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BIOPHYSICAL APPLICATIONS OF NMR TO PHOSPHORYL TRANSFER ENZYMES AND METAL NUCLEI OF METALLOPROTEINS

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INTRODUCTION

In the past few years exciting new applications of nuclear magnetic resonance (NMR) spectroscopy have been made in the study of biological macromolecules. This review is intended to highlight two areas of current NMR research that have mainly been concerned with elucidating the mechanism of action of enzymes. The first area deals with the newly discovered effect of the isotopic shift of ^{18}O on the ^{31}P-NMR spectrum of phosphorus-containing compounds (15); the second area deals with the study of metal nuclei in metalloenzymes.

Until recently mass spectrometry was the method used to study ^{18}O-^{16}O exchange reactions in enzymes that carry out various phosphoryl transfer reactions. The method has the advantage of requiring only small amounts of material for analysis (nanomoles), but volatile phosphate esters must be prepared, which is very time consuming, and continuous observation of the enzymatic process is not possible. The NMR method permits the continuous study of an enzymatic reaction and affords kinetic and isotopic distribution data simultaneously. However, micromoles of material are required. Selected examples of a wide

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variety of enzymatic reactions are covered in this review to stimulate further research in the area.

The study of metal nuclei by NMR is not new but the application to biological systems is. Most metal nuclei have low gyromagnetic ratios, some have low natural abundance, and many are quadrupolar (spin > 1/2). The advent of fourier transform technology to NMR spectroscopy can overcome these disadvantages, and this review deals with some theoretical considerations in applying NMR studies to metalloenzymes. Future applications of NMR to such systems are foreseen and some of the more recent work with metal nuclei binding to macromolecules is reviewed.

NMR STUDIES OF PHOSPHORYL TRANSFER ENZYMES

^{18}O-^{31}P-NMR Shift

Recently, Cohn & Hu (15) and others (46, 48) have demonstrated that there is a small but easily measured chemical shift when ^{18}O is substituted for ^{16}O. The chemical shift is ~0.021 ppm upfield for each ^{18}O

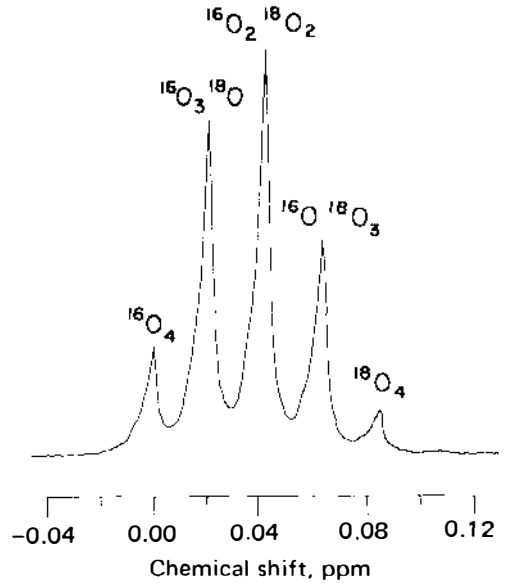


Figure 1 ^{31}P NMR of a randomized sample of [$^{16}O^{18}O$]P_i [From Cohn & Hu (15)].

Table 1 Effect of ^{18}O on the ^{31}P chemical shifts of ATP (16)

P of ATP	Oxygen position	$\Delta\delta$(ppm) per ^{18}O atom
α	α-β bridge	0.017
β	α-β bridge	0.017
	nonbridge	0.028
	β-γ bridge	0.016
γ	β-γ bridge	0.022
	nonbridge	0.022

substituted for ^{16}O and is additive. Thus, the chemical shift for $HP^{16}O_4^{=}$ is 0.084 ppm downfield from $HP^{18}O_4^{=}$. Shown in Figure 1 is the ^{31}P-NMR spectrum at 145.7 MHz of inorganic phosphate containing 44% ^{18}O. Each of the five possible species can clearly be distinguished and each species appears in the expected ratio for a random distribution of ^{18}O and ^{16}O. In phosphate-containing species such as ATP where the phosphorous (α, β, and γ) and oxygen (bridge and nonbridge) atoms are in different environments the effect of ^{18}O substitution on the ^{31}P chemical shift is dependent on the oxygen environment. The shift is largest for those oxygens with most double bond character (16). Shown in Table I is the effect of ^{18}O on the ^{31}P chemical shifts of ATP (16). The 235 MHz spectrum of the β-P of ATP labeled with 63% ^{18}O is shown in Figure 2. Each of the six possible ^{18}O-^{16}O species can be clearly resolved.

As pointed out by Cohn & Hu (15) this phenomenon can be used in two ways: (*a*) for labeling phosphate groups in much the same way as ^{32}P is used, and (*b*) for determining oxygen-phosphate exchange reactions and the site of bond cleavage in phosphate esters such as ATP.

The advantages of this spectroscopic method over the currently used methods are many. Since the method is nondestructive the labeled compounds can be recovered and reused if necessary. The need for using radioactive materials is eliminated. In the case of exchange reactions the kinetics of the reaction can be followed continuously without increasing the amount of material needed to generate a large number of data points. There is also no need for a complicated workup of reaction mixtures to isolate the compound of interest. The main disadvantage appears to be the larger amounts of material needed owing to the insensitivity of the NMR method. The amount of material

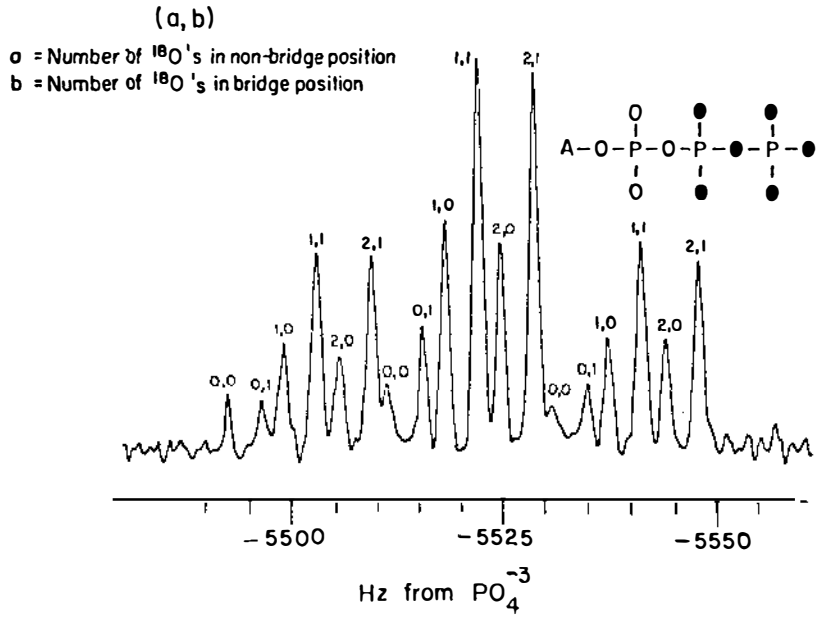


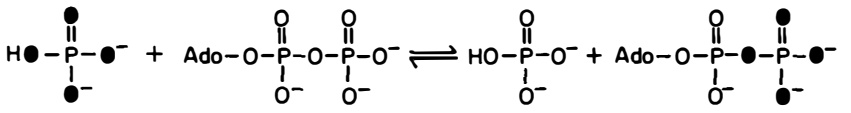
Figure 2 ^{31}P NMR spectrum of the βP of ~63% ^{18}[0]ATP-β, $\gamma^{18}O_6$ recorded at 235 MHz by correlation spectroscopy [from Cohn & Hu (16)].

required is about 10 μmoles, which in most cases is not excessive. If oxygen-phosphorus exchange is to be monitored, there is also the need for a high field spectrometer in order that sufficient resolution be obtained to detect changes in each individual species.

This technique has already found widespread use with a number of different enzymes. The salient results are summarized below.

Uses in Individual Enzyme Systems

POLYNUCLEOTIDE PHOSPHORYLASE Cohn & Hu (15) found that upon the incubation of [^{18}O]P_i and ADP with polynucleotide phosphorylase from *Micrococcus luteus* an exchange of the β-P of ADP with P_i occurred. It was also shown that all four oxygen atoms of P_i were incorporated into the ADP as follows:



The site of bond cleavage is thus between the α-P and the α-β bridge oxygen.

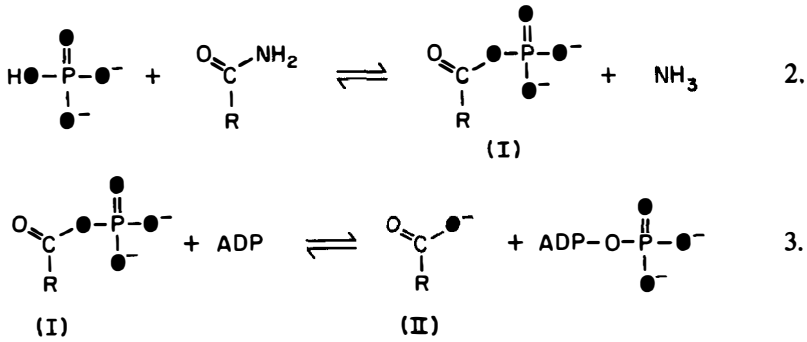
INORGANIC PYROPHOSPHATASE Inorganic pyrophosphatase catalyzes the hydrolysis of pyrophosphate to two moles of inorganic phosphate. Cohn & Hu (15) found that upon incubation of inorganic phosphate (93.4% ^{18}O) and pyrophosphatase the enzyme catalyzed the exchange of ^{18}O out of P_i and into H_2O and that the reaction could be followed at 25 MHz, although the resolution was not good enough to get precise rate constants. This exchange reaction is consistent with the reversible formation of enzyme-bound pyrophosphate and H_2O from two molecules of P_i. No detectable formation of pyrophosphate occurs in solution.

