# Manganese(II) as Magnetic Relaxation Probe in the Study of Biomechanisms and of Biomacromolecules

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## Contents



## I. Introduction

This review considers the use of Mn(II) (manganous ion) as a paramagnetic probe in solution in the study of structures and mechanisms that involve metal ions in biological systems. The treatment focuses on both electron and nuclear spin relaxation studies, but the water proton relaxation rate "enhancement" (PRE) investigations are not included since several reviews[1-4] have discussed this approach thoroughly.

Magnetic resonance techniques provide a powerful tool of investigation in the field of inorganic biochemistry and in clarifying the role of metal ions in biological systems.[1,5-8] Structure and formation of biological metal ion containing compounds of low and high molecular weight, metabolism and transport of metal ions and their complexes, biomodels, and kinetic mechanisms in solution have been recently studied by ESR and NMR, and the use of paramagnetic probes has been rapidly developed.

The manganous ion is present in several enzyme reactions, and its presence is essential in some tissues; in addition many publications refer to the use of manganous ion as a probe for diamagnetic metal ions.

This kind of approach is particularly significant in light of the functional role of metals in biological mechanisms; in fact enzyme regulation is associated with ligand-induced conformational changes; metal ions influence macromolecular conformations (stabilization of protein structure, effects on the double helix of nucleic acids, etc.); metals, by modulating the equilibrium between closely related states of proteins that differ only in conformational energy, may influence enzymatic activities, protein–protein interactions, or turnover rates of proteins in biological systems, thereby affecting important regulatory processes and biomechanisms. Generally metals that are positioned at the active sites of enzyme display distinctive physical chemical characteristics and participate in the catalytic processes; their unusual properties are reflected in dynamically sensitive parameters such as electron and nuclear spin relaxation times.

The role of metals in the mechanisms of enzyme-catalyzed reactions[5,6] is often related to the formation of a ternary complex in which an enzyme–metal–substrate bridge (EMS) is formed. These metal bridge complexes possess kinetic and thermodynamic properties consistent with their participation in the catalytic process. The detection and the study of EMS complexes may be performed by ESR and NMR spectroscopy; both methods require the presence of a paramagnetic metal such as the manganous ion.

Another mechanism that is particularly relevant in biology is the outer-sphere interaction in which a water molecule is bridged between the metal and the biomolecule; generally the so-called second coordination sphere of metal complexes is of direct relevance to catalysis.[6] In this case an Eigen–Tamm[9] mechanism does occur in soluton where the first step is the diffusion-limited approach and the second step is the ligand penetration: the different rates are very sensitively reflected in the correlation times governing the electron and nuclear spin relaxation. Since enzymatic processes proceed by a series of ligand exchange processes, the outer-sphere ligands are likely to be of great significance and even very weak second sphere complexes display preferred molecular structures that may or may not be favorable to subsequent ligand exchange.

Manganous ion is a particularly suitable probe for outer-sphere relaxation studies from the points of view of both the ESR method[10-12] and the water proton relaxation[1] (e.g., the quaternary Mn(II)–creatine–ADP–creatine kinase complex).

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## II. Nature of the Electron Spin Relaxation of the Mn(II) $^6S$ Ion

### A. Introduction

High-spin Mn(II) ions ($S = {}^5/_2$, $I = {}^5/_2$), having $^6S$ spectroscopic ground states, are widely employed as paramagnetic probes in magnetic resonance. The manganous ESR patterns are relatively well resolved and are usually very sensitive to permanent and/or transient changes in the environmental symmetry. The ESR time scale ($\omega_S \simeq 10^{10}$ rad/s) lies in the range of intermolecular motions in solution. Thus the ESR relaxation allows a suitable approach to the study of solute–solvent and metal–ligand interactions.

The theory of the electron spin relaxation process has been reviewed in many papers and books, and only the basic principles will be summarized below in order to allow a comprehensive analysis of the Mn(II) complexes with biomolecules.

### B. The Relaxation Model

In isotropic solutions the random tumbling motions average out all anisotropic interactions, and this limits the structural information obtained compared to single crystals or powdered samples. However the anisotropic couplings are very important in determining the relaxation process. As was first discussed by McGarvey,[13] the ion in solution with its solvation sphere and/or bound ligands may be considered as a microcrystalline tumbling unit in solution, provided that its lifetime is long enough for the structure to be maintained during the tumbling period. If there is any anisotropy in the crystalline resonance of the ion, the tumbling of the microcrystal in solution broadens the resonance line. The extent of broadening is determined by the tumbling rate and the size of the anisotropy.

#### 1. The Spin Hamiltonian

The ESR spectra of Mn(II) are adequately described by the spin Hamiltonian:

$$\mathcal{H} = g\beta HS + AIS + D[S_z^2 - {}^1/_3 S(S+1)] + 2E(S_x^2 - S_y^2) \quad (1)$$

The first term is the Zeeman interaction, the second is the electron–nucleus hyperfine interaction, and the last two represent the zero-field splitting (ZFS), which is a second-rank traceless tensor described by the components $D$ (axial parameter) and $E$ (orthorhombic parameter). The ZFS accounts for the separations of the spin levels whenever the ligand field on the ion is asymmetric, and it therefore represents the major structure-sensitive term. Moreover, the first two terms are isotropic and give the line positions, while the ZFS is anisotropic and its time-dependence governs the electron spin relaxation.[13-15] The spin Hamiltonian is written as

$$\mathcal{H} = \mathcal{H}_0 + \mathcal{H}'(t) \quad (2)$$

where $\mathcal{H}'(t)$ is the fluctuating perturbation which provides the modulation of the ZFS tensor.

Since the ZFS is expected to vanish for highly symmetric systems such as the $Mn(H_2O)_6^{2+}$ ion, it was assumed that a widespread distribution of sites with different crystal-field energy is created either by the impact of solvent molecules on the complex[16] or by lattice fluctuations.[17] The molecular motion averages together the sites with different ZFS values and a resultant ZFS arises to which the ESR line width is attributed. The ambiguity lies in the identification of the actual correlation time, either the characteristic time for the fluctuation, $\tau_v$,[16,18] or the reorientational correlation time, $\tau_R$, of the entire complex.[18]

The distinction between the two models is very subtle in the "extreme narrowing" limit since the details of the time dependence are unimportant when the Redfield relaxation matrix[19] is constructed: the total intensity converges toward the central $|-{}^1/_2\rangle \Leftrightarrow |+{}^1/_2\rangle$ transition and a single Lorentzian with line width proportional to 6.4 $(D{:}D)$ is observed. Here $(D{:}D)$ represent the inner product of the ZFS tensor with itself. However, for the case of Mn(II) complexes with macromolecules, the two models bring about different pictures:

*(i)* $\tau_v$ *Important (Fluctuational Model).* The line widths can be expected to be broad when bound Mn(II) is accessible to efficient solvent collision and narrow when Mn(II) is inaccessible to this fast fluctuating motion. The electron Larmor frequency at which ESR spectra are carried out determines the line width of the transitions. The $|\pm{}^5/_2\rangle \Leftrightarrow |\pm{}^3/_2\rangle$, $|\pm{}^3/_2\rangle \Leftrightarrow |\pm{}^1/_2\rangle$ transitions are broadened beyond detectability, so that the observed intensity arises only from the central transition. Only a small part of the spectral intensity is shared between the "normal" ($\Delta M_S = \pm 1$, $\Delta m_I = 0$) and "forbidden" ($\Delta M_S = \pm 1$, $\Delta m_I = \pm 1$) transitions. Large static deviations from cubic symmetry produce transitions outside the isotropic $g = 2$ region. In this case the metal–ligand interactions coupled with the librational motions of the whole large molecule provide additional contributions to the line width.

*(ii)* $\tau_R$ *Important (Rotational Model).* Only anisotropies as large as about $3\tau_R^{-1}$ are expected to be averaged out, so that the spectrum usually displays a rigid lattice ESR pattern characterized by a widespread distribution of crystal-field sites with ZFS greater and smaller than the Zeeman term $\hbar\omega_S$. For the ZFS $< \hbar\omega_S$ sites, the only observable $|-{}^1/_2\rangle \Leftrightarrow |+{}^1/_2\rangle$ transition gives a relatively intense and narrow sextet, which is inhomogeneously broadened by second-order terms in the electron–nuclear hyperfine interaction. For the ZFS $> \hbar\omega_S$ sites, fine splitting patterns are observed spread over a wide field range. Many inhomogeneous features, such as low-field wings and/or large edges, broad background signal which give rise to inclination of the base line, and inversion in the line-width dependence of the hyperfine components, are usually observable in the ESR spectra, making the line-shape analysis puzzling.

#### 2. A Unified Treatment of the Modulation Process

A general treatment of the modulation process has recently been presented.[14] The hexasolvated $Mn^{2+}$ ion is assumed to experience a perfect and stable octahedral symmetry only if isolated from its environment, while the symmetry of the second solvation sphere is lower than cubic and this is reflected in the total ligand field experienced by the spin. Any motion within solvent molecules in the second solvation shell determines a

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configuration of the $Mn(solv)_1(solv)_2$ unit, and the configurational lifetime, $\tau_{f2}$, can be identified with the mean jump time of solvent molecules. The exchange of solvent molecules outside the second sphere allows rotation of the $Mn(solv)_1(solv)_2$ unit, and the rotational time, $\tau_R$, can be identified with the mean time after which a configuration changes its orientation. A rigid lattice situation gives rise to ESR spectra which reveal the corresponding distribution of spin parameters, while, in the extreme narrowing limit, $\tau_R$ or $\tau_{f2}$, whichever is the faster, determines the line width. Two cases are possible:

(*i*) $\tau_R > \tau_{f2}$. The fluctuation is rate determining in modulating the ZFS components. If the ZFS energy is relatively small, the distribution of ZFS converges into a single mean ZFS term. If a distribution function for

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$f$ configurational sites, $P_f$, is defined, then

$$(D{:}D)_{av} = \sum P_f (D{:}D)_f \qquad (3)$$

(*ii*) $\tau_R < \tau_{f2}$. The rotation is rate determining in modulating the ZFS components. The ion moves from one configuration to another at time intervals longer than the rotational time. This involves attractive immobilization of the second solvation sphere; now the distribution of ZFS sites corresponds to a distribution of relaxing sites. Two limiting situations may occur:

(a) $\tau_{f2} > T_{2f}$. Each configuration is "stable" enough that its spin system relaxes as if it was isolated from all others. The distribution of ZFS sites results in a sum of $f$-degenerate lines with different widths.

(b) $\tau_{f2} < T_{2f}$. The individuality of each site is lost and the observed linewidth is the weighted average of the configurational line widths. This case is only formally distinguishable from case i.

Since the line width cannot be calculated directly from the relaxation matrix,[20] a general formulation of the line-shape function $Y(\omega)$ was defined[21] which allows the computation of ESR spectra with the appropriate choice of a single value of $(D{:}D)$ and of the correlation time. A composite line-shape function $Y'(\omega)$ must be taken into account to reproduce the situations where the ZFS is not averaged out.[14,20,22]

$$Y'(\omega) = \sum P_f Y_f(\omega) \qquad (4)$$

As an example, Figure 1[14] shows the spectra computed with $Y(\omega)$ and $Y'(\omega)$ (dashed line) using the same width and height for the fourth hyperfine line.

Poupko and Luz[23] have recently presented a modification of the theory of the ZFS modulation which takes the isotropic hyperfine interaction into account. Explicit equations were derived for the line width when the tumbling rate is slower than the Larmor frequency but still faster than the hyperfine interaction and the second-order ZFS shift. Under these conditions only the $|-^1/_2\rangle \Leftrightarrow |+^1/_2\rangle$ transition is observed. This theory predicts a marked dependence of the line width on the

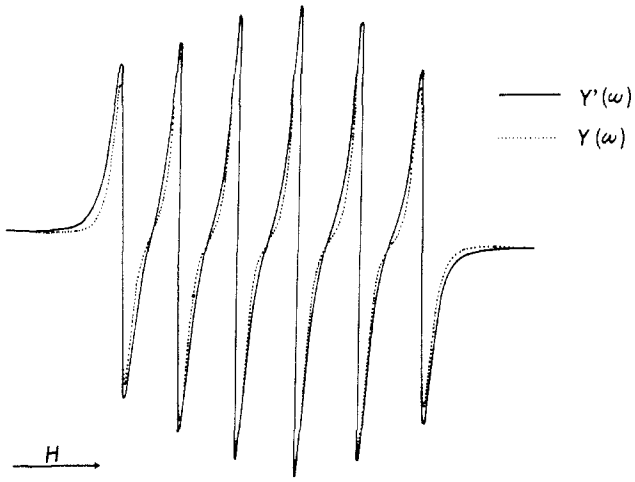



**Figure 1.** Comparison between the single Lorentzian line shape (SL) and the line shape from a sum of Lorentzians with different widths (SLDW) computed from eq 4. Computation has been carried out with equal width and height of the fourth hpf transition. (Reprinted with permission from L. Bulamacchi, G. Martini, M. F. Ottaviani, and M. Romanelli, *Adv. Molec. Relaxation Interact. Processes*, 12, 145 (1978) Copyright 1978, Elsevier Scientific Publishing Co.)

$m_I$ quantum number in both the fast and slow tumbling regions. The analysis of ESR spectra in solution pointed out the following features: (i) contributions to the line width exist from relaxation mechanisms other than the ZFS interaction, e.g., from higher order spin–orbit coupling terms; (ii) the ZFS interaction is temperature dependent, suggesting significant structural changes; (iii) the ESR spectra were simulated with a rotational tumbling mechanism without invoking static distributions of the ZFS interaction.

Well-resolved pattern ESR spectra for Mn(II) in some enzymatic systems could be simulated[24] by considering the perturbing Hamiltonian up to the third order and by using time-independent perturbation theory. Expressions were obtained for the energy levels of Mn(II) for cases of rhombic distortion of the crystal field.

### 3. Effects of Association with Ligands

The disappearance of the hyperfine spectrum of Mn(II) in solution upon complex formation was reported in the pioneer work of Cohn and Townsend.[25] Since then, a lot of research has been dedicated to ESR investigation of Mn(II) complexes in solution. Hayes and Myers[26] first observed the line-width dependence on anionic complexation at various temperatures and concentrations. The formation of both outer-sphere and inner-sphere complexes was considered and the following general equilibrium can be reported in every case:

$$\underset{a}{Mn^{2+}} + X^{n-} \underset{k_{ba}}{\overset{k_{ab}}{\rightleftharpoons}} \underset{b}{Mn^{2+}(aq)X^{n-}} \underset{k_{cb}}{\overset{k_{bc}}{\rightleftharpoons}} \underset{c}{MnX^{(2-n)+}} \qquad (5)$$

The first step (outer-sphere coordination) is usually assumed as a diffusion-limited process which is fast in the ESR time scale. Since the outer species, b, displays some small random distortion from cubic symmetry, line broadening is observed in ligand solutions.[10-12] The second step is slow (at least at room temperature). Thus the line width of species c is not averaged with those of species a and b and gives a separate contribution. Unsymmetrical coordination induces large static ZFS parameters that, when coupled with fast rotation (e.g., in small complexes), give undetectably broad ESR spectra, resulting in a loss in the ESR intensity. However, if the outer equilibrium occurs at a slower rate, the line width may not arise exclusively from a weighted average of the contributions of free ion and outer-sphere complex. For this reason the decrease in intensity may be caused by unaveraging of outer-sphere contributions with larger widths instead of inner-sphere contributions.

When Mn(II) ions are bound to a rigid substrate with a long reorientational time or when the energy separation $\Delta\omega$ of the anisotropic interaction becomes the order of the rate of change of its symmetry axis, $\tau^{-1}$, the spectral features approach the typical glassy situation. The anisotropy itself is no more averaged out and the line shape reveals a wide spread distribution of crystal-field sites. Since at least a part of this distribution in solution is expected to have ZFS energy comparable to the Zeeman energy, inhomogeneous broadening becomes effective in the "slow motion" limit, $\omega_S\tau \geq 1$.[27] The ESR intensity of Mn(II) bound to large molecules is found to be about 9/35 (0.26) times the intensity of an identical concentration of free Mn(II) ions.[28-32] The 9/35 ratio is the relative intensity of the $|-^1/_2\rangle \leftrightarrow |+^1/_2\rangle$ fine structure component, which is therefore the only transition contributing to the observed signal. Also this behavior is typical of rigid lattice situations where the $M_S \neq {}^1/_2$ transitions are smeared out and merge in the background noise.[28,29] The ESR patterns of solution

TABLE I. Scheme of the Conditions Influencing the ESR Spectra of Manganous Ion[a]

| $\omega_S^2\tau^2$ | $D^2\tau_R^2$ | $D/h\omega_S$ | $D$ | ESR spectra |
|---|---|---|---|---|
| | | | Liquid State | |
| $\ll 1$ | $\ll 1$ | $\ll 1$ | single ($\tau_{f_2} < T_{2f}$) | Ia: six hpf lines described by a single Lorentzian shape |
| | | | distributed ($\tau_{f_2} > T_{2f}$) | Ib: sum of degenerate lines described by eq 4 |
| $\ll 1$ | $\ll 1$ | $>0.2$ | | unobservably broad |
| 1 | $\ll 1$ | $\ll 1$ | | maximum observable line width |
| $>1$ | $\ll 1$ | $\ll 1$ | single | IIa: transition $M_S = {}^1/_2$ becomes important; inner hyperfine lines broader than outer |
| $>1$ | | within 0.5 | distributed | IIb: equal to IIa inhomogeneously broadened with background fine splitting wings |
| $>1$ | $>1$ | $>0.5$ | | III: fine splitting patterns |
| | | | Glassy State | |
| $\gg 1$ | | $<0.2$ | single | IVa: six lines inhomogeneously broadened, forbidden transitions |
| | | within 0.5 | distributed | IVb: superposition of differently broadened hpf lines |
| | | within 0.5 | distributed | IVc: equal to $IV_b$ with background fine splitting wings |
| | | $>0.5$ | | V: fine splitting patterns |

[a] From ref 32.

Mn(II)–protein complexes were simulated by using expressions based only on the rigid limit parameters, which were simulating powder spectra as well, provided a Gaussian distribution of ZFS parameters is entered in the formalism.

