Electron Paramagnetic Resonance Parameters and Equilibria of Manganese(II)–Amino-acid Complexes in Aqueous Solution

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Manganese(II) binding to amino-acids is studied, bearing in mind the role that this paramagnetic ion plays in the action of certain enzymes, in order to contribute to a clarification of its interactions with protein molecules. Information on the equilibria of Mn–amino acid complexes is derived from e.p.r. and n.m.r. spectra. Particularly, the following parameters are taken into consideration: the e.p.r. intensity, the Mn^{II} electron spin relaxation time, and the nuclear relaxation time T_2 of the water protons involved in complex formation. Attention is focused on four complexes: Mn–glycine, Mn–valine, Mn–proline, and Mn–α-alanine. Outer sphere co-ordination is taken into account and the dynamic aspects in the solvation shell of the manganese(II) ion are discussed.

It has long been known that interaction with metal ions plays an important role in the activity and properties of proteins and that many enzymes are activated by divalent heavy metal ions. The connection between metal ions, ligands, and cancer was recently underlined by Williams [1] with particular emphasis on complexes of amino-acids and metal ions.[2]

Among these ions Mn^{II} participates extensively in biological systems, particularly by effecting enzymatic processes.[3] Many studies have been carried out on the combination of Mn^{II} with proteins,[4] on the co-ordination complexes of Mn^{II} involved in enzyme action [3] (particularly arginase and carboxylase), and on the mechanism of manganese (or other metal ions) -activated proteolytic enzyme systems.[5,6] Recently the effect of Mn^{II} on the conformational changes of nucleic acids has been described.[7]

There have been several different approaches to the study of Mn^{II} complex equilibria and of the interactions with amino-acids and peptides. Since the pioneer work by Main and Schmidt,[4] much research has been carried out on Mn^{II}–amino-acid complexes by Albert,[8] Monk,[9] Kroll,[10] and others,[11,12] mostly based upon potentiometric titrations. Murphy and Martell [12] have pointed out that the avidity of glycine and glycine peptides for metal ions decreases in the following order: $Cu^{II} \gg Mn^{II} > Mg^{II}$. Datta and Rabin [13] have carried out a study on Mn–peptide chelation. Berezina and Pozigun [14] have investigated, by electrical conductivity, the Mn^{II}–glycine and –alanine systems.

Recently Childs and Perrin [15] have thoroughly analysed from pH titration data the complexes of manganous ion with glycine, L-alanine, L-valine, and L-proline, taking into consideration the many possible species present in solution and thus providing a deeper insight into the understanding of the Mn^{II}–amino acid equilibria.

The species described in all the above studies were inner-sphere complexes with the amino-acid present either in zwitterionic or anionic form. In this paper we draw attention to the presence of ion-pair complexes, that is to the participation of ligands in the second co-ordination sphere of the metal ion. The relevance to catalysis of the second co-ordination sphere has been discussed by Eaton,[16] with particular reference to enzyme-catalysed reactions. Furthermore it is worth noting that even very weak second sphere complexes have some preferred molecular structure, which influences the subsequent ligand exchange.

E.p.r. parameters are extremely sensitive to changes of molecular structure in solution and offer the opportunity of distinguishing between different species, whenever a dynamic situation is present, and of comparing the competitive avidities of the various complex-forming agents.

The aim of this paper is to study the relationship between magnetic resonance spectral parameters, namely e.p.r. intensity and linewidth and n.m.r. relaxation times, and the equilibria of Mn–amino acid complexes in aqueous solution. Particular attention has been devoted to the metal binding powers of amino-acids, in terms of stability constants, bearing in mind that in biological cells and tissues the traces of metallic ions are competed for by the various complex-forming agents and that the binding to the peptide chain occurs through the co-ordinating groups of the amino-acids present.