MYOSIN This enzyme catalyzes the hydrolysis of ATP to ADP and P_i. It has also been shown that myosin will catalyze the slow exchange of oxygens between water and P_i in the presence of MgADP (21). Webb et al (70) have examined this later reaction in more detail by following the ^{31}P NMR spectrum of $[^{18}O]P_i$ at 145.7 MHz. They found that all molecules of $[^{18}O]P_i$ that bound to the enzyme underwent complete exchange with H_2O and were released as $[^{16}O_4]P_i$, with no intermediate formation of $[^{18}O^{16}O]P_i$ in the bulk solution. This is interpreted as occurring by a rapid equilibration of enzyme-MgADP-P_i and enzyme-ATP, with very slow release of P_i from the enzyme, in comparison to the interconversion of the central complexes.

ALKALINE PHOSPHATASE This enzyme catalyzes the hydrolysis of a large variety of phosphate esters and is known to involve a phosphoenzyme intermediate. Eargle et al (25) have shown that Zn^{2+} alkaline phosphatase will catalyze an H_2O-P_i exchange with only one ^{18}O exchanged from $[^{18}O]P_i$ per enzyme encounter. Bock & Cohn (8) have confirmed this observation using ^{31}P NMR and have also shown with the Co^{2+}-substituted enzyme that the exchange pattern is different. On the average more than one ^{18}O is exchanged from $[^{18}O]P_i$ per enzymatic encounter. From a computer analysis of the data it was determined that the ratio of the rates for the formation of the covalent phosphoenzyme from the $E \cdot P_i$ Michaelis complex and the rate for the dissociation of the P_i from $E \cdot P_i$ was 3.0 ± 0.5.

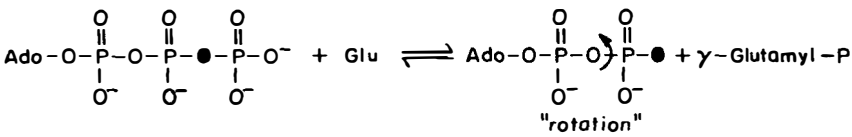
ACID PHOSPHATASE Human prostatic acid phosphatase is another phosphatase that involves a phosphoenzyme intermediate, and Van Etten & Risley (69) have shown by ^{31}P NMR at 40.5 MHz that the enzyme will catalyze an H_2O-P_i exchange. The rate constants for this process are such that only about 1 atom of ^{18}O is exchanged per encounter.

GLUTAMINE SYNTHETASE Glutamine synthetase catalyzes the formation of glutamine from ATP, glutamate, and ammonia. The other products are ADP and P_i. The reaction mechanism is thought to proceed through a γ-glutamyl phosphate intermediate. Balakrishnan et al have shown using ^{31}P NMR that incubation of ADP, glutamine, and [^{18}O]P_i with glutamine synthetase resulted in the loss of ^{18}O from the P_i (6). Analysis of the data showed that only one ^{18}O was lost per encounter and that the rate constant for exchange was 5–7 times faster than net turnover of products. This was also demonstrated by Stokes & Boyer (66) using mass spectrometry.



This exchange reaction is accommodated by assuming that Reactions 2 and 3 are happening on the enzyme surface and that the γ-carboxyl of glutamate (II) is free to rotate, thus allowing, upon reversal of Equation 3, the formation of γ-glutamyl phosphate (I) with an ^{16}O in the bridge position. Reaction of I with NH_3 would then produce P_i with only 3 atoms of ^{18}O instead of the original 4. Since a random distribution of ^{18}O is maintained at all times, only one ^{18}O is lost per encounter with the enzyme and thus P_i must be dissociating from the enzyme faster than glutamine.

The recently introduced positional isotope exchange technique of Midelfort & Rose (50) is also amenable for use by ^{18}O chemical shift technique. Briefly, this technique follows the exchange of label from one part of a substrate to another due to rotational equivalence of some intermediate. The method was first applied to glutamine synthetase in the reaction:



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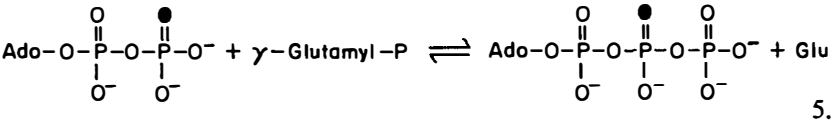


5.

(This is a simplified scheme presented for clarity. In fact all four oxygens of the γ-P were labeled with ^{18}O, but the conclusion is the same for the positional isotope exchange). ATP is synthesized with ^{18}O in the β-γ bridge position. If the enzyme catalyzed the formation of γ-glutamyl-P from ATP and glutamate in the absence of NH_3, then ADP would be formed; because of rotational equivalence it would allow, upon the reversal of the above reactions, the formation of ATP with ^{18}O in the nonbridge position of the β-P. To determine if any isotope exchange occurred they degraded the ATP using acetyl CoA synthetase and glycerokinase and then derivatized the resulting phosphate with diazomethane. The trimethylphosphate derivative was then analyzed for ^{18}O content by mass spectrometry. This reaction is easily detected by the ^{18}O chemical shift technique because the γ-P of ATP will have lost its ^{18}O and thus will give rise to a new resonance for the γ-P of ATP. There will also be a shift in the resonance of the β-P because of different shifts caused by a bridge or nonbridge ^{18}O. This experiment has not, as yet, been repeated using NMR to detect the ^{18}O exchange.

CARBAMYL PHOSPHATE SYNTHETASE Carbamyl phosphate synthetase catalyzes the synthesis of carbamyl-P from HCO_3^-, glutamine, and 2 moles of ATP. The enzyme also catalyzes the HCO_3^--dependent hydrolysis of ATP. Raushel & Villafranca (unpublished observations) followed the exchange of ^{18}O from the bridge to the nonbridge position of [γ-^{18}O]ATP after incubation with enzyme and bicarbonate. The exchange rate was 0.5 times the rate of ADP formation. Shown in Figure 3 is the resonance for the γ-P of ATP before reaction with carbamyl phosphate synthetase and after the chemical reaction had proceeded to ~50% completion. The increase in the [$^{16}O^{18}O_3$] peak shows that there is loss of one ^{18}O from the γ-P. Under somewhat different reaction conditions and using a mass spectral analysis of the [^{18}O] ATP, Wimmer et al (73) have obtained an exchange rate that is 1.4–1.7 times the chemical rate. The experiments described above using ^{31}P-NMR were done in the presence of 10 mM L-ornithine, a positive allosteric effector of the enzyme which tightens the binding of ATP to the enzyme but does not affect the V_{max}. The experiments of Wimmer et al were done without any ornithine. Our experimental findings differ from those of Wimmer et al in that the ratio of the exchange rate to the chemical rate is ~0.5 in the presence or absence of ornithine. This is inconsistent with a slower rate for dissociation (k_{off}) of ATP from the enzyme relative to

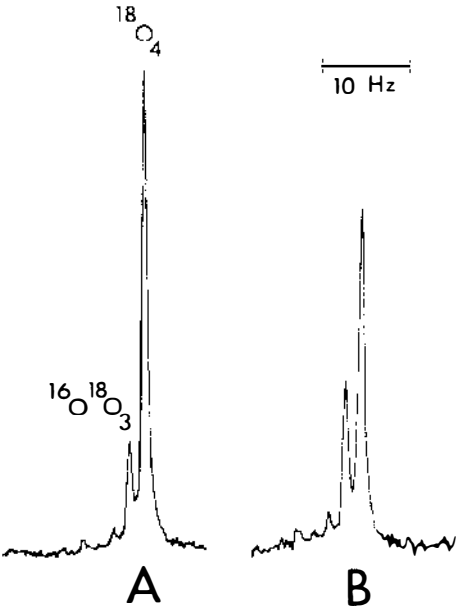


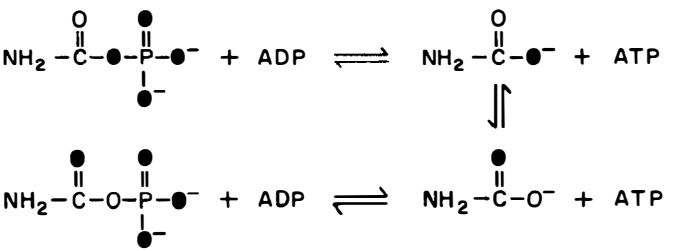
Figure 3 ^{31}P NMR spectra of the γ-P of [γ-^{18}O]ATP at 81 MHz. Only one half of the doublet is shown. (*A*) Before incubation with carbamyl phosphate synthetase and HCO_3^-. (*B*) After incubation with carbamyl phosphate synthetase. The reaction was stopped after 50% completion and the spectrum recorded.

the k_{cat} for the ATPase reaction. The rate of formation of intermediates in the reaction was studied using rapid reaction techniques by Raushel & Villafranca (56) and the data agree with the ^{31}P-NMR studies presented above.

