Coordination Chemistry Reviews, 12 (1974) 407–430.

MAGNETIC RESONANCE STUDIES OF METAL-ENZYMES

P.J. QUILLEY and G.A. WEBB*

Department of Chemical Physics, University of Surrey, Guildford, Surrey (Gt. Britain)

(Received July 23rd 1973)

CONTENTS

A. INTRODUCTION

Enzymes are proteins which, due to their particular structures, act as very specific biochemical catalysts. The oxidative enzymes containing heme iron were the first recognised examples of metal ion participation in biological systems. Since then a large number of enzymatic reactions requiring a metal ion as an essential component have been documented[1–5].

In this review we consider a metal-enzyme to be one in which the metal ion is an essential participant in the catalytic process. An unequivocal demonstration of such participation is usually extremely difficult to achieve and various types of evidence are required[5]. It is convenient to divide metal-enzymes into two classes: (1) Enzymes with strongly bound metal ions. These are called metallo-enzymes[4]; (2) enzymes with dissociable metal ions. These are referred to as metal-ion-activated-enzymes[5]. Enzymes in these two classes show distinct properties. Quantitatively the two classes may be distinguished by means of their stability constants. For metallo-enzymes[6] the stability constants are $\geq 10^8\ M^{-1}$ while for metal-ion-activated-

* To whom correspondence should be addressed.

enzymes they are $\leqslant 10^8\ M^{-1}$. Metalloc-enzymes can be isolated with the metal attached to the protein of the enzyme whereas the the metal ion often dissociates during the purification of the metal-ion-activated-enzymes. This leads to a loss of activity which is usually only regained upon the addition of metal ions of the correct size[4]. In general the metal ions in metallo-enzymes have unusual stereochemistries which most probably contribute to their catalytic behaviour.

Each enzyme has an Enzyme Council classification, e.g. pyruvate kinase (E.C.2.7.1.40) which indicates the substrate upon which the enzyme acts, the products of the enzyme-catalysed reaction and which metal ions or other co-factors are required for the specific activity of the enzyme. To date fifteen metal cations have been reported to activate one or more enzymes each.

Since enzymes are proteins their stability is pH and temperature dependent, consequently these parameters have to be carefully controlled during experimental studies. This can often be achieved much more readily in magnetic resonance experiments than in those employing many other physical techniques.

This review is not intended to be fully rigorous, its main aim is to present a view of the present state of development of the field and attempt to suggest further experiments. It is hoped that the reader will approach the original literature on this subject with added interest.

B. NUCLEAR RELAXATION RATES

In diamagnetic environments the relaxation of nuclei with $I = \frac{1}{2}$ is usually controlled by nuclear dipole–dipole interactions, whereas in the case of nuclei with $I > \frac{1}{2}$ quadrupolar relaxation is usually dominant.

The effect of a paramagnetic centre on the relaxation rates of a nucleus with $I = \frac{1}{2}$ depends upon the distance between the two interacting centres and the motional freedom that they experience. Consequently changes in nuclear relaxation rates concomitant with the introduction of a paramagnetic centre permit the estimation of internuclear distances in the neighbourhood of the active site and characterize the local molecular motion close to this site[9].

Nuclear quadrupolar relaxation depends upon the interaction between the nuclear electric quadrupole moment and the electric field gradients within its close proximity[7]. The electric field gradients are very sensitive to changes in electron distribution around the nucleus such as occur in bonding. Consequently localized molecular motions can also be studied by means of the NMR spectra of quadrupolar nuclei[8].

In addition to local geometrical and motional parameters nuclear relaxation rates can contain information relating to the rate of substrate exchange with an enzyme (ref. 9, 10). Consequently substrate exchange may also be studied by means of nuclear relaxation rate measurements.

(i) Nuclei with $I = \frac{1}{2}$ in a paramagnetic environment

Two types of electron–nucleus interaction can influence the relaxation rates of nuclei with $I = \frac{1}{2}$ in a paramagnetic environment. These are contact and dipolar interactions which, for first row transition metal ions, may be described in terms of the isotropic shifts that they produce in NMR spectra by eqns. (1) and (2) respectively[11,12].

$$\frac{\Delta H}{H_0} = -\frac{a_N g_e^2 \beta_e^2 S(S+1)}{g_N \beta_N \cdot 3KT} \tag{1}$$

$$\frac{\Delta H}{H_0} = -\frac{(3\cos^2\theta - 1)}{r^3}(g_{\parallel}^2 - g_{\perp}^2)\beta_e^2 \frac{S(S+1)}{9KT} \tag{2}$$

where a_N represents the electron–nucleus hyperfine interaction expressed in gauss, g_e is the rotationally averaged electronic g value with major components $g_{\parallel}$ and $g_{\perp}$ in the case of axial symmetry, β_e is the Bohr magneton, g_N and β_N are the corresponding nuclear parameters, S denotes the spin of the unpaired electrons, r represents the separation between the resonating nucleus and unpaired electrons and θ is the angle between the vector defined by r and the principal symmetry axis of the molecule. It has recently become apparent that more complicated expressions than those given by eqns. (1) and (2) are necessary to explain some isotropic shifts, however these need not concern us here[12].

The large perturbation of a nuclear spin system produced by the presence of unpaired electrons not only produces isotropic shifts but also changes in the magnitudes of the nuclear longitudinal, T_1, and transverse, T_2, relaxation times. Bloembergen and Morgan[13] and Solomon[14] have developed expressions to describe T_{1M} and T_{2M} the paramagnetic contributions to T_1 and T_2 for protons within the first hydration sphere of a paramagnetic ion, eqns. (3) and (4),

$$\frac{1}{T_{1M}} = \frac{2S(S+1)}{15\hbar^2} g_e^2 \beta_e^2 \left[\frac{g_N^2 \beta_N^2}{r^6} \left(\frac{3\tau_c}{1+\omega_I^2 \tau_c^2} + \frac{7\tau_c}{1+\omega_S^2 \tau_c^2} \right) + \frac{5a_N^2 \tau_e}{1+\omega_S^2 \tau_e^2} \right] \tag{3}$$

$$\frac{1}{T_{2M}} = \frac{S(S+1)}{15\hbar^2} g_e^2 \beta_e^2 \left[\frac{g_N^2 \beta_N^2}{r^6} \left(4\tau_c + \frac{3\tau_c}{1+\omega_I^2 \tau_c^2} + \frac{13\tau_c}{1+\omega_S^2 \tau_c^2} \right) + 5a_N^2 \left(\frac{\tau_e}{1+\omega_S^2 \tau_e^2} + \tau_e \right) \right] \tag{4}$$

where ω_I and ω_S are the Larmor precession frequencies of the nuclear and electronic spins respectively, τ_c is the correlation time for the dipolar interaction and τ_e that for the contact interaction. Equations (3) and (4) relate to the general case

in which it is assumed that the g tensor is isotropic; modification is required for g tensor anisotropy[15]. The first term in each of these equations represents the dipolar interaction between nuclei and unpaired electrons whilst the second term represents the contact interaction. It is widely assumed that these expressions are also applicable to macromolecular complexes.

The correlation times for the dipolar and contact interactions are related to the correlation times characterising the rotational motion of the separation vector between the paramagnetic centre and the nucleus being studied, τ_r, the electron spin relaxation time τ_s and the residence time of the observed nucleus in the first coordination sphere of the paramagnetic centre, τ_M, by eqns. (5) and (6).

$$\frac{1}{\tau_c} = \frac{1}{\tau_r} + \frac{1}{\tau_s} + \frac{1}{\tau_M} \tag{5}$$

$$\frac{1}{\tau_e} = \frac{1}{\tau_s} + \frac{1}{\tau_M} \tag{6}$$

Equation (5) shows that the magnitude of τ_c is determined by whichever of τ_r, τ_s or τ_M is the shorter. For first row transition metal ions τ_s varies from 10^{-8} to 10^{-13} sec, for unbound aquo-ions τ_r is of the order of 10^{-11} sec and τ_M is usually several orders of magnitude larger than either τ_r or τ_s. For Fe^{II}, Co^{II} and Ni^{II}, $\tau_s < \tau_r$ and for Cr^{III}, Mn^{II} and Cu^{II} $\tau_r < \tau_s$. Consequently proton relaxation data contain information about the molecular dynamics of these ions in solution (ref. 16, 17). At high temperatures $\tau_M < \tau_s$ for metal ions; thus τ_e becomes controlled by τ_M(ref. 18).