The various possible situations which may occur in solutions of Mn(II) ions are summarized in Table I.[33]

## III. Nuclear Relaxation Studies with Mn(II) as Paramagnetic Probe

In solutions of paramagnetic Mn(II) ions the electron spin–nuclear spin interaction is the most efficient mechanism for nuclear relaxation. Both the direct dipole–dipole coupling and the contact hyperfine interaction provide relaxing fields due to modulation by different time-dependent processes. Namely, the correlation time of the Brownian motion, $\tau_R$, the residence time of the nuclear spin in the neighborhood of the electron spin, $\tau_M$, and the electron spin relaxation time, $\tau_S$, account for the time dependence of the IS dipolar interaction, while only $\tau_M$ and $\tau_S$ may modulate the contact interaction.

The information gained by the use of the Mn(II) probe is poor in the absence of chemical exchange between the different environments experienced by the nuclear species; the relaxation rates $T_{iM}^{-1}$ ($i$ = 1,2) of nuclei directly bound to the paramagnetic ion are so fast as to give undetectably broad lines. Fortunately, the chemical exchange carries information from the ion coordination sphere to the bulk. The exchange rate, $\tau_M^{-1}$, defines the limiting conditions reported in Table II,[34] where $P_M$ is the fraction of bound nuclei and $T_{ip}^{-1}$ is the paramagnetic contribution to the relaxation rate defined as

$$\frac{1}{T_{ip}} = \left(\frac{1}{T_i}\right)_{obsd} - \left(\frac{1}{T_i}\right)_{blank} \quad (6)$$

The approach given by Swift and Connick[34] for two sites (free and bound) is easily extended to three or more environments.[35,36] A dual role is therefore envisaged for $\tau_M$: modulation of the relaxing field and determination of the exchange conditions.

Using a point-dipole approximation and assuming the existence of only one $\tau_{1s}$ and one $\tau_{2s}$, the full Solomon–Bloembergen (SB) equations for $T_{iM}^{-1}$ can be written[1,37-39] as

$$\frac{1}{T_{1M}} = \frac{C}{r^6}\left(\frac{3\tau_{c1}}{1+\omega_I^2\tau_{c1}^2} + \frac{7\tau_{c2}}{1+\omega_S^2\tau_{c2}^2}\right) + \frac{2}{3}\frac{A^2}{\hbar^2}S(S+1)\left(\frac{\tau_{e2}}{1+\omega_S^2\tau_{e2}^2}\right) \quad (7)$$

$$\frac{1}{T_{2M}} = \frac{C}{2r^6}\left(4\tau_{c1} + \frac{3\tau_{c1}}{1+\omega_I^2\tau_{c1}^2} + \frac{13\tau_{c2}}{1+\omega_S^2\tau_{c2}^2}\right) + \frac{1}{3}\frac{A^2}{\hbar^2}S(S+1)\left(\tau_{e1} + \frac{\tau_{e2}}{1+\omega_S^2\tau_{e2}^2}\right) \quad (8)$$

The constant terms appearing before the dipolar term are approximately two orders of magnitude greater than those before the scalar term. Thus, the significance of the scalar interaction depends on the interplay of correlation times and it is expected to be valuable for $T_{2M}^{-1}$ when $\tau_{e1} >> \tau_{c1}$. Assuming $\tau_{1S} = \tau_{2S} = \tau_S$ and $\omega_I^2\tau_c^2 << 1$, the limiting conditions in Table III can be obtained. The $T_{1M}/T_{2M}$ ratio therefore yields information on the relaxation mechanism; namely, $T_{1M}/T_{2M} = 7/6 = 1.17$ for a 100% dipolar interaction, while $T_{1M}/T_{2M} = 7/3 = 2.33$ for a 50% dipolar and 50% scalar contribution. Thus, ratios greater than 2.33 indicate that the dipolar term no longer dominates the paramagnetic line broadening.[40,41] Other theoretical aspects have been treated elsewhere.[42-49]

TABLE II. Limiting Exchange Conditions

| region | limiting condition | $1/T_{ip}$ | comments |
|---|---|---|---|
| fast exchange | $\tau_M^{-1} >> T_{iM}^{-1}$ | $P_M/T_{iM}$ | structural information is possible |
| slow exchange | $\tau_M^{-1} << T_{iM}$ | $P_M/\tau_M$ | kinetics of the ligand and/or solvent exchange process may be studied |

TABLE III. SB Equations: Limiting Conditions[a]

| $\omega_S^2\tau_e^2$ | $\omega_S^2\tau_c^2$ | $1/T_{1M}$ | $1/T_{2M}$ |
|---|---|---|---|
| <<1 | <<1 | $(10C/r^6)\tau_c + (2/3)K\tau_e$ | $(10C/r^6)\tau_c + (2/3)K\tau_e$ |
| | 1 | $(6.5C/r^6)\tau_c + (2/3)K\tau_e$ | $(6.75C/r^6)\tau_c + (2/3)K\tau_e$ |
| | >>1 | $(3C/r^6)\tau_c + (2/3)K\tau_e$ | $(3.5C/r^6)\tau_c + (2/3)K\tau_e$ |
| 1 | <<1 | $(10C/r^6)\tau_c + (1/3)K\tau_e$ | $(10C/r^6)\tau_c + (1/2)K\tau_e$ |
| | 1 | $(6.5C/r^6)\tau_c + (1/3)K\tau_e$ | $(6.75C/r^6)\tau_c + (1/2)K\tau_e$ |
| | >>1 | $(3C/r^6)\tau_c + (1/3)K\tau_e$ | $(3.5C/r^6)\tau_c + (1/2)K\tau_e$ |
| >>1 | <<1 | $(10C/r^6)\tau_c$ | $(10C/r^6)\tau_c + (1/3)K\tau_e$ |
| | 1 | $(6.5C/r^6)\tau_c$ | $(6.75C/r^6)\tau_c + (1/3)K\tau_e$ |
| | >>1 | $(3C/r^6)\tau_c$ | $(3.5C/r^6)\tau_c + (1/3)K\tau_e$ |

[a] $K = (A/h)^2S(S+1)$. $C = h^2S(S+1)\gamma_I^2\gamma_S^2$.

In addition, the dipolar interaction can affect the nuclear relaxation of bulk molecules not actually coordinated to the Mn(II) ion (outer-sphere, OS, relaxation). In this case the time dependence of the dipolar field may arise either from the relative diffusional motions of the ion and the bulk ligand or from motions characterized by $\tau_S$. The magnitude of OS relaxation depends on the geometry of the binding site and on the magnetic field, but it is usually small. It has been shown that when the scalar interaction is not important, the OS mechanism contributes about 15%.

If Mn(II) is bound to a large molecule, the entire relaxation of several nuclei may occur via OS relaxation, e.g., for solvent water protons, when all the water molecules are excluded from the solvation sphere by the bound macromolecule.

It must be noted that OS complexes display electron spin relaxation times that are shorter than that of the free ion by at least one order of magnitude.[10-12,51] This is consistent with the idea that different scalar terms have to be taken into account even in the simplest cases (see Table III).

The modes of using the theory to get the required structural information have been thoroughly treated elsewhere.[1,51]

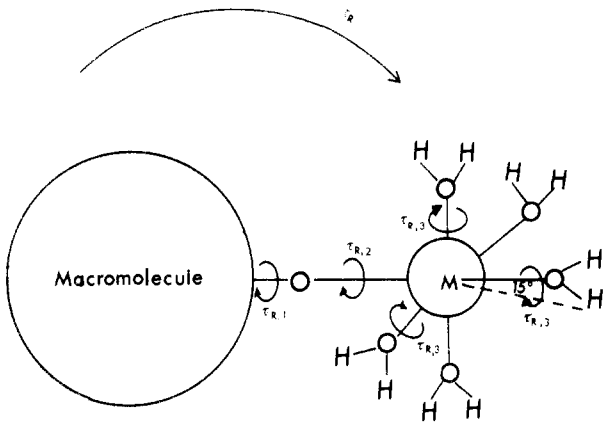



**Figure 2.** Schematic representation of binding of metal ion to a macromolecule via one ligand. Various rotational correlation times are indicated. (Reprinted with permission from R. A. Dwek, "NMR in Biochemistry", Clarendon Press, Oxford. Copyright Clarendon Press.

The applicability of the SB equations is inappropriate when one of the following situations occurs: (a) ZFS $> \tau_c^{-1}$, $\omega_S$; the zero-field splitting is larger than the inverse of the relevant correlation time and the electron Larmor frequency. Expressions were derived for very low fields where the ZFS is much greater than the Zeeman splitting.[52] (b) The distribution of unpaired electron density within the metal orbitals or its delocalization into the ligand orbitals (covalency) cannot be neglected; the SB equations were extended by Waysbort and Navon[49,53] to include this effect. Both (a) and (b) were directly included into the dipolar term when the SBM equations were "ex novo" derived by regarding $1/T_{1M}$ as a dissipation term resulting from a linear interaction of the nucleus with the magnetic susceptibility of the ion[54] rather than by relating $1/T_{1M}$ to the rates of the transitions produced by the time-dependent local field at the nucleus. (c) The hyperfine interaction is of the same order of magnitude as the Zeeman interaction. (d) The tumbling motion is not isotropic; if the rotation is anisotropic, the overall rotational correlation time, $\tau_R$, is a complex function of the reorientational times of the different axes. This situation is commonly encountered when Mn(II) is bound via only one ligand to a macromolecule since many possibilities remain for internal rotational motions, as exemplified in Figure 2. (e) The electronic g factor is not isotropic.

## IV. Paramagnetic Relaxation Studies of Ligands in Mn(II) Solutions

### A. The Mn(II)–ATP Complex

The Mn(II)–ATP complex is used as the primary example, since ATP (i) is a universal cell component, (ii) exists in biological systems as a mixture of variously ionized, metal complexed, and structurally conformed species, and (iii) is a relatively small biomolecule, and its metal complexes can be studied directly without the use of model systems.

Intuitively Szent-Györgyi[55] proposed that metal ions form a bridge between the phosphate end of the ATP molecule and the adenine ring to give a "backbound" complex. In the first NMR studies, Cohn and Hughes[56,57] reported the NMR spectra of ATP ($^{31}P$ and $^{1}H$) and also qualitatively studied the effects of Mn(II)

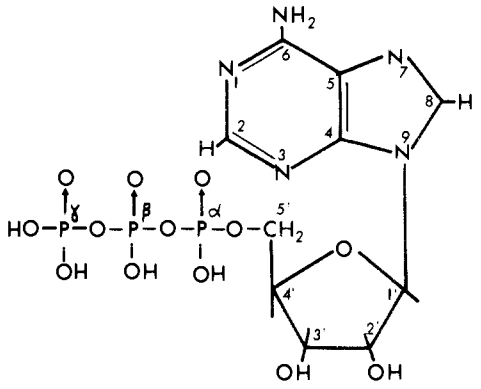


**Figure 3.** Standard numbering of the 5′-ATP molecule.

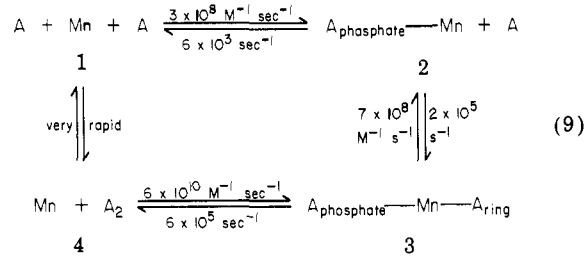
(and also Mg(II)) on the $^{31}P$ and $^{1}H$ NMR line widths. Their conclusion was that Mn(II) interacted with all three phosphate groups and also with the ring, while Mg(II) interacted with the $\beta$- and $\gamma$-phosphate only. Measurements of the $^{31}P$ NMR of the $\alpha$-, $\beta$-, and $\gamma$-phosphates were also made[58] in the presence of Mn(II) at different metal/ATP ratios between 0 and 95 °C. The approximately equal $fT_{1p}$ value observed for the $\alpha$, $\beta$, and $\gamma$ $^{31}P$ nuclei was assumed to indicate that the Mn–P distances were essentially equal. This would imply simultaneous binding to the three phosphates only if one complex existed. $T_{2M}$ values were calculated assuming $\tau_S = 1 \times 10^{-8}$ s, which was a gross assumption. The number of water molecules in the first coordination sphere was also given ($q$ = 2–3) with little precision by assuming the same hyperfine coupling constant for MnATP and Mn–$H_2O$. The phosphorus rotational correlation time was calculated as $\tau_c = 1 \times 10^{-9}$ s at 25 °C for $r$ = 3.3 Å, which is the $r_{Mn-P}$ in the $LiMnPO_4$ crystal. The proton relaxation rate enhancement of water protons was measured, and this gave the water rotational correlation time $\tau_c \sim 2\text{–}3 \times 10^{-10}$ s.

Similar work was reported immediately after using proton NMR studies.[59] $T_1$ and $T_2$ of H-8 and H-2 (Figure 3) were found equal within 20%, while $T_1 \simeq 2.5T_2$ for H-1′, although with somewhat greater uncertainty. This equality was taken to suggest that the system was in fast chemical exchange limit with $fT_{1p} = T_{1M} = T_{2M} = fT_{2p}$ and with the dipolar contribution dominating the scalar contribution to $T_{2M}$. Below room temperature the H-8 resonance narrowed because $fT_{2p}$ was entering the $\tau_M$ region. The fitting was made taking into account the $T_{2M}$ values of H-8 at higher temperatures and the $\tau_M$ values obtained from $^{31}P$ data in ref 58 and was found to be excellent. It was concluded that a single complex exists with the Mn(II) interacting simultaneously with the ring and the phosphate groups. The proton–Mn(II) distances were calculated from the room temperature data with the assumption of a common ATP proton $\tau_c$ which was taken to be either the phosphorus value or the water ligand value.[58] In the water ligand $\tau_c$ gave more consistent distances with those obtained from other paramagnetic complexes. However the binding site in the ring could not be determined because of the experimental error. Among all the possible complexes the single intramolecularly "back-bound" species was most consistent with the experimental results.

These conclusions were in disagreement with earlier UV spectral data at low total ATP concentration ($1 \times 10^{-4}$ M),[60] which showed a very small degree of back-

binding. Moreover the $\tau_M$ values were about one order of magnitude shorter than the dissociation lifetimes obtained from temperature-jump studies.[61]

From $^1H$ NMR measurements on Mn(II) solutions at low concentrations and from competition NMR studies in a mixture of AMP, ATP, and Mn(II)[62] the existence of a 1:2 metal–ligand complex at high total ATP concentration was proposed, and the kinetic scheme at room temperature put forward (eq 9), which was also taken to reconcile the results of the different experimental techniques. By using the rate constants for 1




(9)

$\rightleftharpoons$ 2 obtained from temperature-jump studies and an estimate of the equilibrium constant for the metal-independent step 1 $\rightleftharpoons$ 4, the other rate constants and equilibrium constants were determined or evaluated, as shown in the scheme. In the $MA_2$ complex the simultaneous binding of Mn(II) to the phosphate moiety (AP) of one nucleotide and to the adenine ring (AR) of the second nucleotide was shown. The $MA_2$ complexes in which the metal ion binds to the $N_7$ position predominate. Moreover from the $fT_{2p}$ value for H-8 at room temperature and low nucleotide concentration, where the MA complex becomes accessibile to the NMR technique, the Mn(II)–H-8 distance was calculated by assuming a purely dipolar contribution and a correlation time $\tau_c = 1 \times 10^{-10}$ s ($r$ = 3.8 Å ± 15%). This value was in close agreement with that found at total ATP concentration of 0.32M where the $MA_2$ complex dominates, although this finding was labeled "fortuitous". It was also suggested that both direct binding and water-separated interactions were consistent with the $r$ value.

Heller et al.[63] suggested that the Mn(II) binding to ring positions is negligible since from water proton relaxation times the number of binding sites on the nucleotide was calculated to be between 2 and 3. The calculation was based on eq 10. $\tau_e$ and $A$ were assumed

$$T_2 \propto A^2 P \tau_e \tag{10}$$

to remain constant for the complex. $\tau_e = 1 \times 10^{-8}$ s and $A = 3.3 \times 10^6$ Hz were used. It must be recognized that the authors hypothesized change in $\tau_e$ to explain the five sites they found for inorganic pyrophosphate.

The conclusion that the Mn–ATP complex contains a water molecule that is simultaneously coordinated to Mn(II) and hydrogen bonded to N-7 was drawn from two types of NMR measurements.[64] First, the comparison between the Mn(II) complexes of ATP and tubercidin triphosphate, which has C instead of N at position 7, allowed the calculation of the various Mn–H distances from line-broadening studies at room temperature.

A purely dipolar line-broadening mechanism was assumed. The $r$ value for the Mn–H-8 interaction was evaluated to be 5.3 ± 0.5 Å for Mn–TuTP, which is to be compared with r = 3.8 ± 0.6 Å for Mn–ATP. This leads to the conclusion that the metal ion is close to N-7 of the adenine ring. The outer character of this interaction was suggested by "counting" the water molecules in the first coordination shell of the Mn–ATP complex as deduced from comparing the relaxation of solvent water of Mn–ATP to that of the Mn(II)–cytidine triphosphate complex. The use of CTP as standard was justified by the magnitude of the stability constant for Mn–CTP. The ratio of the water transverse relaxation rates was near 1 irrespective of the temperature. Since the Mn(II)–ring interaction does not occur in Mn–CTP, a water bridging was suggested for Mn–ATP.

Other properties of the Mn–ATP complex in aqueous solution were checked with $^{17}O$ NMR studies.[65] $^{17}O$ NMR line broadening was observed to be independent of the $[Mn]_{tot}/[ATP]_{tot}$ ratio at any temperature, which suggests that the 1:2 complex may not be present in significant amounts. The temperature-dependent line-broadening data were interpreted in terms of three rapidly exchanging waters, which are, within a factor of 3, kinetically equivalent; however, the possibility of one of the three $H_2O$ molecules being much slower or faster than the others remained.