The positional isotope exchange has also been measured with ^{31}P NMR in the reverse reaction of carbamyl phosphate synthetase:

$$\text{Carbamyl-P} + \text{ADP} \rightarrow \text{ATP} + \text{NH}_3 + \text{HCO}_3^-. \qquad 6.$$

Carbamyl phosphate was synthesized with ^{18}O in all oxygens except the carbonyl oxygen of carbon. The exchange of the bridge oxygen into the carbonyl oxygen was followed by NMR and was evidence for the following series of reactions



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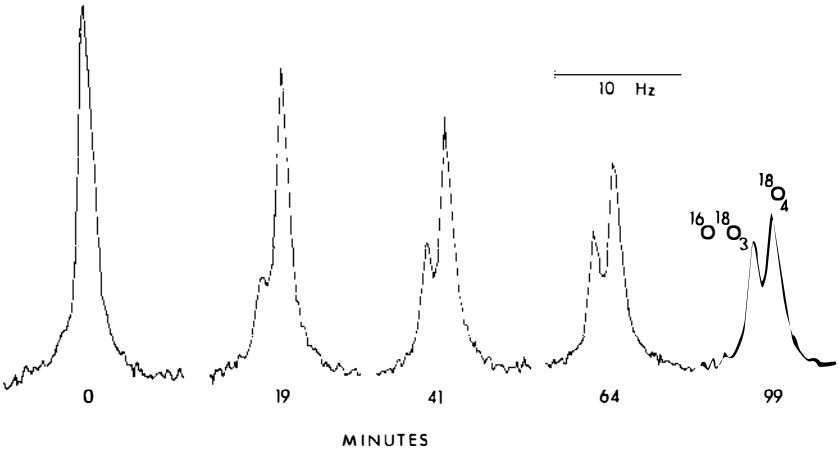
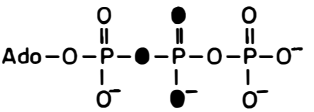


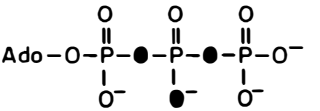
Figure 4 ^{31}P NMR spectra of [^{18}O] carbamyl phosphate at various times after the addition of carbamyl phosphate synthetase and MgADP. Initially all oxygens were labeled with ^{18}O except the carbonyl oxygen.

Shown in Figure 4 is a series of spectra taken at different times showing the exchange of ^{18}O from the bridge to nonbridge position of carbamyl phosphate. The exchange rate was 3.9 times the chemical rate.

PYRUVATE KINASE Using ATP that was labeled in the α-β bridge and β nonbridge positions (III)



III



IV

Lowe & Sproat (46) have shown using ^{31}P NMR at 36.4 MHz that pyruvate kinase catalyzes the exchange of ^{18}O from a β nonbridge position of ATP to a β-γ bridge position (IV) in the absence of any obvious acceptor molecule. They have argued that this is evidence for the formation of a metaphosphate intermediate. However, the possibility of transient transfer of the phosphoryl group to a nucleophile on the enzyme or to compounds such as bicarbonate, acetate (buffer), or even H_2O have yet to be ruled out.

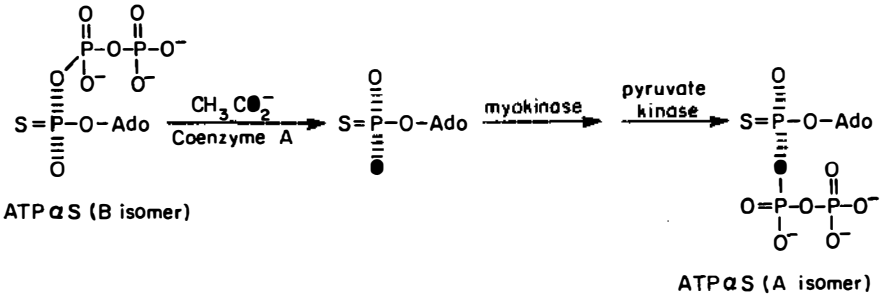
Tsai (67) has developed a technique using ^{17}O substituted phosphate. Since ^{17}O has a spin quantum number of 5/2 and a large electric quadrupole moment, it causes the ^{31}P NMR signal of phosphate to broaden substantially when ^{17}O has been substituted for ^{16}O. Tsai (67)

has used this method to determine whether the attack of acetate on adenosine-5′-(1-thiotriphosphate) proceeded with inversion or retention of configuration at the α-P in the acetyl coenzyme A synthetase reaction. The reaction catalyzed by this enzyme is:

$$\text{Acetate} + \text{coenzyme A} + \text{ATP} \rightarrow \text{acetyl CoA} + PP_i + \text{AMP}.$$

One of the oxygens of acetate is incorporated into AMP. This enzyme was found to use the B isomer of adenosine-5′-(1-thiotriphosphate) (ATPαs), which has the R configuration at the α-P (9, 12).

To determine the stereochemical course of the reaction, Tsai used [^{17}O] acetate and then determined whether the ^{17}O was incorporated into the pro-S or pro-R position of adenosine 5′-thiophosphate (AMPS). The isolated AMPS was reacted with myokinase and pyruvate kinase which are known to make ATP(αS), with the S configuration at the α-P (64). The pro-S oxygen will now be in the nonbridge position and the pro-R oxygen in the bridge position. Thus incorporation of ^{17}O in the pro-S position will decrease the intensity of only the α-P, and incorporation of ^{17}O into the pro-R configuration will decrease the intensity of both the α and β phosphate. Tsai found that both the α and β intensities were reduced 20% using 20% labeled ^{17}O acetate, thus showing that the reaction proceeded with inversion of configuration. This is shown in the following scheme:



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Midelfort & Sarton-Miller have also reported that acetyl coenzyme A synthetase proceeds with inversion of configuration (51).

The same experiments could have been performed using ^{18}O. The only advantage of using ^{17}O over ^{18}O appears to be that the effect caused by ^{17}O, since only the intensities are reduced, is easily detected by low field spectrometer, whereas a high field spectrometer is needed to get significant resolution to separate the ^{18}O and ^{16}O ^{31}P resonances.

Presumably the ^{17}O method could also be applied to problems analogous to those solved by the ^{18}O shift method, but since the ^{17}O method cannot distinguish cases where more than one oxygen will become labeled it does not appear to be potentially as versatile as the ^{18}O method. This is especially true if the phosphate-water exchange were to be measured because the ^{17}O method cannot distinguish the number of ^{17}O atoms bonded to a particular phosphate atom. Therefore it could not be determined if all of the oxygens were exchanged with solvent per enzyme encounter or if they were exchanged one at a time.

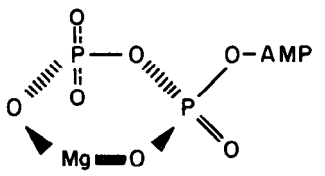
Stereochemistry of MgATP Complexes

Since the α and β phosphorous atoms of ATP are prochiral they are potentially asymmetric centers once they are complexed to divalent cations. There will be two diastereomers if complexation by Mg^{2+} is on the γ and β phosphates and 4 diastereomers if complexation is to all three phosphates. Since Mg^{2+} exchanges ligands very rapidly it is not possible to isolate individual isomers. However, Cr^{3+} and Co^{3+} exchange ligands much more slowly and thus should allow preparation of individual isomers. The Cr^{3+} and Co^{3+} derivatives of ATP have been synthesized in Cleland's laboratory (19, 22).

Since Cr(III) is paramagnetic it is not possible to analyze the products of the synthesis by NMR. However, Co^{3+} is diamagnetic and the β, γ derivative, Co(III) $(NH_3)_4$ATP, distinctly shows that two isomers are present as determined by ^{31}P NMR (20). Cornelius & Cleland were also able to show that yeast hexokinase used only one of the isomers in the following reaction (20):

$$\text{D-glucose} + \text{Co}(NH_3)_4\text{ATP} \rightarrow \text{CoADP} \cdot \text{glucose-6-P}.$$

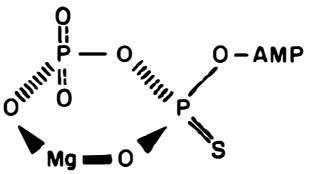
Both the ADP and the glucose-6-P are complexed with the cobalt. The unreacted isomer was degraded and crystallized and its absolute stereochemistry determined by X-ray crystallography (49). The active isomer (substituting Mg^{2+} for Co^{3+}) had the following structure:



Mg ATP

This isomer has been labeled the Λ isomer and the other possible structure, the Δ isomer (20).

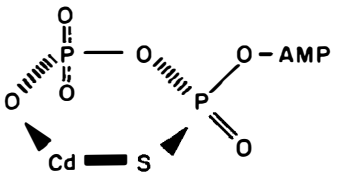
Ekstein & Goody have also prepared derivatives of ATP where sulfur has replaced oxygen in one of the nonbridge positions of the α and β phosphoryls (26). There are two diastereomers for each labeled phosphate. Jaffe & Cohn (36) showed that only the B isomer (nomenclature of Ekstein & Goody (26)) of ATPβS is a substrate for yeast hexokinase. Assuming that only oxygen is a ligand for Mg^{2+} they therefore proposed that the structure of the B isomer had the following configuration:



Mg ATPβS (B)

Thus the B isomer of ATPβS has an R configuration at the β phosphoryl. They also showed that the two diastereomers of ATPβS and ATPαS could be distinguished by their NMR spectra (37). The substitution of S for O results in a 40–50 ppm downfield chemical shift of phosphorus resonance for the phosphorus atom directly bonded to the sulfur atom. In ADPαS the α-P resonances of the A diastereomer are centered 0.4 ppm downfield from those of the B diastereomer (37). The β-P chemical shift of the A diastereomer of ATPβS is 0.14 ppm upfield from that of the B diastereomer (37). The absolute configuration of the ATPαS diastereomers has recently been completed and thus it is now possible to define the geometry of the Mg-ATP complexes that are active with kinases. The A isomer of ATPαS has the S configuration at α-P (9, 12).