THEORY

In aqueous solution of manganese(II) ion it is generally believed that the relaxation mechanism is governed by

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[16] D. R. Eaton in ' Bioinorganic Chemistry,' Amer. Chem. Soc., 1971, p. 174.

the modulation of the anisotropic zero field splitting parameter (ZFS), *i.e.* the e.p.r. linewidth is directly related to the distortion of the ligand field symmetry.[17-19] Thus modifications of the Mn^{II} diamagnetic environment (water and ligand molecules) lead to variation in the electron spin relaxation times. Each paramagnetic species contributes to the overall parameters, namely intensity and linewidth, of the e.p.r. spectrum.[20] This spectrum results from the sum or the average of such contributions, depending on conditions of slow or rapid exchange, whenever dynamic equilibrium among the various complexes is present. This situation has been previously described in other papers.[20-23]

Briefly equilibrium (1) applies in the case where only

$$\underset{(A)}{[Mn(H_2O)_6]^{2+}} + [ligand]^{n-} \underset{\alpha_1}{\rightleftharpoons} \underset{(B)}{[Mn(H_2O)_6;\ ligand]^{-(n-2)}} \underset{\alpha_2}{\rightleftharpoons} \underset{(C)}{[Mn\text{–}ligand]^{-(n-2)}} \quad (1)$$

free ions (A), ion-pairs (B) (outer-sphere complexes), and inner-sphere complexes (C) are present.

In this situation the equilibrium constants, α_1 and α_2, can be easily calculated from e.p.r. intensities and linewidths.[20-23] In fact if a ligand molecule enters the first solvation sphere (unsymmetrical co-ordination), the effect is a distortion of the cubic symmetry and a large ZFS is induced, giving rise to undetectably broad e.p.r. spectra.[20] Due to the relatively slow rate of exchange in the first co-ordination sphere (k 10^7 s^{-1}) with respect to the reciprocal of the electron spin relaxation time (10^9 s^{-1}), the inner species will contribute separately to the e.p.r. signal and the result will be a decrease of intensity.

On the other hand ligand and solvent fluctuations in the second solvation sphere are expected to induce ZFS values of a lower order of magnitude, as a consequence of small temporary distortions of the solvation shell around the metal ion.[20] Thus the fluctuation of the ZFS tensor is associated with the dynamics in the outer co-ordination sphere and line broadening is due to outer-sphere interaction. If rapid exchange conditions exist between the free ion and the ion pair, the experimental linewidth is a linear function of the ion pair mole fraction, x, between the value of the free ion linewidth and the value of the ion pair linewidth [equation (2)].

$$\Delta H_{exp} = (1 - x)\Delta H_{free\text{-}ion} + x\Delta H_{ion\text{-}pair} \quad (2)$$

The nuclear relaxation times of the aqueous protons are directly related to the above conditions. They result from average of the characteristic values of bulk and bound water molecules. Moreover the proton relaxation times of the bound water change from one paramagnetic species to another.[24]

In fact, Mn^{II} drastically shortens the proton spin relaxation times in aqueous solution through proton–electron spin dipole–dipole interaction and through isotropic proton–electron spin exchange. The transverse spin relaxation time of protons within the co-ordination shell of Mn^{II}, T_{2M}, is given by the simplified Solomon–Bloembergen equation (3) where r is the distance between the electron

$$1/T_{2M} = \frac{1}{15} S(S+1)\left[\frac{g^2\beta^2 g_N{}^2\beta_N{}^2}{\hbar^2 r^6}\right]\left[7\tau_c + \frac{13\tau_c}{1+\omega_s{}^2\tau_c{}^2}\right] + \frac{1}{3}\frac{S(S+1)a^2}{\hbar^2}\left(\tau_e + \frac{\tau_e}{1+\omega_s{}^2\tau_e{}^2}\right) \quad (3)$$

and the nuclear spin; ω_s is the electron Larmor angular precession frequency, and a is the spin exchange coupling constant. τ_c and τ_e represent the correlation times for dipolar and exchange interactions respectively. They result, *via* several mechanisms, from the contributions (4) and (5) where τ_s is the electron spin relaxation time,