The spin–lattice relaxation rates of $^{31}P$ of Mn–ATP were measured at 86 MHz as a function of temperature by using pulsed NMR methods.[66] It was found that at low temperatures both $(fT_{1p})^{-1}$ and $(fT_{2p})^{-1}$ are dominated by $\tau_M$, the activation energy of this process being $E_a$ = 11 kcal mol$^{-1}$ and $\tau_M$ = 6.5 μs. At higher temperatures $(fT_{1p})^{-1} = T_{1M}^{-1}$. By assuming the $A/\hbar$ value observed in $LiMnPO_4$ and the fluctuational model for electron spin relaxation, a negligible scalar contribution was calculated for $T_{1M}^{-1}$ from which it was found that the α-phosphate group is more distant from Mn(II) than are the β- and γ-phosphates. The absolute distances were not accurately determined due to the difficulty of measuring the rotational tumbling time of the Mn–ATP complex from both proton relaxation rate enhancement[67] of the water molecules and Stoke's law.

The effects of Mn(II) ions on the PMR spectra of ATP and ATP–AMP mixtures were reinvestigated by Wee et al.[68] It was found that at $[ATP]_{tot} = [AMP]_{tot}$ = 0.25 M and $[Mn]^{2+} = 5 \times 10^{-5}$ M in $D_2O$ at pD = 8 and 27 °C, the ATP H-8 signal was broadened 3.9 times as much as the AMP signal. When the dependence of line broadening on temperature, pD, and concentration was also analyzed, the data were found consistent with an equilibrium between 25% $MnATP^{2-}$ and 75% $Mn(ATP)_2^{6-}$ but the proposed structure for the 1:2 complex was different from that in ref 62. Moreover a drastic change in line broadening was observed when the pD was lowered from 6.4 to 5.4, which was interpreted in terms of a transition in the triphosphate chelation from α–β–γ to β–γ with an accompanying change in the ligand exchange mechanism.

The nature of binding in the Mn–ATP was suitably clarified by using natural abundance Fourier transform $^{13}C$ NMR techniques.[69,70] The most consistent interpretation of the data was reached in terms of the following consecutive binding mechanism:

$$M + L \rightleftharpoons ML_P \rightleftharpoons ML_{P+R} \tag{11}$$

where P and R stand for phosphate and ring. The results are shown in Figure 4 and Table IV.

The temperature dependence of the transverse re-

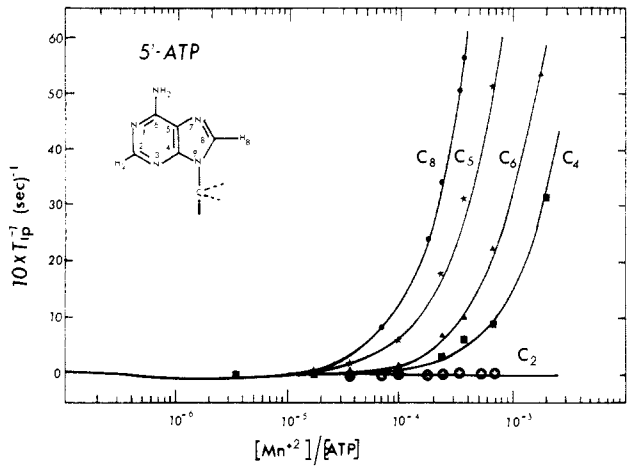



**Figure 4.** Effect of Mn(II) on the $^{13}C$ longitudinal relaxation rates of the adenine base nuclei of ATP. The measurements were carried out in $D_2O$ at a pD of 7.0 at 30 °C. The ATP concentration is 0.3 M. (Reprinted with permission from Y. F. Lam, G. P. Kuntz, and G. Kotowycz, *J. Am. Chem. Soc.*, **96**, 1834 (1974)).

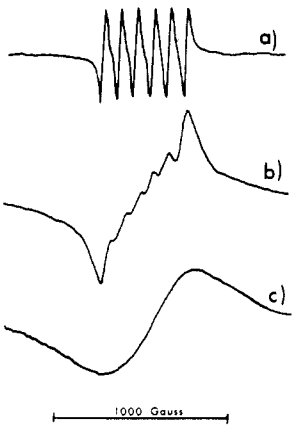



**Figure 5.** ESR spectra of Mn–ATP in water solution at room temperature. (a) Q band ($\omega = 2.16 \times 10^{11}$ rad/s); (b) X band ($\omega = 5.8 \times 10^{10}$ rad/s); (c) S band ($\omega = 1.85 \times 10^{10}$ rad/s). (Reprinted with permission from L. Burlamacchi, G. Martini, and E. Tiezzi, *Chem. Phys. Lett.*, **23**, 494 (1973). Copyright 1973, North-Holland Publishing Co.)

laxation rate was also checked, and showed fast exchange conditions above 30 °C, where the $T_{1p}/T_{2p}$ ratio suggests a purely dipolar contribution to $T_{1M}$ and a purely scalar contribution to $T_{2M}$. The fitting procedure was performed by assuming the exchange rate obtained in ref 58 from $^{31}P$ studies ($2.3 \times 10^5$ s$^{-1}$ at 27 °C) for the low temperature region and applying the theory of Rubinstein et al.[18] for the electron spin relaxation time in the high-temperature region. The rate constant for the interaction of the metal ion with the adenine base was found to be $2.7 \times 10^7$ s$^{-1}$ at 27 °C. From the $T_{1p}^{-1}$ data the distances between Mn(II) and the carbon nuclei of the base were calculated; the results strongly indicated that the metal binds directly to N-7.

Kinetic information was obtained by the same authors[71] studying the ATP concentration dependence of the $^{31}P$ NMR exchange rate, which allowed the assignment of the exchange mechanism either as Eigen–Tamm (dissociation–association) or direct ligand exchange. The data indicated that both exchange mechanisms are required to explain the ATP concentration dependence and the kinetic parameters obtained were found to be in agreement with those derived from other techniques.[72]

Recently[73] $^{17}O$ line-broadening and shift measurements were performed on aqueous solutions containing Mn–ADP complexes by taking into account both mono and bis complexes. The 1:2 complex turned out to have three or four bound waters, suggesting that it is a stacked complex. A comparison with Mn–ATP was made.

The Mn(II)–ATP has also been investigated by ESR. Reed et al.[74] presented the ESR of $2.5 \times 10^{-3}$ M Mn(II) solutions to which excess ATP was added to suppress the equilibrium $Mn(H_2O)_6^{2+}$. From a fitting procedure between experimental and computed spectra at two different frequencies they evaluated the (*D*:*D*) parameter and the correlation time for electron spin relaxation. The temperature dependence was found in agreement with this $\tau_c$ value, which was also consistent with that obtained by Rubinstein et al.[18] from proton relaxation measurements, giving support to a relaxation mechanism produced by solvent collision (fluctuational model).

The ESR spectra obtained at three different frequencies[75] gave rise to severe criticism about the fluctuational model for electron spin relaxation. It was recognized that the observed frequency dependence (Figure 5) was not due to the strong coupling between the ZFS fluctuation and the modulation field but rather to inhomogeneous broadening due to higher terms in the spin Hamiltonian of a "quasi-glassy" representation, in which the ZFS is not averaged to a single value.

These ESR features were further analyzed by extending the implications of the proposed model to the interaction between electronic and nuclear spins.[76] The ESR spectrum was observed to change remarkably with pH, varying from a well-resolved six hyperfine line pattern at pH 5 through a partially resolved spectrum to an unresolved spectrum with a large low-field wing at pH 8. The results were discussed in terms of slow rotation. The $^{13}C$ NMR results pointed out the importance of N-7 as a binding site.

The role of outer-sphere species in the exchange mechanism for the metal–ATP complex was demonstrated by Granot and Fiat[77] and was further checked by nuclear relaxation studies on the Mn(II) complex.[78] The water $T_1$ in a $10^{-3}$ M solution of Mn(II) was measured as a function of $[ATP]_{tot}$ at pH 3 and 7, and the $T_1/T_2$ ratio was measured as a function of pH; the findings were interpreted by means of at least three sites for water binding, with outer-sphere sites being among them, which determine the interplay of dipolar and scalar contributions to the relaxation rates. The

TABLE IV. Summary of $^{13}C$ $T_{1p}^{-1}$ and $T_{2p}^{-1}$ Values for Mn(II)–ATP Solutions[a]

| | C-6 | C-2 | C-4 | C-8 | C-5 |
|---|---|---|---|---|---|
| $T_1$ (pure ATP), s | 6.33 ± 0.25 | 0.17 ± 0.01 | 5.90 ± 0.25 | 0.13 ± 0.01 | 6.54 ± 0.25 |
| $T_{1p}^{-1}$,[b] s$^{-1}$ | 1.1 ± 0.15 | | 0.59 ± 0.07 | 5.0 ± 0.2 | 2.8 ± 0.3 |
| $T_{2p}^{-1}$,[b] s$^{-1}$ | 4.1 ± 0.8 | ≤0.03 | 10.7 ± 0.5 | 28.6 ± 2.4 | 33.4 ± 2.6 |

[a] Experiments were carried out in $D_2O$ at a pD of 7.0 at 30 °C. [ATP] = 0.30 M; [Mn]/[ATP] = $3.7 \times 10^{-4}$ (from ref 69). [b] Average values from five measurements normalized to the [Mn]/[ATP] ratio of $3.7 \times 10^{-4}$.

TABLE V. NMR Relaxation Rates of a Mn(II)-ATP Complex in Aqueous Solution[a]

| nucleus | exchange conditions at room temperature, pH 7 | paramagnetic contribution | further remarks |
|---|---|---|---|
| $^1$H (ligand) | $T_{1M} >> \tau_M$ | $T_{1p}^{-1} = fT_{1M}^{-1}$ | $(fT_{1p})^{-1} \simeq (fT_{2p})^{-1}$ |
| | $T_{2M} >> \tau_M$ | $T_{2p}^{-1} = fT_{2M}^{-1}$ | dipolar contribution only or an equal small scalar contribution |
| $^1$H (water) | $T_{1M} >> \tau_M$ | $T_{1p}^{-1} = fT_{1M}^{-1}$ | $(fT_{1p})^{-1} << (fT_{2p})^{-1}$ |
| | $T_{2M} >> \tau_M$ | $T_{2p}^{-1} = fT_{2M}^{-1}$ | $T_{1p}^{-1}$ dipolar only<br>$T_{2p}^{-1}$ scalar only. At pH 4 $(fT_{1p})^{-1} \simeq (fT_{2p})^{-1}$ |
| $^{31}$P | $\tau_M >> T_{1M}$ | $(fT_{1p})^{-1} = (fT_{2p})^{-1} = \tau_M^{-1}$ | exchange-controlled relaxation rates; kinetic information is possible |
| | $\tau_M >> T_{2M}$ | | |
| $^{13}$C | $T_{1M} >> \tau_M$ | $T_{1p}^{-1} = fT_{1M}^{-1}$ | $(fT_{1p})^{-1} << (fT_{2p})^{-1}$ |
| | $T_{2M} >> \tau_M$ | $T_{2p}^{-1} = fT_{2M}^{-1}$ | $T_{1p}^{-1}$ dipolar only<br>$T_{2p}^{-1}$ scalar only |

[a] From ref 79.

outer-sphere species was also made evident from the temperature dependence of the ESR line width since the smoother high-temperature line narrowing was attributed to the temperature dependence of the mean diffusional jump time of solvent and ligand molecules in the second coordination sphere.

The need of ESR and NMR combined analysis was further underlined[79] with an extension of the ESR theory[14-20] to the Mn–ATP system, by suggesting a dynamic model which accounts for the nuclear relaxation rate analysis for both the ligand and the solvent molecules. A distribution of crystal field sites was apparent, which results in a distribution of $\tau_s$ values. The relevance of outer-sphere coordination was reinforced from ESR measurements at varying temperatures and at two frequencies. The longitudinal and transverse nuclear relaxation rates of water protons in the Mn–ATP system were measured as a function of both concentration and pH, giving prominence to the role of $\tau_s$ in determining the interplay of the dipolar and scalar IS interactions. A reinvestigation of the NMR and ESR results is shown in Table V.

## B. Macromolecular Complexes

### 1. General Introduction

Metallic compounds are adsorbed into all organisms and metabolized. Metal ions (i) are employed at the catalytic sites of many enzymes[80], (ii) show strong and specific combinations mostly with proteins,[2,81-90] (iii) are involved in the reactions of nucleic acids,[91] and (iv) affect the stability of model and biological membranes. The biological activity is usually retained under replacement of naturally occurring metal ions with probes of similar size and chemistry, and this has prompted the wide use of Mn(II) as paramagnetic probe for relaxation studies. The ligand and/or solvent exchange rate, the affinity constants, the identification of binding sites, the geometric and electronic structure of the active site, and the symmetry of the environment surrounding the metal ion can be alternatively elucidated by investigating one or, even better, all the following parameters: (i) nuclear relaxation rates of water protons; (ii) nuclear relaxation rates of ligand nuclei; (iii) ESR properties of the Mn(II) ion; and (iv) ESR and NMR in the presence of two different paramagnetic probes (usually Mn(II) and Cr(III) or Mn(II) and a spin-label).

In many cases, it is possible to elucidate the mechanism of the rate-determining reaction at the catalytic site.

### 2. Nuclear Relaxation of Water Protons

The possibility of obtaining structural and kinetic information in biochemical systems was first recognized by Shulman and co-workers[92] and Cohn and co-workers[93] who noted a proton relaxation rate enhancement (PRE) of solvent water on binding of DNA or proteins to paramagnetic ions. Since then the PRE technique has been extensively used as the basis for conclusions such as the geometry of substrate–metal interactions, the metal hydration number $q$, the affinity constants for metal–enzyme complexes with substrates and inhibitors, and the estimate of the exchange lifetime $\tau_M$.

In the beginning the PRE was attributed to the slower tumbling motion modulating the IS dipolar interaction whenever a macromolecule is bound to the Mn(II) ion. The enhancement parameter was defined as

$$\epsilon^* = \frac{1/T_{1p}^*}{1/T_{1p}} = \frac{(1/T_1^*)_{obsd} - (1/T_1^*)_{blank}}{(1/T_1)_{obsd} - (1/T_1)_{blank}} \quad (12)$$

where the asterisk denotes the presence of a macromolecule. This kind of approach was used to evaluate the dissociation constant $K_D$ and the number of binding sites $n$ for many Mn(II)–enzyme systems. The measurement of the free Mn(II) ion concentration from the intensity of the ESR spectrum was used together or independently of the PRE titrations to get the binding parameters. The PRE analysis was used also to detect multiple equilibria when ternary or higher species were formed in the presence of various substrates and inhibitors. The argument has been thoroughly reviewed elsewhere,[1-4] but the following points are worth noting: (i) A close correlation was usually found between the enhancement factors in ternary complexes with substrates $\epsilon_T$ and kinetic measurements of the enzymatic activity, indicating differing degrees of conformational change at the active site.[94-99] (ii) The approximate graphical methods used to get binding parameters in the early PRE experiments were superseded by computer analysis of the PRE titration data,[100-106] yielding a high degree of accuracy for the enhancement parameters. (iii) The dissociation constants obtained by PRE titration were often compared with $K_M$ and $K_I$ values from kinetic studies on the Mn(II)–enzyme complexes

with substrates and inhibitors, yielding information on the role played by metal complexes in catalysis.[99,102,103,105,107-117] (iv) The ESR intensity has a vague significance in Mn(II)–macromolecule systems due to the presence of unaveraged contributions from differing configurations and to the anisotropic features of the ESR spectra, particularly at X-band. The disagreement between the observed free Mn, determined by ESR, and that calculated with computer programs was recently shown.[118]

For structural and kinetic information, the variable parameters in the SBM equations, $B$, $\tau_v$, $\tau_r$, $\tau_M$, $q$, $r$, $E_v$, $E_r$, and $E_m$, can be deduced from the temperature and frequency dependence of the paramagnetic relaxation rate. If any two of the rate processes represented by $T_{1M}$, $\tau_M$, $\tau_S$, and $\tau_R$ are of the same order of magnitude or if only a limited temperature range can be investigated the ambiguities in interpretation are increased.

The temperature dependence of $T_{1p}^{-1}$ and $T_{2p}^{-1}$ is usually taken into account to decide whether fast or slow exchange conditions hold, but the interpretation is not straightforward due to ambiguities in the temperature dependence of $\tau_S$. The variation of the nuclear relaxation rates with $1/T$ may be either negative or positive depending on the existence of slow or fast exchange conditions, short or long correlation time relative to $\omega_I^{-1}$, and predominance of $\tau_R$ or $\tau_S$ as actual correlation time.

The existence of slow exchange conditions was deduced from negative slopes in $1/T_{1p}$ vs. $1/T$ plots, which were found in ternary or quaternary complexes of some enyzmes.[97,98] Such an interpretation was not consistent with the $T_{1p}/T_{2p}$ ratios or the temperature dependence obtained at more than one frequency. It was instead postulated that fast exchange conditions apply, that $T_{1M}^{-1}$ is a maximum, and that $\tau_S$, which is itself frequency dependent, is the effective correlation time especially at low temperatures and low frequencies.[115,118,119-129] The inclusion of $\tau_M$ in the determination of $T_{1p}^{-1}$ was also excluded on the basis of the small energy of activation obtained from the Arrhenius plot of $T_{1p}^{-1}$ vs. $1/T$[130,131]. $\tau_M$ was shown to become important at high temperature whenever outer-sphere relaxation is the only relaxation mechanism[108,132] since, in that case, the $\tau_M > T_{1M}$ condition is easily reached. However, the usual procedure of best-fitting the PRE data was shown to give rise to ill-defined parameters;[133] in particular the reliability of the $q$ values stems from the uncertainty in the slow-exchange contribution to the relaxation rates.