With yeast hexokinase the preferred isomer of ATPβS was reversed when Cd^{2+} was used at the activating divalent cation (36). Jaffe & Cohn reasoned that this is because Cd^{2+} will bind preferentially to sulfur. However, the active compound still has the same geometric arrangement.



Cd ATPβS (A)

The reversal of specificity when Cd^{2+} is used as the activating divalent cation is a necessary requirement for establishing whether the metal ion is actually ligated to a particular phosphoryl group. It is also possible for side chains of the protein to determine the specificity by hydrogen bonding to one of the oxygens. This appears to be the case in hexokinase with ATPαS. The A isomer is used by hexokinase 20 times faster than the β isomer with either Mg^{2+} or Cd^{2+} and thus the metal ion is probably not coordinated to the α-P during the reaction of hexokinase.

Distance Determinations

In addition to being useful in the elucidation of the stereochemistry of metal ligation to ATP complexes at enzyme active sites, CrATP and CoATP are useful reagents for distance determinations of bound substrates using NMR. CrATP is paramagnetic and thus can be used as a spin-labeled center for distance measurements to other substrate nuclei. CoATP is diamagnetic and can serve as a replacement for MgATP in distance measurements from other paramagnetic centers on the enzyme. This also determines the geometrical conformation of the bound nucleotide. The theory for using paramagnetic molecules to affect the relaxation rates of bound substrates in order to determine intermolecular distances has been adequately reviewed (24).

PYRUVATE KINASE Gupta et al (29) have used CrATP as a paramagnetic probe for the enzyme pyruvate kinase. Using the effect of enzyme bound CrATP on the longitudinal relaxation times (T_1) of pyruvate they were able to determine the distance from the Cr to the methyl protons and to the C-1 and C-2 carbon atoms of pyruvate. The correlation time for the interaction was estimated from the frequency dependence of $1/T_1$ of the water protons. The distances were 6.1 ± 0.4, 6.1 ± 0.3, and 7.9 ± 0.5 Å to the C-1, C-2 carbon atoms, and methyl protons respectively. The results clearly put the pyruvate atoms in the second sphere of the Cr^{3+} and thus establish a close proximity of the phosphate donor substrate (ATP) to the phosphate acceptor substrate on a kinase.

Gupta & Benovic (31) also measured the effect of CrADP on the longitudinal relaxation times of the protons and phosphorus atoms of phosphoenolpyruvate in the enzyme·PEP·CrADP complexes. Using a correlation time of 0.35 nsec they obtained a Cr(III) to phosphorus distance of 5.9 ± 0.4 Å and Cr(III) to proton distances of 8.5 ± 0.6 and 9.6 ± 1.3 Å. The results indicated van der Waals contact between a phosphoryl oxygen of phosphoenolpyruvate (PEP) and the hydration sphere of the nucleotide-bound metal.

Gupta (30) has recently introduced a novel method for determining the distance between two paramagnetic species bound to a protein. This

technique has been applied to pyruvate kinase where it is known that two divalent cations are needed (29). One site is a free divalent cation site and the other is a metal-nucleotide site (29). This technique is based on the assumption that two paramagnetic metal ions with similar electron resonance frequencies may affect the relaxation properties of each other via concerted spin flips. Gupta has used this technique to measure the distance between Mn^{2+} bound at the free divalent cation site and CrATP at the metal nucleotide site. CoATP was used as the diamagnetic control. In this case where one of the spins, such as Cr(III) in CrATP, has a relaxation time (τ_s^{Cr}) much shorter than that of the other spin such as Mn^{2+} (τ_s^{Mn}), and the rotational correlation time of the entire molecule, τ_R, is long compared to τ_s^{Cr}, the cross-relaxation effect shortens the effective spin lattice time of the slowly relaxing spin according to the following equation:

$$\left(\frac{1}{\tau_s^{Mn}}\right)_{Cr} - \left(\frac{1}{\tau_s^{Mn}}\right)_{Co} = \frac{2}{15}\frac{S^{Cr}(S^{Cr}+1)\gamma_{Cr}^2\gamma_{Mn}^2\hbar^2}{r^6}\cdot\tau_s^{Cr} \qquad 10.$$

S^{Cr} and τ_s^{Cr} are the net unpaired spin and the electron spin relaxation time of Cr(III). γ_{Cr} and γ_{Mn} are the electronic gyromagnetic ratios for the Cr^{3+} and Mn^{2+}, and r is the Mn^{2+} to Cr^{3+} distance. $\left(\frac{1}{\tau_s^{Mn}}\right)_{Co}$ is the electron spin relaxation time in the presence of the diamagnetic control, CoATP.

The relaxation times (τ_s^{Mn}) are estimated from the enhancement by Mn^{2+} on the longitudinal relaxation rate of water protons (ε^{Mn}) in the appropriate enzyme complex.

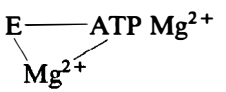
At 24.3 MHz with CrATP and CoATP, the enhancements, ε^{Mn}, by Mn^{2+} are 6 ± 1 and 12 ± 2, which calculates to a distance of 4.8–5.6 Å, depending on the number of assumed water molecules and using $\tau_S^{Cr}=(2.3\pm0.5)\times10^{-10}$ sec. From data also at 8 MHz the calculated Cr^{3+} to Mn^{2+} distance is 5.2 ± 0.9 Å, which indicates van der Waals contact between the hydration sphere of Cr^{3+} and Mn^{2+} in the pyruvate kinase·Mn^{2+}·CrATP complex.

This technique should also be useful in other kinase reactions that require two divalent cations such as glutamine synthetase, carbamyl phosphate synthetase, and PEP carboxy kinase. The chief difficulty with this technique is knowing the exact number of fast exchanging water molecules in the enzyme complexes. However, because of the sixth-root dependence on the distance, this does not introduce unnecessarily high errors. Another important assumption is that the replacement of CrATP with CoATP has no other effect than replacing a paramagnetic complex with a diamagnetic one.

GLUTAMINE SYNTHETASE The distance between two metal ions bound to a protein has also been measured using electron paramagnetic resonance (EPR). This experiment is based on the theory of dipolar electronic relaxation developed by Leigh (40). The theory predicts that if the EPR spectrum of enzyme-bound Mn^{2+} can be observed, the intensity of that signal will diminish upon addition of a second paramagnetic species, such as CrATP, without any change in lineshape. The magnitude of the diminution depends on the distance between the two metal ions. Balakrishnan & Villafranca (37) measured the effect of CrATP on the EPR spectrum of enzyme-bound Mn^{2+} in glutamine synthetase and determined the distance to be 7.1 ± 0.5 Å. The distance between the metal ions of glutamine synthetase was also estimated using the method of Gupta. A distance of 6 ± 1 Å was obtained (S. Ransom and J. J. Villafranca, unpublished results).

BOVINE HEART PROTEIN KINASE Granot et al (27) have used $Co(NH_3)$ ATP to measure the conformation of ATP bound to protein kinase. They had previously determined that in the presence of ATP, protein kinase will bind two atoms of Mn^{2+}. One Mn^{2+} is at the ATP site and one is at an unknown site. In the presence of CoATP only one additional Mn^{2+} was bound, indicating the Co was occupying one site usually held by the other bound Mn^{2+} (24). Using bound Mn^{2+} as the paramagnetic center, the effect of Mn^{2+} on the longitudinal and transverse relaxation times of the proton and phosphorus atoms of CoATP was determined. The results indicated that the CoATP was bound very closely to the Mn^{2+}. The Mn^{2+} was 3.0 Å from each of the phosphorus atoms and 6.4–8.1 Å to the protons on the adenine ring and ribose. From the data gathered it was not possible to determine whether the adenine ring was in an *anti* or *syn* conformation.

On the basis of these data they have suggested the following structure



which indicates that the extra metal is binding to both the enzyme and MgATP.

PRPP SYNTHETASE PRPP synthetase catalyzes the synthesis of phosphoribosyl pyrophosphate (PRPP) from ATP and ribose-5-phosphate. This is an unusual reaction for ATP because of nucleophilic attack at the β-phosphorus. Li et al (41) had previously shown that the Δ isomer of β-γ CoATP is a good substrate for the enzyme. Li et al (42) have

used tridentate CrATP to establish the substrate topography on the enzyme. From the paramagnetic effect of enzyme-bound CrATP on the longitudinal relaxation rates of the anomeric proton and phosphorus atoms of ribose 5-phosphate, they found that both isomers of ribose 5-phosphate bind near the CrATP and that H-1 of the β-isomer is 1.0 Å closer to bound CrATP than is the H-1 of the α-anomer. Therefore the 1-OH group of the α isomer is 1.6 Å closer than the 1-OH group of the β isomer. Assuming that the polyphosphate chain is between the Cr^{3+} and the ribose 5-phosphate, the β-P of ATP is 3.8 ± 0.8 Å from the 1-OH of α-ribose 5-phosphate. This distance approximates the sum of the van der Waals radii and allows no intervening atoms. This is consistent with a direct nucleophilic attack and an associative mechanism as previously deduced from the inversion of configuration of the β-P of $Co(NH_3)ATP$ (41).

