BioMetals **16:** 279–284, 2003.

Organelle specific enzyme markers as indicators of methylmercury neurotoxicity and antidotal efficacy in mice

Cherian Bapu[1], Prem Prakash Sood[2] & Manish Nivsarkar[1,*]
[1]*Department of Pharmacology and Toxicology, B.V. Patel Pharmaceutical Education and Research Development Centre, Thaltej Gandhinagar Highway, Thaltej Ahmedabad. 380 054, Gujarat, India;* [2]*Laboratory of Neurobiology, Department of Biosciences, Saurashtra University, Rajkot 360005, Gujarat, India;* **Author for correspondence (Tel: 91-79-7439375; Fax: 91-79-7450449; E-mail: manishnivsarkar@yahoo.com)*

Received 11 March 2002; accepted 22 April 2002; published on line September 2002

Key words: methylmercury chloride, monothiols, toxicity, vitamins

Abstract

The efficacy of two monothiols, N-acetyl-DL-homocysteine thiolactone (NAHT) and glutathione (GSH) either alone or in combination with two vitamins, vitamin B complex and vitamin E were studied in 7 days methylmercury chloride (MMC; 1 mg kg) intoxicated male Swiss albino mice. Thirteen groups of animals, each containing 6 animals were used for the study. Three groups of animals were kept as control (treated either with vehicle, normal saline or olive oil). Rest of the ten groups were kept as treatment groups. All the animals were treated subcutaneously for 7 days with MMC and one group was sacrificed on the 8th day. The second group was kept without toxicant for another 7 days and were sacrificed on the 15th day. Two MMC pretoxicated groups were treated either with vitamin B complex (20 mg kg) or vitamin E (60 mg kg) and two other groups were treated with N-acetyl-DL-homocysteine thiolactone (40 mg kg) or glutathione (50 mg kg) for another 7 days. The rest of the four groups were treated with either N-acetyl-DL-homocysteine thiolactone or glutathione in combination with either vitamin B complex or vitamin E. All the animals were sacrificed on the 15th day, brain and spinal cord were dissected and estimated for acid phosphatase, alkaline phosphatase, succinic dehydrogenase and α mannosidases. Some of the antidotes showed significant recovery of the enzymes in one tissue while some showed significant recovery in the other tissue depicting the need for treating methylmercury poisoned animals with multi-chelation therapy rather than as a monotherapy.

Introduction

Methylmercury chloride (MMC) is a widely distributed environmental pollutant which has a profound effect on developing as well as adult neuronal systems (Bullet & Cui 1998). Only a few antidotes especially Dimercapto Succinic Acid (DMSA) substantiated the efficacy and safety as a premier metal chelation compound depicted by increased urinary excretion, as compared to other antidotes (Miller 1998). The greater scientific interest in the toxic action of methylmercury chloride (MMC) at the cellular and sub-cellular level and their recovery during antidotal therapy has been focused on its adverse and recovering effects on macromolecule biosynthesis (Miura & Imura 1987). In the past, a number of compounds both synthetic and herbal have been tried to ameliorate the toxic actions of MMC (Bapu *et al.* 1994; 1998; Lee *et al.* 1999). Some have shown encouraging results (Ballatori *et al.* 1998) but at the same time, some antidotes showed a redistribution of metal from one organ to another (Aaseth *et al.* 1982; Wannag & Aaseth 1980).