$$\frac{1}{\tau_c} = \frac{1}{\tau_s} + \frac{1}{\tau_h} + \frac{1}{\tau_r} \quad (4)$$

$$\frac{1}{\tau_e} = \frac{1}{\tau_s} + \frac{1}{\tau_h} \quad (5)$$

deducible directly from Mn^{II} e.p.r. linewidth (*ca.* 10^{-9} s), τ_h is the mean time for which a proton remains in the solvation sphere (*ca.* 10^{-8} s), and τ_r is the rotational correlation time for the Brownian motion of the complex (*ca.* 10^{-11} s). The effective correlation times, τ_c and τ_e, are determined by the interplay of these mechanisms, whichever is the shortest.

If water molecules exchange rapidly between the bulk solution and the co-ordination sphere, the experimental $1/T_2$ is the weighted average of $1/T_{2w}$, due to bulk protons and $1/T_{2M}$ according to the equation (6) where n is the

$$\frac{1}{T_2} = \left(1 - \Sigma_i \frac{N_i n_i}{N_w}\right)\frac{1}{T_{2w}} + \Sigma_i \frac{N_i n_i}{N_w}\frac{1}{T_{2M_i}} \quad (6)$$

number of water molecules co-ordinated to each Mn^{II}, and N and N_w are the molar concentrations of manganous ion and water respectively. The first term in equation (6) is usually negligible. Since each complex has its own e.p.r. linewidth, *i.e.* its own τ_s, the second term contains one contribution from each individual paramagnetic species present in solution.

EXPERIMENTAL

The source of manganese(II) ion was $Mn(ClO_4)_2,6H_2O$ (Merck), in order to minimize anionic complexation. Each 7×10^{-3}M-Mn solution for e.p.r. and n.m.r. measurements was freshly prepared from degassed, glass-distilled water. The pH values of the solution were measured with a Metrohm model E-388 potentiometer and the readings were corrected according to an equation given by Childs and Perrin:[15] $p[H] = pH - \log f$ ($f = 0{\cdot}75$). All solutions were 0·15M in potassium nitrate. For calculations the pK_a values for amino-acids, at 37° in 0·15M-KNO_3, were taken from Table 2 of ref. 15. Complex concentrations were

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calculated by means of simple programs on an Olivetti model 101 system. Merck 99% glycine, alanine, and valine, and B.D.H. proline were used without further purification.

E.s.r. spectra were obtained with a Varian V-4502 X band spectrometer with a dual-sample cavity for linewidth calibration and signal intensity measurements. The first cavity was modulated at 1000 kHz and the second one at 400 Hz. Fremy's salt was used as the reference standard. Flat sample cells for aqueous solution or U-shaped capillaries were used to avoid interaction with the electric field. The temperature was varied with a Varian temperature controller model E-4557. The temperature of the heating nitrogen stream was measured with a copper–constantan thermocouple, with an accuracy of $\pm 1°$.

The linewidth parameter ΔH was evaluated as the peak-to-peak distance of the first derivative spectrum. The fourth line from the low field side of the manganese(II) ion spectrum was chosen for ΔH measurements, since this line does not present uneven broadening due to fine splitting.[25] When overlapping of the hyperfine components was present, comparison was made with computer simulated spectra, as described in ref. 25. At the concentration of manganese(II) ion used in the e.p.r. experiments the dipolar and spin exchange intermolecular effects were

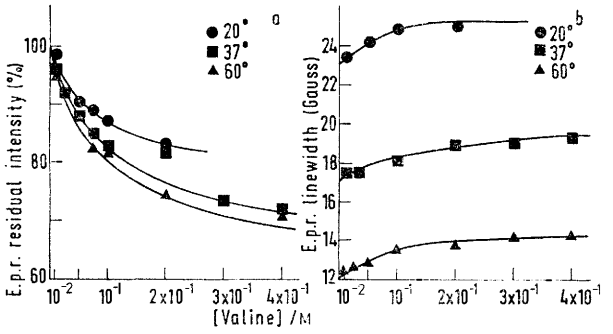

FIGURE 1 E.p.r. parameters of aqueous 0·007M-Mn^{+2} against valine concentration at pH 6: a, e.p.r. residual signal intensity; b, experimental e.p.r. linewidths

negligible. The intensities were calculated by the known relation $\Delta H^2 h$, where ΔH is the experimental linewidth and h is the peak-to-peak height of the derived signal.