In the majority of reported cases of interaction between a paramagnetic centre and a macromolecule, τ_e is sufficiently large such that $\omega_s^2 \tau_e^2 \gg 1$; consequently the contact contribution to T_{1M} becomes negligibly small whilst remaining significant for T_{2M}. Thus by measuring T_{1M} as a function of the concentration of paramagnetic centres in solution a value of r can be obtained from eqn. (3) by assuming that $a_N = 0$ and that a value for τ_c is known for each proton in the macromolecule. This technique has been applied to some complexes of Cr^{III}, Mn^{II}, Fe^{III}, Co^{II}, Ni^{II} and Cu^{II} with some amino acids and AMP (ref. 38).

(ii) Effect of chemical exchange on relaxation rates

If the molecule containing the nucleus under observation exchanges very rapidly between the bulk solvent and the first coordination sphere of the paramagnetic metal ion, then the rate of exchange, τ_M^{-1}, can become fast compared with the relaxation rate in the first coordination sphere, T_{1M}^{-1}, i.e. $T_{1M} \gg \tau_M$. Under these circumstances the paramagnetic contribution to the observed relaxation rate, T_{1P}^{-1} is the weighted average of the nuclear relaxation rates when the molecule is in the bulk solvent and when it is in the first coordination sphere of the metal. If N is the

number of ligand molecules in the coordination sphere of the metal ion and n is the metal: ligand concentration ratio, then

$$\frac{1}{T_{1P}} = \frac{Nn}{T_{1M}} \tag{7}$$

The conditions of fast exchange implicit in eqn. (7) apply to transition metal ions in aqueous solution; if the metal is bound to a macromolecule they may no longer be applicable. Since ligand molecules may reside on the metal longer than the minimum time required for relaxation to occur, inefficient relaxation of the bulk solvent molecules results. The paramagnetic contribution to the relaxation time becomes correspondingly longer. The residence time, τ_M, of the nucleus in the first coordination sphere is taken into account in eqn. (8) (refs. 19, 20)

$$\frac{1}{T_{1P}} = \frac{Nn}{T_{1M} + \tau_M} + \frac{1}{T_1(o)} \tag{8}$$

where $T_1(o)^{-1}$ is the outer sphere contribution arising from interactions between the paramagnetic centre and all of the nuclei beyond its first coordination sphere. For fast exchange $T_{1M} \gg \tau_M$ and eqn. (8) becomes

$$\frac{1}{T_{1P}} = \frac{Nn}{T_{1M}} + \frac{1}{T_1(o)} \tag{9}$$

if $T_1(o)^{-1}$ is small compared with T_{1M}^{-1} then eqn. (9) reduces to eqn (7). For slow exchange $\tau_M \gg T_{1M}$ and eqn. (8) becomes

$$\frac{1}{T_{1P}} = \frac{Nn}{\tau_M} + \frac{1}{T_1(o)} \tag{10}$$

and if $T_1(o)^{-1}$ is small compared with τ_M^{-1},

$$\frac{1}{T_{1P}} = \frac{Nn}{\tau_M} \tag{11}$$

Equations similar to (7) – (11) hold for T_{2P}^{-1} of some transition metal ions[19]. As can be seen from eqn. (8) the observed value of T_{1P} depends upon N, n, τ_M, T_{1M} and possibly $T_1(o)$. Equation (3) shows that T_{1M} is a function of r and τ_c which could be dominated by τ_r, τ_s or τ_M. Hence in order to interpret experimental values of T_{1P} we require independent estimates of limits of the range of values taken by N, r, τ_r, τ_s and τ_M (ref. 9). To obtain these it is often necessary to consider the relaxation rates as a function of temperature and frequency.

If the temperature coefficient of T_{1P}^{-1} is negative it is unlikely that chemical exchange is the rate-limiting process since the rates of chemical reactions have positive

temperature coefficients. Consequently for fast exchange T_{1P} is thus controlled by T_{1M} as shown by eqn. (7), where T_{1M} can be determined by either τ_r or τ_s. The value of τ_r usually decreases with increasing temperature whilst τ_s may either be increased or decreased in magnitude[26].

If the temperature coefficient of T_{1P}^{-1} is positive then further data are required in order to distinguish between three possible situations: (a) fast exchange is taking place as described by eqn. (7) and T_{1M} is controlled by τ_s which has a positive temperature coefficient. This may be verified by obtaining an estimate of the lower limit of τ_s from EPR data if available. If τ_c is found to be shorter than the lower limit of τ_s then situation (a) does not occur. However, if τ_s is unobtainable from independent data or if $\tau_s < \tau_c$ then the situation is less clear since (b), the chemical exchange rate could be sufficiently slow for T_{1P}^{-1} to be dominated by τ_M^{-1} as indicated by eqn. (8). Measurement of T_{2P} could clarify this situation, since if T_{1P} is exchange limited T_{2P} must also be exchange limited*. Frequency dependence measurements of T_{1P}^{-1} may be used to distinguish between situations (a) and (b), if no frequency dependence is observed then (b) is the relevant situation. Finally, (c) if $\tau_c > 10^{-9}$ sec then $\omega_1^2\tau_c^2 > 1$ and the dipolar term in eqn. (3) becomes a function of τ_c^{-1}. Thus an increase in temperature will lead to smaller values of τ_c and a positive temperature coefficient T_{1P}^{-1}. This situation can be distinguished from (b) by frequency dependence studies.

Experimentally, relaxation times may be obtained from either continuous wave or pulsed NMR techniques[27]. Both have been used to provide estimates of N and r for metal-enzyme systems[9]. The type of interpretational problem that can arise is illustrated in Fig. 1 for solutions of *E. coli* ribosomal RNA containing Mn^{II} ions[39]. It is considered that the chemical exchange rate is fast over the entire temperature range considered and that τ_s is the dominant correlation time at the lower end of this range[39]. Consequently, the data presented in Fig. 1 can be accounted for on

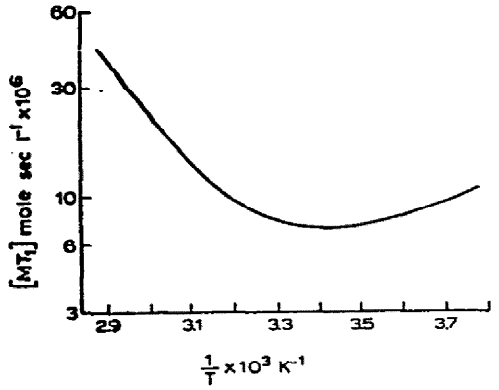

Fig. 1. A plot of MT_1 against reciprocal temperature for solutions of *E. coli* ribosomal RNA containing Mn^{II} ions at 20 MHz (ref. 39).

* We are very grateful to a referee for bringing this point to our attention.

the basis of rapid chemical exchange provided that τ_s for Mn^{II} has a positive temperature coefficient. However, there are conflicting views on this point in the literature[13,40]. Variable-temperature EPR data could decide the sign of the temperature coefficient of τ_s and consequently whether the reported[39] interpretation of the NMR data is correct.

(iii) Relaxation rate enhancement in paramagnetic systems

The effect of Cr^{III}, Mn^{II} and Cu^{II} on the relaxation rate of water protons is increased in the presence of nucleic acids[21]. The major contribution to τ_c arises from τ_r in the aquo-complexes of these metal ions. Upon binding to a macromolecule, τ_r shows a significant increase, consequently the nuclear spin relaxation becomes more efficient and the proton relaxation rate (PRR) is enhanced.

The presence of nucleic acids has little effect on the PRR of the aquo-complexes of Fe^{II}, Co^{II} and Ni^{II} since the dominant contribution to τ_c for these ions arises from τ_s which most probably does not undergo great changes upon binding to macromolecules. A large PRR enhancement is to be expected for metal ions with $\tau_s > 10^{-11}$ sec, organic free radicals may also be suitable[22]. However, free radicals may provide structural changes of unknown magnitude[23].

The dominant correlation time for a system showing PRR enhancement could be τ_r, τ_s or possibly τ_M. In both binary, enzyme-metal, and ternary, enzyme–metal substrate, complexes of Mn^{II} $\tau_r > \tau_s$ thus τ_s is the dominant correlation time in these systems[24,25]. Much of the early work on PRR enhancement due to Mn^{II} bound to macromolecules has been interpreted[9–11] on the assumption that $\tau_s > \tau_r$, this work requires reappraisal[25].

The enhancement factor, ϵ_1, for the longitudinal relaxation time is defined[21] as

$$\epsilon_1 = \frac{\frac{1}{T_{1P}}^*}{\frac{1}{T_{1P}}} = \frac{\frac{1}{T_1(\text{obs})}^* - \frac{1}{T_1}^*}{\frac{1}{T_1(\text{obs})} - \frac{1}{T_1}} \qquad (12)$$

where T_{1P} is the contribution of the paramagnetic ion to the observed relaxation time T_1(obs) and T_1 is the relaxation time in the absence of a paramagnetic ion. The asterisk indicates data obtained in the presence of a macromolecule. A similar enhancement factor, ϵ_2 has been defined for the transverse relaxation time[21].

