MANGANOUS ION AS A SPIN LABEL IN STUDIES OF MITOCHONDRIAL UPTAKE OF MANGANESE

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ABSTRACT Manganous ion (Mn^{2+}) has been used as a spin label for studies of divalent cation uptake by rat liver mitochondria. Spin exchange, observed in the electron paramagnetic resonance (EPR) spectrum of a fraction of the transported Mn^{2+}, shows that this fraction is bound in regions of high local concentration within the mitochondria. The average separation of manganese ions in that fraction is estimated to be 4.0 ±0.6 A at the time of greatest concentration.

INTRODUCTION

Nitroxide radicals have been used as spin labels in biochemical and biophysical experiments for several years (1, 2). Paramagnetic ions have received much less attention as spin labels. In particular, they have almost never been used as spin labels in vivo, probably because the spectra obtained from these ions are often quite broad and complex. Nevertheless, the EPR spectrum of a paramagnetic ion is a sensitive function of the ion's environment and, consequently, these ions can be good spin "probes." This is particularly true in membrane studies where one is interested in either divalent cation transport or the influence of these ions on membrane structure.

Mn^{2+} is known to be a good analogue of Ca^{2+} or Mg^{2+} in a variety of biological systems (3–6). This is undoubtedly related to its spherically symmetric charge distribution, and to the fact that its ionic radius of 0.80 A lies between that of Mg^{2+} and Ca^{2+}, 0.65 A and 0.98 A respectively.

One important system in which Mn^{2+} acts as an analogue of Ca^{2+} is the respiration-dependent uptake of divalent cations by mitochondria (3, 4). One might expect the EPR spectrum of Mn^{2+}-treated mitochondria to provide information on the ways in which Mn^{2+} (and probably Ca^{2+}) ions are bound to outer membrane surfaces or to components inside the mitochondria after transport.

From the physical and chemical literature, it is learned that Mn^{2+} in aqueous

solution is in the form of a "hexahydrate" complex of high symmetry (7). The EPR spectrum (shown in Fig. 1) shows six hyperfine lines due to interaction of unpaired manganese electrons with its $I = 5/2$ nuclear spin. These "free" Mn^{2+} lines show a temperature and frequency dependence which allows them to be classified as "relaxation broadened." The line width is determined from the very short time in which the electrons remain in a given state in accordance with the Heisenberg uncertainty relation.

When complexes are formed which disturb the hydration shells around the manganous ions, the relaxation rate is increased and the six hyperfine lines each become broader. This produces EPR spectra similar to the one seen in Fig. 3 *c*. When complex formation breaks up the high symmetry of the six water molecules in the inner hydration shell this "spin lattice relaxation" generally broadens the lines to the point where they are very difficult to see. Relaxation broadening and other mechanisms of broadening have given rise in the literature to reports of "invisible lines" (8).

A physical mechanism also exists by which a certain class of line (inhomogeneously broadened) may be narrowed. This mechanism, "exchange narrowing," may in the extreme case narrow the set of lines to a width much narrower than the hyperfine envelope of the free manganese lines (See Fig. 3 *e*). This spin exchange phenomenon occurs only when the paramagnetic ions are so closely packed that overlap of electronic orbitals occurs. It is a manifestation of magnetic field "averaging" produced by exchange of electrons between different atomic systems; therefore, it is an indication of a high local concentration of paramagnetic ions or molecules.

Because these and other interactions affect the EPR spectra of Mn^{2+} and other paramagnetic ions, spin-labeling studies provide several types of information about the ionic environment.

The extremely high sensitivity of EPR makes spin labeling possible even with very small amounts of paramagnetic ion. This is a great advantage for experiments performed in vivo.

EXPERIMENTAL PROCEDURE AND RESULTS

The Mn^{2+}–Ethylenediaminetetraacetic Acid (EDTA) Complex

The EPR spectrum of the very broad Mn^{2+}-EDTA chelate at pH 7.3 is shown in Fig. 2. The formation of this strong complex produces quite strong anisotropy in the magnetic resonance spectrum and results in "powder spectra" for samples studied in solution. This terminology refers to cases where all possible orientations of the complex with respect to the external magnetic field are simultaneously observed.

