# Chelation in Metal Intoxication II: *In vitro* and *In vivo* Effect of Some Compounds on Brain, Liver and Testis of Rats Treated with Manganese Sulphate

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Further in our search for a more effective therapeutic agent (TANDON et al, 1975; TANDON & SINGH, 1974) for the treatment of manganese poisoning (BROWNING, 1969; TOLONEN, 1972) some structurally different compounds containing oxygen, nitrogen and sulphur as electron donar atoms were examined for their ability to remove manganese as metal-ligand complexes from some vital organs (in vivo) and their sub-cellular fractions (in vitro) of manganese treated rats. Some earlier investigations (EYBL et al, 1969a, 1969b) mainly aimed at the study of the influence of some chelating agents on the excretion of manganese in urine and faeces and its distribution in the organs of experimental animals have proved the efficiency of a few aminopolycarboxylic acids.

Excess of manganese is known to inhibit the activity of succinic dehydrogenase in the organs examined (SINGH et al, 1974) and therefore, the restoration of the activity of this enzyme as a result of the treatment with the chelating agents may also serve as a measure of their effectiveness. This study was, therefore, undertaken with a view to understand if there exists any interrelationship between the structure of the metal binding agents, the removal of manganese and the restoration of enzyme activity and pathomorphology of the organs of experimental animals poisoned with manganese.

## Materials and Methods

In vitro experiment - Twenty male albino rats of I.T.R.C. colony weighing approximately 150 g, were equally divided into five groups. All the animals were injected intraperitoneally, 6 mg Mn/kg as $MnSO_4 \cdot 4H_2O$ (B.D.H., A.R.) dissolved in 1 ml of 0.9% NaCl, daily for six weeks. The animals were sacrificed 48 hours after the last injection, brain and liver were removed, washed free of extraneous material and weighed. Similar organs from two animals of a group were pooled together so as to get two sets of tissues



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from each group. The homogenates of brain (10%, W/V) and liver (15%, W/V) were prepared in phosphate buffer-KCl solution (0.15 M KCl in 0.05M $Na_2HPO_4$-$NaH_2PO_4$ buffer, pH 6.8). Mitochondria and post-mitochondrial supernatant were prepared from brain (BRODY & BAIN, 1952) and liver (SCHNEIDER & HOGEBOOM, 1950) homogenates. The mitochondrial pellets equivalent to 1 g of brain and liver were suspended in 5 and 3 ml of the buffered solution respectively. A measured volume of fractions from each set were dialysed separately in duplicate using visking dialysis tubing (GallenKamp, England) against buffer-KCl medium (ten times the volume of the fraction) containing a chelating agent in the final concentration of $1x10^{-3}$M for 3 hours at 37°C. The pH of the aqueous solution of chelating agents was adjusted to neutral before taking up in the buffered-KCl medium. The dialysing solution was changed after 1.5 hour. For control, an equal volume of fraction was dialysed similarly without the chelating agent. After dialysis, the whole material of the bag was subjected to manganese estimation (CHRISTIE et al, 1957).

In vivo experiment - Thirty six male albino rats (150 ± 10 g) were injected 6 mg Mn/kg body weight i.p. in the form of manganese sulphate dissolved in 1 ml of normal saline, daily for 25 days. The animals were weighed every fifth day and the amount of manganese injected was adjusted accordingly. Forty eight hours after the last injection the rats were divided equally into 6 groups. Animals from each of the five groups were given i.p. 0.11 m mole/kg of a chelating agent dissolved in 1 ml of normal saline (pH adjusted to neutral), daily for 8 days. The remaining group received an equal volume of saline which served as control. A separate group of 6 rats was injected 1 ml of normal saline throughout the experiment which comprised a set of normal animals. The animals were sacrificed, brain, liver and testis taken out, washed free of extraneous material and processed for the following studies.

Estimation of manganese - The metal was estimated in the fresh tissues according to the procedure of CHRISTIE et al. (1957).