The observed enhancement factor ϵ_1 is a weighted average of the enhancement due to paramagnetic ions bound to macromolecules, ϵ_{1b}, and that arising from free paramagnetic ions in solution, ϵ_{1f}. In the absence of macromolecules the value of ϵ_{1f} is unity from eqn. (12). However, since the structure of bulk water can be modified close to macromolecules, T_1 and T_1^* are not necessarily equal, hence ϵ_{1f} may differ slightly from unity. By measuring ϵ_1 for a series of macromolecule

concentrations, M, in the presence of a constant total concentration of paramagnetic centres it is possible to obtain ϵ_{1b} from a plot of $(\epsilon_1 - 1)^{-1}$ against M^{-1} by assuming ϵ_{1f} to be unity. At infinite macromolecule concentration, all of the paramagnetic centres are bound to the macromolecule and ϵ_{1b} tends to ϵ_1 as M^{-1} tends to zero. Some of the applications of ϵ_{1b} measurements to the determination of metal-enzyme parameters are discussed in Section C.

(iv) Nuclei with $I > \frac{1}{2}$ in a diamagnetic environment

Nuclear relaxation measurements of atoms with quadrupolar nuclei provide a sensitive indication of weak molecular interactions. This technique has been used to study metal-enzyme systems by means of the following quadrupolar nuclei, ^{35}Cl, ^{79}Br, ^{81}Br and ^{39}K with $I = \frac{3}{2}$, ^{25}Mg with $I = \frac{5}{2}$ and ^{43}Ca with $I = \frac{7}{2}$.

Usually a solution of protein in an aqueous sodium halide solution is titrated with a solution containing metal ions which bind to specific protein sites and subsequently with halide ions from the solution. In the majority of halide ion probe studies on proteins the number and availability of metal ion binding sites have been determined from continuous wave ^{35}Cl NMR line width measurements.

Abragam[28] has described an extreme narrowing approximation which occurs when

$$\tau \ll \frac{1}{\omega_0} \tag{13}$$

where τ is the correlation time for molecular Brownian motion and ω_0 is the nuclear Larmor frequency. Equation (13) is frequently satisfied by quadrupolar nuclei[29]. Under these conditions the influence of the quadrupolar moment on the nuclear relaxation times is given by[30]

$$\frac{1}{T_Q} = \frac{1}{T_1} = \frac{1}{T_2} = \frac{3(2I+3)}{40I^2(2I-1)}\left(\frac{eQ}{\hbar}\right)^2\left(\frac{d^2v}{dz^2}\right)^2 \tau \tag{14}$$

where eQ is the nuclear quadrupole moment and d^2v/dz^2 is the maximum electric field gradient at the nucleus. When eq. (14) is obeyed the quadrupolar relaxation time, T_Q, is related to the observed NMR line width at half-height, Δ, for a Lorentzian line by

$$\Delta = \frac{1}{\pi T_Q} \tag{15}$$

From eqns. (14) and (15) it follows that

$$\Delta = \frac{3(2I+3)}{40\pi I^2(2I-1)}\left(\frac{eqeQ}{\hbar}\right)^2 \tau \tag{16}$$

where eq, the electric field gradient at the nucleus, is assumed to have cylindrical symmetry. For the ^{35}Cl nucleus, eqn. (16) becomes[30]

$$\Delta = \frac{2\pi}{5}\left(\frac{eqeQ}{\hbar}\right)^2 \tau \tag{17}$$

In aqueous solutions the ^{35}Cl NMR signal is usually fairly narrow, 12.5 ± 1Hz, due to the symmetrical environment within its hydration shell[31]. If the rate of exchange of chloride ions between various sites is rapid, compared with the width of the broadest ^{35}Cl signal from any of these sites, then a composite ^{35}Cl NMR signal is given by a weighted average[32]

$$\Delta = \sum_i \Delta_i P_i \tag{18}$$

where Δ_i is the linewidth correponding to site i and P_i is the probability of the ^{35}Cl nucleus being at this site.

In order to compare the effect of metal ions in various environments on the ^{35}Cl relaxation rate, a quadrupolar enhancement parameter ϵ_q is defined by[33]

$$\epsilon_q = \frac{\Delta^* - \Delta^*(o)}{\Delta - \Delta(o)} \tag{19}$$

where Δ and $\Delta(o)$ are the line widths in the presence and absence respectively of the metal ions, the asterisk indicates the presence of a chelating agent. For chloride ions bound to macromolecules the line width is often greater by a factor of 10^5 than it is for chloride ions in aqueous solution due to enhanced values of eq and τ. Hence relatively low concentrations of metal-enzymes produce measurable line broadening. This technique has been applied to ^{35}Cl NMR studies in the presence of Zn^{II}, Cd^{II}, Hg^{II}, Mn^{II}, Cu^{II}, Pb^{II} and Tl^{III} (refs. 32–38).

C. APPLICATIONS OF NUCLEAR MAGNETIC RELAXATION DATA FROM METAL-ENZYME SYSTEMS

Metal ions of similar size are able to replace each other in enzymes, usually without loss of activity. Consequently Mg^{II}, which is the natural activator for many enzymes, can be replaced by Mn^{II} which produces a PRR enhancement. Similarly for the peptidases, Mn^{II}, Co^{II}, Ni^{II} and Zn^{II} are often interchangeable. The resulting enhancement factors can provide information on metal-enzyme association constants, the appropriate binding sites and the mechanisms of metal-enzyme catalysis. In some cases quadrupolar enhancement parameters also provide relevant data.

(i) Determination of association constants

If a hydrated metal ion binds to a macromolecule the resulting value of ϵ_1, for the water protons, depends upon the mole fraction of the metal ion in the bound state. Therefore graphical treatment of the titration data obtained from solutions containing suitable concentrations of appropriate metal ions and enzymes provides estimates of binary association constants and the number of binding sites per macromolecule[9].

A number of ternary complexes of the type enzyme–Mn^{II}–substrate (EMS) have been investigated. Association constants for the binding of EM with S and of E with MS have been reported following both graphical and computer analysis of the experimental data[9]. In the case of adenylate kinase–Mn^{II}–ATP significantly different results have been obtained from these two forms of analysis[41,42]. The iterative solutions of the equations describing the system are supported by other data, thus a reinvestigation of the earlier results obtained by graphical extrapolation methods is called for.

(ii) Qualitative identification of binding sites

By comparing the PRR enhancement of water protons in the presence of the binary complexes MS, ϵ_{1a}, and ME, ϵ_{1b}, with the enhancement of the ternary complex EMS, ϵ_{1t}, Mildvan and Cohn have classified 26 metal-enzymes according to three coordination schemes[9]. Type I enzymes produce little or no enhancement of the PRR in binary complexes of Mn^{II}; however, when a substrate is added a large enhancement is observed and therefore $\epsilon_{1b} < \epsilon_{1t}$. This is consistent with the formation of a substrate bridged complex, E–S–M, in which the substrate is responsible for bringing the hydrated Mn^{II} ion into the environment of the macromolecule. The relatively small values of ϵ_{1a} and ϵ_{1b} indicate weak binding between Mn^{II} and either the enzyme or the substrate in binary complexes. Hence type I enzymes are usually taken to be metal-ion-activated.

Both type II and type III enzymes produce significant enhancements of the PRR in hydrated complexes of Mn^{II}. Addition of a substrate to a type II enzyme – Mn^{II} complex reduces the enhancement, this differs from a type III enzyme – Mn^{II} complex where the addition of a substrate does not significantly influence the PRR enhancement[9]. In comparison with type I enzymes, type II behaviour indicates an increase in metallo-enzyme character. This is consistent with the presence of a bridged structure for the ternary complex which could be either linear, E–M–S, or cyclic $E\begin{smallmatrix}S\\|\\M\end{smallmatrix}$.

Type III enzymes probably form a ternary complex of the type M–E–S in which the metal is not involved in any bonding changes which may occur when the substrate is added. Since the tabulation of Mildvan and Cohn was published[9] other metal-enzymes have been classified according to their coordination schemes. Aconitase[43–45], DNA polymerase[46], yeast aldolase[48] and fructose 1,6-diphos-

phatase[49] are reported to be type II enzymes.

^{13}C relaxation rate studies on the quaternary complex pyruvate kinase – Mn^{II} – phosphate – pyruvate show that the Mn^{II} ion is approximately the same distance from the carboxyl and carbonyl carbon atoms of pyruvate[47]. The estimated distances, 0.71 – 0.85 nm, are consistent with the presence of pyruvate in the second coordination sphere of Mn^{II}.

