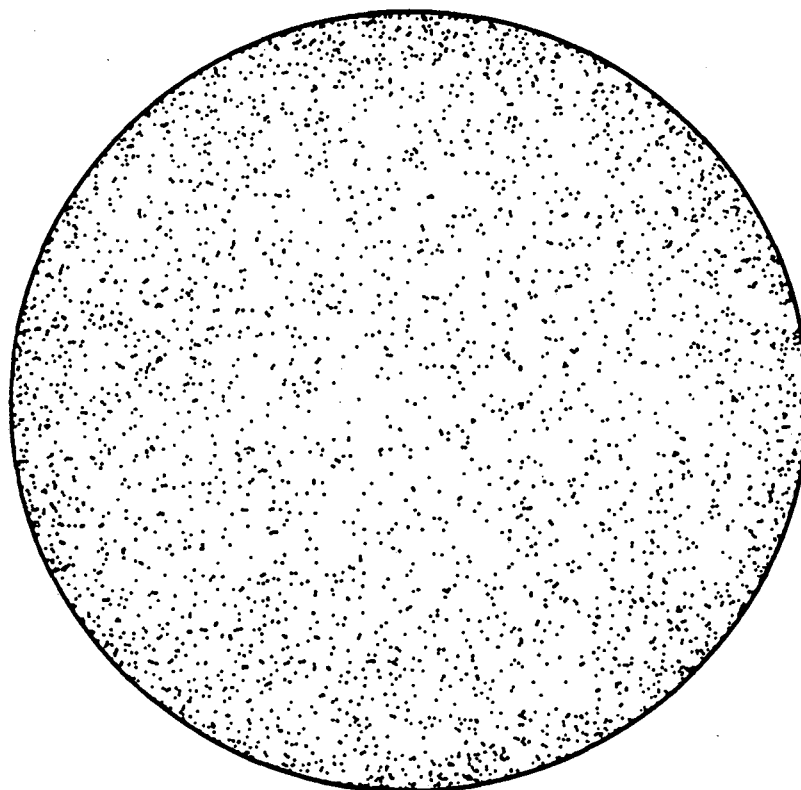


Figure D.1. The first 4096 points of the lattice of γ and β angles used in the Monte Carlo integration of Eq. 1.39. The points are randomly distributed over a spherical surface and have been projected onto a circle. The center nominally represents $\beta = 0^\circ$ and the boundary $\beta = 90^\circ$. For a typical integration of an axially symmetric problem, which involves only the γ and β angles, a lattice of 35000 points was used. The random generation of the α angles used in the general problems is shown in Figure 3.

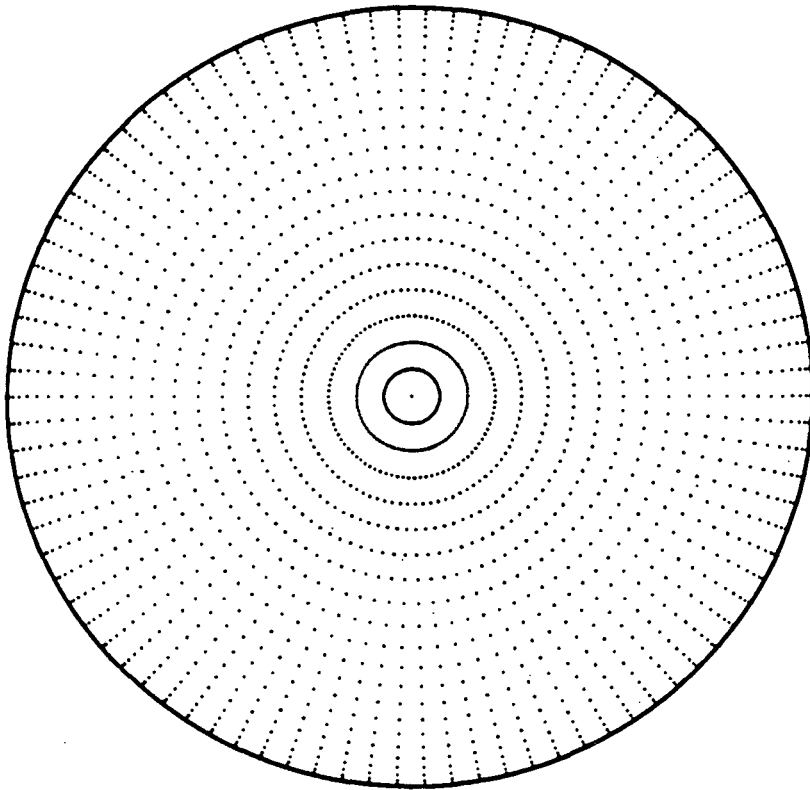


Figure D.2. The first 4050 points of the lattice of γ and β angles used in the Trapezoid rule integration of Eq. 1.39. The points are distributed over a spherical surface and have been projected onto a circle. The center represents $\beta = 0^\circ$ and the boundary $\beta = 90^\circ$. For a typical integration of an axially symmetric problem which involves only the γ and β angles, a lattice of 35000 points was used. The same number of points form each of the concentric circles which represent circles of constant β angle. Each point is scaled by Jacobian, $\sin \beta$, in the integration.

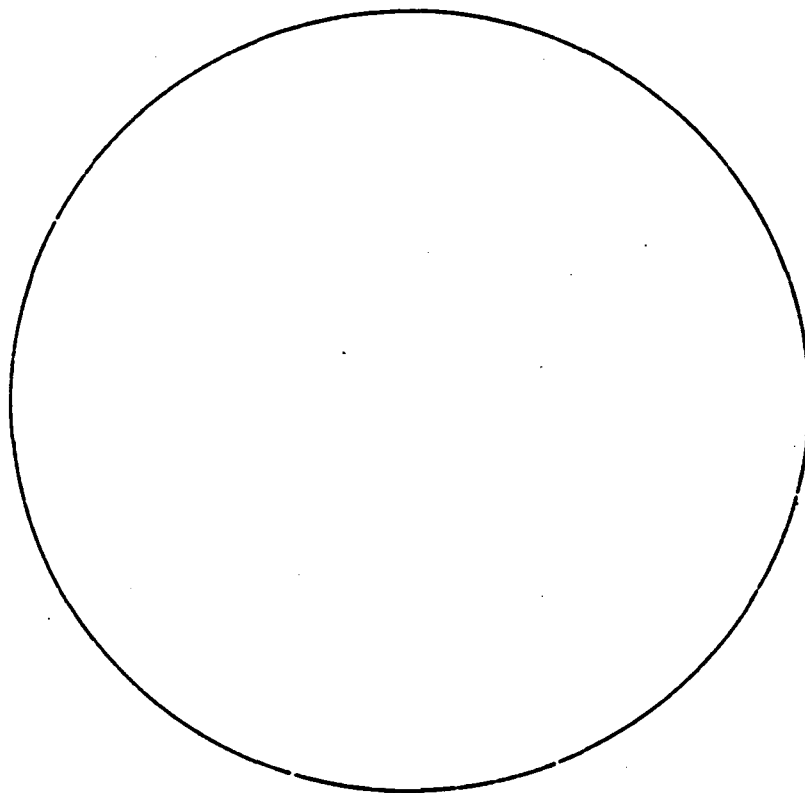


Figure D.3. The first 4096 α angles generated randomly for the Monte Carlo integration of Eq. 1.39. The points are randomly distributed over a circle.

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