ENVIRONMENTAL FACTORS INFLUENCING THE HYPERFINE STRUCTURE OF MANGANOUS LOW-TEMPERATURE ELECTRON PARAMAGNETIC RESONANCE SPECTRA

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ABSTRACT Hyperfine structure is observed in low temperature ($T = -180°C$) EPR (electron paramagnetic resonance) spectra of a number of solutions containing Mn^{++} ions 13, 15) which have characteristics in common with low temperature EPR spectra from biological substances such as mitochondria and microsomes (1–4). This investigation is an attempt to understand the features of these signals in terms of the molecular environment of the manganous ion, and a qualitative explanation for the observations reported here is advanced in terms of the amount of axial distortion of a manganese hydrate in different environments.

INTRODUCTION

It has been shown that manganese has a characteristic and consistent distribution, and can assume an important functional role in biological systems (5–8). An understanding of the EPR (electron paramagnetic resonance) spectra from Mn^{++} ion in biological environment (1–4) is therefore very desirable. To approach this problem, however, necessitates the use of model systems.

Previous work (9–11) has demonstrated that the EPR signal from some samples containing Mn^{++} ion have features that depend quite strongly upon the ion's environment. A good basis for models of biological systems, i.e. $MnCl_2$ in water, however, exhibits only a broad structureless line in its EPR spectrum at low temperatures (where most biological EPR spectra are recorded). Some approximate information only may be derived from this signal (12). Addition of substances (13, 15) to an aqueous solution of $MnCl_2$, such as HCl results in the appearance of a hyperfine structure in the low temperature EPR spectrum, the features of which appear to be quite labile to solution conditions.

In this paper, the EPR spectra from various frozen solutions containing Mn^{++} ion are presented, and a discussion is made with a view to understanding the

molecular conditions affecting them. It is hoped that such interpretation as is made will help in the elucidation of the EPR spectra from the Mn^{++} ion in biological environments.

EXPERIMENTAL PROCEDURES

All water was doubly distilled and then passed through a Barnstead standard mixed bed ion exchange column; the conductivity was then less than 1 μmho. All articles of glassware were thoroughly rinsed in water which has been doubly distilled and deionized. PH was checked against Coleman pH standards, and acids and bases used in all experiments were American Chemical Society reagent grade.

The $MnSO_4$ and KCl were obtained from Mallinckrodt Chemical Works. Sucrose and $MnCl_2$ were supplied as a reagent grade from Baker and Adamson. Methanol and ethanol were purchased from Matheson, Coleman and Bell Co. Protamine sulfate (salmine) was obtained from Nutritional Biochemicals Corporation, Cleveland. Bovine albumin and ovalbumin were purchased from Mann Research Labs, New York. Cytochrome *c* and purified yeast RNA were supplied by Sigma Chemical Company, St. Louis, Missouri. N, n-dimethylformamide was obtained from Eastman Organic Chemicals, Rochester, New York, and β-lactoglobulin was obtained from the Bureau of Agricultural and Industrial Chemistry, Philadelphia.

In the experiments involving $MnCl_2$ in concentrated HCl, the manganous salt $MnCl_2 \cdot 4H\ O$ was dissolved directly into the acid to make a 10^{-3}M solution; similarly, $MnSo_4 \cdot H_2O$ and $Mn_3(PO_4) \cdot 7H_2O$ were dissolved into concentrated H_2SO_4 and H_3PO_4, respectively. For experiments in which the Cl^-/Mn^{++} ratio was varied, an aqueous solution of 2×10^{-3}M $MnCl_2$ was prepared and mixed with an equal volume of HCl or KCl of the appropriate concentration.

The phosphoric acid titration curve was established by measuring the pH of 900 μmole of H_3PO_4 before and during the stepwise addition of 2700 μmole of NaOH. A final volume of 1.0 ml base was added to a starting volume of 10.0 ml H_3PO_4. This procedure was repeated for samples containing small amounts of $Mn_3(PO_4)_2$, and small volumes were removed at various points in the titration for EPR study.