Proton NMR data are consistent with carbonyl and phosphoryl coordination in the type II ternary complexes yeast aldolase – Mn^{II} – acetal phosphate and yeast aldolase – Mn^{II} – fructose diphosphate[48]. ^{31}P NMR measurements on the ternary complex fructose 1,6-diphosphatase – Mn^{II} – fructose - 1 - phosphate and ^{13}C data on the binary Mn^{II} – fructose - 1 - phosphate have led to the proposed Mn^{II} environment shown in Fig. 2, (ref. 49).

(iii) Determination of some structural parameters

As indicated by eqn. (3), (4) and (7) – (11), geometrical information concerning metal-enzyme systems may be obtained from nuclear relaxation rate measurements. By assuming that τ_c is dominated by τ_r for small rigid complexes of Mn^{II} and that $\tau_c \simeq 3 \times 10^{-11}$ sec, the dipolar term in eqn. (3) provides an estimate of metal-ligand nucleus separation[9,50]. In this manner, distance values have been reported which are comparable to those obtained by X-ray diffraction techniques[9,50]. However, when the relaxation rate of the coordinated ligand is too rapid or when it is not possible to obtain a high concentration of the complex in solution, as occurs with many complexes involving macromolecules, then eqn. (8) has to be considered. In general, the calculated distances are not obtained with such accuracy as they are for small

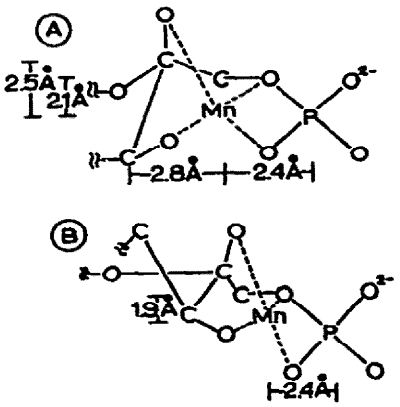

Fig. 2. Some distances between Mn^{II} and ^{31}P and ^{13}C nuclei in a fructose-1-phosphate complex with Mn^{II}, obtained from NMR data. For clarity, protons are omitted[49]. (A) The 1-C β-fructopyranose complex; (B) the twist β-fructofuranose complex.

rigid molecules. Errors of ± 30% are present in some distance values[9,47,49,50]. These are generally due to uncertainty in the value of τ_c. If τ_c is evaluated independently from a frequency-dependent study of T_{1P}, errors of less than 5% may be found[47].

An alternative approach is to employ a metal ion which is less effective than Mn^{II} in producing nuclear relaxation. This technique has been used in the case of carbonic anhydrase where the natural activator, Zn^{II}, is replaced by Co^{II}. The Co^{II} proton separation in the binary complex is reported to be 0.25 – 0.29 nm (ref. 51).

By means of eqn. (7)–(11) it is possible to estimate the number of water molecules, M, in the first coordination sphere of a metal ion in a binary or ternary complex with a macromolecule from relaxation studies. A necessary prerequisite is the availability of acceptable values for τ_c and the metal-nuclear separation, r. A value of τ_c has been obtained for the protons of water molecules coordinated to Mn^{II} from frequency-dependent relaxation data[24]. By taking this together with $r = 0.29$ nm a value of 3 has been estimated[24] for N for the binary complex formed between pyruvate kinase and hydrated Mn^{II}. In a similar investigation on the quaternary complex, creatine kinase–Mn^{II} – ADP–creatine, when r is taken to be 0.286nm N is found to be less than one half[25]. Frequency and temperature dependent measurements of T_{1M} show that this low value of N cannot be attributed to slow exchange. It seems likely that outer sphere relaxation, described in eqns. (8)–(10), plays an important role in this system. However, since the metal–proton separation for protons residing beyond the first coordination sphere can only be crudely estimated, it is not at present possible to decide how important outer sphere contributions to the relaxation data are[25].

(iv) Some studies of the role of metal-enzymes in biochemical reactions

Information relating to the electronic and geometric structures of the active sites of an enzyme as well as the chemical processes involved in the catalytic reaction may be obtained from NMR data. We consider here the results of some paramagnetic and quadrupolar probe studies for a number of metal-enzymes treated under six classifications.

1. Transferases

These enzymes catalyse the transfer of a functional group, e.g. carbonyl, amino, between molecules. Included amongst them are a group of phosphotransferases, the kinases, most of which employ Mg^{II} as an essential activator. Creatine kinase and pyruvate kinase have been studied extensively, the results obtained show that there is no general mechanism for the action of enzymes in this group.

Creatine kinase (E.C.2.7.3.2) catalyses the reaction

ATP + creatine ⇌ ADP + phospho-creatine

and has a dimeric structure with one essential thiol group per subunit[52,53]. The results of paramagnetic probe NMR studies on the interaction of creatine kinase and various substrates have been comprehensively reviewed[9,10]. Recently a nitroxide radical, *N*-(1-oxyl-2,2, 5, 5-tetramethyl-3-pyrrolidinyl) iodoacetamide has been used in a study on the active site of creatine kinasse [54]. The modified enzyme lacks phosphotransferase and ATPase activities but retains the ability to bind nucleotide substrates. The PRR enhancement for water in ternary complexes enzyme–Mn^{II}–nucleotide are found to be 13 ± 2 for the ADP complex and 11 ± 2 for the ATP complex. These enhancements are lower by 38% and 15% respectively than those for the unmodified enzyme. From EPR data the dipolar interaction between Mn^{II} and the nitroxide radical corresponds to a separation of 0.75 ± 0.15 nm for the ADP complex and 1.16 ± 0.06 nm for the ATP complex[55]. This suggests different conformations for the two complexes analogous to that reported for the corresponding Mg^{II} complexes[56].

NMR and EPR experiments have shown that the addition of creatine kinase to solutions of creatine kinase–Mn^{II}–ADP and creatine kinase–Mn^{II}–ATP produces a rearrangement at the active site which results in a less symmetrical environment for the Mn^{II} ion[57]. The addition of nitrate or chloride ions produces further changes in the EPR spectrum and in the PRR of water in the complex creatine kinase–Mn^{II}–ADP-creatine[57]. These observations have been accounted for by the binding of creatine with Mn^{II} whilst the anions are assumed to bind at the vacant phosphoryl site in the quaternary complex.

Modification of the thiol groups of the enzyme reduces its ability to undergo conformational changes when creatine is added to the ternary complex creatine kinase–Mn^{II}–ADP. This loss of conformational adaptability of the enzyme appears to be related to the inability of the modified enzyme to catalyse the phosphoryl transfer reaction[56].

Pyruvate kinase (E.C.2.7.1.40) requires both Mg^{II} and K^{I} as natural activators. It catalyses the reaction

ATP + pyruvate ⇌ ADP + phosphoenol pyruvate

Pyruvate kinase is reported to show type II behaviour towards all substrates so far studied[9].

Rabbit muscle pyruvate kinase consists of four sub-units of the same molecular weight. It has four binding sites for Mn^{II} (refs. 10, 61). The addition of urea is reported to transform the tetramer into an active dimer whilst the number of Mn^{II} binding sites remains unchanged[61].

The presence of four bound Zn^{II} ions per mole of enzyme has been established

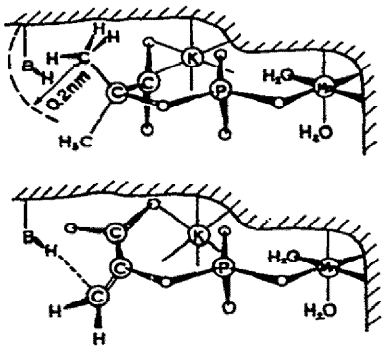

Fig. 3. Comparison of a composite model of the ternary analogue complexes of pyruvate kinase (upper figure) with the ternary complex formed with P-enolpyruvate (lower figure)[59].

by ^{35}Cl NMR data[58]. The role of the K^I ion is uncertain; it could be important in determining the proper conformation of the binary and ternary complexes or it may be directly involved in the binding of the carbonyl group of phosphoenolpyruvate to the enzyme[60].

NMR relaxation studies on the interactions between muscle pyruvate kinase and three competitive analogs of phosphoenolpyruvate reveal that the immobilization of the bound analogs increases as the reaction centre is approached[59]. This immobilization of bound substrates at the reaction centre could allow orientational effects to be operative in enzyme catalysis[59]. A composite model of the ternary analog complexes has been derived from NMR data[60]. This is given in Fig. 3 where it is compared with the ternary complex formed with P-enolpyruvate[59]. A corresponding model of the phosphoenolpyruvate analogs at the active site of pyruvate kinase including the nuclear correlation times is presented in Fig. 4 (ref. 59). ^{13}C nuclear relaxation studies of the quaternary complex pyruvate kinase–Mn^{II}–phosphate–pyruvate indicate that enzyme bound pyruvate is oriented differently with respect to the Mn^{II} ion than it is in the Mn^{II}–pyruvate complex[47]. A possible mechanism for the enolization reaction of pyruvate kinase which is consistent with ^{13}C and ^{31}P NMR data, is shown in Fig. 5.