From the SB equations it follows that the paramagnetic relaxation rates exhibit frequency dependence due to the presence of dispersion terms at both $\omega_I$ and $\omega_S$. The frequency-dependent $T_{1p}^{-1}$ data are usually considered due to the almost exclusive predominance of the dipolar contribution. The $\omega_S$ dispersion is not to be taken into account unless experiments at very low fields ($H \simeq 2000$ Oe, $\nu \simeq 100$ kHz) are performed. The $\omega_I$ dispersion should result in frequency independence of $T_{1p}^{-1}$ when $\omega_I^2\tau_{c1}^2 << 1$ ($\tau_{c1} = \tau_{c2}$ is assumed here) and in a linear increase of $T_{1p}$ with increasing $\omega_I$ when $\omega_I^2\tau_c^2 \simeq 1$. The linear plot should allow the evaluation of $\tau_c$ with the slope/intercept method.[119] Actually, this linearity was verified only in few cases[113,119,120,123,126,134,135] while it was commonly found that the $T_{1p}$ vs. $\omega_I^2$ plot exhibits a minimum; that is, $T_{1p}$ initially decreases with increasing $\omega_I^2$. The existence of this minimum was checked in various Mn(II)–macromolecular systems (ref 52, 102, 103, 105, 115, 118, 121, 123–136, 128, 136–139), and it was ascribed to a frequency dependence of the correlation time, $\tau_c$, itself. Hence, it was deduced that the dominant term in $\tau_c$ was $\tau_S$, which is the only frequency-dependent rate process contributing to the IS correlation times, at low frequencies, while the slow rotational tumbling motion of the macromolecular complex leaves the exchange time as the dominant correlation time at high frequencies.[113,118,124] This situation does not allow an unambiguous quantitative treatment of the data due to the ambiguities inherent in the frequency dependence of $\tau_S$. The evaluation of $\tau_c$ was performed either by graphical extrapolation of the linear portion of the frequency-dependent $T_{1p}^{-1}$ data[102,103,123] or by a computer-fitting procedure (ref 105, 115, 121, 124, 125, 127–129, 137, 142) assuming the validity of BM equations for the frequency dependence of $\tau_S$. The calculated $\tau_c$ values were in the range $10^{-8}$–$10^{-9}$ s, which is not consistent with the complex ESR patterns shown in Mn(II)–macromolecular systems. The BM equations do not account for the frequency dependence of the ESR spectra, which is due to inhomogeneous broadening produced by higher terms in the spin Hamiltonian in the quasi-glassy representation.

Once $\tau_c$ has been evaluated, the assumption of a Mn(II)-H distance allows the evaluation of $q$, the metal hydration number.

$q$ is very sensitive to the value of $r$ (e.g., the uncertainty of 0.1 Å for $r$ introduces a factor of 0.83 in $q$.[1,89,138] Usually $r = 2.87$ Å is assumed from crystallographic values on $Mn(H_2O)_6^{2+}$ and $q$ is consequently evaluated. The error in $r$ makes the uncertainty in $q$ quite large. The inverse procedure was sometimes used; that is, $q$ was assumed to get $r$ values. This was used to demonstrate the exclusion of water molecules from the metal hydration sphere.[113,122,135]

From best-fitting the frequency-dependent $T_{1p}^{-1}$ and $T_{2p}^{-1}$ data, Navon[137] deduced a procedure to calculate the hydration number ($r$ is to be assumed) using $T_{1p}$ and $T_{2p}$ at only one frequency:

$$q = \frac{3C}{r^6} \frac{(T_{1p}/T_{2p}) - 0.5}{[(T_{1p}/T_{2p}) - 1.19]^{1/2}} \frac{\omega_I}{NT_{1p}} \qquad (13)$$

where $N$ is the molar concentration of bound $Mn^{2+}$ ions. This method has been widely used (e.g., ref 114, 127, 143) and its applicability relies on the following conditions: (i) fast exchange conditions hold; (ii) $\omega_I$ is chosen so that $\omega_I^2\tau_c^2 << 1$,[137,133] and (iii) the scalar contribution to $T_{2p}^{-1}$ is negligibly small.

$\tau_c$ can be evaluated also by the ratio of $T_{1p}$ values at any two frequencies, provided fast exchange conditions prevail[122,131] or by $T_{1p}/T_{2p}$ ratios[131] if contact contributions to $T_{2p}^{-1}$ can be neglected. In those cases, a range of $\tau_c$ values is usually given since only upper and lower limiting values can be obtained by assuming no frequency dependence or maximal frequency dependence, respectively.

Koenig et al.[52,136] observed three distinct dispersive regions in $T_{1p}^{-1}$ data of water in Mn(II)–protein systems when the covered frequency range was extended to

frequencies as low as 10 kHz. The low frequency dispersion ($\omega_I \sim 7 \times 10^6$ rad/s) was attributed to the $\omega_S\tau_S$ term. The normal SBM theory was shown to fail in fitting all the data, and the interpretation was that the SBM theory underestimates the magnitude of the dipolar interaction. However the data were fitted by a theory modified to allow for the case where the zero-field splitting of the $Mn^{2+}$ electronic levels becomes of the order of magnitude of the Zeeman splitting. It was found that an unusually low $r$ value (~2.2 Å) resulted from fitting to both theories, while the usual $r$ value (~2.7 Å) was obtained when data for frequencies lower than 10 MHz were excluded. It was suggested that the water molecule is bound to the metal ion asymmetrically so that one of the two protons is nearer than the other.

The pH dependence[127,143-145] and the time dependence[144,146-148] of the water paramagnetic relaxation rates added information on exchangeable protons and on the kinetics of conformational transitions of some macromolecules.

Although the qualitative conclusions obtained from water relaxation studies do not seem to be open to doubt, the following limitations must be carefully considered if quantitative parameters are sought: (1) the distribution of the unpaired electrons over the ligand orbitals brings about an increase of the dipolar interaction relative to that calculated by the SB approach; (2) the ZFS of the Mn(II) electronic levels has the same effect as in (1); (3) internal rotational motions such as those of a part of the macromolecule, of the metal ion, or of the water molecules may reduce the electron proton dipolar interaction; and (4) the interpretation of the electron spin relaxation time as well as of its temperature and frequency dependence is difficult in macromolecular systems. While efforts have been made to include the first three points in the usual approach, it seems hard to overcome the ambiguities arising from the last point unless many pieces of information can be collected from different techniques.

### 3. Nuclear Relaxation of Ligand Nuclei

The theoretical approach and hence its limitations are the same as those outlined in the preceding section for nuclear relaxation of water protons. However, advantage can be taken from the possibility of performing NMR relaxation studies with many different nuclei, such as $^{13}C$, $^{31}P$, etc. Since structural information can be gained only if the nuclear relaxation rates are not exchange limited, nuclei which are undergoing fast exchange between bulk and bound environments can always be chosen. In fact, the exchange limitation reflects the magnitude of $T_{1M}$ and $T_{2M}$ compared to $\tau_M$. In varying nuclei on the same ligand, $T_{1M}$ and $T_{2M}$ will change, since the factors $\gamma_I^2/r^6$ in the SB equations change.

The paramagnetic relaxation rates depend on five parameters: the lifetime of the complex, $\tau_M$, the coordination number, $q$, of the ligand in the complex, the correlation time for the dipolar interaction, $\tau_c$, the distance, $r$, from the electron spin to the nucleus, and the magnitude of the contact contribution. The last one can be neglected for the longitudinal relaxation rate, $T_{1p}^{-1}$, whenever the measured $T_{1p}/T_{2p}$ is much greater than one. The paramagnetic transverse relaxation rate, $T_{2p}^{-1}$, may be used to set a lower limit to the rate constant for dissociation of the ligand from the complex, $\tau_M^{-1}$.

The temperature dependence of $T_{2p}^{-1}$ may, sometimes, allow the evaluation of $\tau_M^{-1}$ and provide information on the kinetics of the ligand exchange process. When $T_{1p}$ is much greater than $T_{2p}$, the contribution of $\tau_M$ to $T_{1p}^{-1}$ may be neglected. The correlation time, $\tau_c$, has been measured by one of the following methods:[89] (i) Frequency dependence of $T_{1p}$ or ratio of $T_{1p}^{-1}$ values at any two frequencies is used. This approach requires that $T_{1p}^{-1}$ must not be exchange limited, which can be checked either from the $T_{1p}/T_{2p}$ ratio or by carrying out a study of the temperature dependence of $T_{2p}^{-1}$ in order to determine $\tau_M^{-1}$ or its lower limit. As in the case of water protons, however, the ambiguities arising from the contributions from $\tau_S$ are not easily overcome. (ii) The $\tau_c$ value determined for water molecules bound to Mn(II) in the same complex may be assumed. In this way further approximations come out since the contribution from $\tau_R$ to $\tau_c$ may be quite different for the water molecules and for the ligand. (iii) In some cases $\tau_c$ was evaluated by the ESR line width of Mn(II) in the same complex. This method is very approximative and can give only a rough estimate of $\tau_c$. In fact contributions from $\tau_R$ and $\tau_M$ are not considered. Furthermore, the ESR line shape in macromolecular systems is characterized by many inhomogeneous features that do not allow a straightforward interpretation. (iv) $T_{1p}/T_{2p}$ ratio is determined. In this case an estimate of $\tau_c$ is obtained provided that $T_{2p}$ is not exchange limited and does not contain a valuable contact contribution.

Once $\tau_c$ has been evaluated the $q/r^6$ parameter can be derived, and if the stoichiometry and dissociation constants of the ligand are known independently, $r$ can be determined.

The following points are to be stressed: (1) the $T_{ip}^{-1}$ ($i$ = 1, 2) value is evaluated by measuring the paramagnetic relaxation rates and the relaxation rates in a diamagnetic control in which Mg(II) or Zn(II) replace Mn(II). (2) The $T_{ip}^{-1}$ ($i$ = 1, 2) must be corrected for the concentration of the species considered, since the ligand may be found in different coordination environments. (3) The $T_{ip}^{-1}$ ($i$ = 1, 2) of any ligand nucleus may be sensitive to the addition of other competitive ligands, or metal ions, allowing the detection of the kinetics of the ligand displacement process or the relative stability of metal complexes.

### 4. ESR Properties of the Mn(II) Ion

The direct use of ESR spectroscopy in structural studies of macromolecular complexes of Mn(II) has been initially prevented by the observation that the ESR signal was virtually disappearing in solution. For this reason the ESR measurements have been used for distinguishing between bound and free Mn(II) in measuring binding constants.[24,149] However, it was soon recognized that solid-state character of the line shape rather than rapid homogeneous relaxation of the electron spin states of the bound ion accounts for the loss of amplitude of the Mn(II) signal accompanying macromolecule binding.[150] Up to now, a great number of more or less well-resolved ESR signals arising from Mn(II)–macromolecule complexes have been reported.

In most of the cases only signal around $g = 2$ were observed,[27-31,113,115,123,126,150-157] although in some cases signals at lower fields also were observed.[115,129,151,158-165] The spectra were usually interpreted in terms of an ESR powder pattern of $Mn^{2+}$ with a spin Hamiltonian given in eq 1. An axially symmetric ZFS tensor was usually found to be consistent with the spectra, and values for $D$ ranging from 50 to 100 G were given.[28,30,31,115,150-154,166] In other cases, however, a predominantly rhombic symmetry was suggested.[27,126,155,163]

The ESR techniques are very appropriate in examining the effects of ligand binding to the Mn(II) ion, since the magnetic anisotropies arising from asymmetry in the electronic environment of the ion are apparent in the spectra. Thus, changes in the position and shape of ESR lines of bound Mn(II) reflect changes in the ligand composition and geometry of the complex. By taking advantage of the paramagnetic properties of the Mn(II), used as the metal ion cofactor, the effects of substrate and/or inhibitor binding on the environment of enzyme-bound metal ion have been widely investigated (ref 113, 115, 123, 126, 139, 151, 153, 155–158, 160–162, 164, 165, 167). Two main questions involved in enzymatic catalysis may be approached in this way: (i) determination of the specific structural features of the enzyme–substrate complex that is relevant to the catalytic function may be attempted with substrate analogues of varying activities and (ii) the structural constraints imposed on the active complex in the transition state may be clarified by studying special complexes mimicking the transition state. By working with high concentration of enzyme, changes in the low-intensity spectrum of the enzyme–$Mn^{2+}$ system were monitored upon progressive addition of various ligands. Qualitative information was gained concerning ligand substitutions, rearrangements, and structural or conformational changes in the vicinity of the paramagnetic probe.

The addition of both diamagnetic and paramagnetic metal ions to Mn(II)–macromolecule systems provides information either on the relative binding strength of the metals or on conformational changes when the macromolecule has different metal binding sites. The change in ESR line shape and anisotropic parameters[153,154] or the variation of the ESR signal intensity (e.g., ref 27, 153, 164, 168–171) were alternatively used. The ESR intensity was used also to determine the binding parameters of Mn(II) both to proteins[129,156,162,164,170,171] and nucleic acids.[168,169] It is worth noting that an extensive line broadening was commonly observed (see, for example, ref 113, 115, 126, 139, 155, 156, 161), which, however, was not adequately accounted for. It was noted that, at X band, the intensity is shared between normal and forbidden transitions. For this reason, the ESR spectra were usually run also at Q band, where a better resolution was obtained (ref 27–29, 31, 115, 126, 129, 150, 151, 154, 155, 160, 163). It seems unlikely, however, that the line narrowing of the ESR lines, observed at Q band, can be explained by the field dependence of the electron spin relaxation time, as is sometimes suggested, since the ESR patterns resemble those obtained for powder samples. The better resolution of the forbidden transitions at Q band has been used to get structural information about the geometry of the binding site.

An unambiguous indication of direct bonding and the identification of binding sites could be obtained through the investigation of superhyperfine coupling between the unpaired electron spins and the $^{17}O$ nuclei of the ligand. In fact in cases where the line width of the electron spin transitions were relatively narrow, the superhyperfine interaction with the ligand $^{17}O$ nuclei affects the ESR signal with a consequent inhomogeneous broadening. Due to the relative size of scalar and dipolar contributions to the superhyperfine coupling, an $^{17}O$ nucleus can exert an appreciable line broadening only if in the first coordination sphere of the Mn(II). By using selectively labeled water or ligand molecules the six coordinating groups to Mn(II) were identified in a transition-state analogue of creatine kinase[172] and in the complex with elongation factor Tu and guanosine diphosphate.[173]

### 5. ESR and NMR in the Presence of Two Different Paramagnetic Probes

So that the Mn(II) binding site can be mapped out, information can also be obtained from the ESR and NMR parameters when a second paramagnetic probe, either a spin label or a metal ion, is bound or near to the macromolecule. For two relatively rigid paramagnetic centers which reorientate slowly compared with the electron spin relaxation time of the added paramagnetic probe, the line width of the ESR signal of the observed probe is given by the Leigh's theory of the dipolar interaction[174]

$$\Delta H = \frac{g\beta\mu^2\tau_{s1}}{\hbar r_0^6}(1 - 3\cos^2\theta_R')^2 + \Delta H_o \qquad (14)$$

where $\mu$ and $\tau_{s1}$ are the magnetic moment and the longitudinal electron spin relaxation of the added probe, $\Delta H_o$ is the residual line width of the observed probe in the absence of the other, $\theta_R'$ is the angle between the magnetic field direction and the vector joining the two dipoles, and $r_0$ is the probe to probe distance.

In practice, the dipolar effect results in a quenching of the observed signal amplitude due to the angular dependence of eq 14. For most $\theta_R'$ values the dipolar contribution is large and the resulting lines are undetectably broad: at $\theta_R' \simeq 54°$ the line width is unperturbed and represents the observed "diminished" signal. Advantage has been taken of this effect either by adding Mn(II) ions to macromolecules covalently spin-labeled with stable free radicals[162,175-178] or by adding paramagnetic metal probes (mostly Cr(III) but also Co(II) to macromolecule-bound Mn(II).[155,179-183] To calculate $r$, it is necessary to know $\Delta H$, $\Delta H_o$ (which are obtained from the relative amplitudes of the ESR signals in the presence and in the absence of the added probe), and $\tau_{s1}$. When Mn(II) was added to spin-labeled macromolecules, $\tau_{s1}$ was given as a "lower limit" from the PRE titration data. From the effect of adding Cr(III) or Co(II) to bound Mn(II), a dual divalent cation requirement was assayed for some enzymes.[155,179,180,182,183] The probe to probe distance was shown to depend on the presence of substrates and inhibitors,[182] allowing a direct proof of induced conformational changes.

A method was suggested by Gupta[181] to determine the distance between two paramagnetic metal ions on a macromolecule from water proton relaxation measure-

ments. The technique is based on the existence of "cross-relaxation" of electronic magnetizations of the two paramagnetic metal ions. When one of the two ions (e.g., $Cr^{3+}$) has a relaxation time ($\tau_s^{Cr}$) much shorter than that of the other ion (e.g., $Mn^{2+}$) ($\tau_s^{Mn}$) and the rotational time of the entire molecule is long compared to $\tau_s^{Cr}$, then the cross-relaxation phenomenon affects the effective $\tau_s^{Mn}$ and hence the longitudinal relaxation rate of water protons bound to Mn(II). To deduce $r_0$ it is then necessary to obtain the difference between $\tau_s^{Mn}$ in the presence of the other ion and $\tau_s^{Mn}$ in a system identical with the first except that the fast relaxing spin is replaced by a similar diamagnetic metal.

The frequent incorrect use of magnetic dipole–dipole formulas for ESR distance estimation was emphasized in a general analysis of the various spin–spin interactions[184] when both exchange and dipole–dipole operators are present and comparable in magnitude. It was shown that in the presence of an isotropic exchange interaction, which was actually observed in the room-temperature solution ESR spectra of copper complexes with ligands containing nitroxyl radicals,[185] a single determination of the distance is not unique and "very likely incorrect".[184]

### 6. Some Applications

**1. Carbonic Anhydrase.** Carbonic anhydrase (CA) catalyzes the reversible hydration of $CO_2$ as well as that of aldehydes and the hydrolysis of esters. It has a molecular weight of 30 000 and contains one zinc atom per protein molecule; this is essential for activity and may be replaced by various divalent cations. A basic group, whose chemical nature is not yet identified, closely linked to the metal ion and having a $pK_a$ of ~7, is critically involved in catalysis.