For experiments where the effect of organic solvents was studied, an aqueous solution of 10^{-3}M $MnCl_2$ was mixed in varying proportions with solutions of 10^{-3}M $MnCl_2$ solutions in methanol, ethanol, or n, n-dimethylformamide.

In the protein experiments, a 2×10^{-3}M aqueous solution of $MnCl_2$ was mixed in equal volume with a protein solution of appropriate concentration.

To observe the EPR spectra, a Varian V-4500 EPR spectrometer (Varian Associates, Palo Alto, California) with 100 kc/sec field modulation was used. Klystron frequencies were determined with a Hewlett-Packard K-532B frequency meter (236 East 75th St., New York), and magnetic field strength were determined with a Hewlett-Packard 524C frequency counter in conjunction with a Varian F-8 fluxmeter. For low temperature spectra, a Varian V-4547 variable temperature attachment were used. All spectra shown are the first derivative of the actual absorption curve.

To allow a rough comparison between the intensities of the different EPR spectra, a magnification factor (M.F.) is introduced in the results. This is merely the product of the gain and modulation settings of the Varian machine used in the experiments, the recorded intensity of a given signal increasing in a fairly linear fashion with the value of this product in the absence of modulation broadening of the lines.

EXPERIMENTAL RESULTS

In the spectra reported, no effect of microwave power saturation of the Zeeman levels, or of modulation broadening of the EPR signal was observed.

Many of the results quote anion/Mn^{++} ratio values. These values are for reference only and refer to the total number of atoms present; they would be true figures only under conditions of complete dissociation. This is not true for high anion (i.e. Cl^-) concentrations, for instance, but, on the other hand, the ratios such as CH_3OH/Mn^{++} are probably quite valid.

Biological Materials. Typical spectra from microsomes such as those mentioned previously (1–4), are shown in Fig. 1. In Fig. 1 *A* is the EPR spectrum of beef liver microsomes, and, in Fig. 1 *B* is the EPR spectrum of the separated fraction after trypsin digestion and emasol treatment.

Inorganic Anions. The EPR spectrum of the Mn^{++} ion in frozen aqueous solution, upon the addition of excess anion has been reported before (13, 15), and here further observations upon this signal are reported.

The basic form of this signal is taken to consist of 6 allowed transitions split by

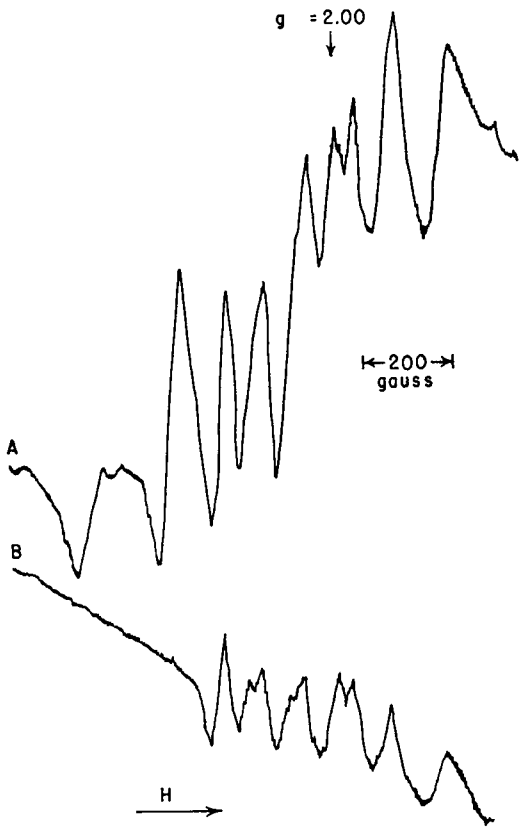

FIGURE 1 Typical low temperature EPR spectra of beef liver microsomes (*A*) and the microsomal manganese aqueous fraction (*B*) after trypsin digestion and emasol solubilization. Protein concentrations are 53.5 and 5.0 mg/ml, respectively. M.F. = 1.6×10^8.