Rhodanese (E.C.2.8.1.1.) catalyses the reaction

thiosulphate + cyanide ⇔ sulphite + thiocyanate

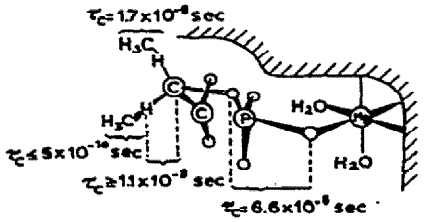

Fig. 4. A composite model of the phosphoenolpyruvate analogues at the active site of pyruvate kinase including the correlation times[59].

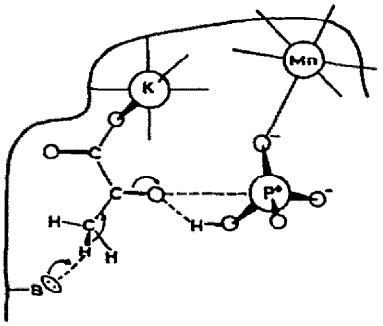

Fig. 5. Mechanism of the enolization of pyruvate kinase consistent with ^{13}C and ^{31}P NMR data[47].

^{35}Cl NMR data has been reported for beef liver rhodanese[90]. Although the enzyme binds one equivalent of Zn^{II} it can also be prepared zinc-free in which case it retains its activity. The activity is found not to be enhanced by the addition of Zn^{II} ions[90]. It is thus unlikely that Zn^{II} takes part in the enzyme-catalysed cleavage of the sulphur–sulphur bond of the substrate. Obviously the function of the strongly bound zinc atom in this enzyme requires further investigation.

2. *Lyases*

These enzymes catalyse reactions in which a complete group of atoms is removed from a molecule, e.g. decarboxylation, deamination. Early NMR work on enolase, histidine deaminase and citrate lyase has recently been reviewed[9].

Aldolase (E.C.4.1.2.6.) catalyses the reaction

dihydroxyacetone phosphate + glyceraldehyde 3 phosphate ⇔ fructose diphosphate

Yeast aldolase contains Zn^{II} which can be removed to give an apoenzyme which is reactivated by Mn^{II}, Co^{II} or Zn^{II} (ref. 9). PRR enhancement, EPR and kinetic data on the apoenzyme have revealed two catalytically active sites which bind Mn^{II} tightly as well as some weaker binding sites[48]. From PRR enhancement measurements it has been reported that enzyme-bound Mn^{II} coordinates with acetal phosphate dihydroxyacetone phosphate, dihydroxyacetone phosphate hydrate and fructose diphosphate[48]. Some probable structures and mechanisms for yeast aldolase–substrate complexes based upon these observations are given in Fig. 6. The high dissociation constant for the binary Mn^{II}–fructose diphosphate complex suggests independent binding of Mn^{II} to the phosphoryl groups. As indicated in Fig. 6, carbonyl group coordination to the enzyme-bound Mn^{II} ion reveals that the metal ion plays the role of electrophile[62].

Aconitase (E.C.4.2.1.3.) catalyses the dehydration of citric and isocitric acids to form *cis*-aconitic acid, the reverse reactions and the interconversion of citric and

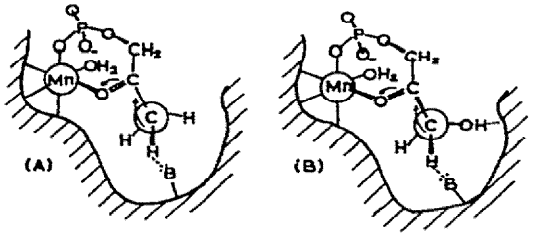

Fig. 6. Some probable structures and mechanisms of yeast aldolase – substrate complexes[48]. (A) The Mn^{II} bridge complex of acetol phosphate with yeast aldolase; (B) the Mn^{II} bridge complex of dihydroxyacetone phosphate with yeast aldolase.

isocitric acids. Its activity depends upon the presence of Fe^{II}.

The Fe^{II} free-enzyme has an EPR spectrum which has been attributed to the presence of high-spin Fe^{III} (ref. 43). This spectrum is unchanged when either Fe^{II} or citrate ions are added implying a structural rather than a catalytic role for Fe^{III}. Although aconitase specifically requires Fe^{II} for its activity it also binds with Mn^{II} but this does not activate it. There are reported to be two tight binding sites for Mn^{II} together with five to seven weaker ones in aconitase[44].

Relaxation time measurements have demonstrated that Mn^{II} induces type II behaviour in aconitase suggesting the formation of ternary metal bridged complexes[45] The Mn^{II}-to-proton separation in the ternary complex aconitase–Mn^{II}–citrate is reported from NMR data to be 0.4 ± 0.05 nm and the Fe^{II}-to-proton distances in the aconitase–Fe^{II}-monodeuterocitrate complex to be 0.38 ± 0.02 nm ($-CH_2-$) and 0.31 ± 0.02 nm (–CHD–) which are in good agreement with X-ray diffraction data[45]. A contact interaction is found in the proton spectrum of citrate ions when enzyme-bound Fe^{II} is present. This is consistent with direct coordination between citrate and the bound Fe^{II} ions.

Three possible mechanisms for the action of aconitase in the conversion of citrate to isocitrate, which are consistent with the NMR data, are presented in Fig. 7 (ref. 45). Following the elimination of water to form the *cis*-aconitate complex (B) the substrate may change from a citrate-like to an isocitrate-like conformation by routes I, II or III. Route I comprises the ferrous wheel mechanism[63], route II the reverse ferrous wheel mechanism and route III the Bailar twist mechanism[62]. Finally water is added to form isocitrate.

Soft π bonding ligands such as sulphur, coordinate to Fe^{II} and undergo the Bailar twist mechanism which is associated with a rapid change from high-spin to low-spin Fe^{II} (refs. 12, 64, 65). The fact that Fe^{II} is a unique activator for aconitase together with the formation of 42% low-spin Fe^{II} upon combination between the enzyme and Fe^{II} (ref. 44) and the requirement of a reducing agent as a co-factor which could produce a sulphur donating centre on the enzyme favours route III. However, none of the routes described in Fig. 7 can be ignored at present[45].

Carbonic anhydrase (E.C.4.2.1.1.) catalyses the hydration of carbon dioxide at high pH. It contains one firmly bound zinc atom per molecule which is neces-

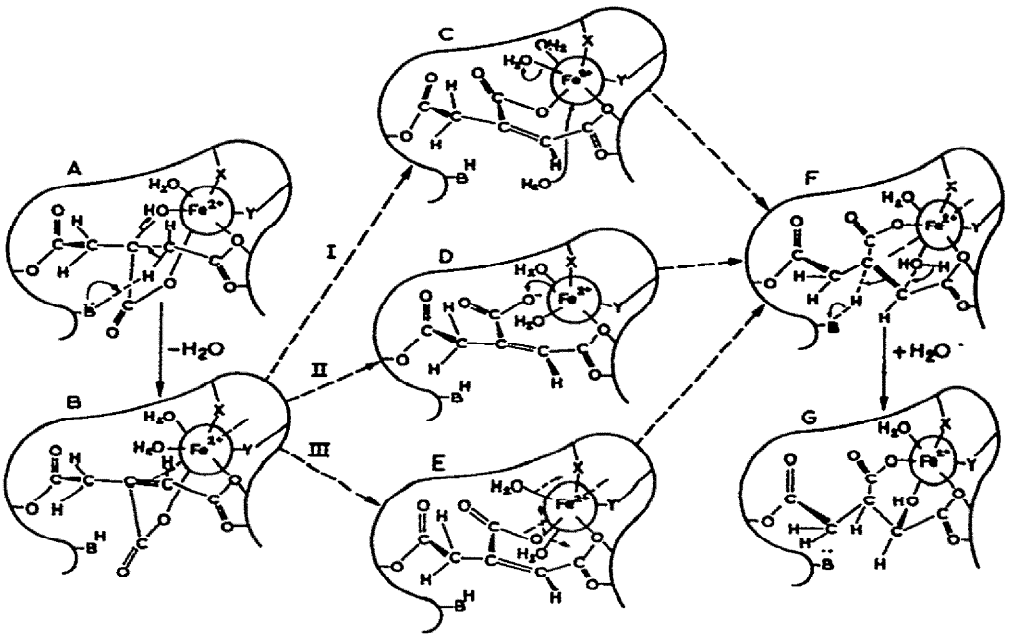

Fig. 7. Three alternative mechanisms for the conversion of citrate to isocitrate by aconitase which are consistent with NMR data[45].

sary for its activity. Variation in the ^{35}Cl NMR line width during titration demonstrates the presence of one coordination site which is available for chloride or cyanide binding, but which can be inhibited by acetazolamide[33].