Since sulfonamides are potent inhibitors of the enzyme, the effect of Mn(II)–CA on the $T_1^{-1}$ and $T_2^{-1}$ values of sulfacetamide protons was studied.[186] From these the Mn–H distances were evaluated, which suggested a model in which the sulfonamide nitrogen is directly bound to the metal ion. The effect of Mn(II)–CA on the $T_{1p}^{-1}$ of water protons was shown to depend on pH, decreasing noticeably below pH 7.[143] Also the sulfonamide inhibitor acetazolamide affected the proton relaxation rate of water, while azide and nitrate ions did not. From the $T_{1p}/T_{2p}$ ratio it was calculated[143] that 0.98 ± 0.05 water molecules are titratable in the pH range 9–6.7, suggesting that the basic form of the group which catalyzes the hydration reaction is not a metal-bound hydroxide ion.[187] It seems more likely that an ionization on the protein moiety is responsible for the activation of the enzyme. The single proton NMR line of the acetate ion was shown to be appreciably broadened in a solution of Mn(II)–CA.[188] By titration of the line width of the acetate NMR signal with azide and sulfonamide ligands two anionic acetate binding sites were distinguished, only one of which gave rise to inhibition of the enzymatic activity. With the assumption of the value of $5 \times 10^{-9}$ s for $\tau_c$[186] the Mn(II)–H distances was calculated[188] ($r$ = 4.3 Å), suggesting a direct bond between the acetate anion and the metal ion. The Mn(II)–enzyme was further characterized by using the temperature (2–35 °C) and frequency (5–100 MHz) dependencies of the $T_{1p}^{-1}$ and $T_{2p}^{-1}$ of water.[127] The pH dependence of $T_{1p}^{-1}$ was fitted to a sigmoidal curve with $pK$ = 7.8. The hydration number was derived by using either the frequency dependence of $T_{1p}$ or the $T_{1p}/T_{2p}$ ratio; $q$ = 0.96 was calculated at high pH, whereas at low pH the relaxation data were consistent with no $H_2O$ molecules in the first coordination sphere. $^{13}C$ $T_1$ values for $^{13}C$-enriched acetate ion were determined in the presence of Mn(II)–CA.[189] The drastic reduction of $T_1$ upon addition of apocarbonic anhydrase gave evidence of acetate binding to the polypeptide chain of the protein, and the further reduction in the presence of Mn(II)-CA was interpreted in terms of two acetate binding sites, one in close proximity to the metal ion. By use of the $\tau_M$ and $\tau_c$ values reported in ref 188 the Mn–$^{13}C$ distances were calculated in agreement with those from proton data.[188] The ESR spectra of the Mn(II)–enzyme were reported;[158] they suggested relatively large rhombic distortion from spherical symmetry. The analysis of the spectrum[27] was found to be consistent with typical powder samples in which the main anisotropic term is a quadratic ZFS interaction. From the frequency dependence of the spectrum, an approximately rhombic symmetry ($E/D \simeq 1/3$) was inferred. This was characterized by a considerable extent of distribution in the ZFS parameters, which might reflect a distribution in the distance between the Mn(II) ion and its ligands in the binding sites. Further information on the coordination geometry was deduced from titration of the $T_1^{-1}$ of the water protons with mono- and bidentate inhibitors,[145] suggesting the possibility of a coordination number of five or six. The data on Mn(II)–CA have been recently summarized.[190]

**2. Malic Enzyme.** Malic enzyme catalyzes the oxidative decarboxylation of L-malate to pyruvate, thereby generating the NADPH needed for fatty acid synthesis

$$\text{L-malate} + \text{NADP}^+ \xrightarrow[M^{2+}]{\text{malic enzyme}} \text{pyruvate} + CO_2 + \text{NADPH} \quad (15)$$

The enzyme has four equivalent subunits ($M_r$ 260 000) and a divalent metal ion requirement for activity, which is satisfied by $Mg^{2+}$ or $Mn^{2+}$.

The ESR titration of the ME complex[141] showed 2 tight and 3 ± 1 weak Mn(II) binding sites, which suggests half-site stoichiometry and half-site reactivity for Mn(II), since four NADPH molecules were found to bind per tetramer with equal affinity.[191] A possible strong negative cooperativity between the four subunits of equal size was suggested.

The $T_1^{-1}$ data on solvent water[141] suggested that while the carboxylic acid substrates interact with the enzyme-bound Mn(II) to decrease the number of fast exchanging water ligands, the pyridine nucleotide substrates do not, although the affinity of the ME complex for certain substrates, above all L-malate, is profoundly affected. As shown in Table VI, the addition of Mn(II) to a solution of enzyme and saturating levels of 1-$^{13}C$- and 2-$^{13}C$-enriched pyruvate increased the relaxation rates (experiments 1–4). $T_{1p}^{-1}$ values were approximately halved by the addition of a saturating level of $NADP^+$ (experiment 5) and were abolished by the addition of an excess of the inhibitor oxalate (experiment 6). The Mn(II)–$^{13}C$ distances were calculated by using $\tau_c = 2.64 \times 10^{-9}$ s, determined for the Mn(II)–$H_2O$ interaction in the same complex from the frequency dependence of $T_{1p}^{-1}$ of water protons. This assumption

TABLE VI. Paramagnetic Effects of Malic Enzyme-$Mn^{2+}$ on Relaxation Rates of Carboxyl and Carbonyl $^{13}C$ Atoms of Pyruvate[a,b]

| expt | [1-$^{13}$C]-pyruvate + [2-$^{13}$C]-pyruvate, mM | enzyme tetramer,[b] $\mu$M | [$Mn^{2+}$], $\mu$M | $^{13}$COO $1/T_1$, $s^{-1}$ | $^{13}$COO $1/fT_{1p}$, $s^{-1}$ | $^{13}$COO $1/fT_{2p}$, $s^{-1}$ | $^{13}$C=O $1/T_1$, $s^{-1}$ | $^{13}$C=O $1/fT_{1p}$, $s^{-1}$ | $^{13}$C=O $1/fT_{2p}$, $s^{-1}$ |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 105.1 | 24.3 | | 0.040 | | | 0.045 | | |
| 2 | 104.4 | 24.2 | 0.635 | 0.145 | 17.260 | 215.000 | 0.125 | 13.150 | 192.000 |
| 3 | 103.8 | 24.0 | 1.261 | 0.171 | 10.780 | 211.000 | 0.149 | 8.560 | 284.000 |
| 4 | 102.5 | 23.7 | 2.491 | 0.290 | 10.290 | 135.000 | 0.282 | 9.750 | 216.000 |
| 5 | 101.5[c] | 23.5 | 2.468 | 0.160 | 4.940 | 128.000 | 0.145 | 4.110 | 162.000 |
| 6 | 99.7[d] | 23.1 | 2.423 | 0.023 | 0 | | 0.033 | 0 | |

[a] The effects of $Mn^{2+}$ on the relaxation rates of pyruvate are assumed to result entirely from paramagnetic interactions, in accord with theoretical considerations[30,141] and with data previously obtained on other pyruvate utilizing enzymes.[17,18,141] Solutions also contained 46.3 mM triethanolamine-Cl, pH 6.9 (measured), 23.1 mM KCl, 0.8 mM dithiothreitol, and 38.3% $D_2O$ for field frequency locking. $T$ = 22.2 °C. [b] Preparation I was used for these studies (from ref 141). [c] Solution also contained 278 $\mu$M TPN. [d] Solution also contained 4.5 mM potassium oxalate and 273 $\mu$M TPN.

was justified by the inference that $\tau_s$ is the dominant correlation time for the dipolar interaction. The calculated distances of 4.6 and 4.8 Å for the carboxyl and carbonyl carbon atoms, respectively, were appropriate for a second-sphere enzyme–Mn(II)($H_2O$)–pyruvate complex. The formation of the abortive quaternary complex with NADP, which approximates the catalytically active complex, further increased both distances by 0.8 Å assuming an equal $\tau_c$ value. It was estimated that at least 77% of the pyruvate is in the second sphere ($r$ = 6.3 Å) and at most 23% is directly coordinated to Mn(II) ($r$ = 3.5 Å) in the ternary complex, while at least 93% is second-sphere interacting in the quaternary species. The decrease in the number of fast exchanging water ligands suggested that the intervening ligand was a water molecule (Figure 6). The role of the metal-bound water in polarizing the C-2 carbonyl group of pyruvate was supported by the observation that D-malate does not alter the number of hydrated waters, suggesting that the substituent at C-2 of the substrate occludes a water ligand of the enzyme-bound Mn(II).

**3. Carboxypeptidase.** Carboxypeptidase A catalyzes the hydrolysis of the carboxyl terminal peptide in polypeptide chains. The enzyme has a molecular weight of 34 000 and has a tightly bound zinc ion essential for activity, which can be substituted by Mn(II) retaining some enzymatic activity.

Different studies of the effect of Mn(II)-substituted enzyme on $T_1^{-1}$ and $T_2^{-1}$ of solvent water protons showed that there is at least one water ligand on the Mn(II)[192] and that the inhibitor $\beta$-phenylpropionate markedly reduces the water interaction[194] and affects the $F^-$ interaction. Measurements of $T_1^{-1}$ of water in the Mn(II)–enzyme, in the frequency range 0.01–100 MHz[136] were interpreted by assuming that only one water molecule is bound to Mn(II), with the same Mn–O bond length as in the hexaaquo ion. It was shown that the water orientation must be such that at least one proton is much closer to the Mn(II) ion than protons in the first hydration shell of the free aquo ion.

In another study,[137] the frequency dependence of the $T_1^{-1}$ and $T_2^{-1}$ of water in a more limited frequency range (10–100 MHz) was also used to evaluate the number of exchangeable water molecules, but with the different conclusion that the one exchangeable water molecule is positioned from the metal at the same distance as in the aquo ion.

However it was pointed out[14] that internal rotation

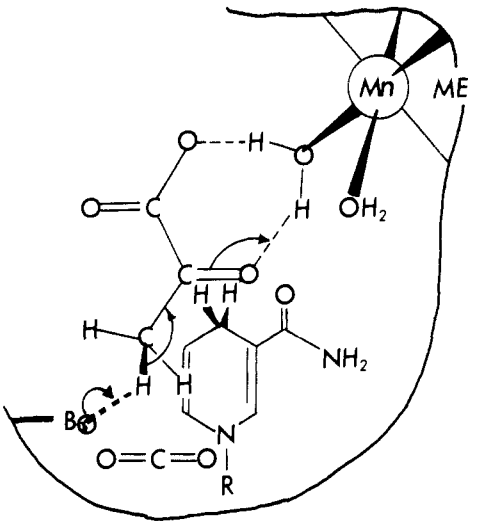



**Figure 6.** Role of Mn(II) in the enolization of pyruvate catalyzed by malic enzyme. (Reprinted with permission from R. Hsu, A. S. Mildvan, G. C. Chang and C. H. Fung, *J. Biol. Chem.*, **251**, 6574 (1976). (Copyright 1976, American Society of Biological Chemists, Inc.)

or other complicating features could give rise to errors in estimates of the number of hydrating water molecules. A revision of the usual form of the Solomon–Bloembergen–Morgan theory, taking into account the zero-field splitting of the Mn(II) electronic levels, was suggested[52] to be necessary to interpret the data in the greater frequency range of 0.02–100 MHz. It was shown[52] that the two theories yield the same fitting parameters when the comparison is restricted to frequencies above 10 MHz, while at low frequencies a total disagreement is observed. Therefore the conclusions reported in ref 137 were questioned. The argument has been further discussed in a recent paper[54] where it was shown that the application to the $T_1^{-1}$ and $T_2^{-1}$ dispersion of water in Mn(II)–carboxypeptidase A of more generalized equations, describing the spin–lattice relaxation of nuclear spin moments caused by magnetic dipolar interactions with neighboring paramagnetic ions, reinforces the suggestion that the apparent strength of the dipolar interaction is a factor of 5 larger than expected. In other words, the number of exchangeable water ligands and the Mn–proton distance are not independent parameters: if one water molecule is assumed, an $r \simeq 2$ Å is obtained, which is smaller than $r$ = 2.8 Å of the hexaaquo ion; if $r$ = 2.8 Å is

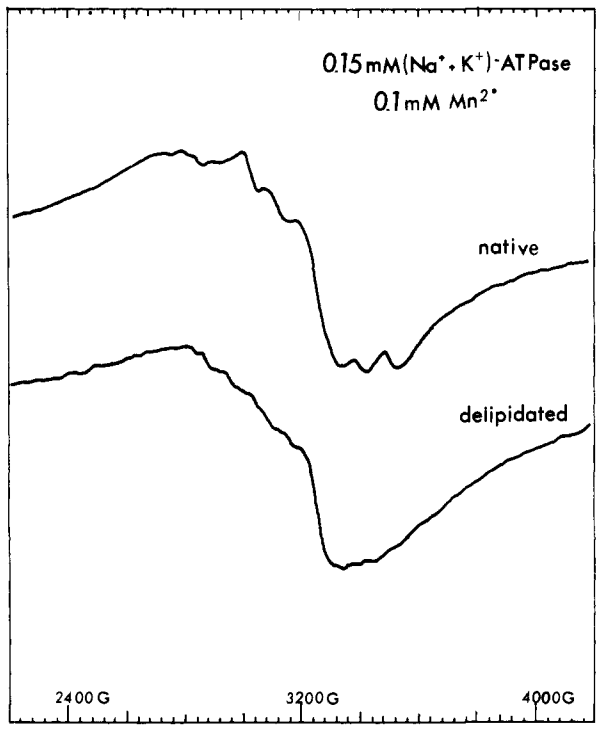



**Figure 7.** X band ESR spectra of Mn(II) bound to a single site on $(Na^+ + K^+)$-ATPase. (Reprinted with permission from S. E. O'Connor and C. M. Grisham, *Biochemistry*, *18*, 2315 (1979)).

assumed, five water molecules are observed, which is in contrast with the hinted tetrahedral symmetry.[158] Failure of the assumption of a point-dipole model for the magnetic moment of the Mn(II) ion, which is the basis of the SBM theory, was believed,[54] probably because of the delocalization of the magnetic moment.

$T_1^{-1}$ and $T_2^{-1}$ of solvent water were also measured in solution of Mn(II)–substituted carboxypeptidase B[114] in the presence of various competitive inhibitors. L-Argininic acid and acetyl-L-arginine were found to bind specifically to the active site of the enzyme, displacing the single water molecule at the Mn(II) ion.

**4. $(Na^+ + K^+)$-ATPase.** The sodium and potassium ion activated adenosine triphosphatase, $(Na^+ + K^+)$-ATPase, is a plasma membrane bound enzyme responsible for $Na^+$ and $K^+$ transport in mammals. Besides the monovalent cation, the enzyme requires $Mg^{2+}$ or $Mn^{2+}$ for the catalytic activity. The enzyme can be phosphorylated by either ATP or inorganic phosphate, and a divalent cation is also required for these phosphorylations. The $Mn^{2+}$ binding site on the enzyme has been characterized in relation to both the divalent cation activation and the transport system,[103,195,196] indicating that protonation of an enzyme-bound phosphoryl group converts a $K^+$ binding site to a $Na^+$ binding site, that different types of $Mn^{2+}$ binding sites exist, and that removal of the essential phospholipids in the enzyme preparations abolishes the weaker binding sites. NMR studies using $^{205}Tl^+$, $^{31}P$, and $^7Li^+$ nuclei have also shown that the $Mn^{2+}$ site is close to (a) a $Na^+$ site ($r \simeq 4$ Å) involved in enzyme activation and ion transport,[196] (b) a $K^+$ site ($r \simeq 7$ Å) that is probably involved in ATP hydrolysis and/or transport,[197] and (c) a noncovalently binding phosphate site.[195] The investigation of the interactions of $Mn^{2+}$, inorganic phosphate, and nucleotide substrate and substrate analogues with ATPase by means of ESR (165) is worth examining. The X band ESR spectrum of the Mn–enzyme complex, either native or delipidated, displays a powder or quasi-solid-state lineshape (Figure 7). ATP, ADP, and high concentrations of $PO_4^{3-}$ produce broadening of the lines, while AMP causes a substantial narrowing. It was suggested that ATP, and not MnATP, is the true substrate. The changes in line shape caused by AMP on the native and delipidated enzyme were taken as a proof of strong alterations of the ability of the enzyme to interact with nucleotides upon removal of lipids: the divalent metal and substrate sites appear to be preserved, but their conformation is substantially altered. Figure 8 collects all the structural information currently available on the active site of the enzyme, including the results obtained from the paramagnetic Cr–ATP complex on $Li^+$ at the $K^+$ site by $^7Li$ NMR.[165]

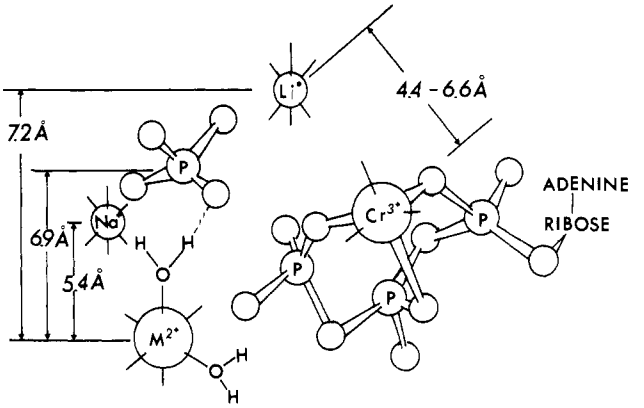



**Figure 8.** Active-site structure of $(Na^+ + K^+)$-ATPase as determined by $^1H$, $^{205}Tl^+$, $^{31}P$, and $^7Li^+$ NMR, Mn ESR, and kinetic studies. (Reprinted with permission from S. E. O'Connor and C. M. Grisham, *Biochemistry*, 18, 2315 (1979)).

**5. Yeast Aldolase.** This yeast enzyme is the prototype of class II aldolases, which catalyze the reversible aldol cleavage of fructose bisphosphate to give dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. In contrast to class I aldolases, this class II enzyme is constituted by two subunits and contains one essential zinc ion per subunit. The metal ion can be removed to produce an inactive apoenzyme retaining the dimeric structure which tightly binds other divalent cations, such as Mn(II) and Co(II), to produce an active enzyme.