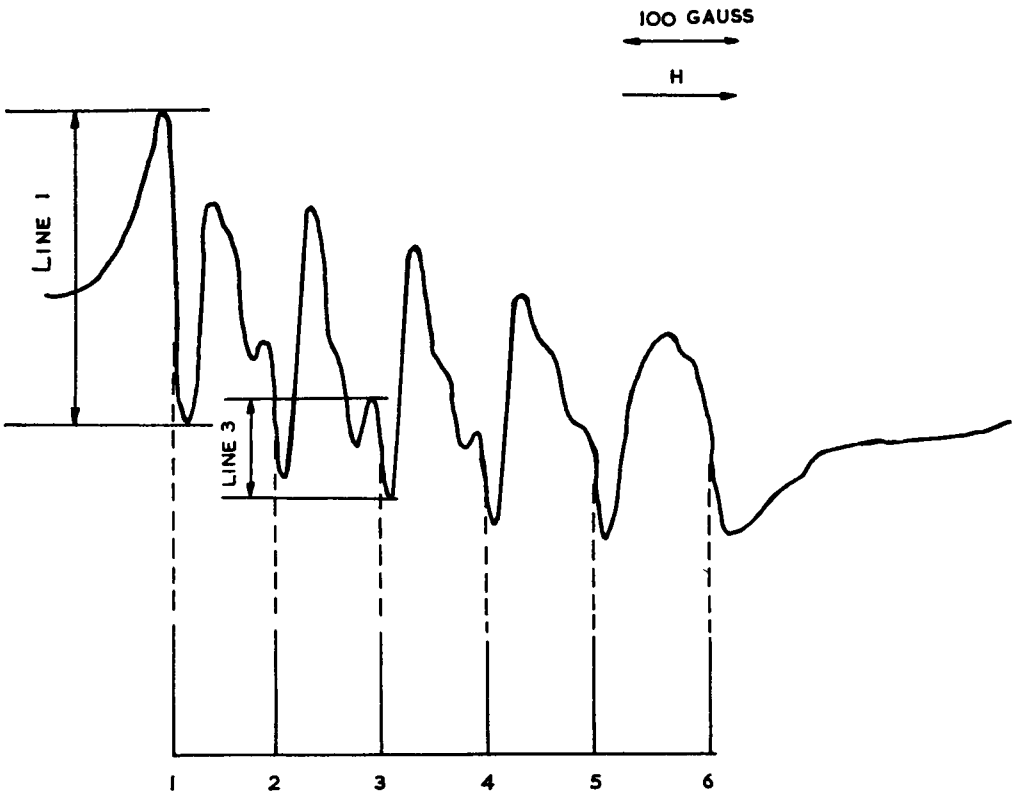

FIGURE 2*a* EPR spectrum of $MnCl_2$ in concentrated HCl at low temperatures. The lines designated 1, 2, ... 6 from low field are allowed transitions centered on g = 2.00, the lines between them are so called forbidden transitions. The line 1/line 3 ratio is the ratio of the lengths of the lines designated line 1 and line 3, in the figure.

90 to 100 gauss and centered upon $g = 2.00$. Between each of these lines occurs a forbidden doublet (not always fully resolved as a doublet) (Fig. 2*a*). Allowed lines correspond to the transitions $M = -\frac{1}{2} \rightarrow +\frac{1}{2}$, for $\Delta m = 0$, and forbidden lines to the transitions $M = -\frac{1}{2} \rightarrow +\frac{1}{2}$, for $\Delta m = \pm 1$, where M is the magnetic quantum number for the 3*d* electrons, a M is the nuclear magnetic quantum number. It is possible to take a measurement from such a spectrum designated the line 1/line 3 ratio (Fig. 2*a*), the significance of which will be discussed later in the paper.

The variation of the line 1/line 3 ratio with CL^-/Mn^{++} ratio, for $MnCl_2$ in HCl is shown in Fig. 2*b*. Addition of a given Cl^- ion concentration to aqueous $MnCl_2$ solution in the form of HCl, LiCl, or NaCl give very similar EPR spectra at the lower Cl^-/Mn^{++} ratios (<1000). In these samples the positive cation (H^+, Li^+, Na^+), appeared to have little influence on the spectra, their appearance in each case depending upon the Cl^-/Mn^{++} ratio.