Biochemical lesions are considered to be the most primary effect of methylmercury intoxication (Unnikumar & Sood 1985) and numerous reports have detailed the effects of it on the biochemical machinery of the cell system (Bapu *et al.* 1994; Bapu & Sood 1998). It has also been reported that mercury binds competitively to Zn^{++} and Mg^{++} dependent enzymes (Kozik *et al.* 1977), inhibits various organelle

specific marker enzymes (Vijayalakshmi *et al.* 1992), sulphydryl containing enzymes like succinic dehydrogenase (Raghu *et al.* 1992) and decreases or alters the normal concentration of essential elements in the body (Bapu *et al.* 1994; Webb & Cain 1982). With chelation therapy, a different scenario exists in the toxicated animals. Some of the antidotes remove mercury from the body while some antidotes cause a redistribution of the metal in different tissues (Thomas & Smith 1982). Thyroxine, pyridoxine, methionine and ascorbic acid are some of the compounds which are effective in preventing heavy metal toxicity (Flora & Tandon 1995). In cadmium toxicity, the supplementation of vitamin B complex simultaneously with cadmium has been effective in preventing the appearance of signs of cadmium intoxication (Tandon *et al.* 1984).

With this background knowledge and our earlier conclusions that treatment of methylmercury intoxicated animals with vitamins like vitamin B complex and vitamin E and monothiols like N-acetyl-DL-homocysteine thiolactone and glutathione could remove mercury (Sood *et al.* 1993), restore various enzymes (Bapu *et al.* 1998), vitamins (Sood & Vijayalakshmi 1995) and various essential elements (Bapu *et al.* 1994) in the brain and spinal cord as well as repair various histopathological alterations in the brain (Bapu & Sood 1998) of mice, it prompted us to check their efficacy in the restoration of certain organelle specific marker enzymes and sulphydryl containing enzymes after mercury intoxication.

Materials and methods

Seventy eight three months old Swiss male albino mice (inbred strain) weighing about 30 ± 5 gm bred at our animal house obtained from the original stock from National Centre for Laboratory Animal Sciences, National Institute of Nutrition, Hyderabad were used for the present study. The animals were kept in highly hygienic conditions under controlled environmental conditions like temperature of 25 ± 2 °C and relative humidity 55 ± 5% and were kept in polypropylene cages with 12 h of light and darkness. They were divided into 13 groups and each group contained six animals.

The experimental protocol was approved by the Institutional animal house ethics committee.

Control groups

Three groups of animals received subcutaneous injections of respective vehicle (10 mM Na_2CO_3-$NaHCO_3$, pH 9.2; normal saline or olive oil). The mode of administration, volume and interval between injections were similar in all the groups.

Methylmercury treated groups

Ten groups of animals were used for this purpose. They were injected sub-cutaneously with methylmercury chloride (MMC; Wako Pure Chemicals Ltd., Japan) dissolved in 10 mM Na_2CO_3-NaH CO_3, pH 9.2 at a daily dose of 1 mg kg body weight for 7 days. One group was sacrificed on the 8th day and the second group was kept without drug treatment for the next seven days and were sacrificed on the 15th day. The rest of the groups were utilized for therapeutic treatments.

Vitamin treated groups

Two groups of animals were used for this purpose. They were injected sub-cutaneously with vitamin B complex (Neurobion, Ranbaxy Laboratories Ltd., New Delhi, 20 mg kg; diluted in normal saline) or vitamin E (Evion; Merck, India, 60 mg kg; diluted in olive oil) to 7 days MMC pre-toxicated animals for another 7 days.

Monothiols treated groups

Two groups of animals, pre-treated with methylmercury chloride for 7 days were used for this purpose. They were injected sub-cutaneously with glutathione (50 mg kg) and N-acetyl-DL-homocysteine thiolactone (40 mg kg) for another 7 days.

Monothiols and vitamins treated groups

Four groups of 7 days MMC pretoxicated animals were utilized for this purpose. They were injected subcutaneously with glutathione (50 mg kg) and after half an hour were injected either with vitamin B complex (20 mg kg) or vitamin E (60 mg kg). Another two groups of animals were injected with N-acetyl-DL-homocysteine thiolactone (40 mg kg) for another 7 days.

All the therapeutic groups were sacrificed on the 15th day.