The intensity of the ESR spectrum, as well as the nuclear relaxation rate measurements of water protons, was used[108] to demonstrate competition between the Zn(II) and Mn(II) for the same site. It was also shown[108] that the enzyme-bound Mn(II) affected the nuclear relaxation rates of protons on substrates and competitive inhibitors. Metal–proton distances in binary and ternary complexes of Mn(II) were calculated by evaluating upper and lower limits for the expected correlation time. The frequency dependence of $^1H$, $^{31}P$, and $^{13}C$ nuclear relaxation times in ternary complexes, the metal–ligand distances, and the conformation of the enzyme-bound substrate were recently calculated from the frequency dependence of $^1H$, $^{31}P$, and $^{13}C$ nuclear relaxation rates in the ternary complex of the Mn(II)–enzyme with the substrate acetol phosphate; the correlation times for the metal–substrate protons interaction (about $1 \times 10^{-9}$ s), the metal–phosphorus interaction (about $2.2 \times 10^{-9}$ s), and the metal–water protons interaction (about $2.3 \times 10^{-9}$ s) were all sig-

nificantly lower than the protein tumbling time, showing that $\tau_s$ modulates the dipolar interaction. The configuration of the active site could be sketched and the number of fast-exchanging water ligands on Mn(II) calculated ($q$ = 1). From the temperature and frequency dependencies of $^{31}P$ transverse relaxation rates the exchange parameters for acetol phosphate dissociating from the ternary complex were calculated ($k$ = 1.1 × $10^5$ $s^{-1}$, $E_a$ = 9.5 kcal/mol), indicating that such a ternary complex is kinetically competent to function in catalysis. The mechanistic role of the bound metal was suggested in which an enolate intermediate is stabilized through an intervening imidazole ligand, which transmits the electrophilic effect of the metal.

**6. Glutamine Synthetase.** Glutamine synthetase (GS) catalyzes the synthesis of glutamine from glutamate and $NH_4^+$ with the consumption of a high-energy phosphate bond:

$$\text{glutamate} + NH_4^+ + \text{ATP} \rightarrow \text{glutamine} + \text{ADP} + \text{P} + H^+ \quad (16)$$

The unmodified enzyme, unadenylylated GS, has a molecular weight of 600 000 and consists of 12 identical subunits. The modification of each of these subunits gives the fully adenylylated enzyme. This last one requires Mn(II) for the biosynthesis of glutamine, while the transfer of the glutamyl group is catalyzed in the presence of Mn(II) for both forms of the enzyme.

From the frequency and temperature dependencies of the $T_{1p}^{-1}$ and $T_{2p}^{-1}$ of water in the binary fully adenylylated enzyme–Mn(II) complex[125] three exchangeable water molecules ($\tau_M$ = 1.3 × $10^{-7}$ s) were detected by using $\tau_c = \tau_s \simeq 2\text{–}10 \times 10^{-9}$ s. The presence of L-glutamate reduced the number of water ligands to two, suggesting a coordination to the enzyme-bound Mn(II). With Mn(II) bound at the active site of the unadenylylated enzyme, the same analysis[128] gave two rapidly exchanging $H_2O$ ligands in the binary ME complex, which were reduced to one in the presence of glutamine. A study of Mn(II) binding revealed[128] a first set of 12 "tight" binding sites ($K_D$ = 0.5 M) and 12 "weak" binding sites ($K_D$ = 45 M). In enzyme samples with Mn(II) bound at both sites the data were interpreted by means of two rapidly exchanging water molecules at each bound Mn(II) ion. The substitution into the coordination sphere of glutamine or ADP reduced the solvent accessibility to the bound Mn(II). It was suggested that this was the case for Mn(II) at the weak metal ion site. The results were also consistent with the conclusion that the metal ion sites are 6 Å apart. Therefore, one of the metal ion sites is involved in binding the nucleotide substrate, while the other site was implicated as being near the glutamate binding site by ESR studies.[115,139] The high-affinity Mn(II) binding sites were examined as a function of Mn(II) concentration and the state of adenylylation by ESR,[199] suggesting that the ions remain magnetically isolated at different states of adenylylation and in the presence of the inhibitor L-methionine sulfoximine. The small axial distorsion was found consistent with nearly isotropic environment for Mn(II), which underwent drastic change in the presence of the inhibitor. For the intermediate-affinity binding sites it was found[200] that, in the absence of nucleotides, the Mn(II) environment is nearly isotropic, the Mn(II) bonds are highly ionic and the interaction distance is >12 Å. Nucleotide binding gave rise to disappearance of the ESR signal while affecting the amplitude and the line-broadening of the high-affinity binding site; this was taken as demonstrating a conformational change in the enzyme, making the two binding sites near each other. By analyzing the effect of the additions of Cr(III)–ATP in the ESR spectra of the ternary complex with the competitive inhibitor L-methioninesulfoximine, the metal–metal distance was calculated from the paramagnetic electron–electron relaxation theory.[174,182] This distance was ~7 and ~6 Å for the enzyme–Mn(II)–Cr(III)–ATP and the enzyme–Mn(II)–Cr(III)–ADP, respectively. The binding of substrates or inhibitors gave rise to slight changes in the distance, demonstrating conformation changes at the metal ion sites. The distinction of the two metal ion binding sites was demonstrated also by incorporating Co(III) at the first site and observing the ESR features when Mn(II) was at the second site,[183] ratifying the preceding conclusions.[174,182] By using $^{13}C$-enriched ATP it was also possible to follow the effects of Mn(II) on $^{13}C$ and also $^{31}P$ signals from the [2-$^{13}C$]AMP–enzyme NMR signal.[201] From the $T_{1p}^{-1}$ and $T_{2p}^{-1}$ values for two different metal stoichiometries the metal–carbon distances were evaluated by using a value of 1 × $10^{-8}$ s for $\tau_c$ ($r$ = 11.7 and 11.2 Å for the first and second metal sites, respectively). The addition of 0.5 and 1.0 equiv of Mn(II) per subunit also increased the $^{31}P$ NMR line width of fully adenylylated enzyme. The correlation time was evaluated at 1.4 × $10^{-8}$ s from the $T_{2p}/T_{1p}$ ratio (the metal–$^{31}P$ distances were 9.7 and 7.5 Å, respectively).

**7. Formyltetrahydrofolate Synthetase.** Formyltetrahydrofolate synthetase catalyzes the reaction

$$l\text{-tetrahydrofolate} + \text{MgATP} + \text{formate} \rightleftharpoons l\text{-}N\text{-10-formyltetrahydrofolate} + \text{MgADP} + P_i \quad (17)$$

Kinetic and water proton relaxation rate[96] studies were used to indicate that the metal–nucleotide rather than free nucleotide is the substrate for the enzyme. The dissociation constants of the ternary complex with ATP and ADP were obtained from $T_{1p}^{-1}$ and $T_{2p}^{-1}$ of water protons,[106] consistent with a stoichiometry of four sites per tetramer. The addition of the second substrate tetrahydrofolate resulted in noticeable changes in the $T_{1p}^{-1}$ and $T_{2p}^{-1}$ values, giving evidence for synergism of substrate binding. The ESR spectra gave further information about the conformational properties of the active site of the enzyme.[126] As shown in Figure 9, the addition of tetrahydrofolate (THF) to the ternary complex E–Mn–ADP results in substantially different ESR line shapes. The addition of formate to the ADP quaternary complex induced a further significant change, although, in the absence of THF, formate does not affect the ESR spectrum of the E–Mn–ADP species. From the frequency dependence of both ESR line shape and NMR water $T_{1p}^{-1}$ the effective correlation time was shown to be the electron spin relaxation time,[126] which was evaluated at 1.6 × $10^{-9}$ s. From this assumption it was inferred that for the ternary enzyme–Mn–nucleotide complexes, some exchangeable water ligands are in the first solvation sphere (2–3), while for the quaternary complexes with THF no water ligand can be observed. The conclusion was drawn that the active site becomes enclosed, being Mn(II) protected from

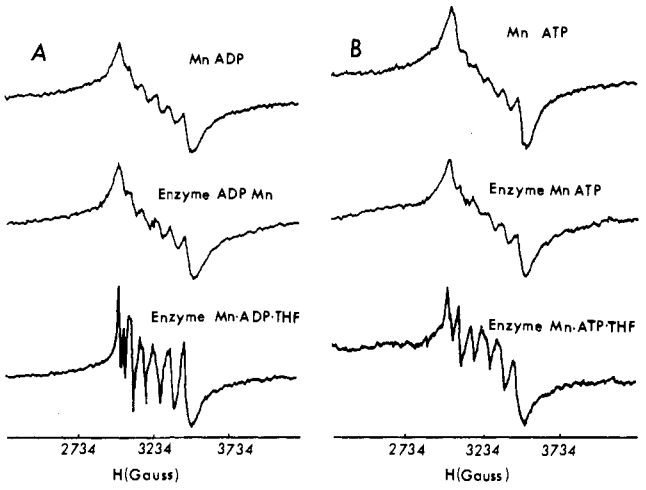



**Figure 9.** (A) X-band ESR spectra for various Mn-ADP complexes. All solutions contained KCl, 100 mM, $MnCl_2$, 0.4 mM, ADP, 2.5 mM, dithiothreitol, 1 mM, Tris-HCl, 25 mM; pH 8.0; $T$ = 1 K. Additional components were formyltetrahydrofolate, 900 M enzyme sites, *dl*-tetrahydrofolate, 3.7 mM, 2-mercaptoethanol, 92 mM. (B) X-band ESR spectra for various Mn-ATP complexes. All solutions contained KCl, 100 mM, $MnCl_2$, 400 M, ATP, 2.0 mM, dithiothreitol, 1 mM, Tris-HCl, 25 mM; pH 8.0; $T$ = 1 K. Additional components were formyltetrahydrofolate syntetase, 900 M enzyme sites, *dl*-tetrahydrofolate, 5.3 mM, 2-mercaptoethanol, 135 mM. (Reprinted with permission from D. H. Buttlaire, G. H. Reed, and R. H. Himes, *J. Biol. Chem.* **250**, 261 (1975). Copyright 1975, American Society of Biological Chemists, Inc.)

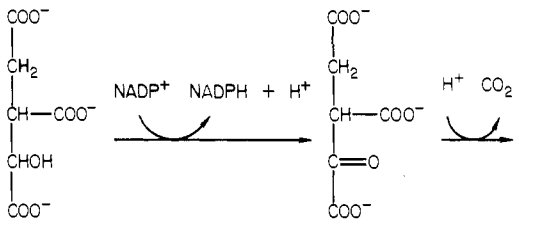
motional perturbations of the bulk solvent.

**8. Phosphoribosylpyrophosphate Synthetase.** Phosphoribosylpyrophosphate synthetase catalyzes the transfer of the $\beta,\gamma$-pyrophosphoryl group from ATP to ribose 5-phosphate, requiring both a nucleotide-bound metal as a substrate and an enzyme-bound metal as an essential activator. The strength of binding of Mn(II) to the enzyme was estimated from ESR intensity measurements, suggesting that the tight binding site on the enzyme is functionally active.[202]

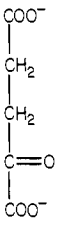
Since substitution-inert complexes of ATP such as the $\alpha,\beta,\gamma$-tridentate $Cr^{III}(H_2O)_3ATP$ and the $\beta,\gamma$-bidentate $Co^{III}(NH_3)_4ATP$ or its analogues act as slow substrates for the enzyme, determining distances from the paramagnetic Mn(II) probe to substrate atoms by NMR relaxation measurement could be considered. In fact, by measurements of the paramagnetic effects of Mn(II) on the $^{31}P$ nuclear relaxation rates of the bound substrates $Co^{II}(NH_3)_4AMPCPP$, the products AMP and adenosine 5′-O-(thiophosphate), and the activator inorganic phosphate, the conformation of the enzyme-bound nucleotide was determined.[203]

The dipolar correlation time was calculated by the $T_{1p}$ value at two frequencies and was used to calculate distances which were found in the range 4.5–6.0 Å for the various complexes, indicating second-sphere phosphoryl coordination. The preferred conformation was inferred with the help of a computer search program[89] showing a significant difference between the conformations of bound and free substrate. Upon binding of ribose 5′-phosphate a conformational change was detected in the polyphosphate chain. The exchange rates of the substitution-inert ATP complex and of AMP were given a lower limit from the $^{31}P$ $T_{2p}^{-1}$ values, assigning the complexes detected by NMR kinetic competence to function in catalysis. The role of the essential activator inorganic phosphate was elucidated.

**9. Isocitrate Dehydrogenase.** Isocitrate dehydrogenase (ICDH) catalyzes the oxidative decarboxylation of isocitrate to $\alpha$-ketoglutarate, oxalosuccinate being formed as an intermediate. The $NADP^+$-dependent ICDH is a monomer of $M_r$ 60 000 which requires not only $NADP^+$ as coenzyme but also a divalent metal ion for activity. Mn(II) is the most effective metal ion.[139]




isocitrate oxalosuccinate



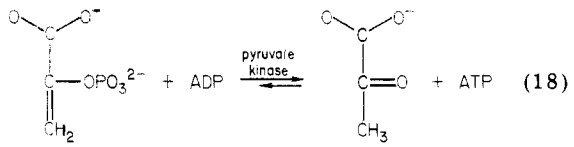

α-ketoglutarate

With use of ESR for the free Mn(II) titration it was evaluated[204] that 1 mol of Mn(II) per mol of enzyme is bound with $K_D$ (dissociation constant) = 45 $\mu$M, which does not change in the presence of $NADP^+$, NADPH, and $\alpha$-ketoglutarate but is lowered to 2.2 $\mu$M in the presence of isocitrate. Isocitrate, however, did not affect the inactive *N*-ethylmaleimide-modified enzyme, suggesting that modification of critical SH groups disrupts the interaction between the Mn(II) and isocitrate sites. The formation of metal-bridge EMS ternary complex with isocitrate was suggested[204] from NMR relaxation of solvent water.[2] The frequency and temperature dependence of the longitudinal relaxation rate of water protons in the ME complex were used[138] to calculate the number of water ligands in the coordination shell of Mn(II), $n$ = 2, which was reduced to 1 in the ternary complex with either isocitrate or $\alpha$-ketoglutarate.

The $T_{1p}^{-1}$ and $T_{2p}^{-1}$ values for $\alpha$-ketoglutarate and isocitrate protons were found consistent[138] with binding of the keto moiety of $\alpha$-ketoglutarate and chelation of succinate in the respective ternary complexes. The ESR spectra of Mn(II) in the various complexes were obtained[157] in the solution state and were simulated according to the Reed and Ray procedure,[151] yielding the largest amount of axial distortion (~450 G) for the ternary complex with isocitrate and the maximum amount of rhombic distortion for that with $\alpha$-ketoglutarate. This was interpreted in terms of direct coordination of isocitrate and of a different mode of binding for $\alpha$-ketoglutarate. The effects of $CO_2$, $HCO_3^-$, formate, and thiocyanate on the ESR spectrum were also shown to present evidence for $CO_2$ rather than $HCO_3^-$ as the actual reactive species. Titrations performed by following the proton longitudinal relaxation rate of water protons suggested[118] that the competitive inhibitor oxalylglycine and the nucleotides $NADP^+$ and NADPH bind near, but not directly, to the metal ion

site while the substrates isocitrate and α-ketoglutarate probably bind directly to Mn(II). Furthermore $NADP^+$ and NADPH were shown to interact with the enzyme in a different way: NADPH, rather than $NADP^+$, weakens the Mn(II) binding to the enzyme and alters the accessibility of the Mn(II) to the bulk solvent. All the magnetic resonance investigations provide some evidence for Mn(II) acting as an electrophilic center on the enzyme to bind substrate and stabilize an enzyme-bound intermediate enolate of α-ketoglutarate.

**10. Pyruvate Kinase.** Pyruvate kinase catalyzes the transfer of a phosphoryl group from phosphoenolpyruvate (PEP) to ADP in the final step of the glycolytic pathway (eq 18). The rabbit muscle enzyme is




(18)

a tetramer ($M_r$ 237 000). Each subunit has a high affinity site for divalent cation, $Mg^{2+}$, and a lower affinity site for monovalent cation, $K^+$. The enzyme shows an absolute requirement for both types of cation. $Mg^{2+}$ can be replaced by $Mn^{2+}$ with about 50% retention of activity. The paramagnetic Mn(II) ion binds competitively to the Mg(II) binding sites; the dissociation constants evaluated from ESR and PRE measurements[205] were found in agreement with the kinetically determined activator constants of Mn(II) and Mg(II).