Since the pK for the dissociation of the Mn^{2+}-EDTA chelate is 13.5 (9), chelation is complete for all Mn^{2+} which comes into contact with free EDTA. Hence the sharp six-line spectrum of the hexahydrate complex seen when Mn^{2+} is dissolved in

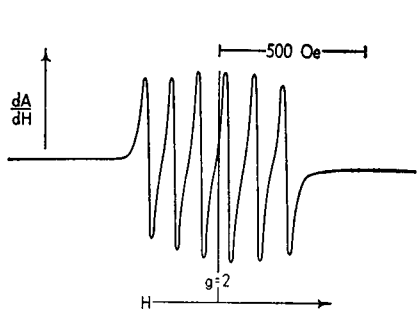

FIGURE 1

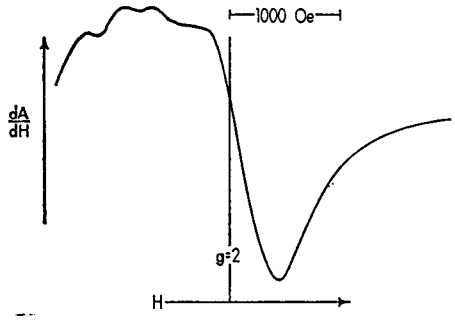

FIGURE 2

FIGURE 1 Derivative of absorption vs. field for 10^{-4} M Mn^{2+} in distilled water.
FIGURE 2 Derivative of absorption vs. field for Mn-EDTA chelate.

water can be entirely eliminated from the spectra of external mitochondrial bathing solutions simply by adding small amounts of EDTA. The spectrum of Mn^{2+} in the bathing solution then changes very quickly to the very broad spectrum of the type seen in Fig. 2 and attention is focused on spectra of Mn^{2+} inside the mitochondria.

Details of Limited Loading Experiments

Mitochondria were prepared in 0.33 M sucrose by a modification of the method of Hogeboom et al. (10). After the final centrifugation, the mitochondrial preparation was suspended in a medium containing 0.33 M sucrose, 5 mM succinate, and 10 mM Tris buffer, pH 7.3. Concentration of mitochondrial protein was determined by the Lowry (11) technique.

EPR spectroscopy was carried out at X band frequencies in a Varian E-12 system (Varian Associates, Palo Alto, Calif.). Quantitative work with solutions was done in a TE 104 mode "dual" cavity system. Samples were held for various experiments in aqueous solution (flat) cells.

In a typical experiment an aliquot of mitochondrial suspension prepared at 0°C was allowed to come to room temperature. Then manganese chloride solution was stirred into the sample in small amounts such that no more than 20 nmoles Mn^{2+}/mg mitochondrial protein were added at one time. This process could be continued until about 200 nmoles Mn^{2+}/mg mitochondrial protein were added in this way. The final density of mitochondrial protein was 5–10 mg/ml. After 10 min time allowed for uptake, a slight excess of EDTA to total Mn^{2+} was added, and the EPR spectrum was taken.

Analysis of Spectra

Fig. 3 *a* shows the EPR spectrum of a sample prepared as described above. Fig. 3 *b* shows the background (a spectrum taken of the sample holder filled with sucrose, succinate, Tris medium). The first step in the analysis was the subtraction of 3 *b* from 3 *a*. The spectrum was then analyzed by computer into the following components:

(*a*) A spectrum made up of six lorentzian lines. For the bulk of the data considered in this paper, the width of these lines was determined to be 52 ±3 oersteds

(Oe) peak-to-peak. (What is meant by "peak-to-peak" line width is shown in Fig. 3 *e*.) This will be referred to as the 52 Oe spectral component and the Mn^{2+} producing it as the 52 Oe fraction. Under conditions of maximum uptake a hyperfine sextet of narrower line width (30–35 Oe) is seen as well.

(*b*) The spectrum of the manganese-EDTA complex.

(*c*) A broad symmetrical line which will be referred to as the spin exchange spectrum. The manganese producing this spectrum will be termed the spin exchange fraction.

Figs. 3 *c*, *d*, and *e* show these respective components of the experimental spectrum 3 *a*. Fig. 3 *f* shows the small fraction of the experimental spectrum left unaccounted for by the computer analysis.

The measurement of absolute number of spins is difficult for a number of reasons. Wyard and Cook (12) and Zimmer et al. (13) discuss many of these reasons and describe how to calculate number of spins. Special problems involved with the use of transition metal ions are treated by Levanon et al. (14).