On the scheduled day, the animals were sacrificed by cervical dislocation in the morning hours to prevent diurnal changes and the brain and spinal cord were dissected out quickly, washed with normal saline (4 °C), blotted and weighed. They were minced with a sharp scissors and homogenized in a glass mortar with pestle in double distilled water for the estimation of alkaline phosphatase, acid phosphatase and succinic dehydrogenase and in sodium citrate (1 mg ml) for α mannosidase. Complete homogenization was obtained by adding non-acidic sand to the medium. This was followed by centrifugation in a Remi (RC24) refrigerated centrifuge (4 °C). The supernatant was divided into two portions. One portion was used for the spectrophotometric assay of alkaline and acid phosphatase (Shinowara *et al.* 1942) and succinic dehydrogenase (Kun & Abood 1949) and the other portion was partially purified with chilled acetone and resubjected to centrifugation. The supernatant was discarded and residue was suspended in sodium citrate and estimated for α mannosidase (Tettamanti & Masserini 1984).

The specific activities of the enzymes are expressed as amount of inorganic phosphate liberated/mg protein/hr (for alkaline and acid phosphatase), amount of formazan formed/mg protein (for succinic dehydrogenase) and μmol 4-nitrophenol liberated/mg protein/hr (for α mannosidase).

Statistical Analysis

The results were evaluated using a two way ANOVA according to the method of Sokal and Rohlf, 1969, for calculating the significant difference between the treatments.

Results

Biochemical alterations during methylmercury intoxication

Methylmercury chloride treatment for 7 days caused a significant decrease in acid phosphatase, $\propto$ mannosidase, alkaline phosphatase and succinic dehydrogenase in brain and spinal cord (M: Figure 1 A–D). When the 7 days MMC pretoxicated animals were kept without intoxicant for the next 7 days and sacrificed on the fifteenth day, a further decrease in acid phosphatase, $\propto$ mannosidase, alkaline phosphatase and succinic dehydrogenase activity was found in all the tissues (W: Figure 1 A–D).

Effect of vitamins therapy

The post-therapeutic treatment of the animals with vitamins B complex and E significantly recovered the alkaline phosphatase activity in the nervous tissues (B,E; Figure 1 B). The activity of acid phosphatase recovered significantly in the brain during vitamin E application (E; Figure 1 A) and succinic dehydrogenase also recovered completely in brain during vitamin B complex and E application (B.E; Figure 1 C). $\propto$ mannosidase showed a significant recovery in its activity in both the tissues during both vitamin B complex and E therapy (B, E; Figure 1 D).

Effect of monothiols therapy

NAHT and GSH application recovered the alkaline phosphatase activity in the brain while in spinal cord, both monothiols inhibited its activity (N,G.; Figure 1 B). The acid phosphatase activity on the other hand recovered in all the tissues which was found to be more suitable than the other monothiol, glutathione (N,G.; Figure 1 A) The recovery of succinic dehydrogenase was found in all the tissues during GSH therapy (G.; Figure 1 C).

Glutathione application revealed a recovery of succinic dehydrogenase activity in all the tissues while NAHT treatment showed a reverse effect (N,G.; Figure 1 C). NAHT and GSH therapy recovered the enzyme level in all the tissues but α mannosidase on the other hand, showed a recovery in both brain and spinal cord in both the nervous and non-nervous tissues but the degree of recovery was different. Both these antagonists showed more recovery in brain compared to spinal cord (N,G; Figure 1 D).

Effect of combined therapy of monothiols and vitamins

The application of glutathione and vitamin B complex recovered alkaline phosphatase level significantly in spinal cord (B2, Figure 1 B). The combined application did not show a significant recovery in acid phosphatase in the nervous tissues (E2, Figure 1 A). In spinal cord, NAHT application with vitamin E showed the best results (E1; Figure 1 A). The level of succinic dehydrogenase enzyme recovered in brain with a combination of vitamin B complex and NAHT or GSH (B1, B2; Figure 1 C) while monothiols and vitamin E showed insignificant recoveries (E1, E2; Figure 1 C) as compared to the monothiols alone group. In spinal cord, none of the combinations showed better results