^{1}H N.m.r. spectra were taken with a Varian A-56-60 high resolution spectrometer operating at 60 MHz, equipped with a Varian model V-60-40 variable temperature assembly, N.m.r. linewidths were evaluated at half-height of the absorption signal. Care was taken to obtain symmetrical curves and to avoid saturation.

RESULTS

The first series of experimental results derives from e.p.r. spectra of Mn^{II} in water, in the presence of different amino-acids as ligands. Two parameters have been taken into account: the signal intensity and the electron spin relaxation time. These two parameters have been measured by varying, in turn, the temperature, the ligand concentration, and the pH of the solutions.

Figure 1 shows the behaviour of the Mn^{II} e.p.r. linewidth and of the residual absorption intensity percentage (I) of the original Mn^{II} intensity as a function of ligand concentration for valine complexes.

Many results were obtained at the biological temperature, 37°. Mn^{II} Intensity at pH 5·6 is plotted against glycine concentration in Figure 2a, at four different temperatures,

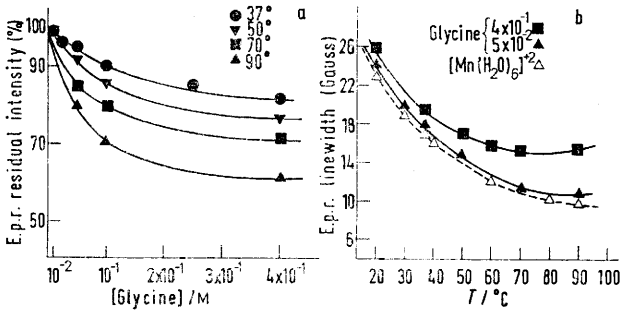

FIGURE 2 a, E.p.r. residual signal intensity of aqueous 0·007M-Mn^{+2} against glycine concentration at pH 5·6; b, experimental e.p.r. linewidths of 0·007M-Mn^{+2} against temperature

as an example of equilibrium dependence on temperature whereas Figure 2b refers to the temperature dependence of the Mn^{II} electron spin relaxation time in the case of the glycine complex. Proline and α-alanine behave in a very similar fashion.

It is worth noting that the curves of ΔH against temperature in Figure 2b differ remarkably from the curve due to the free ion (dotted line) and that e.p.r. data are sensitive to the dependence of equilibria on temperature (Figure 2a). Furthermore, as expected for amino-acids, the complexation is greater at higher pH.

Figures 3a and b display the decreases of the n.m.r. linewidth of water protons with increasing ligand concentration, for valine, glycine, and α-alanine at various pH values and temperatures. The 100% value refers to the

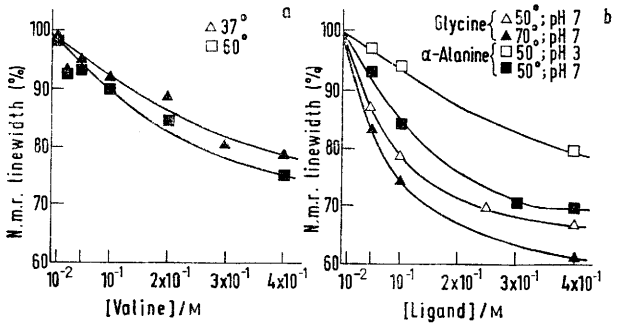

FIGURE 3 N.m.r. parameters of aqueous 0·007M-Mn^{+2} against ligand concentration: a, at pH 6; b, at various pH values

linewidth of water protons in the presence of free manganese(II) ion.