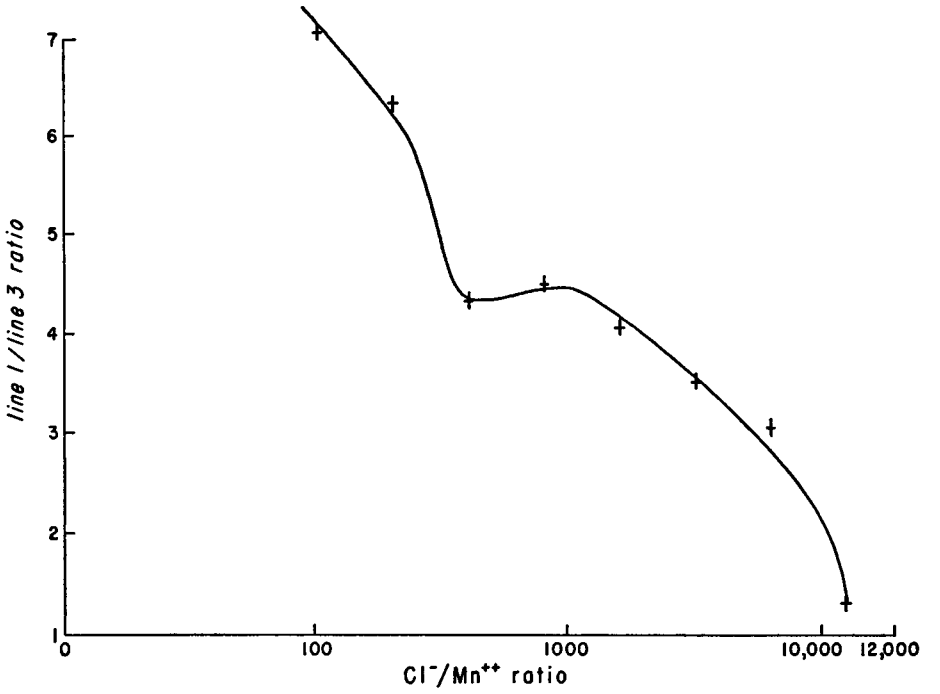

FIGURE 2*b* A plot of the line 1/line 3 ratio, against the CL^-/Mn^{++} ratio, for $10^{-3}M$ $MnCl_2$ in HCl.

For $MnCl_2$ in KCl solutions (Fig. 3), the situation is different. Unlike the HCl, LiCl, and NaCl solution, the hyperfine spectrum appears well resolved at low Cl^-/Mn^{++} ratios, and as this ratio is raised from 100 to 1000, the line 1/line 3 ratio remains constant at about 2 (compare with Fig. 2*b* for HCl). The over-all intensity of this hyperfine spectrum also increases markedly as the Cl^-/Mn^{++} ratio is raised over this range, which is not the case for the smaller anions.

$MnSO_4$ in H_2SO_4 and $Mn_3(PO_4)_2$ in H_3PO_4 also give hyperfine spectra (Fig. 4) of the same basic form described above. In an experiment with a $Mn(PO_4)_2$ solution in H_3PO_4 being titrated against NaOH, the results shown in Figs. 5 and 6 were obtained. Fig. 5 shows the titration curve of a sample containing 50 μmole of $Mn_3(PO_4)_2$ in 900 μmole of H_3PO_4, against NaOH. The shift of this titration curve from that of pure phosphoric acid is probably due to the basic effect of the $Mn_3(PO_4)_2$ when it dissociates. In this figure, the titration points (A, B, C . . .) represent the points where small samples from a titration mixture of 900 μmole of H_3PO_4 containing 5 μmole of $Mn_3(PO_4)_2$ were taken and examined with EPR. The EPR spectra from these samples are shown in Fig. 6, the individual spectra being labeled (A, B, . . .). Together Fig. 5 and 6 suggest that the hyperfine structure remains present as long as $H_2PO_4^-$ exists in the solution.

