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EPR Detection and Characterization of Manganous-(II) Ion in Subcellular Fractions of the Liver and Kidney and Thyroid Homogenate of Mice Treated with Manganese Chloride

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Manganese is indispensable to all animals and plants [1–4]. Animals with manganese deficiency tend to suffer from such symptoms as malformation of neonates, infertility and ataxia. Excessive manganese intakes may result in retardation of growth, interference of calcium and phosphorous uptakes, reduction of hemoglobin formation and neurological disorders. The highest amounts of manganese are reportedly found in the liver, kidney and pancreas of human beings and several animals [1]. Manganese distribution in the rat subcellular fractions is shown to be in the order of nuclear > mitochondria > microsomes > supernatant [5], whereas in rats treated with ^{56}Mn in the order is supernatant > mitochondria ≈ microsomes > nuclear [6]. However, neither the discrepancy of manganese distribution in cells nor the *in vivo* valence state of the metal have not yet been resolved.

Manganese is known to exist in eleven oxidation states, ranging from +7 to −3. Divalent manganous ion (Mn(II)) with five unpaired 3d electrons and the relatively long spin relaxation time is readily observed on EPR measurements for Mn(II) solution, not only at liquid nitrogen but also at room temperature. Therefore, EPR measurements are successfully used for the detection and characterization of Mn(II)-containing proteins or enzymes to elucidate the coordination geometry around Mn(II) [7, 8].

In view of the intrinsic biochemical importance of manganese ion, we attempted to obtain further fundamental information about the nature of manganese in animals. During investigations on these problems by EPR spectroscopy, after subcutaneous administration of $MnCl_2$ for two weeks we detected Mn(II) in subcellular fractions of mouse liver and kidney, thyroid homogenate and skin (necrotized due to injection). This paper reports studies on the EPR detection and characterization of Mn(II) in a living system.

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Materials and Methods

Male ddY mice weighing 30–35 g were maintained on standard laboratory pellets and tap water *ad libitum*. Based upon consumed food and water, which were subjected to atomic absorption analysis, approximately 41.2 μg/day of manganese were ingested by each animal. Manganous ion was subcutaneously (s.c.) injected; 0.2 ml of 10 mg $MnCl_2 \cdot 4H_2O$/ml in physiological saline solution was given to each animal for two consecutive weeks. Thus, the cumulative dosage of Mn(II) given to an individual animal was 7.77 mg. When animals were sacrificed, major organs (including the liver, kidney, thyroid and aliquots of the skin) were removed. The subcellular fractionation of liver and kidney was carried out by the method of Hogeboom [9], and the crude homogenates of other organs were prepared by a glass-fitted homogenizer. The skin was minced finely by scissors, followed by a Polytron homogenizer. EPR spectra of Mn(II) were measured with a JES-ME-3X (X-band) spectrometer with 100 KHz field modulation. The second derivative display was obtained with 80 Hz field modulation. As standards, DPPH powder and MgO powder doped with Mn(II) were used. Measurements were carried out at 293 K in solution and at 77 K in liquid nitrogen in frozen state.

Results and Discussion

In electrostatic field of cubic symmetry of Mn(II), the spectrum shows a hyperfine structure of six lines arising from hyperfine coupling of the unpaired electron with Mn(II) nucleus (I = 5/2). The additional lines in the spectrum of the frozen state have also been observed in the spectrum of $Mn(H_2O)_6^{2+}$ at 77 K. The latter are attributed to 'forbidden' transition, originated from the change of both the nuclear and spin quantum number [10].