In the quantitative work reported here, a dual cavity technique similar to that developed at Karlsruhe (15, 16) was used. This allowed Q changes due to sample loading and position to be monitored and corrections made. The standard was manganese chloride dissolved in distilled water. The conditions under which the sample and standard were run were as nearly identical as possble with regard to dielectric constant, sample cells, etc.

The spin exchange spectra were shown by computer analysis to fit lorentzian line shape functions quite well in the limit of greatest exchange narrowing. The total number of spins was obtained by double integration (12) of a lorentzian line of the same width and height as the experimental spectral lines.

Evidence for Exchange

It was determined that the spectrum referred to as the spin exchange spectrum exhibited narrowing to a width much less than that of the over-all hyperfine splitting. Furthermore, as the amount of manganese taken up by the mitochondria increased, the width of this component of the spectrum decreased. This result leads to the firm conclusion that this fraction of the manganese is held in a region of high local manganese concentration (17).

Fig. 4 shows the results of an experiment in which calcium and manganese were added simultaneously to a mitochondrial preparation as described above. The total amount of calcium plus manganese was kept constant but the composition was varied from 33 to 100% manganese. As the amount of manganese increased, the width of the line produced by this fraction of the manganese decreases, demonstrating increased spin exchange and, consequently, a higher local Mn^{2+} concentration.

In Fig. 4 the results of a related study in which the only divalent cation added to the mitochondrial preparation was manganese, are also given. The amount of manganese was varied in the same range as in the calcium-manganese experiment

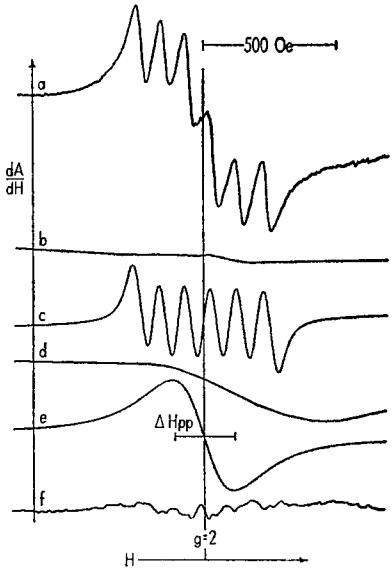

FIGURE 3

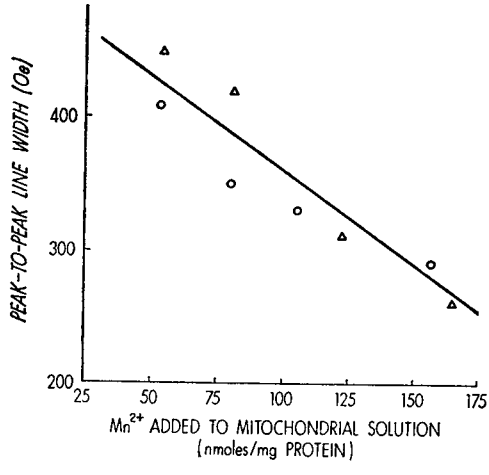

FIGURE 4

FIGURE 3 Mn^{2+}-mitochondria spectral analysis. (*a*) Derivative of absorption vs. field for manganese taken up by mitochondria after treatment of the preparation with a slight excess of EDTA. (*b*) Background spectrum taken with mitochondrial medium in the same sample holder as used in *a*. (*c*) Computer simulation of spectrum of high symmetry complex made up of six lorentzian lines of 52 Oe peak-to-peak line width. (*d*) That fraction of spectrum *a* subtracted out by the computer as being caused by the manganese-EDTA chelate. (*e*) The computer-fit lorentzian line described as the spin exchange fraction. (*f*) The difference in *a* and the sum of *b*, *c*, *d*, and *e*. All spectra are on the same scale. ΔHpp represents the peak-to-peak line width of the exchange spectrum.

FIGURE 4 Peak-to-peak line width vs. total manganese added to each mitochondrial preparation per milligram mitochondrial protein. The line widths plotted here were those representing the best fits of lorentzian lines to the spectral data after the 52 Oe spectrum and Mn-EDTA chelate spectrum had been subtracted off. △, Mn^{2+} added in presence of Ca^{2+}; ○, Mn^{2+} added without Ca^{2+}.

described above. The trend shown in this case, like that of the latter case, shows that the larger the amount of manganese present the narrower the width of the spin exchange type spectrum, again supporting the conclusion that exchange narrowing is observed.