Bovine carbonic anhydrase is found to be a thousand times more effective than hydrated Zn^{II} ions in broadening the ^{35}Cl signal whereas the zinc-free apoenzyme is twenty five times less effective[33,67]. These observations have been interpreted in favour of a chloride ion exchange process and a molecular rotational interaction with correlation times of $\simeq 10^{-8}$ sec for carbonic anhydrase and $\simeq 10^{-11}$ sec for hydrated Zn^{II} ions[33,67].

PRR enhancement data have been obtained by replacing Zn^{II} by Co^{II} (ref. 51). The Co–proton separation is estimated to be between 0.25 and 0.29 nm by assuming that the correlation time for the Co^{II} enzyme binary complex is $\simeq 10^{-11}$ sec and that only one water molecule is present in the first Co^{II} coordination sphere[51]. However, if the Co^{II} ion has only one bound water molecule then it is most probably four coordinate, the electronic spectrum does not provide unambiguous information on this point[71,72]. Some ligand binding studies have been recently reported[66] on carbonic anhydrase with encouraging results.

3. Lygases

These comprise a small group of less than fifty known enzymes which catalyse

condensation reactions. Over half of the lygases require Mg^{II} as the natural activator and are thus amenable to paramagnetic probe studies using Mn^{II}.

Pyruvate carboxylase (E.C.6.4.1.1.) from chicken liver was the first naturally occurring Mn^{II} metalloezyme to be discovered. Consequently it has been the focus of much research activity[9,47]. Pyruvate carboxylase catalyses transcarboxylation reactions. When purified from calf liver it is found to contain both Mg^{II} and Mn^{II} bound in a combined stoichiometry equivalent to the biotin content of the enzyme which implies a different structure to that found in the enzyme obtained from chicken liver. However, it is possible to substitute Mg^{II} for Mn^{II} with retention of catalytic activity in the pyruvate carboxylase purified from chicken liver. Further studies are required in order to differentiate between a number of possible models which have been proposed to describe the mechanism of this enzyme[9,47,68,69].

4. Oxidoreductases

As implied by the title, these enzymes catalyse oxidation–reduction reactions. The results of NMR studies on cytochrome C, some nonheme-iron proteins and some copper enzymes belonging to this category have been recently reviewed[9,12].

Liver alcohol dehydrogenase (E.C.1.1.1.1.). The role of zinc in the binding of the co-enzyme NADH and in maintaining the structural integrity of horse liver alcohol dehydrogenase (LADH) has been the subject of numerous investigations[9,79]. LADH consists of two sub-units each containing two zinc atoms, it is rendered inactive if zinc is either removed or chelated[73].

The presence of LADH in sodium chloride solutions produces an increase in the width of the ^{35}Cl NMR signal which increases with protein concentration[74,75]. A similar study involving ^{81}Br NMR has not revealed any line broadening in the presence of LADH[76]. However, the addition of NADH to a solution containing LADH in aqueous sodium chloride produces a decrease in the ^{35}Cl NMR signal width as demonstrated in Fig. 8. The differences in the titration curves could arise from a heat denaturation step employed in the preparation of the enzyme[77]. Lindman et al.[75] report the NADH : LADH stoichiometry ratio to be 1 : 1 at the end point of the titration, whereas Ward et al.[74] report a ratio of 2 : 1 from spectrophotometric data taken under identical conditions to those used in the ^{35}Cl NMR experiments[78]. From the data available it is not possible to imply that NADH binds to zinc in LADH but merely that the presence of NADH prevents the zinc–chloride interaction from taking place in aqueous sodium chloride solutions. Since the ^{35}Cl line broadening produced by LADH could arise from specific $Zn^{II} - {}^{35}Cl$ interactions, non-specific protein $- {}^{35}Cl$ interactions or possibly from viscosity effects[33,67] the role of zinc in LADH remains undecided.

Other dehydrogenases which should be amenable to study by means of the paramagnetic ion probe technique include the Mn^{II} activated enzymes, malic enzyme (E.C.1.1.1.40) and isocitrate dehydrogenase (E.C.1.1.1.41).

Xanthine oxidase (E.C.1.2.3.2.). Although molybdenum metallo-flavoproteins

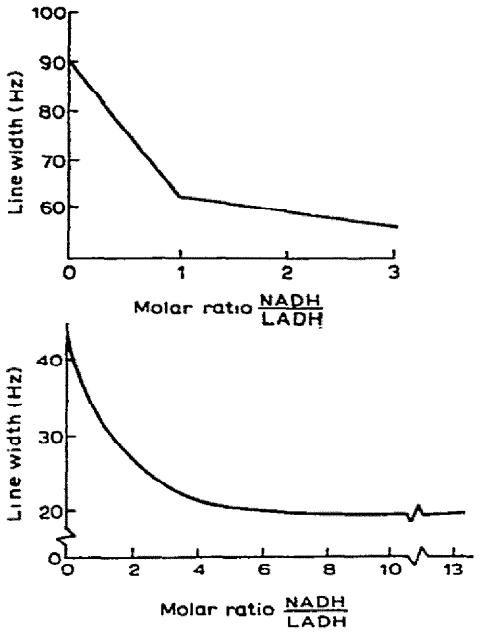

Fig. 8. Plots of ^{35}Cl NMR line width against the molar ratio of NADH to LADH (refs. 74 (bottom), 75 (top)).

are not readily amenable to paramagnetic probe studies, due to long electronic relaxation times, they may be investigated by EPR. With the exception of milk xanthine oxidase, molybdenum enzymes have not been frequently investigated by magnetic resonance techniques. It appears that the molybdenum ion plays a direct role in the redox catalysis. In both xanthine oxidase and aldehyde oxidase (E.C.1.2.3.1.) the metal is involved in the interaction of the enzymes with reducing substrates, while in nitrate reductase (E.C.1.6.6.3.) it apparently interacts with the oxidising substrate[70].

5. Hydrolases

These enzymes catalyse hydrolysis reactions.

Carboxypeptidase A (E.C.3.4.2.1.) is a zinc metallo-enzyme. The results of paramagnetic probe studies on this enzyme have been recently reviewed[9]. It contains two half-cystine residues whose function in the native enzyme has been the subject of much discussion[81]. Protein thiol groups have been investigated by ^{35}Cl NMR probe studies[31,34] and recently this technique has been used to assay carboxypeptidase A for active thiol groups by titration with $HgCl_2$ (ref. 86). Although Hg^{II} does bind to the enzyme it is not removed by dialysis indicating that the binding is unlikely to be through a sulphur atom[86]. This implies the absence of free thiol groups and lends support to the proposal of a disulphide bond linking the two half cystine residues[87].

^{19}F NMR data have been reported for solutions containing fluoride ions and carboxypeptidase A in which Zn^{II} has been replaced by Mn^{II} (ref. 88). An increase in the ^{19}F relaxation rate implies binding between fluoride ions and Mn^{II} which is inhibited by β-phenylpropionate[88]. From a consideration of the PRR enhancement studies on the Mn^{II} substituted enzyme[89] and X-ray diffraction data[87] on the native enzyme it has been reported that in both cases the number of metal-to-ligand bonds is five or more[88].

Alkaline phosphatase (E.C.3.1.3.1.) catalyses the hydrolysis of orthophosphoric monoesters. It is a zinc metallo-enzyme considered to contain four zinc atoms per mole of protein[80]. The role of the zinc atoms has been studied by various authors (ref. 81, 82). Recently it has been demonstrated that the zinc-free apoenzyme is unable to bind phosphate[83] which suggests that zinc plays an important part in the phosphate binding of the native enzyme.