NMR STUDIES OF METAL NUCLEI

Background on Nuclei With Spin >1/2

The direct study of metal ions (other than Na^+) in biological systems by NMR techniques is a field in its adolescence. Table 2 presents the properties of a number of metal nuclei that either have been shown to have a direct biological function or can serve as substitutes for naturally

Table 2 Properties of nuclei[a]

Nucleus	Spin	Quadrupole moment	Frequency, MHz	Natural abundance	Relative sensitivity[b]
^{1}H	1/2	—	200.1	99.99	1.00
^{6}Li	1	0.00046	29.45	7.42	6.31×10^{-4}
^{7}Li	3/2	−0.042	77.77	92.58	2.71×10^{-1}
^{13}C	1/2	—	50.32	1.11	1.75×10^{-4}
^{23}Na	3/2	0.11	52.93	100	9.25×10^{-2}
^{25}Mg	5/2	0.22	12.25	10.13	2.70×10^{-4}
^{39}K	3/2	0.055	9.34	93.08	4.73×10^{-4}
^{43}Ca	7/2	0.06[c]	13.46	0.145	9.28×10^{-6}
^{63}Cu	3/2	−0.15	53.04	69.09	6.43×10^{-2}
^{67}Zn	5/2	0.18	12.52	4.11	1.17×10^{-4}
^{113}Cd	1/2	—	44.39	12.26	1.34×10^{-3}
^{133}Cs	7/2	−0.003	26.25	100	4.74×10^{-2}
^{139}La	7/2	0.21	28.27	99.91	5.91×10^{-2}

[a]From the *Handbook of Chemistry and Physics* (39).
[b]The sensitivity of a nucleus (from Reference 39) multiplied by the natural abundance and normalized to ^{1}H.
[c]Calculated from the T_1 data in Reference 43.

occurring metal ions. The Table includes NMR frequencies at constant field for a spectrometer operating at 200 MHz for ^{1}H. A number of companies offer spectrometers operating at this field strength (47 kG) that have multinuclear capabilities; thus many scientists will be able to study some or all of the nuclei listed in Table 2 in the near future. This section of the review explores practical and theoretical aspects of high resolution fourier transform NMR studies of metal ions and reviews some of the current literature.

One of the most important considerations in the design of NMR experiments is the sample (nuclei) concentration, as this directly determines the signal-to-noise (S/N) ratio that one can hope to achieve. All of our applications deal with metal ions binding to proteins. The solubility of a protein in a buffer at a certain pH, temperature, salt concentration, etc., is usually a limiting factor, since the practical range for protein concentrations in NMR experiments is 10^{-4}–10^{-2}M. Certain enzymes have been studied extensively by high resolution ^{1}H and ^{13}C NMR, in part because of their unusually high solubility and low molecular weight (e.g. lysozyme and ribonuclease). Solubility of a protein governs the S/N problem for a metal ion bound to a protein, since only 1–2 metal ions are usually bound per monomer. However studies of metal ion binding to proteins can be carried out by using excess metal ion and titrating with protein (for fast exchange conditions), thus permitting information on the bound metal ion to be evaluated from the data. However, direct observations of the bound metal ion is always desirable, since changes in chemical environment can be directly followed by changes in chemical shifts.

In Table 2 the relative natural abundance sensitivities of metal nuclei at constant field are compared to ^{1}H and ^{13}C. The comparison of sensitivities for various nuclei is presented in this way so that one can judge how practical a particular experiment would be (time-wise) to achieve the same S/N for the same "concentration" of material. This assumes that all other conditions are equal e.g. relaxation rates, nuclear Overhauser effect (NOE), instrumental conditions, quadrupole effects, chemical environment, exchange, etc., which is never the case. However the comparison provides an idealized starting point. Isotopic enrichment of ^{6}Li, ^{25}Mg, ^{43}Ca, ^{67}Zn, and ^{113}Cd will substantially improve the desired S/N conditions, and enriched samples of these nuclei are available commercially or from government laboratories.

Of the metal nuclei listed only ^{113}Cd has a nuclear spin of one half (other metal nuclei with a spin of one half, e.g. Pt, Hg, Sn, Pb, are not discussed). All the other nuclei have spin greater than one half, which means that they possess a nuclear quadrupole moment. These values are also listed in Table 1.

One example will suffice. For a sample of 0.05 M lithium formate in water, a single scan in a fourier transform spectrometer is all that is needed to observe the ^{1}H of formate and the $^{7}Li^{+}$. Several thousand spectra would have to be averaged to achieve a spectrum with good S/N for ^{13}C and $^{6}Li^{+}$ however. Incidentally, the time required for 1000 scans for the ^{13}C spectrum is roughly 4 hr since the T_1 is about 5 sec and the time between pulses should be $\sim 3 \times T_1$. However, the T_1 for aqueous samples of $^{6}Li^{+}$ is $\sim$170 sec at 30°C, and 1000 scans would take $\sim$6 days (!) under the same conditions. Isotopic enrichment would seem to be a must for biological experiments with ^{6}Li.

Since most of the metal nuclei in Table 2 have quadrupole moments, a discussion of quadrupolar relaxation and its important aspects with regard to biological applications are presented before a discussion of each nucleus. An excellent compilation of physical properties of the alkali (44) and alkaline earth (45) ions has recently appeared, and the purpose of the current review is to stress applications to biomacromolecules.

Hertz has presented a detailed mathematical model for quadrupolar relaxation (33, 34). The magnetic relaxation of nuclei with spin greater than one half is caused by the interaction of their electric quadrupole moment with an electrical gradient at the nuclear site, which is the result of the local charge distribution. This charge distribution can be due to ions or dipoles or both in the first or outer hydration shells of metal ions. For solvated ions Hertz has presented a "fully random distribution model" formulated as follows

$$\frac{1}{T_{1(o)}} = \frac{1}{T_{2(o)}} = \frac{24\pi^3}{5} \cdot \frac{2I+3}{I^2(2I-1)} \left(\frac{PeQ(1+\gamma^{\infty})}{h} \right)^2 \frac{M^{2} \cdot C \cdot \tau}{r^5} \qquad 11.$$

The longitudinal $(1/T_1)$ and transverse $(1/T_2)$ relaxation rates are equal for infinite dilution, and definitions of the pertinent quantities for our discussion are: I, spin quantum number; eQ, nuclear quadrupole moment; and $(1+\gamma^{\infty})$, the Sternheimer antishielding factor (47). A common misconception about nuclei with quadrupole moments is that they have excessively broad NMR spectra that will make high resolution experiments unobtainable. This fallacy can be overcome by examining the data for some ions. T_1 values in parenthesis for some aqueous ions at $\sim$25°C are $^{7}Li^{+}$ (25 sec), $^{6}Li^{+}$ (170 sec), $^{23}Na^{+}$ (0.07 sec), $^{133}Cs^{+}$ (15 sec), $^{43}Ca^{+2}$ (1.3 sec), $^{25}Mg^{2+}$ (0.14 sec), $^{139}La^{3+}$ (0.003 sec) (44, 45, 60, 71, 72). The corresponding line widths (not considering instrumental broadening) are $^{7}Li^{+}$ (0.01 Hz), $^{6}Li^{+}$ (0.002 Hz), $^{23}Na^{+}$ (4.5 Hz), $^{133}Cs^{+}$ (0.02 Hz), $^{43}Ca^{2+}$ (0.25 Hz), $^{25}Mg^{2+}$ (2.3 Hz), $^{139}La^{3+}$ (106 Hz). Thus, with the exception $^{139}La^{3+}$, narrow lines are expected in this limited

selection and are actually observed for metal ions with I values from 1 to 7/2. The major structural difference between metal ions and compounds involving $^{14}N(I=1,\ Q=0.071)$ and $^{17}O(I=5/2,\ Q=0.004)$ is that metal ions have "ionic" bonding, whereas compounds of ^{14}N and ^{17}O are covalently bonded to other atoms. The paramagnetic effect of the p electrons involved in bonding produces a large asymmetric electric field gradient at the nucleus, making quadrupole relaxation quite efficient and hence the lines very broad (100–3000 Hz). Comparison of the spin quantum number, the Sternheimer antishielding factor, and the quadrupole moments will point out why narrow lines are observed for $^{133}Cs^+$ and broad lines for $^{139}La^{3+}$ in dilute ionic solutions. For both nuclei $I=7/2$, so this factor is not important. The Sternheimer antishielding factor is larger for Cs $[(1+\gamma^\infty)\simeq 104)]$ compared to La $[(1+\gamma^\infty)\simeq 66]$, and since this enters Equation 11 as a squared term this factor is ~2.5 times larger for Cs. However, the ratio of the quadrupole moments squared differs by about 5000, which is the dominant factor in the narrow lines widths for Cs^+ compared to La^{3+}.