Location of Spin Exchange Fraction

In an effort to establish that the spin exchange fraction and 52 Oe fraction were *inside* the mitochondria, the following experiments were performed.

When mitochondria were treated with Mn^{2+} and then EDTA, the spin exchange spectrum remained visible for 30 min to 1 hr. This demonstrates that the Mn^{2+} in these fractions was held in compartments relatively inaccessible to EDTA. When

samples were treated with detergent, the spin exchange spectrum immediately disappeared; subsequent treatment with EDTA left only the Mn^{2+}-EDTA chelate spectrum. Mitochondria preloaded with Mn^{2+} and then exposed to a burst of Ca^{2+} before treatment with EDTA showed a decrease of the spin exchange component correlated with the magnitude of the Ca^{2+} burst and the time lag before EDTA treatment. Mn^{2+}-loaded mitochondria, twice washed in sucrose medium at 0°C, showed a spin exchange spectrum while nearly all free manganese outside the organelles was removed. The 52 Oe spectrum behaved in essentially the same way as the spin exchange spectrum in each of the above experiments.

Relationship to Active Transport

In order to elucidate the connection between respiration-dependent divalent cation uptake and the existence of the 52 Oe and exchange type signals, a series of transport inhibitors were added before treating the mitochondrial samples with Mn^{2+}. In separate experiments the mitochondria were treated with (*a*) La^{3+}, which is believed to act at the carrier site to prevent divalent cation binding; (*b*) 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation; and (*c*) electron transport inhibitors rotenone and antimycin used simultaneously. Each of these, in sufficient quantity, is known to inhibit or block divalent cation transport (18–21).

The effect of these inhibitors on the spin exchange spectral component and the 52 Oe component was that if enough of any one of them was added, the amount of the spin exchange component and the 52 Oe component was very small or zero. As the amount of inhibitor was increased, the amplitude of both spectral components decreased while the line width of the spin exchange spectrum increased.

The spectra showed just the opposite dependence on substrate (succinate). Samples were prepared as usual but with varying amounts of succinate present. The intensities of the spin exchange and 52 Oe spectra increased from zero to maximum as the succinate concentration was increased from zero to about 5 mM. The Mn^{2+} concentration was 0.6 mM in each sample, corresponding to approximately 200 nmoles Mn^{2+} mg mitochondrial protein. When mitochondria were not present, neither succinate nor the inhibitors, in the concentrations employed in the mitochondrial experiments, modified the hexahydrate spectrum characteristic of manganese in the bathing solution.

These observations, that the spin exchange fraction is inside the mitochondria, that the amount of this fraction observed is greatly affected by inhibitors, and that this fraction shows a succinate requirement, lead to the conclusion that the manganese making up the spin exchange fraction is transported into the mitochondria by the widely discussed respiration-dependent process of manganese and calcium uptake.

Estimation of Mn^{2+} Ion Separation in Spin Exchange Fraction

An estimate of the local separation of Mn^{2+} necessary to show spin exchange to the extent observed in mitochondria under limited loading conditions can be made

with the aid of Fig. 5. Here the peak-to-peak line width of the manganese hyperfine envelope, as affected by spin exchange, is plotted vs. manganese ion separation. This separation is taken as nearest manganese-manganese spacing in the case of crystalline samples (calculated from crystallographic data). For amorphous and liquid samples an "average" separation is calculated by assuming a simple cubic geometry for the manganese and using known values of density and molecular weight.

The minimum line width of the spin exchange spectral component was found to be around 200 Oe. The minimum average separation of neighboring manganese ions for the mitochondrial spin exchange fraction is then estimated from Fig. 5 to be 4.0 ±0.6 A.

Measurement of Amount of Mn^{2+} Taken Up

Several spectra similar to that shown in Fig. 3, where uptake of manganese under limited loading conditions was greatest, were analyzed. The number of nanomoles of Mn^{2+} taken up in the spin exchange fraction was calculated from the height and width of the lorentzian curve which best fit the spin exchange fraction. Each of these analyses yielded an uptake in the range of 60–80 nmoles Mn^{2+}/mg protein. The error in measurement is estimated to be ±25%.