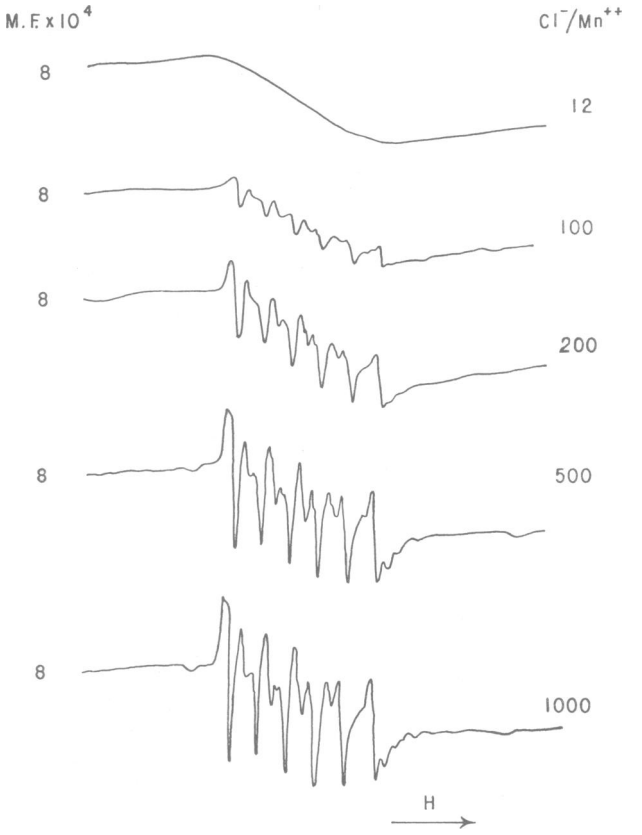

FIGURE 3 EPR spectra of $10^{-3}M$ $MnCl_2$ in varying concentrations of KCl.

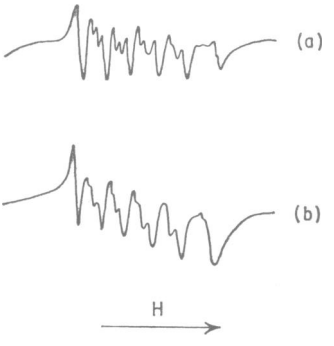

FIGURE 4 (*a*). EPR spectrum of $10^{-3}M$ $MnSO_4$ in concentrated H_2SO_4. (*b*). $10^{-3}M$ $Mn_3(PO_4)_2$ in concentrated H_3PO_4. M.F. $= 4 \times 10^4$.

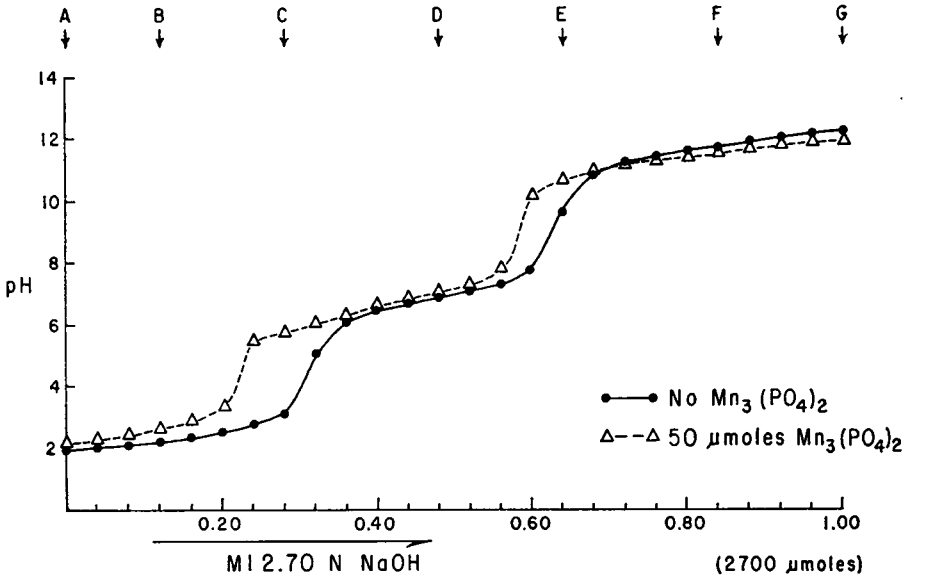

FIGURE 5 Experimental phosphoric acid titration curve with and without the presence of magnanous ion. Arrows designate the points along the titration curve at which samples of the solution were taken for examination by EPR.