For consideration of NMR experiments of metal ions with quadrupole moments bound to macromolecules the following expression holds:

$$1/T_{1,\text{obs}} = P_f(1/T_{1f}) + P_b(1/T_{1b}). \qquad 12.$$

This equation is valid for rapid chemical equilibrium between free (f) and bound (b) sites where P is the fraction of nuclei in each environment. An analogous expression obtains for T_2. Titration experiments of protein into metal ion solutions are usually performed for weakly binding ions, and both binding constants and $1/T_{1b}$ (or $1/T_{2b}$ or both) can be obtained. One can expect the symmetries of the free and bound states to be different, and hence their quadrupole coupling constants, since the free ion is hydrated and the bound ion is ligated by amino acid residues and perhaps also by water molecules. Ideally such titration experiments would reach an end point for the fully bound metal and this "bound" species could then be examined in detail. For the case of enzymes, which are of most interest to the authors, substrate and inhibitor binding at the metal ion site could then be explored to look for chemical shift differences in tightly bound metal ion-enzyme complexes. Examination of Table 2 again reveals that this may be practical, in the high resolution sense, for the quadrupole nuclei $^{43}Ca^{2+}$, $^{133}Cs^+$, $^7Li^+$, and $^6Li^+$. But only Ca^{2+} is known to bind very tightly to proteins and such experiments would have to be performed with enriched $^{43}Ca^{2+}$.

In addition to changes in the quadrupole coupling constant when a metal ion binds to a macromolecule, there is a drastic change in the correlation time, τ_c. This arises because in the hydrated ion τ_c is 10^{-11}–10^{-12} sec, and when the ion is bound to a protein τ_c is the

tumbling time for the macromolecule (10^{-9}–10^{-8} sec for small proteins of $<60{,}000$ mol wt). Bull has treated this problem for the $I=3/2$, $5/2$, and $7/2$ cases (10, 11). For these nuclei and nonextreme narrowing conditions (when $\omega\tau_c \sim 1.5$ as would be the case for many macromolecules), a single T_1 and T_2 value is still obtained since the magnetization decay is approximately exponential. For fast exchange conditions the difference in relaxation rates in the presence ($1/T_{i,obs}$) and absence of ($1/T_i$) of macromolecules are

$$1/T_1 = 1/T_{1,obs} - P_f(1/T_{1f})$$
$$= \frac{3(2I+3)\tau_c}{40I^2(2I-1)} \cdot K^2 \cdot P_B\left(\frac{0.8}{1+4\omega^2\tau_c^2} + \frac{0.2}{1+\omega^2\tau_c^2}\right) \quad 13.$$

$$1/T_2 = 1/T_{2,obs} - P_f(1/T_{2f})$$
$$= \frac{3}{40}\frac{(2I+3)\tau_c}{I^2(2I-1)} \cdot K^2 \cdot P_B\left(0.3 + \frac{0.5}{1+\omega^2\tau_c^2} + \frac{0.2}{1+4\omega^2\tau_c^2}\right) \quad 14.$$

where K^2 is the quadrupole coupling constant. When $\omega^2\tau_c^2 \gtrsim 1$, τ_c can be obtained from a frequency dependence of T_1 or T_2 or from the T_1/T_2 ratio. The above equations are both valid for $I=3/2$; but for $I=5/2$ and $7/2$, $1/T_1$ is approximated by a single exponential for the case of two-site exchange, whereas $1/T_2$ is the sum of three and four exponential for $5/2$ and $7/2$ respectively. This could in practice produce one relatively narrow line and one very broad line or a non-Lorentzian line shape. This has been recently observed (S. Forsén, personal communication).

For $^{43}Ca^{2+}$ bound to a protein of $\sim$40,000 mol wt with $\tau_c \sim 1.5 \times 10^{-8}$ sec, one can expect a line of the bound ion of $\geqslant 250$ Hz. If all of the $^{43}Ca^{2+}$ is bound, one could observe this resonance with modern fourier transform spectrometers. However, if the relaxation is also modulated by chemical exchange (between several protein conformers, each having a different chemical environment), then the spectrum may be broadened beyond detection. This will be shown for some $^{113}Cd^{2+}$-proteins in a later section.

^{7}Li NMR Studies

There have been relatively few studies of this nucleus with biological macromolecules. ^{7}Li is the most abundant naturally occurring isotope; its narrow line width in aqueous solution and high sensitivity make it an ideal NMR probe. Li^+ has been shown to substitute for Na^+ or K^+ with many enzymes that require a monovalent cation for catalytic activity. Perhaps the best studied enzymes that are activated by monovalent cations are pyruvate kinase, the Na^+-K^+ ATPases, and the Ca^{2+}-ATPases.

Li^+ provides a low level of activation of pyruvate kinase and has a weak binding constant of ~11 mM. Two groups have studied the interaction of $^7Li^+$ with this enzyme (3, 35). Hutton et al (35) conducted studies of the $1/T_1$ relaxation rates of $^7Li^+$ interacting with enzyme in the presence of Mn^{2+} and the substrate phosphoenolpyruvate (PEP). They calculated distances between Mn^{2+} and $^7Li^+$ based on paramagnetic enhancement of the $1/T_1$ rate of $^7Li^+$ by enzyme-bound Mn^{2+}. Their distances (E-Mn^{2+}-Li^+, $r = 11.0$ Å; E-Mn^{2+}-Li^+-PEP, $r = 5.8$ Å) were longer than the corresponding Mn^{2+} to $^{205}Tl^+$ distances (8.2 Å and 4.9 Å respectively) obtained by Kayne & Reuben (38, 59) who conducted $^{205}Tl^+$-NMR ($I = 1/2$) studies with this enzyme. Some of the discrepancies were clarified by Ash et al (3) who carried out $^7Li^+$-NMR experiments with this same enzyme at 5 and 30°C. Previous EPR studies (58) showed that the pyruvate kinase-Mn^{2+}-Li^+-PEP complex exists as an equilibrium between two different enzyme conformers. Low temperature favors a complex characterized by a Mn^{2+}-Li^+ distance of 9 Å, whereas at high temperature this distance is 4.7 Å in agreement with Kayne & Reuben.

Recent $^7Li^+$ data with Cr^{3+}-ATP as the paramagnetic probe show that the Li-Cr distance is 4.9 Å in the absence of Mg^{2+} (there is another divalent metal ion site in addition to the metal-nucleotide site) but changes to 6.0 Å with Mg^{2+} (68). The overall conclusion from these data is that in the active enzyme complex the divalent-monovalent cation distance is the same, whether the monovalent cation gives low or high activation; thus other factors than the spatial relationship between enzyme-bound cations must be responsible for the differences in enzyme activity. One can now see how powerful NMR studies can be for answering fundamental structural questions, especially when two nuclei ($^7Li^+$ and $^{205}Tl^+$) can be studied.

Several other enzymes are in the preliminary stages of investigation by $^7Li^+$-NMR. Very interesting studies on Na^+-K^+ATPase from kidney by Grisham & Hutton (28) suggest that Li^+ will occupy only the K^+ site at 10 mM Na^+. Using this observation, these authors studied the effect of Mn^{2+} and Cr^{3+}-ATP on the $1/T_1$ relaxation rates of $^7Li^+$. A Mn^{2+}-Li^+ distance of 7.2 Å was computed in the presence of Na^+ but the Li^+-Cr^{3+} data were not analyzed. More recent studies with Ca^{2+}-ATPase, with Gd^{3+} as the paramagnetic probe, show two Gd^{3+} binding sites that are 7.0 and 9.1 Å from the Li^+ (K^+) binding site (65). A picture is thus emerging from these data showing that the activating monovalent cation site(s) are close to the active site for ATP hydrolysis.

Carbamoyl-phosphate synthetase is activated by divalent and monovalent cations in addition to having two ATP sites (see earlier section) (55, 57). In preliminary studies from our laboratory a Li^+-Mn^{2+} distance of 7.9 Å was calculated between the two cation activator sites

(F. M. Raushel and J. J. Villafranca, unpublished observations). Further studies are underway to establish the spatial relationship between these sites and the catalytic site.

In future work $^{7}Li^{+}$ relaxation rate studies should be conducted at two magnetic field strengths (for diamagnetic complexes) so that both τ_c and K^2 can be evaluated for the bound $^{7}Li^{+}$. In this way the change in quadrupole coupling constant can be used to evaluate certain aspects of the bonding at the monovalent cation binding site in macromolecules. This parameter should be sensitive to change in local environment when various other substrates or inhibitors bind; these data may then be correlated with the paramagnetic effects already observed.

If only one field strength is available but the spectrometer has the capability for observations of $^{6}Li^{+}$ and $^{7}Li^{+}$, then the T_1 ratio for these two nuclei will provide the ratio of the K^2 values; this could provide a parameter for studying conformational changes at the monovalent cation site of macromolecules, especially since τ_c is expected to stay the same for both bound $^{6}Li^{+}$ and $^{7}Li^{+}$.

Another nucleus that should receive more attention for biological studies is $^{133}Cs^{+}$. As can be seen in Table 2, the quadrupole moment is very small, the natural abundance is 100%, T_1 values are long, and the sensitivity quite reasonable. In practice, only a few scans are required per τ value for a T_1 experiment at a reasonable concentration of ions (10–50 mM) for biochemical studies (F. M. Raushel and J. J. Villafranca, unpublished observations). The crystal ionic radius of 1.67 Å suggests that it would be a good replacement for K^{+} (1.33 Å), considering that Tl^{+} (1.47 Å) has been used as a replacement for K^{+} in pyruvate kinase.

Studies could of course be conducted with $^{23}Na^{+}$ and $^{39}K^{+}$ but would be more difficult because of excess broadening in the bound state.

^{43}Ca, ^{25}Mg, and ^{139}La Studies

Only a few studies on biological molecules have been reported with these nuclei. Robertson et al (62) reported $^{43}Ca^{2+}$ and $^{25}Mg^{2+}$ binding to γ-carboxy-glutamate containing peptides as models of proteins involved in the blood coagulation processes. This newly discovered amino acid is the proposed site for binding the Ca^{2+} and Mg^{2+} ions in the proteins of the blood-clotting process.