From the frequency dependence of $T_{1p}^{-1}$ and $T_{2p}^{-1}$ of water protons, three exchangeable water ligands were calculated.[121] The formation of ternary complexes with PEP affected the water relaxation, indicating displacement or immobilization of water ligands by the substrate.[206] The exchange rate of PEP onto the enzyme–Mn(II) complex was shown to be slow in the NMR time scale,[207,208] and the structural information was gained either from complexes of inactive analogues[200,201] or phosphorylated compounds.[207,209] Studies of the reaction products $FPO_3^{2-}$[202] and phosphoglycolate[207] and of the competitive analogues of PEP, D- and L-phospholactate[207] and α-[(dihydroxyphosphinyl)methyl]acrylic acid[201] allowed the calculation of distances that indicated direct phosphoryl coordination to the metal in every case. Pyruvate and inorganic phosphate ($P_i$) compete simultaneously with PEP for the Mn–enzyme and form an enzyme–$K^+$–$Mn^{2+}$–pyruvate–$P_i$ complex. The carbonyl and carboxyl carbons of pyruvate were found at a distance from Mn(II) (both 7.3 Å), as deduced by frequency-dependent $^{13}C$ nuclear relaxation studies,[203] which is consistent with second-sphere coordination. The $^{31}P$ NMR studies of the phosphate phosphorus led to the calculation of a Mn(II)–P distance (5.3 Å) revealing either a distorted inner-sphere or a second-sphere complex.[210] From $^{205}Tl$ nuclear relaxation studies[211,212] the $Tl^+$–$Mn^{2+}$ distance was shown to change (from 8.2 to 4.9 Å) upon formation of the ternary complex of PEP, demonstrating a substrate-induced conformational change. The ESR spectra showed dramatic changes both in line width and electron symmetry[161] in the ternary complexes of pyruvate and PEP but not in those of ADP or ATP, suggesting the existence of two monovalent ion-dependent forms of the enzyme. From $^{31}P$ and $^1H$ nuclear

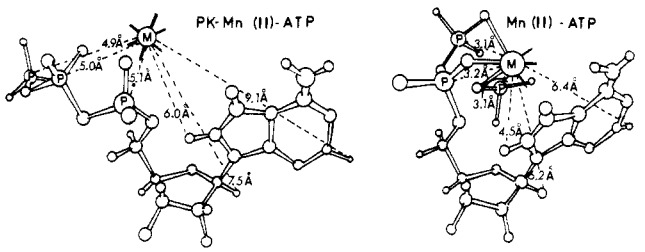



**Figure 10.** Models for the conformations of Mn(II)–ATP and pyruvate kinase–Mn(II)–ATP. (PK–Mn(II)–ATP). (Reprinted with permission from D. L. Sloan and A. S. Mildvan, *J. Biol. Chem.*, **251**, 2412 (1976). Copyright 1976, American Society of Biological Chemists, Inc.)

relaxation studies of ATP[213] in the Mn–enzyme complex the metal–phosphorus (4.9 Å, 5.1 Å) and the metal–proton distances were interpreted as indicating second-sphere interaction; the relative model is displayed in Figure 10. The torsion angle that defines the relative conformation of the enzyme-bound riboside and adenine was determined to be 30° compared with 90° in the binary Mn–ATP complex. Since $CrATP^-$, a substituting inert paramagnetic analogue of $MgATP^{2-}$, occupies the $MgATP^{2-}$ site on the enzyme[179,214,249] and because both metal ions exist at the catalytic site of pyruvate kinase, both being essential for the catalysis, the paramagnetism and nonlability of the Cr(III) ion were also used. The exchange of spin magnetization between the two metals from time-dependent dipolar interactions was suggested to provide a perturbation on the $T_{1p}^{-1}$ of inner-sphere water protons by altering the $\tau_s$ value. From this approach a distance of 5.2 Å was estimated between Mn(II) and Cr(III), indicating a van der Waals contact between the hydration spheres of the enzyme- and nucleotide-bound metal ions.[181] The interaction of Li(I) with substrate and inhibitor complexes of the enzyme was investigated by ESR and $^7Li$ NMR.[215] The data were interpreted in terms of two different forms of the enzyme, characterized by differing Mn(II)–Li(I) distances ($r$ = 4.7 and 9 Å). The 4.7-Å separation agrees with the Mn(II)–Tl(I) distance in the PEP complex.[212]

**11. Adenosine 3′,5′-Monophosphate Dependent Protein Kinase.** Adenosine 3′,5′-monophosphate (cAMP) dependent protein kinase catalyzes by providing a metabolic link between intracellular biochemical functions and the binding of hormones to external receptors. The activation by cAMP occurs through formation of a complex with a regulatory dimer, yielding the active catalytic subunit, which requires a divalent cation for its activity ($Mg^{2+}$ or $Mn^{2+}$) and the transfer of a phosphoryl group from ATP or other nucleoside triphosphates to the hydroxyl group of serine or threonine of protein substrates.

The studies of the mechanism of action and regulation have been recently reviewed.[216] The binding features of $Mn^{2+}$ were investigated with ESR and proton relaxation rate measurements on water protons.[217] Very weak and nonspecific binding was observed ($K_D \simeq 1$ mM). The analysis of enhancement factors in the presence of nucleotides, the substitution-inert complex $Co^{III}(NH_3)_4ATP$ peptide substrates, led to the conclusion[217] that the nucleotide-bound $Mn^{2+}$ activates while the more weakly bound $Mn^{2+}$ is inhibitory, indicating the active form of the enzyme is a nucleotide bridge complex with a stoichiometry of one enzyme-bound

nucleotide per molecule of catalytic subunit. The conformation of enzyme-bound nucleotide substrates was determined from $^1H$ and $^{31}P$ $1/T_{1p}$ measurement when $Co(NH_3)_4ATP$ and $Mn^{2+}$ were used at the single inhibitory site.[218] The $\tau_c$ for the $^1H$–Mn(II) and $^{31}P$–Mn(II)dipole interaction was inferred by the frequency dependence of $1/T_{1p}$ by assuming that $\tau_s$ dominates $\tau_c$ and that it has a maximum frequency dependence. The $^1H$–Mn(II) and $^{31}P$–Mn(II) were thereby calculated by mapping out the conformations of the substitution-inert ATP complex in its binary and ternary complexes with $Mn^{2+}$ and the catalytic subunit. Either $\alpha,\gamma$ or $\alpha,\beta,\gamma$ coordination of the triphosphate chain by the enzyme-bound $Mn^{2+}$ was suggested, in agreement with thermodynamic and kinetic properties of enzyme complexes. A lower limit for the rate of exchange of $Co(NH_3)_4ATP$ into the paramagnetic environment could be evaluated from the $1/T_{2p}$ values in the same experiments,[218] leading to the suggestion that the residence time of $Mn^{2+}$ in the ternary complex was of the same order or shorter than the residence time of the nucleotide in the binary complex.

By investigation of the effects of the regulatory subunits on the interaction of metal ions, nucleotides, and peptide substrates with the catalytic subunit,[219] blockage of binding of the peptide substrate Leu-Arg-Arg-Ala-Ser-Leu-Gly and of its competitive analogue Leu-Arg-Arg-Ala-Ala-Leu-Gly was detected by line-width measurements of the side-chain protons, whereas no interference with the binding of the nucleotide substrate on its metal complex to the active site could be detected. As a consequence it was proposed that the regulatory subunit exerts its inhibitory function by blocking the binding site of the protein substrate.

The extension to include Co(III) and Cr(III) complexes of $\beta,\gamma$-methylene-ATP as substitution-inert substrate analogues[220] led to the evalution of the $Mn^{2+}$–$Cr^{3+}$ distance (4.8 Å) and to the calculation of the distances from $Mn^{2+}$ and $Cr^{3+}$ to the serine methylene protons (9.1 and 8.1 Å, respectively) of the peptide substrate Leu-Arg-Arg-Ale-Ser-Leu-Gly by measuring the paramagnetic effects on the longitudinal and transverse relaxation rates of peptide protons.

From model building all the measured distances to the metal ion ($S$) were used to estimate a distance of 5.3 ± 0.7 Å along the reaction coordinate between the $\gamma$-phosphorus of ATP and the serine hydroxyl oxygen. As a consequence a dissociative mechanism with a metaphosphate intermediate was supported as the preferred phosphoryl transfer mechanism on protein kinase.

**12. DNA and RNA Polymerases.** DNA and RNA polymerases are the catalysts of the replication and transcription of DNA. The role of the divalent metal ions in these enzymes was recently reviewed.[221] DNA polymerase catalyzes the copying of a nucletide template. The reaction pathway is sketched in Figure 11.[221] The divalent cation activator may well be Mg(II) or Mn(II);[101] the ESR titrations revealed 1 tight-binding site for Mn(II) ($K_D \sim 1$ μM), 4 intermediate binding sites ($K_D = 29$ μM), and approximately 20 weak-binding, inhibitor Mn(II) sites ($K_D = 0.8$ μM). Nuclear relaxation studies on water were taken as indicating that nucleoside substrates such as dTTP interact with Mn(II) at both the tight and the intermediate sites,

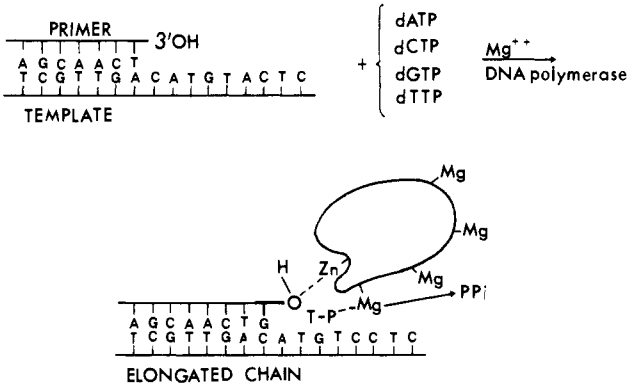



**Figure 11.** DNA polymerase reaction. (Reprinted with permission from A. S. Mildvan and L. A. Loeb, *CRC Crit. Rev. Biochem.*, 6, 219, (1979). Copyright 1979, Chemical Rubber Co.)

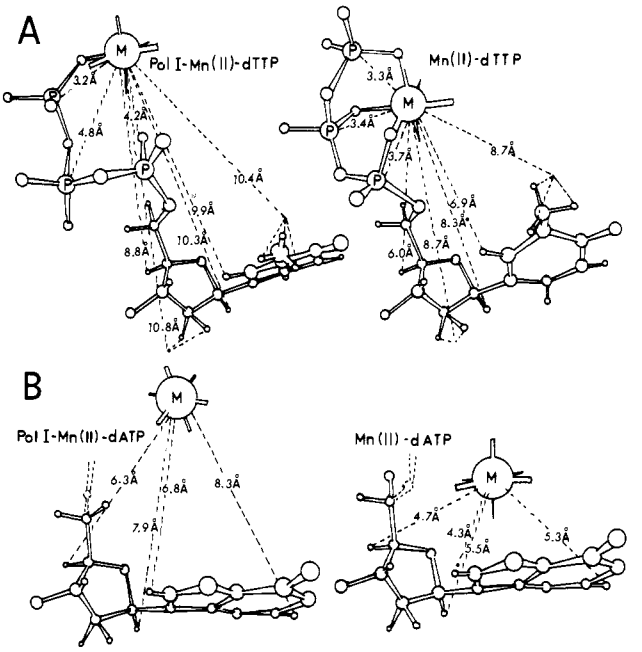



**Figure 12.** Conformations in solution of binary Mn(II) complexes and ternary Pol I–Mn(II) complexes of (A) dTTp and (B) dATP. (Reprinted with permission from A. S. Mildvan and L. A. Loeb, *CRC Crit. Rev. Biochem.*, 6, 219 (1979). Copyright 1979, Chemical Rubber Co.)

displacing one of water ligands in the first coordination sphere of enzyme-bound Mn(II).[101] From the $^1H$ and $^{31}P$ $T_{1p}^{-1}$ and $T_{2p}^{-1}$ values of the bound dTTP substrate[222] the distances from Mn(II) to five protons and three phosphorus atoms of dTTP were calculated in both the Mn–dTTP and the DNA polymerase–Mn–dTTP complexes (Figure 12A): the important role of the divalent cation is to link the $\gamma$-phosphoryl group of the substrate by assisting the departure of the leaving pyrophosphate group and to change the torsion angle from 45° to 90°. Similar effects were shown for a purine substrate dATP (Figure 12B).

RNA polymerases are generally more complex than the DNA polymerases. All require a divalent cation such as Mg(II) or Mn(II) for activity. The ESR and PRR titrations[116] revealed one tight Mn(II) binding site per enzyme molecule ($K_D < 10$ μM) and approximately six weaker Mn(II) binding sites ($K_D \sim 1$ mM). The closer agreement between the kinetic and $K_D$ constants of the elongation substrates than that of specific initiators with the enzyme-bound Mn(II) and the increase

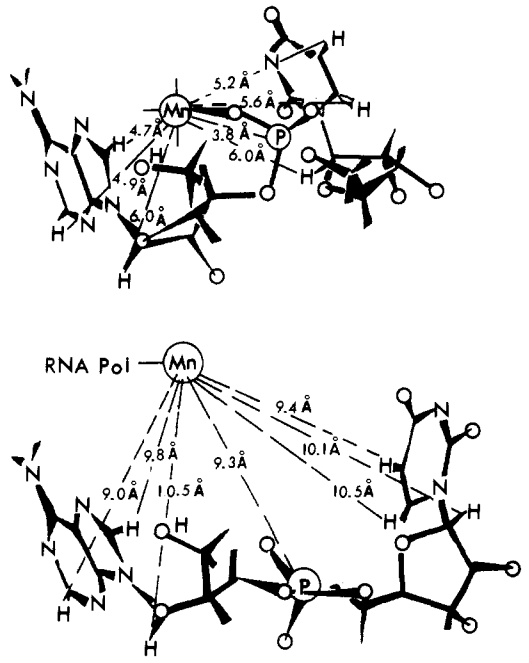



**Figure 13.** Conformations of adenylyl(3′–5′)uridine (ApU) in its binary complex with Mn(II) (upper) and in its ternary complex with RNA polymerase and Mn(II) (lower). (Reprinted with permission from A. S. Mildvan and L. A. Loeb, *CRC Crit. Rev. Biochem.*, 6, 219 (1979). Copyright 1979, Chemical Rubber Co.)

in the affinity of the enzyme-Mn(II) complex for UTP in the presence of the specific initiator ApA were all interpreted[116] as demonstrating that the tight Mn(II) binding site functions as the active site for RNA chain elongation. The paramagnetic effects of RNA-polymerase–Mn(II) on the $T_1^{-1}$ and $T_2^{-1}$ values of $^1H$ and $^{31}P$ atoms of ATP bound at the elongation site were also investigated.[142] By use of the $\tau_c$ value determined from the dispersion of $T_1^{-1}$ of water and ApU protons in the enzyme complexes, the distances from the enzyme-bound Mn(II) to the proton and $^{31}P$ of ApU (9.0 and 10.5 Å) and to the protons of ATP (4.0 for H-8 and 5.7 for H-2 and H-1) were calculated (Figure 13), supporting the tight binding of Mn(II) at the elongation site.

**13. Concanavalin A.** Concanavalin A (Con A) is a metalloprotein of the class of plant lectins, which are specific saccaride-binding proteins. Below pH 6 it is a dimer, $M_r$ 54 000, and above pH 7 it is a tetramer. Both the saccharide binding and agglutination properties require the presence of two divalent cations per monomer: a variety of divalent transition-metal ions, including $Mn^{2+}$, can bind at a site S1, while $Ca^{2+}$ or $Cd^{2+}$ binds at a site S2. This last one is formed only after the S1 site is occupied. The saccharide binding site is formed when both S1 and S2 are occupied.

The ESR spectra were used either for structural information or for titrating the free Mn(II) ion. ESR spectra for Mn(II) bound to the protein were obtained in solution[29,31,150,153] and in the solid state.[29,30,150,154] It was shown that the binding of Ca(II) at S2 alters the characteristic spectrum of Mn(II) bound to S1, while the binding of the methyl α-glucoside does not affect the spectrum anymore. From the line-shape analysis the correlation times associated with the Mn(II) ions in the various forms were derived, suggesting that the release of Ca(II), although not affecting the overall rotational mobility, allows faster motion in the part where Mn(II) is located. The ESR titration method revealed two equivalent binding sites for Mn(II) per monomer ($K_D \simeq 50$ μM)[223] and was further used to evaluate the role of Ca(II) in the functional properties of Con A at different pH values;[171] preincubation of demetalized Con A with Ca(II) causes the nature of Mn(II) binding to change from noncooperative to cooperative, and the effect is more evident at pH 7 than at pH 5. It was shown that the kinetics of Mn(II) binding was also strongly affected.[147] That the addition of Ca(II) to Con A alters the properties of the metal ion at S1 was also pointed out by the decrease of $T_1^{-1}$ of solvent water, which was attributed to a decrease in the off rate of an exchanging water ligand of the Mn(II).[223-225] Slow time-dependent changes in either the properties of the ion at S1 or the activity of the protein upon addition of Ca(II) were also reported.[226,227] The magnetic relaxation dispersion of $T_1^{-1}$ of solvent water at various temperatures[140] was interpreted in terms of one rapidly exchanging water on the Mn(II), having the same geometry as in the hexaaquo ion with protons 2.7 Å from the Mn(II), but a longer residence lifetime (about $3 \times 10^{-6}$ s compared with about $7 \times 10^{-8}$ s). Furthermore, the monosaccharide binding to Mn–Con A had little effect on $T_1^{-1}$, indicating that the sugars do not coordinate directly to the transition metal. The extension of the magnetic relaxation dispersion method to cover a wider range of frequency (0.01–50 MHz) at two pH values and two temperatures and to account for time-dependent effects following the addition or removal of metals[144] led to the postulation of two conformational states of Con A with different metal ion binding properties. The conformation of lower energy ("unlocked") is the same for both apo-Con A and Con A with S1 occupied by Mn(II) ions; it binds Ca(II) at S2 very weakly when Mn(II) is bound at S1. The unlocked ternary complex was shown to undergo a first-order transition to a conformational state ("locked"), separated from the "unlocked" one by only a few kilocalorie per mole but with a fairly high energy barrier (~22 kcal $mol^{-1}$), in which Ca(II) ions and hence Mn(II) ions are very strongly bound to the protein, both at pH 5.3 and 6.4. In the absence of Ca(II), it was also shown[146] that Mn(II) can bind at S2 to form an "unlocked" ternary complex which undergoes a transition characterized by slower time course and higher energy barrier in comparison with the Ca(II)–Mn(II) case. The conclusions were drawn that the occupation of S2 by metal ions is associated with the induction of a change of conformation of the metal–protein complex to that of the native protein, this function being served by Mn(II) ions as well as by Ca(II) ions. A similar analysis[146] was used to give evidence for the formation of a "locked" ternary complex with Ca(II) at both the S1 and S2 sites of the protein and to demonstrate that the saccharide-binding activity is accounted for by the "locked" conformation of ConA, with the function of bound metals being to maintain the "locked" conformational state. The binding of various mono- and oligosaccharides to Ca(II)–Mn(II)–Con A was shown[148] to affect the $T_1^{-1}$ of water protons proportionally to the amount of saccharide bound to the protein, due to an increase of the residence time of the water ligand. This was interpreted as suggesting the same conformational change in the protein upon binding of whichever saccharide. The

sugar–protein interaction was checked by $^{13}C$ NMR of methyl D-glucopyranoside $\alpha$ and $\beta$ anomers[228-230] and by $^{19}F$ NMR of *N*-(trifluoroacetyl)-$\alpha$- and -$\beta$-D-glucosamine.[231] Different Mn(II)–nucleus distances were found for $\alpha$ and $\beta$ anomers, although both were consistent with the $C_1$ chair conformation.