Release of Manganese

When samples were left in the oxygen-deprived environment of the liquid flat cell for 20–30 min, rapid release of Mn^{2+} then ensued. In the absence of EDTA the release appeared as an increase in the six-line Mn^{2+} hexahydrate spectrum. In the presence of EDTA the release led in time to the disappearance of the spin exchange spectrum and the 52 Oe spectrum with the concomitant growth of the Mn^{2+}-EDTA complex signal.

Massive Loading Conditions

Uptake of Mn^{2+} was followed by EPR in media containing phosphate buffer in place of Tris buffer. As is well known from the literature (19), large amounts of Mn^{2+} or Ca^{2+} per milligram of mitochondrial protein can be taken up under these conditions.

Amounts of Mn^{2+} were added to phosphate-buffered mitochondrial suspensions covering the range of a few hundred nanomoles per milligram to approximately 3000 nanomoles/mg mitochondrial protein. The suspension was subjected to low speed centrifugation in order to remove any external precipitate. Again a spin exchange type spectrum was observed. This spectrum was very intense in cases where large amounts of manganese were taken up.

Several differences were noted in the massive loading case from what was seen under limited loading conditions:

(*a*) The total uptake of manganese into the spin exchange fraction was much

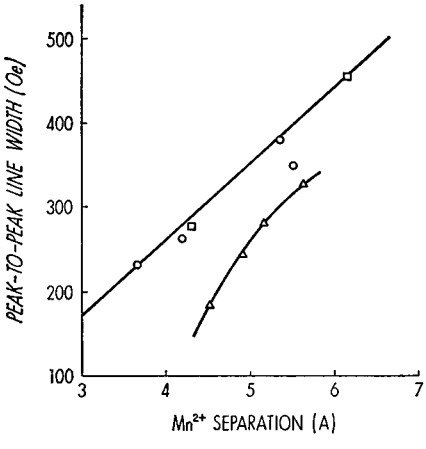

FIGURE 5

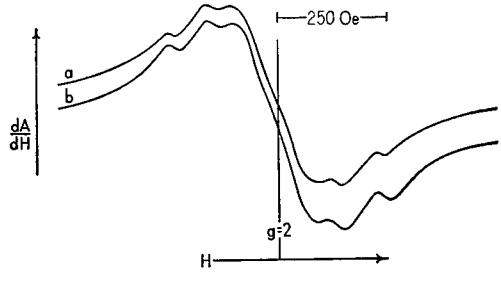

FIGURE 6

FIGURE 5 Peak-to-peak line width across the hyperfine envelope vs. manganous ion separation for a number of manganese salts and for very concentrated manganese aqueous solutions. For the crystalline salts the crystallographic separation of the nearest manganese neighbors to a given manganous ion was determined and plotted. For the aqueous solution data and amorphous salts a simple cubic array was assumed and an "average" separation determined from the molecular weight and density. The salts represented above are manganous chloride, fluoride, phosphate, carbonate, formate, and hypophosphite. ○, polycrystalline powders; □, amorphous powders; △, aqueous solutions (22).

FIGURE 6 (*a*) Derivative of absorption vs. field for a precipitate formed in a concentrated manganese chloride, sodium phosphate solution at pH 7.4. The precipitate was not "washed" and the weak free Mn^{2+} lines seen here are those of the usual Mn^{2+} hexahydrate complex formed in aqueous solution. (*b*) Derivative of absorption vs. field for a mitochondrial preparation after massive loading with Mn^{2+} in phosphate buffer. This spectrum is taken on a mitochondrial preparation which had been washed twice in phosphate-free mitochondrial medium to remove any manganese phosphate which might form outside the mitochondria. (Note: manganese phosphate which forms in solution may be pulled out of suspension much more quickly with a centrifuge than the massive loaded mitochondria.)

greater in this case, up to 2600 nmoles/mg of protein as compared with a maximum of the order of 100 nmoles/mg in the limited loading case.

(*b*) The peak-to-peak line width was always about 280 Oe, while in the limited loading case the line width went as low as 200 Oe. More important, the line width did not vary with the amount of manganese taken up. No variation would be expected from a simple precipitate, and indeed the spin exchange spectrum observed in this case is indistinguishable from a precipitate formed in a concentrated manganese chloride–sodium phosphate solution at pH 7.4. The two types of spectra are compared in Fig. 6.