Preparation of tissue homogenates and assay of succinic dehydrogenase

10% homogenates (W/V) of the tissues, in ice cold 0.25 M sucrose, were prepared using a Potter Elvehjem homogenizer. The activity of succinic dehydrogenase (SDH) [succinate : (acceptor) oxidoreductase, EC. 1.3. 99.1] was determined in the whole homogenate using the method of SLATER and BONNER (1952).

Estimation of protein - The total protein content of the homogenates was determined in trichloroacetic acid precipitate using bovine serum albumin as the standard (LOWRY et al, 1951).

Histopathological studies

A portion of the tissues was fixed in neutral buffered formalin for a week. Blocks of tissues after routine processing were embedded in paraffin, sections were cut at 5 μ and stained with haematoxylin and eosin.

## Results

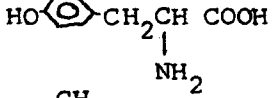
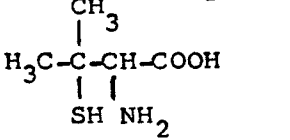
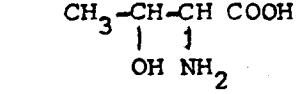
In vitro - There was some removal of manganese from mitochondrial and post-mitochondrial fractions of brain and liver of manganese administered rats by all the chelating agents examined (table 1), the maximum mobilization being achieved by L-Cysteine followed by D-Pencillamine. Since these compounds possess groups containing oxygen, nitrogen and sulphur, it appears that the presence of more than two donar atoms is rather more favourable for the formation of a stable metal-ligand chelate affecting a better mobilization of the excess of the bound metal.

In vivo - The removal of manganese and the restoration of the activity of SDH in intact brain, liver and testis of rats pretreated with manganese sulphate, as a result of the treatment with the five chelating agents have been shown in table 2. While all the compounds affected a significant mobilization of manganese from the three tissues, they could bring about a remarkable recovery of altered activity of SDH due to manganese only in brain. Threonine restored the enzyme activity to 80%, while other chelating agents brought about only a partial recovery in the liver. In testis, there was practically no recovery of the enzyme activity.

No gross abnormality was noticed in liver, testis and brain of all the animals injected with $MnSO_4 . 4H_2O$ for 25 days followed by the treatment with normal saline for 8 days. However, some pathomorphological alterations were observed in the liver and testis, while there was no abnormality in the morphology of the brain. Sections of liver showed dilatation and congestion of central veins and adjacent sinusoids. Kupffer cells showed hyperplasia. A few tiny areas of focal necrosis were seen and at places there was increased cellularity around portal tracts (Fig. 1). Testicular alterations included degeneration of a few seminiferous tubules and depletion of spermatocytes from them. Interstitial cells did not show any abnormality (Fig. 2).

TABLE 1

*In vitro* Removal of Manganese from Sub-cellular Fractions of Brain and Liver of Manganese Administered Rats by Some Compounds.

| Compound | | Removal of Mn (%) | | | |
|---|---|---|---|---|---|
| | | Brain | | Liver | |
| | | Mitochondria (a) | Post-mitochondrial supernatant (b) | Mitochondria (c) | Post-mitochondrial supernatant (d) |
| L-cysteine |  | 50 | 50 | 25 | 23 |
| L-Dopa |  | 33 | 17 | 20 | 17 |
| D-Penicillamine |  | 34 | 33 | 20 | 17 |
| Thioacetamide | $CH_3\ CS\ NH_2$ | 10 | 25 | 25 | 20 |
| Threonine |  | 25 | 13 | 20 | 20 |

Each figure represents the mean of the values from two sets of fractions, each analysed in duplicate and is based on control value taken as 0% removal. Absolute Mn content in control fractions from 5 g of fresh tissue :- $^{a}8\text{-}11\ \mu g$; $^{b}9\text{-}12\ \mu g$; $^{c}14\text{-}19\ \mu g$; $^{d}13\text{-}16\ \mu g$