Among the tissue homogenates of mice injected with $MnCl_2$, the EPR spectra characteristic to Mn(II) in high spin state were detected in kidney, liver, thyroid and necrotized skin. However, in other parts such as testis, spleen, pancreas, adrenal, appendix, lung, serum and normal skin, the Mn(II) signal was not found. Further subcellular examination was carried out with kidney and liver. The EPR spectra in the kidney nuclear fraction at 77 K and 293 K (Fig. 1) show clearly the presence of manganese in a divalent form. This spectral pattern, and the

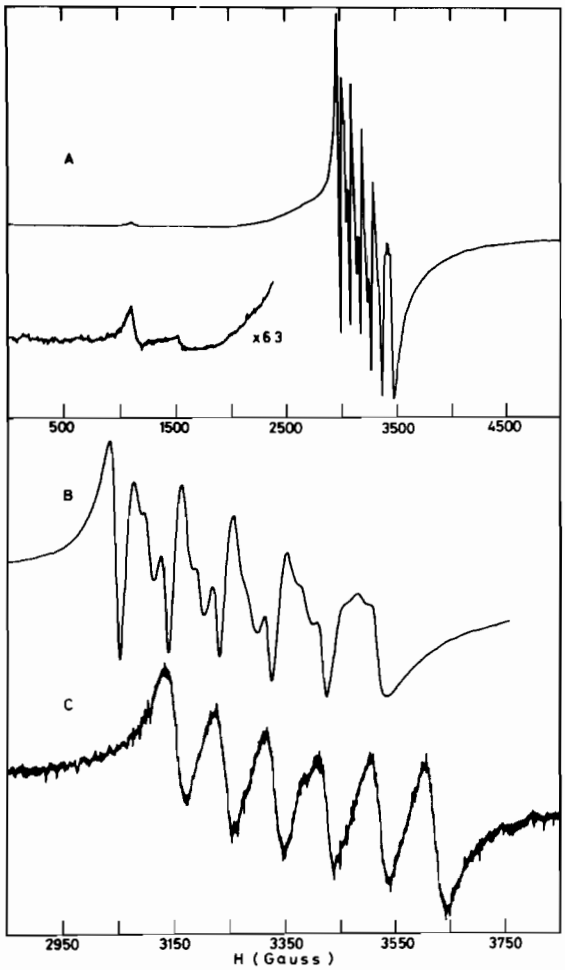

Fig. 1. EPR spectra of the nuclear fraction of kidneys from mice treated with manganese chloride. A and B: 77 K, g = 5.8, 4.2 and 2.020, magnitude of hyperfine structure interval around g = 2.0 (A) = 93.6 Gauss. C: 293 K, g = 2.008, A = 96.4 Gauss.

magnitude of hyperfine structure interval (A), indicate that there is little or no electron delocalization from Mn(II) onto the nitrogen ligand system and that the ligand environment around Mn(II) has high symmetry in this state [10]. The A-value in Mn(II) depends strongly on covalent bonding between Mn(II) and ligand [11]. Evaluation of the data reveals that the magnitudes of A for Mn(II) found in kidney nuclear fraction and other organelles of mice fall in the oxide region, suggesting a ligand field environment largely consisting of oxygen rather than sulfur donors. Judging from the signal intensities, the relative amount of Mn(II) decreased in the following order: nuclear > microsomes > mitochondria > supernatant, thus the highest level of Mn(II) was observed in the nuclear fraction under the applied conditions. In the liver subcellular fractions, the order was found to be nuclear > mitochondria > microsomes. The