(*c*) Release of Mn^{2+} in this massive loading case was not observed. The spectral changes which did occur over a period of hours correlated with settling and probably dying of the mitochondria.

DISCUSSION

The spin exchange fraction under limited loading conditions is clearly not a precipitate of manganese with inorganic phosphate. The maximum amount of Mn^{2+} taken up in this form, at least 70 ±20 nmoles/mg mitochondrial protein, is greater than the total amount of inorganic phosphate present in the mitochondria (20–40 nmoles/mg protein) (3). The gradual decrease in EPR line width with increasing amount of manganese is not what one would expect from a precipitate. It is rather what one would expect from a high local concentration of binding sites filled in random order as the amount of available manganese is increased. Finally, the maximum width of the spin exchange line seen under limited loading conditions is around 200 Oe which is much less than the 280 Oe seen with a manganese phosphate precipitate.

Spin exchange narrowing could not arise from osmotically active Mn^{2+} distributed uniformly throughout the interior of mitochondria. The maximum Mn^{2+} concentration inside the mitochondria under limited loading conditions was less than 0.1 M, about two orders of magnitude too small to permit exchange narrowing.

The spin exchange fraction does not appear to be a simple manganese phospholipid complex either. The intermolecular distance between phospholipid molecules in a bilayer, and consequently the minimum conceivable average separation of Mn^{2+} in the bilayer, has been shown to be about 8 A (23). Such a separation of Mn^{2+} ions would be far too great to provide for the amount of orbital overlap necessary to get the strong exchange narrowing observed. Moreover, binding studies of Ca^{2+} to phospholipid monolayers (24) and Mn^{2+} to phospholipid vesicles[1] indicate that only one divalent cation is bound per five or six units of net charge on a phospholipid surface. Therefore, the average local separation of Mn^{2+} on such a surface would be at least 16 A. Moreover, the spectra of Mn^{2+} bound to phospholipid vesicles showed no evidence of the strong spin exchange seen in mitochondria.[1] Since much of the transported manganese is in the spin exchange fraction, these conclusions do not support the common assumption that the only major binding sites for divalent cations inside mitochondria are on phospholipid molecules.

Other workers have reached the conclusion that divalent cation binding in mitochondria was either to proteins or phospholipids (3). These observations leave mitochondrial protein as the most likely candidate for binding the spin exchange fraction. Which protein is responsible for the binding is unknown, but possibly mitochondria contain a protein, with many divalent cation sites, similar to the one recently isolated from sarcoplasmic reticulum (25).

There does not appear to be a strong correlation between amounts of manganese held in the 52 Oe fraction and that held in the spin exchange fraction although

[1] Puskin, J. S., and T. E. Gunter. Unpublished results.

increasing the amounts of transported manganese generally increases the amounts in both forms.

The 52 Oe fraction is not held in the form of a manganese phosphate precipitate; the manganese phosphate spectrum is, of course, quite different. It seems very likely from the work of Burlamacchi et al. (7) that the 52 Oe fraction exists in a high symmetry complex, probably the hexahydrate form, but with a faster spin lattice relaxation than free manganese. The observed hyperfine splittings for this fraction are also very close to those of the aqueous solution hexahydrate complex. These observations suggest a weakly bound or unbound complex in which the inner hydration shell is not disturbed.

Mn^{2+} observed inside the mitochondria under massive loading conditions is believed to be in an osmotically inactive precipitate form in agreement with the conclusions of others (18, 19).

SUMMARY

The use of manganous ion as a spin probe has allowed the observation of up to approximately 100 nmoles Mn^{2+}/mg protein of that fraction of manganese transported by the mitochondria under limited loading conditions. Of this, less than 10% is in the form of a fraction showing six-line hexahydrate type signal with a line width of about 52 Oe. The remainder is in a form showing strong spin exchange. Removal of substrate for respiratory activity or the addition of transport inhibitors prevents the appearance of both types of signals.

It is argued that the spin exchange fraction is probably bound to mitochondrial protein. The minimum average separation of manganous ion in this spin exchange fraction is estimated to be 4.0 $\pm$0.60 A.

Under massive loading conditions EPR data are shown to support the view that manganese is in a phosphate precipitate.

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