TABLE 2

Restoration of the Activity of Succinic Dehydrogenase and Removal of Manganese from In vivo Brain, Liver and Testis of Rat Pretreated i.p. with 6 mg Mn/kg Daily for 25 days, by Treatment with Some Chelating Agents for 8 days.

| Experiment Regimen | Brain | | | | Liver | | | | Testis | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | SDH | | Mn | | SDH | | Mn | | SDH | | Mn | |
| | Enzyme activity | Restoration (%) | n moles/g | Removal (%) | Enzyme activity | Restoration (%) | n moles/g | Removal (%) | Enzyme activity | Restoration (%) | n moles/g | Removal (%) |
| 1 | 13.0±0.22 | | 16.4±0.18 | | 33.1±0.22 | | 41.8±0.91 | | 6.6±0.22 | | 10.9±0.72 | |
| 2 | 8.4±0.52 | | 40.7±4.55 | | 16.1±0.73 | | 82.9±4.91 | | 3.6±0.23 | | 41.8±5.27 | |
| 3 | 13.3±0.73[a] | 106 | 23.6±1.45[b] | 70 | 19.8±0.73[b] | 22 | 41.0±2.00[a] | 101 | 3.6±0.29 | - | 23.6±0.91[b] | 59 |
| 4 | 13.2±0.66[a] | 104 | 21.1±0.91[b] | 81 | 19.8±0.36[b] | 22 | 54.5±1.45[a] | 70 | 3.2±0.44 | - | 25.4±1.10[c] | 53 |
| 5 | 13.6±0.73[a] | 112 | 18.9±2.18[b] | 90 | 18.6±0.14[b] | 15 | 45.1±4.18[a] | 92 | 3.1±0.29 | - | 20.0±1.27[b] | 70 |
| 6 | 11.7±0.73[b] | 71 | 24.7±2.00[b] | 67 | 19.0±0.73[b] | 17 | 42.5±2.00[a] | 98 | 4.4±0.15[b] | 27 | 20.4±1.27[b] | 69 |
| 7 | 13.2±0.73[a] | 104 | 21.0±1.27[b] | 81 | 29.3±0.73[a] | 80 | 42.9±2.54[a] | 97 | 3.6±0.36 | - | 20.7±0.91[b] | 68 |

Numbers in the Experiment Regimen's column; 1 = Normal; 2 = Control; 3 = L-cysteine; 4 = L-Dopa; 5 = D-Pencillamine; 6 = Thioacetamide; 7 = Threonine.
Each value represent the mean ± standard error (S.E.) of 6 rats. $^{a}P < 0.001$, $^{b}P < 0.01$, $^{c}P < 0.05$ when compared with control as evaluated by the student 't' test. P value for control compared to normal was < 0.001. Enzyme activity is expressed as n moles of $K_3Fe(CN)_6$ reduced/min/mg, fresh tissue protein.

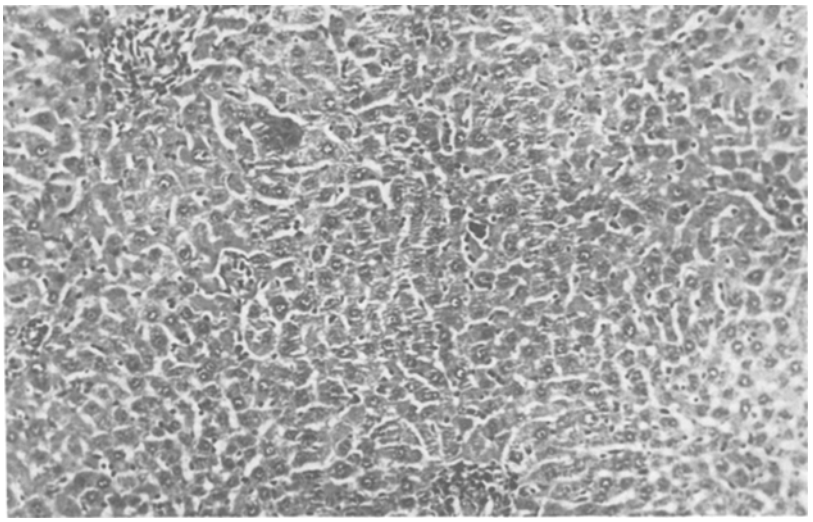


Figure 1. Liver section from a rat, treated with $MnSO_4.4H_2O$ for 25 days followed by normal saline for 8 days, showing congestion and minute areas of focal necroses. Haematoxylin & Eosin X 145.