Organic Solvents. The addition of small amounts of methanol to aqueous $MnCl_2$ solutions again brings about the appearance of hyperfine structure in the EPR signal as shown in Fig. 7. Increasing the methanol/Mn^{++} ratio above 250 does not appear to improve the resolution of the signal (i.e. decrease the line 1/line 3 ratio). Very similar spectra to these are produced by addition of 1 part in 10 by volume of ethyl alcohol and n, n-dimethylformamide to aqueous 10^{-3}M $MnCl_2$ solutions.

Proteins. Addition of protein to an aqueous $MnCl_2$ solution also produces a hyperfine structure in the EPR spectrum. The resolution of the signal improves as more protein is added, but with these large molecules, as might be expected, the line 1/line 3 ratio does not depend only upon their molarity. In Fig. 8, EPR spectra was taken of Mn^{++} ion containing solutions which also contained 10^{-3} M protein. The interesting point to note here is the increasingly good resolution of the hyperfine structure with the molecular weight of protein. On the basis of this result, an experiment was tried with protein of 3 different mol wt, which were added to $MnCl_2$ solutions in molarities so as to produce roughly equal surface areas of protein in each sample. This was done, using a crude assumption that the protein molecules were all spherical and had the same density. The results shown in Table I demonstrate that the signals, as classified by line 1/line 3 ratios, are all quite similar.

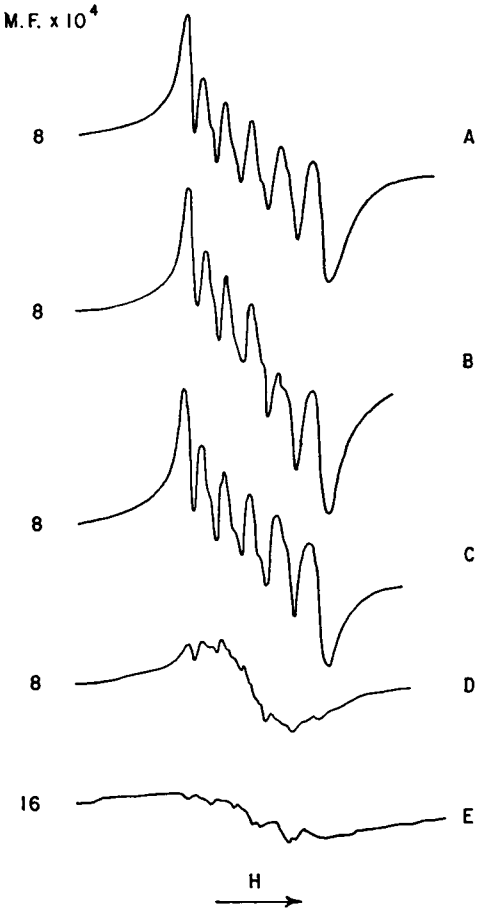

FIGURE 6 EPR spectra of samples taken from the titration experiment. The letters labeling the spectra correspond to the labeled arrows in Fig. 5.

It may be remarked here that the addition of RNA to a $MnCl_2$ solution also produces a well resolved hyperfine structure in the EPR signal.

DISCUSSION

As shown in Fig. 1, once the paramagnetic, hence iron compound, and the free radical content of the microsomal fraction have been separated by trypsin digestion and emasol treatment, an EPR spectrum characteristic of the Mn^{++} ion is seen more clearly (14). The spectrum, *B*, in Fig. 1, however is somewhat more complex than the simple 6 line spectrum observed from the Mn^{++} ion at room temperature, and could well have the basic structure proposed by Allen and Nebert (13) for the signal from Mn^{++} ion in frozen aqueous HCl solutions.