DISCUSSION

It seems useful to compare these experimental results with other data available in previous papers. As far as we know, the only magnetic resonance approach to this study is due to the pioneer work of Cohn and Townsend,[26] who studied similar systems, namely histidine and glycylglycine complexes. Since their

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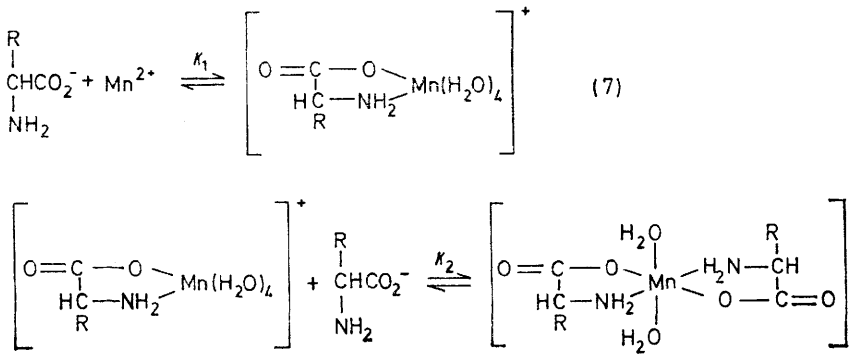
[26] M. Cohn and J. Townsend, *Nature*, 1954, **173**, 1050.

dissociation constants, in the form $K = [Mn^{+2}][L]/[MnL]$, where [L] stands for ligand concentration, do not discriminate either between the anionic and zwitterionic form of the amino-acid or between the various species of complexes, it is not possible to make a direct comparison.

On the basis of pH measurements, Monk [9] and Kroll [10] suggest the formation of bidentate complexes according to the stepwise process (7). These authors reach the conclusion that the predominant complex is



(7)

the 1 : 1 combination; and accordingly give only K_1 values, which are 700 and $2{\cdot}2 \times 10^3$ for glycine– and proline–Mn^{II} respectively.[10] We have performed K_1 calculations based on the data reported in Figures 1 and 2, assuming that the bidentate monoanionic species is the only complex present. These calculations produce misleading conclusions, in that the K_1 values are not constant, and depend upon concentration. Agreement with the results of Monk and Kroll is confined to a narrow amino-acid concentration range. Thus an equilibrium of the type suggested by Monk and Kroll does not explain the experimental e.p.r. data, and the Mn–anion complex MnL is not sufficient to account for the complex concentration. Furthermore, the remarkable decrease of the e.p.r. intensity even at isoionic or acid pH strongly suggests that the zwitterionic form of the amino-acid is involved in complex formation.

$\bar{n}$ functions, the stability constants related to equilibria (8), where L and HL are the anionic and zwitterionic

$$Mn + L \overset{K_1}{\rightleftharpoons} MnL;\quad Mn + 2L \overset{\beta_2}{\rightleftharpoons} MnL_2;$$
$$Mn + HL \overset{K_1'}{\rightleftharpoons} Mn(HL);$$
$$MnL + HL \overset{K_2'}{\rightleftharpoons} MnL(HL) \qquad (8)$$

forms of the amino-acid and the notation of Childs and Perrin is used for the equilibrium constants. E.p.r. and n.m.r. relaxation rate data do not allow the calculation of the above constants, since these methods do not distinguish among different types of inner-sphere complexes. Also, equilibrium (1) is not sufficient to describe the system in this case, and the usual calculation [21,22] of α_1 and α_2 is misleading whenever various inner-sphere species are present, as in the equilibria (8). However it is possible to make a quantitative comparison between our results and those of Childs and Perrin. Table 1 shows this comparison in the case of valine. The relative amounts of the major species present in solution [namely MnL, Mn(HL), MnL_2, MnL(HL)], calculated on the basis of the values of K_1, K_1', β_2, and K_2' given in ref. 15, are reported together with the residual intensity I% and the total amino-acid concentration [AA]. From a comparison of the e.p.r. intensity decrease and the summation of the four