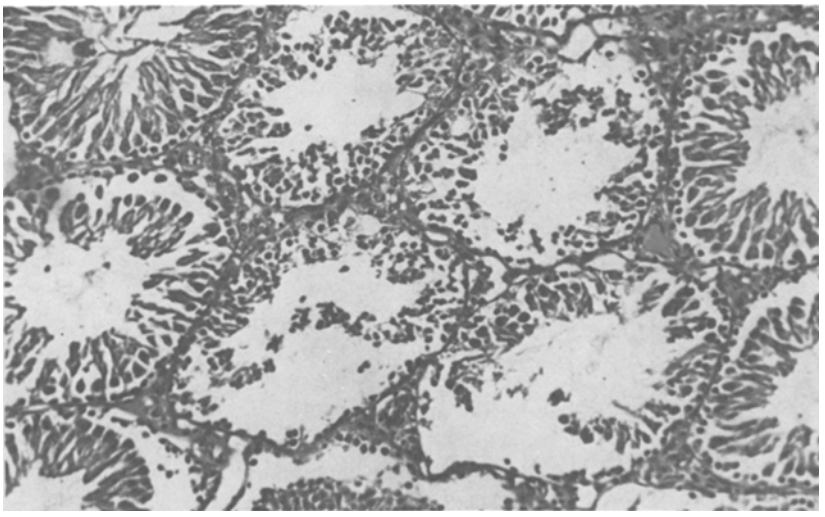


Figure 2. Testicular tissue from a rat, treated with $MnSO_4.4H_2O$ for 25 days, followed by normal saline for 8 days showing degeneration of epithelial cells in a few tubules. Haematoxylin & Eosin X 145.

The sections of liver and testis of manganese poisoned rats treated with five chelating agents for 8 days, did not show an appreciable improvement in the pathology of the organs.

## Discussion

The present results indicate that there is apparently no definite inter-relationship between the structure of the compounds examined, their ability to remove manganese and the recovery of the biochemical or histopathological changes in the tissues. However, the greater removal of the metal from the *in vivo* system, than that of the *in vitro* system, may be attributed to a different mechanism of *in vivo* situation compared to that of the *in vitro*. The constant removal of manganese-ligand chelates from the cell environment into the plasma and thence out of the system will lead to a highly favourable diffusion gradient for a greater mobilization of the metal.

The pathological changes in brain are known to occur quite late in the manganese poisoning (SINGH et al, 1974) and therefore, the decreased activity of SDH in the brain of manganese administered rats may be due to the increased concentration of the metal in the organ. Thus the activity of this enzyme in the brain was restored completely following the removal of the metal by the chelating agents. Such a recovery of the enzyme could not, however, be observed in the liver and testis, though the metal was removed significantly from these organs. This may presumably be due to the unaltered morphological damages which were not reversible in 8 days of treatment by the five metal binding agents. The recovery of histopathological changes may rather be slow and require more time after the removal of the metal from the site (TANDON et al, 1975).

## Summary

Some structurally different compounds were examined for their ability to remove manganese as their chelates from brain, liver and testis and the sub-cellular fractions of brain and liver of manganese administered rats. An attempt was also made to co-relate the removal of the metal with the recovery of altered activity of succinic dehydrogenase in these organs. While all the compounds were able to remove the metal significantly from *in vivo* brain, liver and testis, they could fully restore the enzyme activity only in brain. However, there was no significant improvement in the altered morphology of the tissues in eight days of treatment with the chelating agents.

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