Appearance of hyperfine structure in the low temperature EPR spectrum of aqueous solutions of $MnCl_2$, upon addition of a number of salts and organic liquids

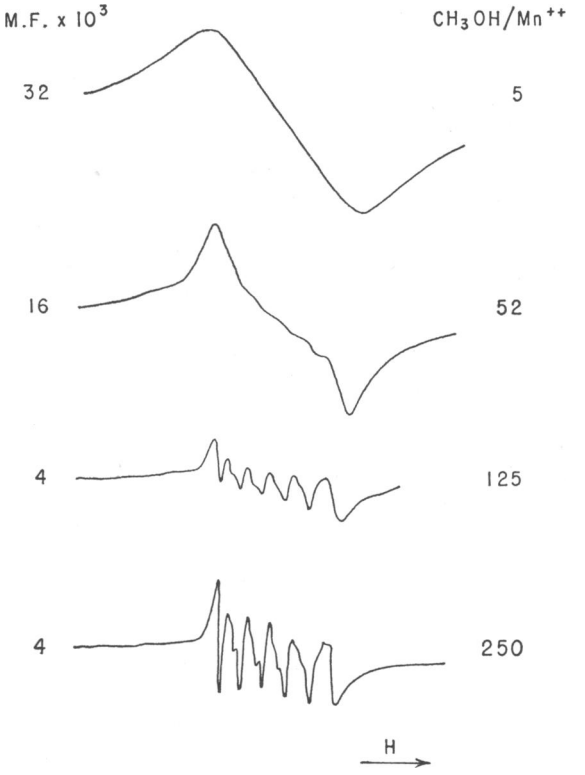

FIGURE 7 The effect of addition of methyl alcohol to an aqueous $10^{-3}M$ solution of $MnCl_2$ upon the EPR spectrum.

has been reported by Ross (15). The resolution of the hyperfine structure in these spectra depended upon the anion/Mn^{++} ratio, and the explanation for the disappearance of this hyperfine structure depended upon the existence of a dipolar broadening term in the spin Hamiltonian for the Mn^{++} ion. However Allen and Nebert (13) proposed that the change in the appearance of the hyperfine spectrum with changing anion/Mn^{++} ratio was due to a change in the magnitude of an axial zero field-splitting term in the spin Hamiltonian for the Mn^{++} ion.

Well resolved spectra from these types of sample can be explained quite well by the effect of an axial zero field-splitting term. The line 1/line 3 ratio has been related quantitatively to such a term for smaller values of this ratio (i.e. < 3) (16), and in general it is possible to say that an increase in this ratio can mean an increase in the zero field-splitting term. For Mn^{++} ion in salt solutions however, when the anion/Mn^{++} ratio is lowered to within the region of a 100, the inner allowed transitions (i.e. 2, 3, 4, 5 in Fig. 2*a*) are not resolved or have diminished to zero, leaving a 6 line hyperfine spectrum, the inner 4 lines of which consist of

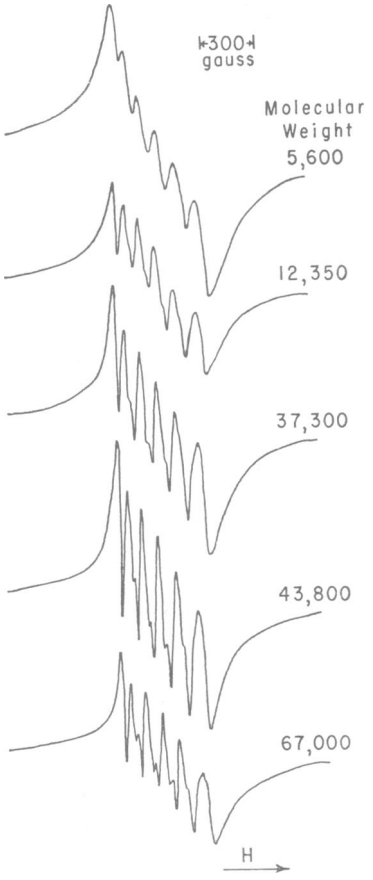

FIGURE 8 EPR spectra of $10^{-3}M$ $MnCl_2$ in aqueous solution containing $10^{-3}M$ protein. The spectra change as the molecular weight of the protein changes. The proteins used in order of increasing molecular weight were: protamine sulfate, cytochrome *c*, lactoglobulin, ovalbumin, and bovine albumin. M. F. $= 4 \times 10^4$.