^{35}Cl NMR linewidth data have been used in the investigation of the zinc–alkaline phosphatase interaction by two research groups with apparently conflicting results (refs. 84, 85). The differences are most probably due to the different enzyme preparations used and their residual zinc content. The titration curve of apo-alkaline phosphatase with Zn^{II} obtained from ^{35}Cl NMR data, together with changes in specific activity[84] is shown in Fig. 9. The dramatic increase in ^{35}Cl NMR linewidth when the Zn^{II} : protein ratio increases beyond four is in agreement with the presence of four zinc atoms per mole of protein in the enzyme[80]. However, it is noteworthy that the maximum specific activity of the enzyme occurs before the ^{35}Cl linewidth increases. This could be accounted for either by the protein providing all of the donating centres to satisfy the coordination of Zn^{II} or by the observed NMR line not being a composite ^{35}Cl signal due to the slow exchange of chloride ions between the bulk solution and a coordinated position on the metallo-enzyme[84]. If the

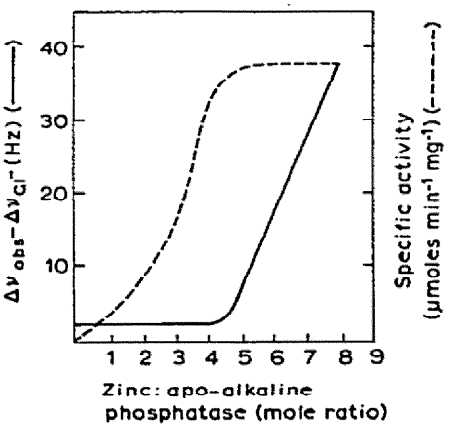

Fig. 9. Data obtained from the titration of apo-alkaline phosphatase with Zn^{II} ions[84].

former is correct then the primary function of zinc in alkaline phosphatase appears to be to hold the protein in the active conformation rather than in promoting the formation of a bridged enzyme–metal–substrate complex. However, the possibility remains that the substrate could displace one or more metal-enzyme linkages to form such a complex[84]. PRR enhancement data on binary and ternary Mn^{II} alkaline phosphatase complexes could provide valuable information on this matter. Studies on the rate of chloride exchange with the metallo-enzyme would also help to clarify the function of zinc in alkaline phosphatase.

There are a number of metallo-enzymes in this class which are candidates for future NMR investigations using paramagnetic probes, these include the Ca^{II} activated enzymes lipase (E.C.3.4.4.13) and myosin (ATPase) (E.C.3.6.1.3). Some further hydrolases are activated by Mg^{II}, Mn^{II}, Fe^{III}, Co^{II} and Zn^{II}.

6. Isomerases

The members of this small group of enzymes catalyse isomerisation reactions. D-xylase isomerase is a Mg^{II} activated enzyme which catalyses the interconversion of D-xylose and D-xylulose, the results of NMR studies on this enzyme have recently been reviewed[9]. Some other Mg^{II}-activated enzymes in this class which could lend themselves to paramagnetic probe studies using Mn^{II} include arabinose isomerase (E.C.5.3.1.3.) and isopentyl pyrophosphate isomerase (E.C.5.3.3.2.).

D. CONCLUDING REMARKS

It has been our aim in this review to report on magnetic resonance data from metal-enzyme systems which have extended or revised opinions and conclusions reached earlier[9,10,50]. The results obtained from PRR enhancement and EPR data are largely complementary. PRR enhancement experiments are best suited to the determination of equilibrium data for ternary and quaternary complexes, while some structural information may be obtained if sufficient knowledge of the system is available. On the other hand EPR measurements using spin labels can be more incisive in distinguishing subtle structural and dynamic perturbations in the immediate environment of the metal ion activators.

Until recently little progress has been reported in the utilisation of alkaline or alkaline earth metal cations as NMR probes. The increasingly widespread utilisation of pulse and Fourier transform NMR techniques[27] will doubtless lead to studies on these and other quadrupolar nuclei in biochemically interesting systems[91]. Bryant and his co-workers have reported the results of some initial investigations on ^{25}Mg, ^{39}K and ^{43}Ca NMR in biochemical systems[92–94].

The addition of pyruvate kinase to a 2 *M* solution of potassium chloride results in an approximate doubling of the ^{39}K NMR line width. Control experiments with bovine serum albumin signify that direct binding occurs between potassium and

pyruvate kinase. The results of a ^{205}Tl NMR investigation[95] indicate that Mn^{II} and Tl^{I} occupy positions in close proximity to one another on the enzyme. Obviously further information of this nature would be of great interest.

Of the non-metallic quadrupolar nuclei so far used as probes in enzyme systems, ^{35}Cl has proved to be the most fruitful. It is particularly useful in investigating the number and strength of metal ion binding sites in zinc-enzymes and as a reporter in the study of conformational changes occurring during the formation of binary and ternary complexes.

Recently the ^{79}Br and ^{81}Br nuclei have been used as NMR probes of protein conformation using pulsed NMR with promising results[96] and ^{79}Br relaxation measurements have been used to study the interaction of DNA with the Zn^{II} metalloenzyme DNA polymerase[100]. ^{17}O and ^{23}Na are other quadrupolar nuclei of biochemical interest[97–99]. It is to be hoped that further advances in NMR instrumentation will permit the omnipresent ^{14}N nucleus to be studied in macromolecular systems in the near future[101].

REFERENCES

1 B.L. Vallee, in P.D. Boyer, H. Lardy and K. Myrbach (Eds.), *The Enzymes*, Vol. 3, Academic Press, New York, 1959, p. 225.
2 J.E. Coleman, *Progr. Bio-org. Chem.*, 1 (1971) 159.
3 O. Lehninger, *Physiol. Rev.*, 30 (1950) 393.
4 B.L. Vallee, *Advan. Protein Chem.*, 10 (1955) 317.
5 B. Malmström and K. Rosenberg, *Advan. Enzymol.*, 21 (1959) 131.
6 A.S. Mildvan, in P.D. Boyer (Ed.) *The Enzymes*, Vol. 2, 3rd Edn., Academic Press, New York, 1970, p. 447.
7 M. Witanowski and G.A. Webb, in E.F. Mooney (Ed.) *Annual Reports on NMR Spectroscopy*, Vol. 5A, Academic Press, London, 1972, p. 395.
8 O. Jardetzky and J.E. Wertz, *J. Amer. Chem. Soc.*, 82 (1960) 813.
9 A.S. Mildvan and M. Cohn, *Advan. Enzymol.*, 33 (1970) 1.
10 M. Cohn, *Quart. Rev. Biophys.*, 3 (1970) 61.
11 G.A. Webb, in E.F. Mooney (Ed.), *Annual Reports on NMR Spectroscopy*, Vol. 3, Academic Press, London, 1970, p. 211.
12 G.A. Webb in E.F. Mooney (Ed.), *Annual Reports on NMR Spectroscopy*, Vol. 6, Academic Press, London, in press.
13 N. Bloembergen and L.O. Morgan, *J. Chem. Phys.*, 34 (1961) 842.
14 I. Solomon, *Phys. Rev.*, 99 (1955) 559.
15 H. Sternlicht, *J. Chem. Phys.*, 42 (1965) 2250.
16 H.G. Hertz, in J.W. Emsley, J. Feeney and L.H. Sutcliffe (Eds.), *Progress in NMR Spectroscopy*, Vol. 3, Pergamon Press, Oxford, 1967, p. 159.
17 G.H. Reed, J.S. Leigh and J.E. Pearson, *J. Chem. Phys.*, 55 (1971) 3311.
18 N. Bloembergen, *Nuclear Magnetic Relaxation*, Benjamin, New York, 1961, p. 90.
19 T.J. Swift and R.E. Connick, *J. Chem. Phys.*, 37 (1962) 307.
20 Z. Luz and S. Meiboom, *J. Chem. Phys.*, 40 (1964) 2686.
21 J. Eisinger, R.G. Shulman and B.M. Szymanski, *J. Chem. Phys.*, 36 (1962) 1721.
22 M. Cohn, in E.B. Chance, C.P. Lee and J.K. Blaise (Eds.), *Probes of Structure and Function of Macromolecules and Membranes*, Vol. 1, Academic Press, New York, 1971, p. 97.