As expected the $^{25}Mg^{2+}$ line width broadened from a few Hz in free solution to ~160 Hz in the peptide complex. This is due to the drastic change in τ_c upon complexation and also perhaps to an increase in K^2.

For this study 98.25 atom % enriched $^{25}Mg^{2+}$ was used, and the authors report that from 1000-30,000 scans were acquired per spectrum in a titration experiment to achieve a S/N of 5–10 to 1.

When $^{43}Ca^{2+}$ (79.98 atom %) binding was studied with the peptide, the authors report a shift upon binding to the peptide with little broadening (~2 Hz). The line widths were sharp (as expected for the small Q value) and the reported shift was about 14 Hz for full complexation. No discussion is given of whether fast exchange prevails under these experimental conditions, however.

Lindman's laboratory has presented preliminary NMR data on $^{25}Mg^{2+}$ (13), $^{43}Ca^{2+}$ (54), and $^{113}Cd^{2+}$ (13, 23) binding to parvalbumin, a Ca^{2+}-binding protein of 11,500 mol wt. When parvalbumin was added to a 0.1 M solution of $^{43}Ca^{2+}$ (61.63 atom %), no significant variation of the line width (2–3 Hz) was observed up to 58°C. This is attributed to slow exchange of Ca^{2+} on the NMR time scale ($k_{off} \leqslant 10^3$ sec^{-1}). Broadening of the $^{43}Ca^{2+}$ signal was observed from 65 to 95°C (up to ~20 Hz) and the protein is known to undergo a disorganization of tertiary structure at these elevated temperatures. Rapid exchange conditions are thus present and the authors argue that exchange of metal ion from the native Ca^{2+}-binding sites is being monitored under these conditions. An increase in the $^{43}Ca^{2+}$ line width was also observed at 54°C when the pH was varied from 7 to 12. This phenomenon was reversible, but none of these studies allows a distinction between the two Ca^{2+}-binding sites.

$^{113}Cd^{2+}$ NMR studies of parvalbumin have been conducted by this group. These studies show two distinct $^{113}Cd^{2+}$ resonances, with parvalbumin at about −94 and −98 ppm. These signals of equal intensity are nearly invariant when the pH is changed from 7 to 9. An interesting competition experiment was performed in which Gd^{3+} was titrated into a solution of $^{113}Cd_2^{2+}$-parvalbumin. The two signals selectively and progressively disappear without broadening, which is consistent with the stepwise displacement of the two $^{113}Cd^{2+}$ ions.

Experiments with $^{25}Mg^{2+}$ binding to parvalbumin were not as definitive as those with $^{43}Ca^{2+}$ and $^{113}Cd^{2+}$. When Ca_2^{2+}-parvalbumin is titrated into a solution of $^{25}Mg^{2+}$ (0.1 M, 97.9 atom %), broadening of the 25 Mg^{2+} signal is observed. It is unclear whether Mg^{2+} is displacing the Ca^{2+}. Since the broadening implies that fast exchange conditions exist, it is possible that $^{25}Mg^{2+}$ is binding to ancillary metal ion sites on the protein. Further experimentation seems necessary. Overall, one can see from these experiments by Lindman's group that the use of multiple NMR nuclei in the study of metal ion binding to proteins is indeed a powerful technique.

Reuben has reported binding of $^{139}La^{3+}$ to bovine serum albumin (60). In all cases the line widths were broad and the T_1 values very short (61), as was expected for the Q value (Table 2) and large Sternheimer antishielding factor (47). Nonetheless, when $^{139}La^{3+}$ replacement for Ca^{2+} or Mg^{2+} is a desirable experiment, one may choose to study this nucleus to provide additional information on the metal ion binding site. The $^{139}La^{3+}$ binding study to bovine serum albumin was conducted at two magnetic field strengths and the τ_c value obtained, 2.25×10^{-8} sec, was in good agreement with the tumbling time of the macromolecule.

^{63}Cu and ^{67}Zn

No NMR studies of these nuclei have been reported for biomacromolecules. $^{67}Zn^{2+}$ should possess many of the NMR properties of $^{25}Mg^{2+}$ but would have to be obtained in enriched form for studies to be practical. The line widths of bound nuclei would be ≥ 350 Hz but may be observable for some small proteins. ^{63}Cu could be studied in the diamagnetic states as Cu^{+} or Cu^{3+} (with tetragonal distortions). Many small molecule complexes have been synthesized and could be studied with respect to their chemical shift differences (expected to be large due to the difference in charge); NMR could be used to evaluate whether some Cu^{2+}-containing proteins change oxidation state in their catalytic reactions (32) to Cu^{+} or Cu^{3+}.

^{113}Cd NMR Studies

Since ^{113}Cd has a nuclear spin of one half it does not have a quadrupole moment. Recently many studies of $^{113}Cd^{2+}$ binding to macromolecules have appeared, mainly from the laboratories of Coleman at Yale (18) and Ellis at the University of South Carolina (4). $^{113}Cd^{2+}$ compounds have a shift of over 800 ppm which is attributable to distortions of the electron cloud of the filled orbitals and the paramagnetic contribution of these electrons to the "shielding" at the nucleus. Coleman's laboratory has observed a chemical shift range of $\sim$200 ppm for $^{113}Cd^{2+}$ bound to enzymes where the protein ligands are nitrogen and/or oxygen.

In Figure 5*A* there is the spectrum of $^{113}Cd_2^{2+}$-alkaline phosphatase (14, 17) (2 Cd^{2+} bound to the dimer of 86,000 mol wt from *Escherichia coli*.) One line is observed (with a line width of $\sim$50 Hz) at 170 ppm referenced to cadmium perchlorate. This is interpreted as both Cd^{2+} ions being in the same chemical environment in the protein dimer. Addition of one equivalent of phosphate to the protein gives the spectrum shown in Figure 5*B*. The $^{113}Cd^{2+}$-NMR spectrum now consists of two lines—one at 142 ppm and one at 55 ppm. The conclusion

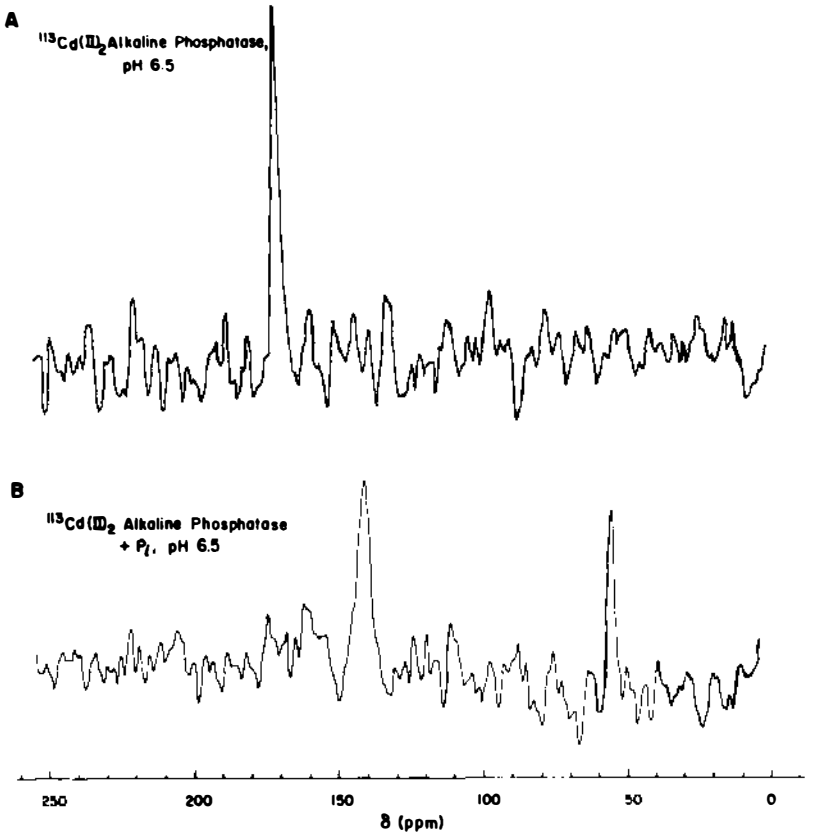


Figure 5 $^{113}Cd^{2+}$ NMR spectra of Cd_2^{2+}-alkaline phosphatase (*A*) and Cd_2^{2+}-phosphoryl alkaline phosphatase (*B*) (from Reference 14, with permission).

drawn from this study is that phosphorylation of one of the two active sites by P_i has altered the chemical environment of both subunits and thus of both metal ion sites. Since neither $^{113}Cd^{2+}$ resonance is at the same resonance position as in the sample with no P_i added, phosphorylation destroys the twofold symmetry of the subunit, and this is interpreted in terms of the negative cooperativity exhibited in catalysis by this enzyme.