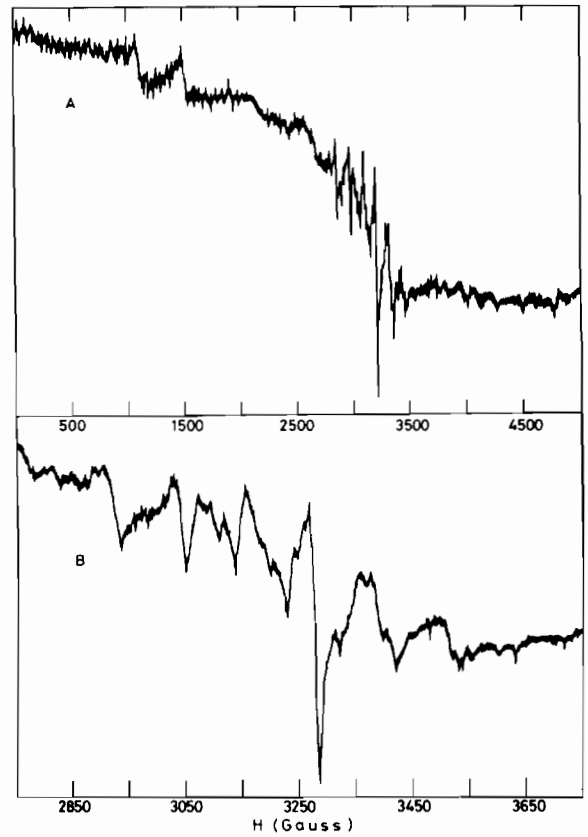

Fig. 2. EPR spectrum at 77 K of the nuclear fraction of the kidneys of untreated mice. g = 2.030, A = 87.9 Gauss.

metal was not detected in the supernatant fraction. These tendencies are fairly compatible with those found in the manganese-treated rats by Thiers and Vallee [5]. Addition of EDTA to the kidney nuclear fraction brought about a new signal at g = 4.2 with remarkable reduction of the signal around g = 2. This indicates the removal of Mn(II) from its binding protein sites and the subsequent complex formation with EDTA, in which considerably deviated axial symmetry around Mn(II) is presumed [12].

The EPR spectrum at 77 K due to Mn(II), although feeble, was also obtained in the kidney nuclear fraction of untreated mice (Fig. 2) suggesting that manganese is naturally accumulated in the kidney and at least some of it is present in the divalent state.

It is interesting to note that Mn(II) was detected in the thyroid homogenate, concomitant with the signal at g = 4.3 at both room and liquid nitrogen temperatures (Fig. 3); the amount being comparable with that in the liver nuclear fraction. Since Mn(II) was not detected in control thyroid, the selective accumulation of manganese is probable. These results agree with the previous observations in which a high partition of manganese was found in the thyroid [13–15].

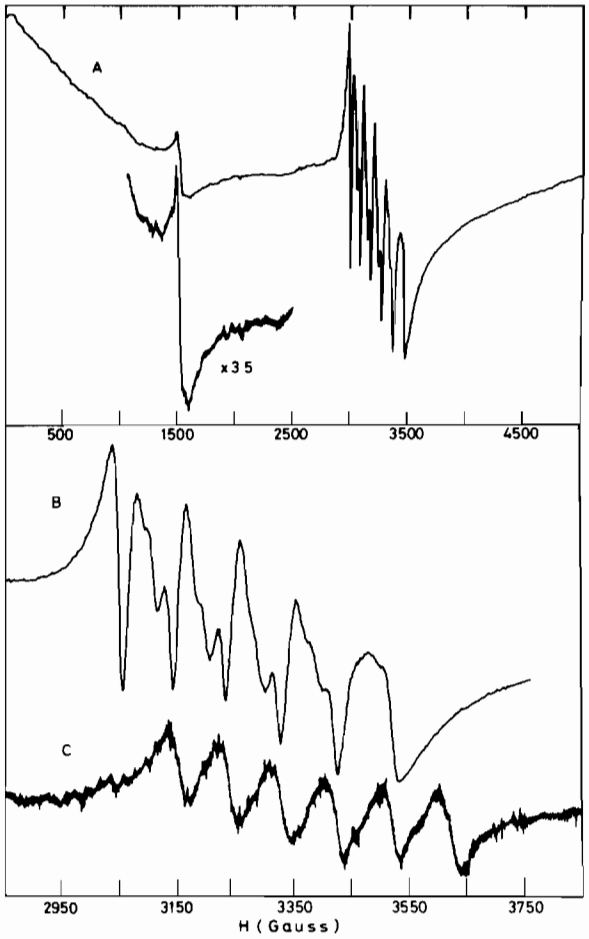

Fig. 3. EPR spectra of the thyroid homogenate of mice treated with manganese chloride. A and B: 77 K, g = 4.3 and 2.030, A = 97.6 Gauss. C: 293 K, g = 2.008, A = 95.6 Gauss.