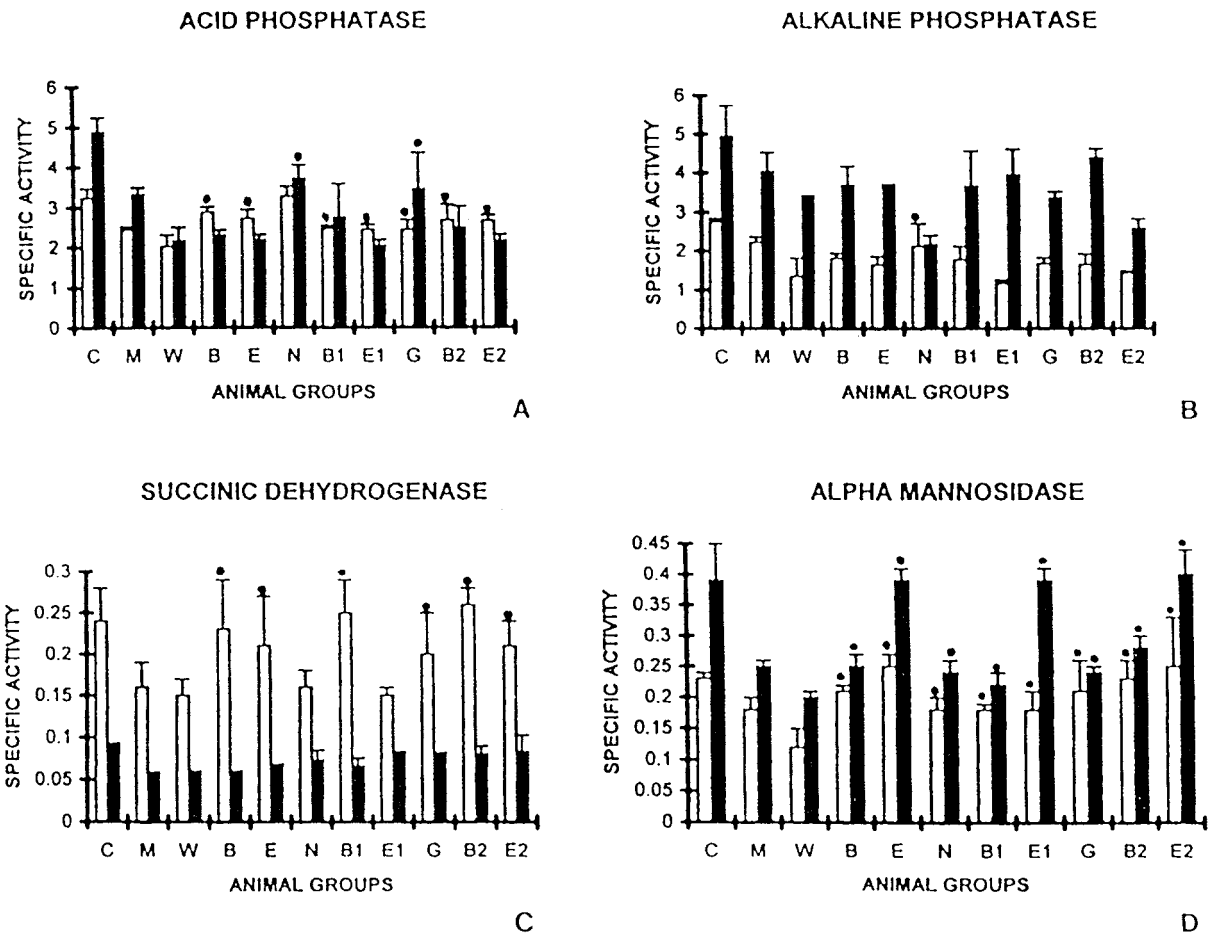

Fig. 1. A, B, C & D represent the percentage changes in acid phosphatase, alkaline phosphatase succinic dehydrogenase and alpha mannosidase in brain (open bars) and spinal chord (closed bars), during methylmercury chloride (M) application as well as during normal withdrawal (W) and N-acetyl-DL-homocysteine thiolactone (N), Glutathione (G), vitamin B complex (B), vitamin E (E), N-acetyl-DL-homocysteine thiolactone and vitamin B complex (B1), N-acetyl-DL-homocysteine thiolactone and vitamin E (E1), glutathione (G), glutathione and vitamin B complex (B2) and glutathione and vitamin E (E2) application. $P < 1\%$.