**14. Nucleic Acids.** Although metal ions mediate all the biological reactions involving nucleic acids, the mechanism of their action is still little understood.[91] Magnetic resonance techniques were used to obtain some information and the pioneer works, describing the ESR and PRE features in macromolecular solutions,[24,92,232] included some experiments on both DNA and RNA. Since then water proton relaxation rate studies[119,120,233-235] have been used to detect the features of the metal interactions. It was shown that RNAs, in contrast with polynucleotides, have two different types of metal binding sites: up to six Mn(II) ions are strongly bound ($K_D$ = 50–25 $\mu$M) in a cooperative way, while many other Mn(II) ions are more weakly bound in a noncooperative way. The parameters are fairly different for RNAs from different sources but the behavior is quite general. From $^{31}P$ $T_{2p}^{-1}$ values in RNA it was seen[236] that the calculated isotropic hyperfine constants for the metal–phosphorus interaction were consistent with the values derived from $^{31}P$ NMR measurements in crystals of $Mn_3(PO_4)_2$, suggesting the direct binding of Mn(II) to the phosphates. An interaction between Mn(II) and the adenine ring of homopolymer poly(A) was suggested from $^1H$ NMR line-broadening studies.[63] The $^{31}P$ $T_{1p}^{-1}$ and $T_{2p}^{-1}$ values of poly(A) were used[237] to evaluate the Mn–P distance ($r$ = 3.3 Å), which was found consistent with that in the Mn–ATP system, and the activation enthalpy for dissociation of the complex. The evaluation of the Mn–H distances from $^1H$ NMR $T_{1p}^{-1}$ values of H-8, H-2, and H-1, of poly(A)[237] allowed the suggestion of a model (shown in Figure 14), in which every Mn(II) is bound directly to two phosphate groups, while a part of the Mn(II) ions are simultaneously bound to the adenine ring, either at N-7 (about 39%) or N-3 (or N-1) (about 13%). The ESR spectra of Mn(II) were identified in samples of RNA,[238,240] indicating that Mn(II) is located at the center of a distorted octahedron. The ESR of Mn(II) was also used to titrate the Mn(II) binding both to DNA and RNA and to evaluate the competition effects of inorganic and organic cations (ref 168, 169, 235, 241, 242, 286, 292–295) which suggested the employment of electron donors groups of the base rings in the metal binding. The ESR study of the coupling of Mn(II) ions with spin-labeled tRNAs[175] allowed the detection of a conformational transition induced by the metal ions in terms of increase in the spin-label mobility, suggesting that this transition destroys some hydrogen-bonding network, such that some bases acquire sufficient sterical freedom to coordinate the Mn(II) ion. The ESR spectrum of the Mn(II)–DNA complex was also shown to be[76] quite sensitive to pH and temperature changes, being characterized by the inhomogeneous features typical of "quasi-glassy" ESR patterns. An ESR spectrum was also recently reported of Mn(II) containing DNA fibers.[243] A considerably line sharpening was also observed in the presence of Cu(II), indicating an interaction of these two ions in their binding to DNA. By studies of the paramagnetic line

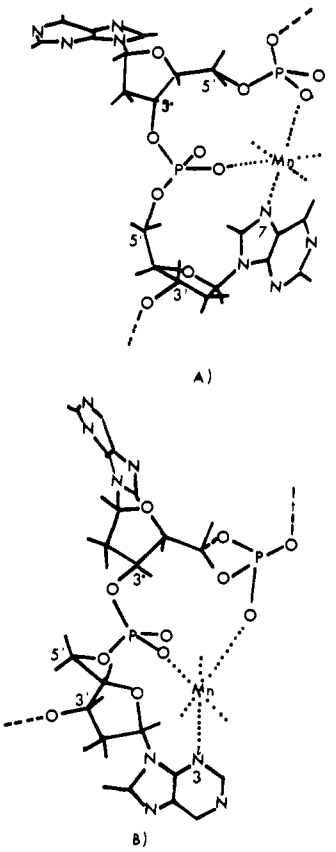



**Figure 14.** Schematic representation of two sterically allowed structures for the ring–Mn(II)–phosphate complex. (a) Mn is coordinated by N-7 from an adenosine group whose adenine ring takes an anti position with respect to rotation about the glycosidic bond and by two phosphate groups at the 5′ side of this adenosine group. (b) Mn is coordinated by N-3 from an adenosine group whose adenine ring takes an "intermediate" position with respect to rotation about the glycosidic bound and by two phosphate groups at the 5′ side of this adenosine group. (Reprinted with permission from A. Yamad, K. Akasaka, and H. Hatano, *Biopolymers*, 15, 1315 (1976). Copyright 1976, John Wiley and Sons).

broadening of tetramethylammonium protons as well as of water[244] the binding of this low molecular weight compound to tRNA was demonstrated in the presence of the Mn(II) ions, allowing the evaluation of the stability constant ($K$ = 13.0 $M^{-1}$) and of the distance of tetramethylammonium protons from Mn(II) ($r$ = 7.3 Å). The value obtained was interpreted as indicating that the ligand molecules adjacent to the tRNA-bound Mn(II) are located at the neighboring phosphate.

**15. Model and Biological Membrane Systems.** Phospholipids are a major constituent of biological membranes and have often been used as model membrane systems. Metal ions are well-known to affect the stability of the lipid bilayer structure, modulating the temperature of the first-order phase transition which involves cooperative rotations of the hydrocarbon chains. Mn(II) has been used to investigate different features of both natural and artificial membranes, most information being derived by using the paramagnetic properties of the Mn(II) ion. The ESR measurements obtained in phospholipid vesicles were used in different ways: the signal amplitude was taken as a measure of free Mn(II) to evaluate the affinity of the phospholipid for Mn(II),[245,246] suggesting that, when different lipids are randomly dispersed in the bilayer, the Mn binding strength is roughly proportional to the weight fraction

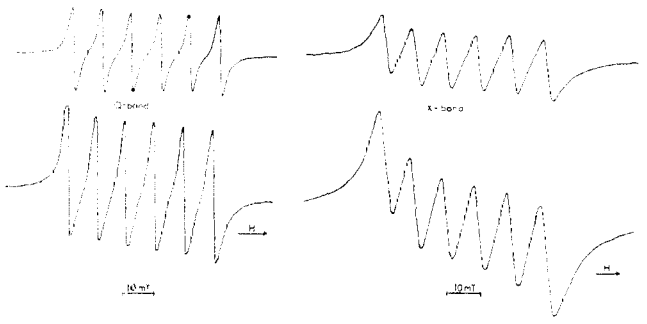


**Figure 15.** Q-band ($\omega = 2.16 \times 10^{11}$ rad/s) and X-band ($\omega = 5.8 \times 10^{10}$ rad/s) ESR spectra of Mn(II) in lecithine in 25% by weight-sonicated aqueous dispersions at room temperature: (a) pH 3, upper spectra; (b) pH 7, lower spectra.

of charged phospholipids. Moreover, a 1:2 divalent cation–phospholipid interaction was inferred, as the cation affinity is more favored when the temperature is raised, which allows an increased lipid fluidity.

The ESR line-shape analysis[247] at different frequencies and pH values was taken as indicating a prominent role of the "through-water" metal–lipid interaction in egg yolk lecithin vesicles, especially at acidic pH. The spectra are shown in Figure 15 (unpublished results) and display strong deviations from a "single Lorentzian" line shape (especially at pH 7), showing the distribution of several Lorentzians due to apparent immobolization of the metal solvation sphere. This statement allowed some inferences about the lipid and solvent dynamics by analyzing the ESR line shape when either Mn(II) is wholly outside the vesicle or when it is wholly inside the vesicle. The binding of the ionophore A 23187 to Mn(II) in sonicated lecithin dispersions was also studied[248] and indicated a distorted octahedral symmetry, a metal–ligand interaction predominantly ionic in character, a 1:2 Mn(II)/ionophore stoichiometry, and an association constant of about $10^{10}$ $M^{-2}$. ESR analysis was also carried out in micelles of sodium dodecyl sulfate[249] and bis(2-ethylhexyl) sulfosuccinate,[250] showing two different environments, the Stern layer and the diffuse layer for Mn(II) in aqueous surfactant solutions and the monomer–micelle equilibrium in dry benzene solutions. The ESR technique was used also in studying Mn(II) transport across the mitochondrial membrane[251-256] by studying the distribution of Mn(II) between the matrix and the extramitochondrial space. The exit of Mn(II) from submitochondrial particles in exchange for a $H^+$ during treatment of the particles with the ionophore A 23187 was also observed.[257] By use of the ESR method it was shown[258] that Mn(II) can be either in a free state inside the submitochondrial particles or complexed with protein and/or lipid membrane components. The desorption of Mn(II) with consequent increase of free Mn(II) inside the particles made it possible to follow the pH changes generated on the mitochondrial membrane. In plant chloroplast Mn forms part of the photosystem 2 in the ratio 4 Mn atoms to 1 reaction center. In native chloroplast, however, only a weak ESR signal was observed, the intensity of which strongly increases after either insonation, heating, treating with 0.8 M Tris-HCl or reductants, or aging of the chloroplast. In all cases the line shape is typical of the hydrated Mn(II) ion,[259,260] which indicates release of the bound functional Mn. An asymmetrical ESR spectrum was observed in chloroplast isolated in Tris-

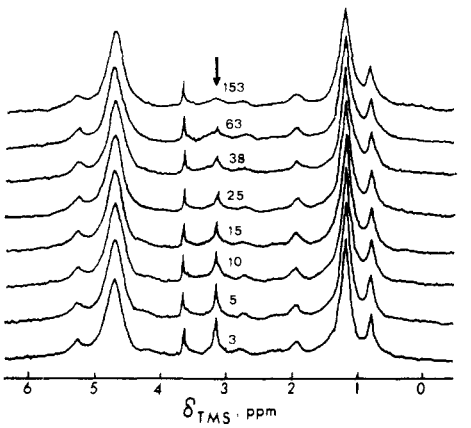



**Figure 16.** 270-MHz $^1H$ NMR spectra of egg phosphatidylcholine vesicles prepared in $^2H_2O$ containing 6.7 mM Tris, in the presence of 30 M X-537A and 1 mM $MnCl_2$ added after the sonication at pH 6.8 and 28 °C. Chemical shifts were referred to an external tetramethylsilane (TMS). The inserted numbers refer to the time in minutes after the addition of $MnCl_2$ to the outer medium. The position of the inner choline signal is indicated by an arrow. (Reprinted with permission from H. Degani, *Biochim. Biophys. Acta*, **509**, 364 (1978). Copyright 1978, Elsevier Scientific Publishing Co.)

HCl buffer in the presence of 0.1 M NaCl and in absence of salts,[257-261] changing to the symmetrical spectrum in the presence of reductants and light. The Mn(II) ESR signal from lettuce chloroplasts was interpreted[262] as indicating that only the $-^1/_2 \leftrightarrow {}^1/_2$ fine structure band is observed in untreated chloroplasts, which suggests an asymmetric environment for Mn(II) as in protein complexes. The ESR signal was light sensitive; its disappearance in the presence of oxygen evolution inhibitors or electron donors and the slowing down of the process by heat treatment were found to be consistent with a relation between the photooxidation of Mn(II) and the phosphosynthetic cycle.

$^1H$, $^{13}C$, and $^{31}P$ NMR studies on lipid molecules in the presence of Mn(II) ions[263-266] provided some inferences about the distribution of different phospholipids in the internal and external surfaces of the bilayer. From the $^1H$ and $^{31}P$ paramagnetic induced line broadening in cosonicated vesicles of phosphatidylglycerol (PG) and phosphatidylcholine (PC) it was shown[265] that the outer surface contains twice as many PG as PC molecules, these being two lipid classes not spatially segregated into patches. A similar analysis was performed in cosonicated vesicles of phosphatidylcholine with phosphatidylethanolamine[267] and phosphatidylserine.[266]

The transport phenomena were also investigated by studying the interaction of Mn(II) with the ionophorous antibiotic X-537A,[268] which yielded a rate of dissociation for the complex in the range from $2 \times 10^4$ to $1 \times 10^6$ $s^{-1}$ as deduced from the paramagnetic effect upon the NMR resonances of three of the proton species in X-537A. By observing the time course of the Mn(II)-induced broadening of the inner choline protons when the ionophore is present[269] (Figure 16) it was possible to calculate the rates of X-537A mediated Mn(II) transport, suggesting that the complex involved in the transport rate-determining step is $MnX_2$. The activation energy for the transport process was also measured from the temperature dependence of the transport rate ($E_a \sim 22$ kcal/mol), indicating that the rate-deter-

mining step is the $MnX_2$ diffusion through the membrane.

The nuclear relaxation rates of solvent water with Mn(II) ions were also taken into account. The water diffusion permeability of both natural and artificial membrane is very high, the exchange time being in the range 1–10 ms. Since the transverse relaxation time is typically of the order of 140 ms, the exchange time cannot dominate the relaxation mechanism and kinetic information could not be obtained. The addition of Mn(II) ions to the external medium or inside the vesicles was used to shorten the natural relaxation time, making the exchange process detectable by NMR measurements. In this way the water transfer across nerve cell membranes,[270] red blood cell membranes,[271-274] and phospholipid vesicle membranes[275,276] was checked with both $^1H$ and $^{17}O$ NMR measurements. Water proton $T_1^{-1}$ and $T_2^{-1}$ were also used to study the metal ion environment in various systems. In ribosomes[277] it was shown that Mn(II) ions are in an aqueous environment, that they require less than 35 s to exchange with Mn(II) ions in free solution, and that water molecules require less than 1 ms to reach the metal-binding sites. In mitochondria[278] the binding of Mn(II) to the inner membrane was suggested by the increase of $T_{1p}^{-1}$, which then decayed to about $^1/_2$ of the initial value as a consequence of energy-driven Mn(II) uptake. Since a linear correlation held between the $T_{1p}^{-1}$ value and the initial velocity of Mn(II) uptake, a binding of Mn(II) to the divalent cation pump was hypothesized. In chloroplasts[278-280] it was shown that treatments which affect the bound-Mn content in the membrane also affect the $T_{1p}^{-1}$ of water protons, with the addition of oxidants and reductants having opposite effects. The $T_{2p}^{-1}$ values were shown to exhibit damped oscillations following a series of intense light flashes, exactly like the oxygen evolution. From the dependence of $T_{1p}^{-1}$ and $T_{2p}^{-1}$ of both $^1H$ and $^{17}O$ on the Mn concentration in dark-adapted chloroplasts, it was concluded that the relaxation rates are determined largely by the loosely bound Mn present in the membranes. Mn frequency dependent studies yielded an evalution of both $\tau_s$ ($1.1 \times 10^{-8}$ s) and $\tau_M$ ($2.2 \times 10^{-8}$ s). The $^{17}O$ $T_{1p}^{-1}$ and $T_{2p}^{-1}$ data in the presence and in the absence of a detergent were taken as an indication of the location of Mn in the thylakoids.

In phospholipid aqueous dispersion[247,281] the $T_{1p}^{-1}$ and $T_{2p}^{-1}$ of water protons were used to determine the number of interacting sites per polar head group ($n$ = $4.7 \times 10^{-3}$ and $6.8 \times 10^{-2}$) and the association constants ($K_A = 4 \times 10^4$ $M^{-1}$ and $9 \times 10^4$ $M^{-1}$) for phosphatidylcholine and phosphatidylserine, respectively.

## V. Glossary of Symbols and Terms

| | |
|---|---|
| $A$ | electron nucleus hyperfine coupling constant |
| $B$ | zero-field splitting parameter in Bloembergen–Morgan equations |
| BM | Bloembergen–Morgan |
| $C$ | constant = $2g^2S(S+1)\beta^2\gamma_I^2/15$ |
| $D$ | axial parameter of zero-field splitting tensor |
| $(D:D)$ | inner product of the zero-field splitting tensor with itself |
| $E$ | rhombic parameter of zero-field splitting tensor |
| $E_a$ | activation energy |
| $E_M$ | activation energy for chemical exchange ($\tau_M$) |
| $E_R$ | activation energy for rotation ($\tau_R$) |
| $E_V$ | activation energy for $\tau_V$ |
| EMS | enzyme–metal–substrate complex |
| ESR | electron spin resonance |
| $g$ | electronic $g$ factor |
| $h$ | Planck's constant |
| $H$ | magnetic field |
| $\Delta H$ | ESR line width |
| $\Delta H^*$ | enthalpy of activation |
| $I$ | nuclear spin |
| $k$ | Boltzmann constant |
| $K_D$ | dissociation constant for metal complex |
| $K_I$ | kinetic constant for inhibition |
| $K_M$ | kinetic constant for activation |
| $m_I$ | nuclear spin quantum number |
| $M_S$ | electron spin quantum number |
| $n$ | number of binding sites |
| NMR | nuclear magnetic resonance |
| OS | outer sphere |
| $P_f$ | configurational probability distribution |
| $P_M$ | fraction of ligand bound to the metal ion |
| PRE | proton relaxation rate enhancement |
| $q$ | coordination number |
| $r$ | metal–nucleus distance |
| $r_0$ | probe to probe distance in Leigh's expression |
| $R$ | gas constant |
| $S$ | electron spin |
| $\Delta S^*$ | entropy of activation |
| SB | Solomon–Bloembergen |
| SBM | Solomon–Bloembergen–Morgan |
| $T$ | temperature |
| $T_{iM}^{-1}$ | relaxation rate of nuclei in the metal coordination sphere |
| $T_{ip}^{-1}$ | paramagnetic contribution to relaxation rate |
| $T_{2f}$ | electron spin relaxation time in the $f$th configuration |
| $Y(\omega)$ | ESR line shape function |
| $Y'(\omega)$ | modified ESR line-shape function |
| ZFS | zero-field splitting |
| $\beta$ | Bohr magneton |
| $\gamma_I$ | nuclear magnetogyric ratio |
| $\gamma_S$ | electronic magnetogyric ratio |
| $\epsilon^*$ | obserbed PRE |
| $\epsilon_T$ | PRE of a ternary EMS complex |
| $\mu$ | magnetic moment |
| $\nu$ | frequency |
| $\tau$ | correlation time for electron spin relaxation |
| $\tau_c$ | correlation time for electron–nucleus dipolar interaction |
| $\tau_e$ | correlation time for electron–nucleus contact interaction |
| $\tau_{f2}$ | configurational lifetime |
| $\tau_M$ | lifetime of a ligand in the metal coordination sphere |
| $\tau_R$ | rotational correlation time |
| $\tau_S$ | electron spin relaxation time |
| $\tau_V$ | fluctuational correlation time |
| $\omega_I$ | nuclear Larmor frequency |
| $\omega_S$ | electron Larmor frequency |

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