TABLE 1

Mn(II)–Valine complexes. Both experimental and calculated data refer to aqueous 0·007M–Mn^{+2} at pH 6 and 37 °C

[AA]	I% residual	[Mn] free ion	[L]/ M	[Mn]/ M	[MnL]/ M	[Mn(HL)]/ M	[MnL(HL)]/ M	[MnL_2]/ M	Σ Complex %
10^{-2}	96	88·2	$4{\cdot}4 \times 10^{-6}$	$6{\cdot}15 \times 10^{-3}$	$5{\cdot}9 \times 10^{-6}$	$8{\cdot}2 \times 10^{-4}$	$6{\cdot}4 \times 10^{-7}$	$1{\cdot}1 \times 10^{9}$	11·8
$2{\cdot}5 \times 10^{-2}$	91·8	75	$1{\cdot}12 \times 10^{5}$	$5{\cdot}26 \times 10^{-3}$	$1{\cdot}27 \times 10^{-5}$	$1{\cdot}76 \times 10^{-3}$	$3{\cdot}44 \times 10^{-6}$	$6{\cdot}16 \times 10^{-9}$	25
5×10^{-2}	88·2	59·2	$2{\cdot}4 \times 10^{-5}$	$4{\cdot}11 \times 10^{-3}$	$2{\cdot}2 \times 10^{-5}$	$2{\cdot}81 \times 10^{-3}$	$1{\cdot}2 \times 10^{-5}$	2×10^{-8}	40·8
$7{\cdot}5 \times 10^{-2}$	85	49	$3{\cdot}44 \times 10^{-5}$	$3{\cdot}45 \times 10^{-3}$	$2{\cdot}57 \times 10^{-5}$	$3{\cdot}56 \times 10^{-3}$	$2{\cdot}14 \times 10^{-5}$	$3{\cdot}81 \times 10^{-8}$	51
10^{-1}	82·7	41·6	$4{\cdot}4 \times 10^{-5}$	$2{\cdot}9 \times 10^{3}$	$2{\cdot}7 \times 10^{-5}$	4×10^{-3}	3×10^{-5}	$5{\cdot}2 \times 10^{-8}$	58·4
2×10^{-1}	82	27	$9{\cdot}18 \times 10^{-5}$	$1{\cdot}85 \times 10^{-3}$	$3{\cdot}68 \times 10^{-5}$	$5{\cdot}09 \times 10^{-3}$	$8{\cdot}17 \times 10^{-5}$	$1{\cdot}45 \times 10^{-7}$	73
3×10^{-1}	73·3	18	$1{\cdot}42 \times 10^{-4}$	$1{\cdot}3 \times 10^{-3}$	$4{\cdot}06 \times 10^{-5}$	$5{\cdot}5 \times 10^{-3}$	$1{\cdot}39 \times 10^{-4}$	$2{\cdot}48 \times 10^{-7}$	82
4×10^{-1}	71	14	$1{\cdot}8 \times 10^{-4}$	$1{\cdot}04 \times 10^{-3}$	$4{\cdot}26 \times 10^{-5}$	$5{\cdot}8 \times 10^{-3}$	$1{\cdot}9 \times 10^{-4}$	$3{\cdot}46 \times 10^{-7}$	86

In the light of the above considerations the more recent results of Childs and Perrin,[15] obtained by means of pH titrations, seem to be the most valid data available. They calculated, applying the SCOGS computer program and using mathematical methods based on major complex species, it appears evident that disagreement exists and increases with increasing ligand concentration. Proline and glycine give rise to similar values. Calculations have also been carried out using the maximum and minimum values given in ref. 15

for the stability constants, without appreciable changes in the Σ (complex %). This can be explained by the fact that the e.p.r. intensity decrease is a measurement of inner complex concentration, whereas the calculation carried out by Childs and Perrin do not distinguish between outer and inner complexation. All the above considerations enable us to suggest the existence of an outer-sphere complex, which can be further justified on the basis of the following points: (*a*) the broadening of the e.p.r. line with ligand concentration; (*b*) the tendency to a limit, and not to zero, of the residual I%; (*c*) the concentration of the outer-sphere species is expected to increase with increasing ligand concentration, as does the difference between the I% decrease and the Σ (complex %).