as compared to the monothiols alone group (B1, B2; Figure 1 C) except NAHT plus vitamin E (E1; Figure 1 C). In the case of α mannosidase, the combined therapy of vitamins and NAHT were not effective in brain and spinal cord (B1, E1; Figure 1 D). None of the other combinations were effective in recovering the inhibited enzyme level.

Discussion

Neuropathology associated with methylmercury chloride is characterized by complete architectural disruption of neuronal elements within the cerebellum where it binds strongly to protein and sulphydryl groups (Philbert *et al.* 2000). The level of different enzymes of various metabolic pathways have to be studied during intoxication and detoxication to prove the efficacy as an antidote during methylmercury poisoning. The decrease in activity of various enzymes like alkaline phosphatase, acid phosphatase, α mannosidase and succinic dehydrogenase during mercury intoxication shows that mercury created serious disturbances in the biochemical machinery involved in various metabolic processes. This may be correlated well with the previous study where mice treated under similar experimental conditions showed the deposition of mercury during intoxication in brain and spinal cord (Sood *et al.* 1993) and disturbances in elemental composition in various tissues (Bapu *et al.* 1994) which may be the reason for severe disturbances in activities of metal and SH dependent enzymes. Alkaline

phosphatase, a plasma membrane marker enzyme has been found to be affected considerably as mercury attacks the membrane transport processes. Mercury gets deposited in the lysosomes during intoxication and various hydrolases have been found to be affected (Webb & Cain 1982; Vinay *et al.* 1990). Acid hydrolases have been shown to increase or decrease during methylmercury intoxication depending upon dose, duration, animal species and target tissue (Vinay *et al.* 1992). In this study, a decrease in the level of acid phosphatase and α mannosidase has been found which may be because mercury, being a labilizing agent and due to the preferential storing of the compound in the lysosomes, causes cell autolysis which causes the release of enzymes released into the circulation. Further, the decreased level of cholesterol (Sood *et al.* 1997) and vitamin E (Sood *et al.* 1995) which are regarded as lysosomal stabilizing agents (Weissman 1967) during methylmercury intoxication, also increases the lysosomal fragility, which may be the reason for decreased level of acid phosphatase and α mannosidase in the present study.

Succinic dehydrogenase, which contains four groups of non-heme iron and SH groups, is more susceptible to methylmercury chloride intoxication, and it has been reported that vitamin E protects SH groups of dehydrogenases and elevate their level (Vijayalakshmi 1993). SDH is inhibited both in the brain and spinal cord clearly indicating that mercurials interfere with glycolysis and Kreb's cycle. Likewise, inhibition of pentose phosphate pathway enzyme, glucose-6-phosphatase have been reported in the brain and spinal cord in a similar experimental condition (Vijayalakshmi 1993). Thus all major energy yielding pathways are disturbed during MMC intoxication.

The treatment of MMC pretoxicated animals with monothiols like GSH and NAHT and vitamin B complex and E either alone or in combination showed varying results. The recovery of the enzyme with one combination in some tissues and the other in some other tissues were found in this study. For instance, glutathione alone and in combination with vitamin B complex showed maximum recovery of the enzyme in spinal cord while in brain, NAHT alone and in combination with vitamin B complex revealed maximum recovery. The recovery of SDH also showed varying results in different tissues. Vitamins and monothiols treated groups showed sufficient recovery of these enzymes in different tissues.