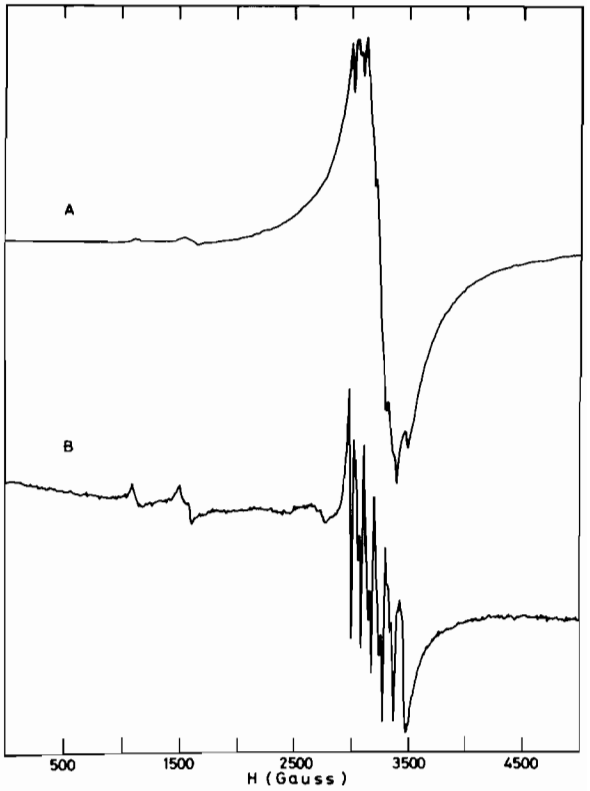

Fig. 4. EPR spectra at 77 K of the necrotized skin part (A) and its homogenate (B). A: g = 5.8, 4.3 and 2.028, A = 96.0 Gauss. B. g = 6.0, 4.3 and 2.029, A = 90.4 Gauss.

Certain pathological symptoms, such as elevated oxygen consumption and reduced thyroidal iodine uptake [13, 16], could be ascribed to incorporated manganese. More recently, manganese itself was identified by the authors as a causative element of goiter in animals [17]. These results suggest the occurrence of specific binding sites for Mn(II) in the thyroid. The appearance of the signals at g = 4.3, whose intensity was 4-fold high compared with the control thyroid, indicates the formation of complexes of the metal and biological materials, with slight deviation from cubic symmetry around Mn(II) [12].

Severe necrosis was observed in the area of skin injected. The characteristic EPR spectra were obtained in the fine mince and homogenate in relatively high amounts. However, the spectral patterns were different (Fig. 4). The principal characteristic of EPR spectrum of mince (Fig. 4A) is the simple and broad curve similar to that in high concentrations of Mn(II) [18]. When the mince was homogenated, the broad curve was completely converted to the typical sextet hyperfine structure characteristic of Mn(II) (Fig. 4B), thus indicating the localized high level of $MnCl_2$ in the injection area where necrosis resulted.

Manganese has already been found in rat serum to be bound to transferrin [19], probably in trivalent form [2]. Manganese superoxide dismutase (Mn–SOD) has been isolated from chicken liver mitochondria and bacteria, and the valence state of manganese bound to Mn–SOD from E. coli is probably trivalent, as indicated by magnetic susceptibility and EPR experiments [20]. As we could not detect Mn(II) in serum nor in other organs than kidney, liver and thyroid, the possibility remains that manganese exists in trivalent (or other EPR-undetectable states) in those organs.

Our further interest is focused on the ratio of Mn(II) to the total manganese concentration in each organ or subcellular fraction, in order to understand the intrinsic role of manganese in biological systems. Full details, including the precise ligand binding character around Mn(II), will be reported.

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