23 B.T. Hooton, *Biochemistry*, 7 (1968) 2063.
24 J. Reuben and M. Cohn, *J. Biol. Chem.*, 245 (1970) 6539.
25 G.H. Reed, H. Diefenbach and M. Cohn, *J. Biol. Chem.*, 247 (1972) 3066.
26 B.B. Garrett and L.O. Morgan, *J. Chem. Phys.*, 44 (1966) 890.
27 T.C. Farrar and E.D. Becker, *Pulse and Fourier Transform NMR*, Academic Press, New York, 1971.
28 A. Abragam, *The Principles of Nuclear Magnetism*, Oxford University Press, Oxford, 1961.
29 J.M. Lehn and J.P. Kintzinger, in M. Witanowski and G.A. Webb (Eds.), *Nitrogen NMR*, Plenum Press, London, 1973, p. 79.
30 A. Abragam and R.V. Pound, *Phys. Rev.*, 92 (1953) 943.
31 T.R. Stengle and J.D. Baldeschweiter, *Proc. Nat. Acad. Sci. U.S.A.*, 55 (1966) 1020.
32 J.A. Happe and R.L. Ward, *J. Amer. Chem. Soc.*, 91 (1969) 4906.
33 R.L. Ward, *Biochemistry*, 8 (1969) 1879.
34 T.R. Stengle and J.D. Baldeschweiter, *J. Amer. Chem. Soc.*, 89 (1967) 3045.
35 A.G. Marshall, *Biochemistry*, 7 (1968) 2450.
36 R.G. Bryant, *J. Amer. Chem. Soc.*, 91 (1969) 976.
37 J.L. Sudmeier and J.J. Pesek, *Anal. Biochem.*, 41 (1971) 39.
38 D.F.S. Natusch, *J. Amer. Chem. Soc.*, 95 (1973) 1688.
39 A.R. Peacocke, R.E. Richards and B. Sheard, *Mol. Phys.*, 16 (1969) 177.
40 M.C. Scrutton and A.S. Mildvan, *Biochemistry*, 7 (1968) 1490.
41 B. Sheard and E.M. Bradbury, *Progr. Biophys. Mol. Biol.*, 20 (1970) 187.
42 G.H. Reed, M. Cohn and W.J. O'Sullivan, *J. Biol. Chem.*, 245 (1970) 6547.
43 J.J. Villafranca and A.S. Mildvan, *J. Biol. Chem.*, 246 (1971) 772.
44 J.J. Villafranca and A.S. Mildvan, *J. Biol. Chem.*, 246 (1971) 5791.
45 J.J. Villafranca and A.S. Mildvan, *J. Biol. Chem.*, 247 (1972) 3454.
46 J.P. Slater, T. Tamir, L.A. Loeb and A.S. Mildvan, *J. Biol. Chem.*, 247 (1972) 6784.
47 C.H. Fung, A.S. Mildvan, A. Allerhand, R. Komonoski and M.C. Scrutton, *Biochemistry*, 12 (1973) 620.
48 A.S. Mildvan, R.D. Kobes and W.J. Rutter, *Biochemistry*, 10 (1971) 1191.
49 S.J. Benkovic, J.J. Villafranca and J.J. Kleinschuster, *Arch. Biochem. Biophys.*, 155 (1973) 458.
50 A.S. Mildvan, in E.B. Chance, C.P. Lee and J.K. Blaise (Eds.), *Probes of Structure and Function of Macromolecules and Membranes*, Vol. 1, Academic Press, New York, 1971, p. 109.
51 M.E. Fabry, S.H. König and W.E. Shillinger, *J. Biol. Chem.*, 245 (1970) 4256.
52 D.M. Dawson, H.M. Eppenberge and N.O. Kaplan, *Biochem. Bioohys. Res. Commun.*, 21 (1965) 346.
53 R.E. Benesch, H.A. Lordy and R. Benesch, *J. Biol. Chem.*, 216 (1955) 663.
54 J.S. Taylor, J.S. Leigh and M. Cohn, *Proc. Nat. Acad. Sci. U.S.A.*, 64 (1969) 219.
55 M. Cohn, H. Diefenbach and J.S. Taylor, *J. Biol. Chem.*, 246 (1971) 6037.
56 J.S. Taylor, A. McCloughlin and M. Cohn, *J. Biol. Chem.*, 246 (1971) 6029.
57 G.H. Reed and M. Cohn, *J. Biol. Chem.*, 247 (1972) 3073.
58 G.L. Cottam and R.L. Ward, *Arch. Biochem. Biophys.*, 132 (1969) 308.
59 T. Nowak and A.S. Mildvan, *Biochemistry*, 11 (1972) 2813.
60 T. Nowak and A.S. Mildvan, *Biochemistry*, 11 (1972) 2819.
61 G.L. Cottam and A.S. Mildvan, *J. Biol. Chem.*, 246 (1971) 4363.
62 J.C. Bailar, *J. Inorg. Nucl. Chem.*, 8 (1958) 165.
63 J.P. Glusker, *J. Mol. Biol.*, 38 (1968) 149.
64 E.L. Muetterties, *Accounts Chem. Res.*, 3 (1970) 266.
65 L.H. Pignolet, R.A. Lewis and R.H. Holm., *J. Amer. Chem. Soc.*, 93 (1971) 360.
66 A. Lanir and G. Navon, *Biochemistry*, 10 (1971) 1024.

67 R.L. Ward, *Biochemistry*, 9 (1969) 2447.
68 M.C. Scrutton, P. Griminger and J.C. Wallace, *J. Biol. Chem.*, 247 (1972) 3305.
69 M.C. Scrutton and A.S. Mildvan, *Arch. Biochemistry Biophys.*, 140 (1970) 131.
70 R.C. Bray and J.C. Swann, *Struct. Bonding (Berlin)*, 11 (1972) 107.
71 R.L. Ward and K.J. Fritz, *Biochem. Biophys. Res. Commun.*, 39 (1970) 707.
72 A.E. Dennard and R.J.P. Williams, in R.L. Carlin (Ed.), *Transition Metal Chemistry*, Vol. 2, Arnold, London, 1967, p. 116.
73 D.E. Drum and B.L. Vallee, *Biochemistry*, 9 (1970) 4078.
74 R.L. Ward and J.A. Happe, *Biochem, Biophys. Res. Commun.*, 43 (1971) 1444.
75 B. Lindman, M. Zeppezauer and A. Akeson, *Biochim. Biophys. Acta* 257 (1972) 173.
76 M. Zeppezauer, B. Lindman, S. Forsen and I. Lindqvist, *Biochem. Biophys. Res. Commun.*, 37 (1969) 137.
77 M. Zeppezauer, personal communication.
78 R.L. Ward, personal communication.
79 I. Iweibo and H. Weiner, *Biochemistry*, 11 (1972) 1003.
80 R.T. Simpson and B.L. Vallee, *Biochemistry*, 7 (1968) 4343.
81 J.C. Coleman and B.L. Vallee, *J. Biol. Chem.*, 236 (1961) 2244.
82 C. Lazdunski, C. Petticlerc and M. Lazdunski, *Eur. J. Biochem.*, 8 (1969) 510.
83 R.G. Bryant, personal communication.
84 G.L. Cottam and R.L. Ward, *Arch. Biochem. Biophys.*, 141 (1970) 768.
85 H. Csopak, B. Lindman and H. Lilja, *FEBS Letts.*, 9 (1970) 189.
86 R.G. Bryant, *Biochem. Biophys. Res. Commun.*, 40 (1970) 1162.
87 W.N. Lipscomb, J.A. Hartsuck, G.N. Peeke, F.A. Quiocho, P.H. Bethge, M.L. Ludwig, T.A. Steitz, H. Muirhead and J.C. Coppola, *Brookhaven Symposium*, No. 21, 1968, p. 24.
88 G. Navon, R.G. Shulman, B.J. Wyluda and T. Yamane, *J. Mol. Biol.*, 51 (1970) 15.
89 G. Navon, R.G. Schulman, B.J. Wyluda and T. Yamane, *Proc. Nat. Acad. Sci. U.S.A.*, 60 (1968) 86.
90 R.G. Bryant and S. Rajender, *Biochem. Biophys. Res. Commun.*, 45 (1971) 532.
91 T. Axenrod and G.A. Webb (Eds.), *NMR of Nuclei other than Protons*, John Wiley, New York, 1974.
92 R.G. Bryant, *J. Mag. Res.*, 6 (1972) 159.
93 R.G. Bryant, H.J.C. Yeh and T.R. Stengle, *Biochem. Biophys. Res. Commun.*, 37 (1969) 603.
94 R.G. Bryant, *J. Amer. Chem. Soc.*, 91 (1969) 1870.
95 F.J. Kayne and J. Reuben, *J. Amer. Chem. Soc.*, 92 (1970) 220.
96 T.R. Collins, Z. Starcuk, A.H. Burr and E.J. Wells, *J. Amer. Chem. Soc.*, 95 (1973) 1649.
97 J. Reuben, in J.W. Emsley, J. Feeney and L.H. Sutcliffe (Eds.), *Progress in NMR Spectroscopy, Vol. 9*, Pergamon, Oxford, 1973, p. 1.
98 T.L. James and J.H. Noggle, *Proc. Nat. Acad. Sci. U.S.A.*, 62 (1969) 644.
99 D.H. Haynes, B.C. Pressman and A. Kowalsky, *Biochemistry*, 10 (1971) 852.
100 C.F. Springgate, A.S. Mildvan, R. Abramson, J.L. Engle and L.A. Loeb, *J. Biol. Chem.*, 248 (1973) 5987.
101 M. Witanowski, L. Stefaniak and H. Januszewski, in M. Witanowski and G.A. Webb (Eds.), *Nitrogen NMR*, Plenum Press, London, 1973, p. 163.