The glycosidases which are found in higher concentrations in the myelin sheath (Hafiza & Sood 1980; Vinay *et al.* 1990), associated with carbohydrate and lipid metabolism are highly sensitive to methylmercury (Vijayalakshmi *et al.* 1992) which has been confirmed in this study. α mannosidase being a Zn dependent enzyme (Weissman 1967) are easily affected by methylmercury as this heavy metal has the tendency to knock out other metals during its intoxication. The deficiency of these enzymes causes drastic metabolic defects in the myelin sheath depicted by severe myelin degeneration as has been reported in a similar experimental condition in mice and the application of vitamin B complex alone or in combination with glutathione were found to be helpful in regaining the myelin contour (Bapu & Sood 1998). It has been reported that some of the constituents of vitamin B complex, particularly thiamine, has been found successful both individually and as adjuvants in preventing lead toxicity (Flora & Tandon 1995; Tandon *et al.* 1984). Since different chelators showed different results, the application of chelators as a multi-chelation therapy would be suitable after mercury poisoning as it has been found that the improvement of SH groups, cysteine and GSH in various functional activities would cause the recovery of altered biochemical machinery in the normal cell.

References

Aaseth J, Alexander J, Reknerud N. 1982 Treatment of mercuric chloride poisoning with dimercaptosuccinic acid and diuretics. Preliminary studies. *J Toxicol Clin Toxicol* **19**, 173–186.

Ballatori N, Lieberman MW, Wang W. 1998 N-acetylcysteine as an antidote in methylmercury poisoning. *Env Hlth Perspect* **106**, 267–271.

Bapu C, Sood PP. 1998 Protective effect of vitmain B complex and glutathione against methylmercury induced histopathological alterations in mice brain. An ultrastructural study. In R Prakash, ed. *Heavy Metal Pollution,Toxication and Chelation*. New Delhi: M.D. Publications Pvt. Ltd.; 359–379.

Bapu C, Purohit RC, Sood PP. 1994 Fluctuations of trace elements during methylmercury toxication and chelation therapy. *Human Exp Toxicol* **13**, 815–823.

Bapu C, Rao AP, Sood PP. 1998 Restoration of methylmercury inhibited adenosine triphosphatases during monothiols and vitamins therapy. *J Exp Pharmacol Toxicol Oncol* **17**, 75–80.

Bullet RF, Cui H. 1998 Methylmercury antagonizes the survival promoting activity of insulin like growth factor on developing cerebellar granule neurons. *Toxicol Appl Pharmacol* **153**, 161–168.

Corwin LM.1980 The role of vitamin E in mitochondrial metabolism. In: LJ Machlin, ed. *Vitamin E. A comprehensive treatise*. New York: Marcel Dekker; 333–347.

Flora SJS, Tandon SK. 1995 Adjuvant for therapeutic chelating agents in lead intoxication. *Trace Metals Electrolytes* **12**, 131–140.

Gautam AK, Sood PP. 1982 Significance of glycosidases in myelin lipid metabolism. I. Studies in diencephalon of fresh water turtle *Lissemys punctata*. *J Hirnforsch* **23**, 295–300.

Hafiza B, Sood PP. 1980 Topographic distribution of beta glucuronidase and beta galactosidase in the medulla oblongata of a microchiropteran bat. *Yokohama Med Bull* **31**, 43–51.

Kozik MB, Sosinski E, Szczezech J. 1997 Phosphatases and esterase activity in the brain following acute sublimate intoxication. *Folia Histochem Cytochem* **15**, 87–94.

Kun E, Abood LG. 1949 Colorimetric estimation of succinic dehydrogenase by triphenyl tetrazolium chloride. *Science* **109**, 144–146.

Lee JH, Kang HS, Roh J. 1999 Protective effect of garlic juice against embryotoxicity of methylmercury chloride administered to pregnant Fischer 344 rats. *Yonsei Med J* **40**, 483–489.

Miller AL. 1998 Dimercaptosuccinic acid, a non-toxic, water soluble treatment for heavy metal toxicity. *Alt Med Rev* **3**, 199–207.