An even more dramatic result (for a spectroscopist) is the observation of ^{31}P-^{113}Cd coupling in the ^{31}P spectrum of alkaline phosphatase (52). A doublet with ~30 Hz coupling constant is observed for the ^{31}P resonance assigned to the binary $E \cdot P_i$ complex (Figure 6). This coupling is found in the enzyme-Cd_4^{2+} complex (2 active site metal ions and two structural) and is proof that the metal ion at the active site is ligated to

the P_i in an inner sphere complex. The overall catalytic scheme with alkaline phosphatase and P_i is

$$E + P_i \rightleftharpoons E \cdot P \rightleftharpoons E\text{–}P + H_2O$$

where $E \cdot P_i$ is a non-covalent complex and E–P is covalently attached phosphate. The ^{31}P-^{113}Cd coupling is only seen for the ^{31}P resonance corresponding to $E \cdot P_i$ and these data suggest that when the covalent complex forms, the $^{113}Cd^{2+}$ is no longer binding to the phosphoryl group.

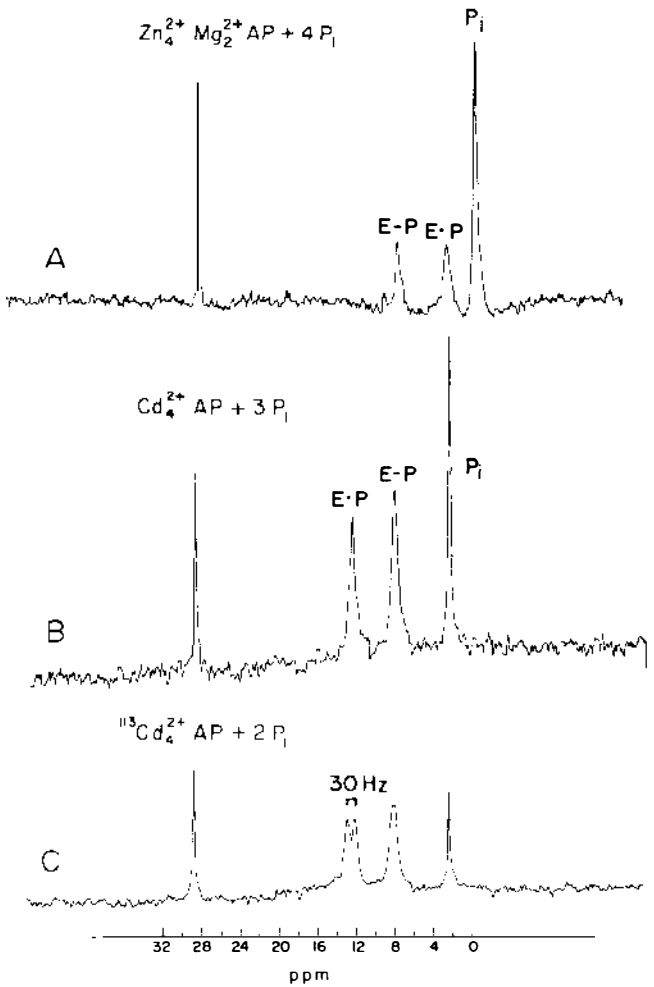


Figure 6 ^{31}P NMR spectrum of $^{113}Cd_4^{2+}$-alkaline phosphatase; conditions in Reference 52. The 30 Hz coupling constant in *C* is from $^{113}Cd^{2+}$ (enriched).

$^{113}Cd^{2+}$-NMR has also been applied to the study of carbonic anhydrase by Coleman's laboratory (18, 63). These data point out some of the unique advantages and disadvantages in studying metal ion-NMR spectra of protein-bound metal ions. One consequence of the large range of chemical shifts of $^{113}Cd^{2+}$ bound to macromolecules is that if the protein is fluctuating between various conformers at rates between 10^2–10^4 sec^{-1} and the chemical shift of the $^{113}Cd^{2+}$ in these

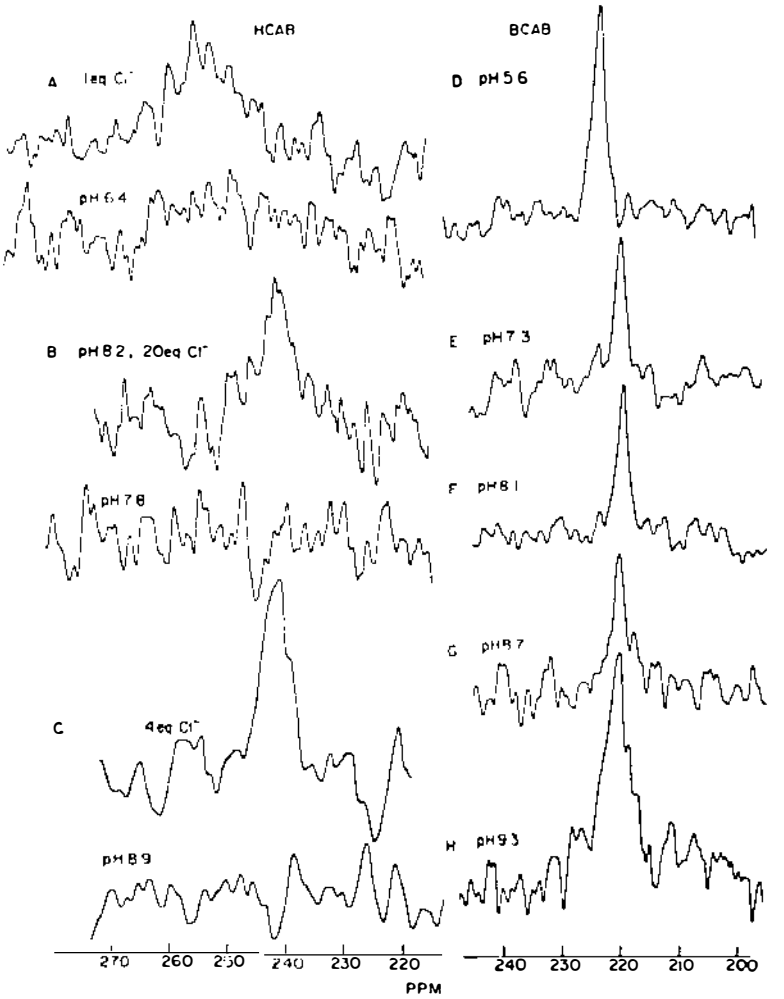


Figure 7 $^{113}Cd^{2+}$ NMR spectra of human carbonic anhydrase (HCAB) and bovine carbonic anhydrase (BCAB) (from Reference 18, with permission).

different states is 50–200 ppm, this corresponds to the slow or intermediate exchange condition encountered in NMR experiments. The result is severe broadening of the $^{113}Cd^{2+}$ resonance, which in practice leads to a lack of observation of the bound spectrum. Coleman has treated this problem and his calculations show that the populations of the two (or three) different protein conformers need not be equal in order to give broadening of peaks that are beyond detection in conventional spectrometers (17). This consideration along with the NOE and possibly long T_1's of $^{113}Cd^{2+}$ make quantitation of various observed $^{113}Cd^{2+}$ resonances problematical. Figure 7 shows the spectrum of human carbonic anhydrase B (HCAB) and bovine carbonic anhydrase B (BCAB) at various pH values and with and without Cl^- (63). The differences in line width and chemical shift are interpreted in terms of varying degrees of chemical exchange broadening between different conformational states of the enzyme, each with a different chemical shift. The near complete lack of spectrum for Cl^--free HCAB at all pH values is taken as an intermediate (10^3 sec^{-1}) chemical exchange process of a monodentate ligand (24) giving two discrete species. A broad resonance appears when the temperature is lowered, as is expected in going from an intermediate to slow exchange process. In BCAB this slow exchange process either is absent or is faster than the difference in chemical shift of various enzyme species in solution. Cl^- alters the exchange rate such that spectra are observed, but the line widths are still dependent on pH. This example shows how difficult it may be to observe the NMR spectrum of bound metal ions and that one should carry out many experiments before attempting to correlate chemical environments of the metal ion with catalytic functions. This field is fresh and new and many exciting results can be anticipated.

Ellis and coworkers (4, 53) have been studying $^{113}Cd^{2+}$ binding to concanavalin A and have obtained data showing a large difference in chemical shift between the S1 (46 ppm) and S2 (-125 ppm) metal ion sites of this protein. The original report assigned the resonance at 68 ppm to another protein site but this was later shown to be due to a cadmium chloride complex in solution. Competitive experiments with Zn^{2+} show that it can compete with Cd^{2+} at both the S1 and S2 sites whereas Ca^{2+} competes for binding at the S2 site. The chemical shift of $^{113}Cd^{2+}$ at S2 changes to -118 ppm when Zn^{2+} occupies S1. The chemical shift of $^{113}Cd^{2+}$ at S2 suggests a very shielded environment relative to $Cd(H_2O)_6^{2+}$; this site contains six oxygen ligands in a near octahedral environment.

Other studies of $^{113}Cd^{2+}$ substitution for Zn^{2+} in superoxide dismutase (1, 5) show that the $^{113}Cd^{2+}$ resonance can be observed when

the copper at the Cd-Cu site (~6 Å apart) is reduced to Cu^+, but the $^{113}Cd^{2+}$ resonance cannot be observed when the copper is oxidized to Cu^{2+}. This suggests a paramagnetic broadening of the $^{113}Cd^{2+}$ by Cu^{2+} as expected for two ions at this distance. The $^{113}Cd^{2+}$ resonance in these samples has a line width of 27 Hz and a T_1 of 1.2 sec.

In conclusion, several metal ion nuclei have been studied with proteins. As spectrometers become more sensitive and more available to biophysicists and biochemists, we can expect to see studies of many other metal ion nuclei in very interesting biochemical systems. The purpose of this section of the review was to point out the properties of metal ion nuclei that either have not been previously studied with proteins or have been studied only in a few systems.

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