The presence of an ion pair is to be expected in ionic aqueous solutions, in which the cation largely retains its full solvation sphere and the anion (the anionic form of the amino-acid in this case) has the effect of distorting the ligand field symmetry, if co-ordinated in the second solvation sphere.

An exact calculation of the linewidth characteristic of the ion-pair from equation (2) is not possible, since values of α_1, α_2, and x are not known. However a limit for the $\Delta H_{\text{ion-pair}}$ has been roughly evaluated from graphic extrapolation. Table 2 reports the $\Delta H_{\text{ion-pair}}$ values together with the molar fraction x, calculated from equation (2).

As outlined in the theory section, the proton magnetic resonance relaxation rates are related to the stepwise equilibrium, through equation (6), and to the electron spin relaxation time *via* the correlation time τ_s. The hyperfine term is the dominant one in equation (3) and is determined by the shortest correlation time, *i.e.* the electron spin relaxation time τ_s.

τ_s Is of the order of magnitude of 10^{-9} s for both the free ion and the ion pair, whereas it is $<10^{-10}$ s for the complex species.[24] According to these values the inner-sphere complex contribution to $1/T_2$ is negligible and we can therefore deduce that the n.m.r. linewidth of protons in water is essentially determined by the terms due to $[Mn(H_2O)_6]^{2+}$ and $[Mn(H_2O)_6$; ligand]. Since the e.p.r. residual signal intensity is also attributed only to the free ion and the ion pair, the n.m.r. linewidths are expected to narrow with increasing concentration proportionally to the decrease of the e.p.r. I%

TABLE 2

Molar fraction of the outer-sphere complex (x); experimental e.p.r. linewidths (ΔH) and outer-sphere complex molar concentration [ion-pair]

Valine			Proline		
T = 60°			T = 37°		
ΔH free-ion = 12 G			ΔH free-ion = 17 G		
ΔH ion-pair = 15 G			ΔH ion-pair = 20 G		
x	ΔH/G	[Ion-pair]/M	x	ΔH/G	[Ion-pair]/M
0·15	12·4	$8{\cdot}6 \times 10^{-4}$	0·03	17·1	$2{\cdot}1 \times 10^{-4}$
0·20	12·6	$1{\cdot}2 \times 10^{-3}$	0·10	17·3	$6{\cdot}6 \times 10^{-4}$
0·28	12·9	$1{\cdot}7 \times 10^{-3}$	0·17	17·5	$7{\cdot}7 \times 10^{-4}$
0·50	13·5	$2{\cdot}8 \times 10^{-3}$	0·43	18·3	$2{\cdot}7 \times 10^{-3}$
0·57	13·7	$3{\cdot}0 \times 10^{-3}$	0·47	18·4	$2{\cdot}8 \times 10^{-3}$
0·70	14·1	$3{\cdot}5 \times 10^{-3}$	0·63	18·9	$3{\cdot}6 \times 10^{-3}$
0·77	14·3	$3{\cdot}8 \times 10^{-3}$	0·93	19·8	$5{\cdot}2 \times 10^{-3}$

curves. This fact is experimentally confirmed (Figures 3a and b).

Bearing in mind the deviations possibly due to a small contribution caused by inner-sphere complexes, the agreement between e.p.r. and n.m.r. data is fairly good.

E.p.r. seems to be a suitable method in studying dynamic situations in aqueous solution of complexes, since it distinguishes between outer and inner co-ordination, and between conditions of slow and rapid exchange. Furthermore e.p.r. measurements of Mn^{II} on the one hand and n.m.r. measurements of water proton relaxation times on the other permit an independent check of the equilibria under study.

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