Miura K, Imura N. 1987 Mechanism of methylmercury cytotoxicity. *CRC Crit Rev Toxicol* **18**, 161–188.

Philbert MA, Billingsley ML, Reuhl KR. 2000 Mechanisms of injury in the central nervous system. *Toxicol Pathol* **28**, 43–53.

Raghu KG, Kodi RB, Viajayalakshmi K, Bapu C, Sood PP. 1992 Inefficiency of metal chelators to promote recovery of methylmercury inhibited central nervous system succinic dehydrogenase. *Acta Neurol Belgica* **92**, 157–164.

Shinowara HY, Jones LM, Reinhardt HL. 1942 The estimation of serum inorganic phosphate and alkaline phosphatase activity. *J Biol Chem* **142**, 912–921.

Sokal RR, Rohlf FJ. 1969 Biometry. San Francisco: Freeman and Company.

Sood PP, Vijayalakshmi K. 1995 Vitamin status in mice tissues during methylmercury intoxication and detoxication. *J Nutr Environ Med* **5**, 133–141.

Sood PP, Vijayalakshmi K, Bapu C. 1993 Ameliorative capacities of vitamins and monothiols administered alone or in combination in methylmercury mobilization in nervous and non-nervous tissues of mice. *Cell Mol Biol* **39**, 213–220.

Sood PP, Bapu C, Sinha N, Rao AP. 1997 Cholesterol and triglycerides fluctuations in mice tissues during methylmercury intoxication and monothiols and vitamins therapy. *J Nutr Environ Med* **7**, 155–162.

Tandon SK, Flora SJS, Behari JR, Ashquin M. 1984 Vitamin B complex in the treatment of cadmium intoxication. *Ann Clin Lab Sci* **14**, 487–492.

Tettamanti G, Masserini M. 1984 In: J Bergmeyer, M Graßl, Ed. Methods of Enzymatic Analysis, Vol. IV. Basel: Verlag Chemie; pp. 241–246.

Thomas DJ, Smith JC. 1982 Effect of co-administered low molecular weight thiol compounds on short-term distribution of methylmercury in the rat. *Toxicol Appl Pharmacol* **62**, 104–110.

Unnikumar KR, Sood, PP. 1985 Therapeutic capacities of N-acetyl-DL-homocysteine thiolactone and 2,3 dimercaptosuccinic acid in the restoration of methylmercury inhibited proteins. *Yokohama Med Bull* **36**, 79–87.

Vijayalakshmi K. 1993 Vitamins and monothiols in methylmercury detoxication. A biochemical study. Ph.D. Thesis. Rajkot: Saurashtra University, pp. 1–180.

Vijayalakshmi K, Bapu C, Sood PP. 1992 Differential effect of methylmercury, thiols and vitamins on galactosidases of nervous and non-nervous tissues of mice. *Bull Environ Contam Toxicol* **49**, 157–164.

Vinay SD, Raghu KG, Kodi RB, Vijayalakshmi K, Bapu C, Sood PP. 1990 Glycosidases inhibition with methylmercury vis-a-vis myelin degeneration: A biochemical and electron microscopic study. *Environ Conc Tissue Inj* **4**, 67–75.

Vinay SD, Raghu KG, Sood PP. 1992 Differential therapeutic responses of thiol compounds in the reversal of methylmercury inhibited acid phosphatase and cathepsin E in the central nervous system of rat. *Bull Environ Contam Toxicol* **49**, 78–84.

Wannag A, Aaseth J. 1980 The effect of immediate and delayed treatment with 2,3 dimercaptopropane sulphonate on the distribution and toxicity of inorganic mercury in mice and in fetal and adult rats. *Acta Pharmacol Toxicol* **46**, 81–88.

Webb M, Cain K. 1982 Functions of metallothioneins. *Biochem Pharmacol* **31**, 137–142.

Weissmann G. 1967 Structure and function of lysosomes. *Rheumatology* **1**, 1–28.