TABLE I

Protein	Mol wt	Molarity required for equal surface area	Line 1/line 3
Protamine sulfate	5,600	5.3×10^{-3} M	2.7
Cytochrome *c*	12,350	3.1×10^{-3} M	2.7
Bovine albumin	67,000	1.0×10^{-3} M	3.8

unresolved, forbidden doublets. As the anion/Mn^{++} ratio is lowered still further, this hyperfine spectrum broadens and disappears, and the mechanism of this may well be a dipolar broadening effect. Similarly in Fig. 7, the spectrum for a CH_3OH/Mn^{++} ratio of 52 looks very much like a 6 line hyperfine spectrum that has suffered dipolar broadening, although the difference between the spectra for CH_3OH/Mn^{++} ratios of 125 and 250 could be accounted for by the zero field-splitting effect.

We should like to consider those experiments where the zero field-splitting effect appears to predominate, and present an interpretation based upon the relationship between the line 1/line 3 ratio and the axial zero field splitting parameter (16). If we regard the magnitude of the zero field splitting to be related directly to the magnitude of the crystal field acting upon the Mn^{++} ion, we can say that an increase in the line 1/line 3 ratio means an increase in the crystal field strength.

It would then appear that for HCl, LiCl, and NaCl above a Cl^-/Mn^{++} ratio of approximately 100, the effect of excess Cl^- ion is to decrease the magnitude of the crystal field strength acting upon the Mn^{++} ion. This in turn could be regarded as a decrease in an axial distortion of a manganese hydrate. The K^+ salt is unique here in that the strength of the crystal field acting upon a part of the Mn^{++} ion population in the sample is not changed upon raising the Cl^-/Mn^{++} ratio from 100 to 1000, but that the number of ions in this part of the population is increased.

Addition of methyl alcohol causes a decrease in the crystal field strength (or distortion of the manganese hydrate), up to a CH_3OH/Mn^{++} ratio of 250, whereupon additional CH_3OH molecules have no further effect. Also the amount of protein surface area in a sample appears to determine the strength of the crystal field.

CONCLUSIONS

The diversity of conditions giving rise to hyperfine structure in the low temperature EPR spectrum of aqueous solutions containing Mn^{++} ion, lead us to agree with Ross (15), that the species giving rise to this is common in all samples and is probably a hydrate.

Taking the view that the important perturbing effect upon the Mn^{++} ion, is for the cases discussed here, the crystal field of the surrounding water molecules, and that the strength of this is related to the line 1/line 3 ratios observed in the spectra, we would interpret our results in the following way.

The addition of Cl^- ion, H_2SO_4 and $H_2PO_4^-$ to an aqueous solution containing Mn^{++} ion decreases (in the frozen state) the amount of distortion of the manganese hydrate. This may be an indication of how these ions effect the frozen state of water. An increase in protein surface area has a similar effect.

Addition of Cl^- ion as the potassium salt does not decrease the amount of distortion of the manganese hydrate, it does however increase the amount of manganese hydrate in a specific environment.

The fact that complex hyperfine structure can be detected from Mn^{++} ion contained in microsomes (Fig. 1), would infer that a lower zero field-splitting exists here (<200 gauss) (16) than in pure frozen aqueous solutions of $MnCl_2$ (≈ 300 gauss) (12).

The authors would like to thank Professor H. S. Mason for his active interest in the work, and

acknowledge the financial support from the American Cancer Society and the United States Public Health Service.

Received for publication 21 April 1965.

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