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Saline or Plant-Incorporated Methylmercury Effects on Distribution, Demethylation, and Blood Parameters in Rats

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The influence of diet is recognized as a significant factor in the expression of toxicity (Landry et al. 1979). This applies particularly to toxins like methylmercury (MeHg) which are metabolically incorporated into growing food plants and biotransformed within the plant before ingestion (Czuba and Mortimer 1980).

Methylmercury in this form may influence the early physiological and biochemical events which lead to development of toxicity. In a previous study, a single dose of plant-incorporated methylmercury (MeHg) had a different route of distribution and accumulation in rat organs after 48 h than an equivalent dose of saline MeHg (Czuba et al. 1982) with the greatest accumulation being in red blood cells. The level of total Hg in red blood cells and in cerebrum (mitochondrial and soluble fraction) was reduced by over 50% if a plant MeHg form was ingested.

Clinical cases of acute MeHg poisoning have initially (about 1 month), a very high blood concentration (with no visible outward signs) immediately after exposure (latent or silent period) which develops into a MeHg toxicity by inhibition of protein synthesis and unrepairable damage in the brain, inhibition of aetylcholine binding, and disruption of microtubular structures in some cells (Clarkson 1983). Among the long-term studies, kidney damage was the sensitive indicator of chronic MeHg toxicity (Munro et al. 1980; Verschuuren et al. 1976).

All red blood cells preferentially accumulate alkyl mercury (Burriss, Garrett and Garrett 1974). Significant impairment of blood components including creatine has been found under exposure to halogen compounds (Bosia et al. 1983). Creatine, an important storage form of high energy phosphate in muscles (Swenson 1977) is also a primary indictor of erythropoietic dynamics under hypoxia (Syllm-Rapoport et al. 1981), a sensitive indicator of hemolytic disease (Fehr and Knob 1979), red blood cell aging and impaired marrow efficiency (Bosia et al. 1983). Since changes in creatine levels occur sooner than changes in other blood parameters, itself

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being neither synthesized nor metabolized by red blood cells, it can be used as a sensitive indicator of toxicity.

The present work investigates the differences in a longer term, multiple-dose regime of saline or bean-incorporated MeHg ingestion with special attention being given to various blood parameters.

MATERIALS AND METHODS

Male Wistar rats (205 ± 10 g) (National Research Council breeding laboratories) were divided into 4 groups of 12 animals each and housed in groups of 6 in polycarbonate cages with free access to food and water. Dosing was done 5 times a week with 20 ug (5 uCi) MeHg (Amersham, United Kingdom) per dose for 5 weeks; equivalent to a dose of MeHg of 0.1 mg/kg/day in a volume of 1.5 ml. The four groups included a control group receiving only physiological saline (0.9 M NaCl), a second group receiving bean homogenate in physiological saline, a third group dosed with bean-MeHg (homogenized immediately before dosing to avoid demethylation) and a final group dosed with saline-MeHg.

Bean seeds (Phaseolus vulgaris, var. Pencil Pod Black Wax) soaked up water for 16 h., then incorporated labelled 203-MeHg for 24 h. Samples were checked for total uptake and any demethylation. A quantity of MeHg-containing bean seed was homogenized and diluted to give the required amount (20 ug MeHg in 0.3 g dry weight of bean) in the 1.5 mL dose comparible to the saline-MeHg treatment. The sample preparation was completed immediately before dosing time.

At the end of each week, feces and urine were collected for 24 h from two rats from each group placed in metabolic cages. Food and water consumption were recorded. At the end of each week, 2 animals from each group were anaesthetized with Nembutal and blood (5 to 7 g/rat) was collected with heparinized syringes by heart puncture. Plasma and blood cells (RBC) were separated by centrifuging whole blood at 3,000 rpm for 15 min. at room temperature. Animals were sacrificed by vascular perfusion with 500 ml of physiological saline. Kidneys, liver, spleen, brain (cerebrum, cerebellum) were removed and weights recorded.

All radioactive counting of samples was done in a Tracor Analytical 11197 deep well counter to a 2% counting error. MeHg concentrations were calculated from the counting data corrected for decay and the known specific activity of the MeHg solutions. The amounts of organic and inorganic Hg were determined in organs and excreta by the thin layer chromatography TLC method (Czuba et al. 1981).

Hematological determinations of blood samples were done by the standard procedures used for the determination of hematocrit (Dacie and Lewis 1968), sedimentation rate (Miller and Keane 1969), osmotic fragility (Dacie and Lewis 1968) and creatine in red blood cells (Griffiths 1968). The data were analyzed by

Students t-test; the least significant difference test was used to compare the means of treatments at the .05 and .01% levels of significance. Standard deviation was used where indicated.

RESULTS AND DISCUSSION

The ingestion of toxins and their subsequent accumulation depends on the species gut holding time (Kozak and Forsberg 1979), form of mercury and diet (Rowland et al. 1980) as well as enzymatic capacities in various organs. In the present study, animals in all groups gained weight throughout the study without any significant differences among the 4 groups. Thus, the effects observed were those at the low end of the toxicity range. The total Hg accumulation in kidney, spleen, cerebrum plasma and red blood cells (RBC) was significantly lower at 5 weeks of repeated dosing with bean-MeHg compared with saline-MeHg (Fig. 1). In liver, it occurred sooner - at 2 and 3 weeks. The most variable pattern of accumulation over the 5 weeks was found in liver and plasma. In plasma the pattern of accumulation after bean-MeHg ingestion was the mirror-image of the saline-MeHg treatment. Bean-MeHg ingestion caused a deviation of the linear pattern of accumulation of MeHg with time.

Methylmercury was easily incorporated into bean seeds and distributed in the methyl form among the high molecular weight proteins (60% of total) and low molecular weight proteins and peptides (data not shown). Thus, some of the material would be processed by gut microflora and would be asborbed in complex form as found in other studies (Rowland et al. 1980). Gut microflora are known to influence the incorporation and excretion of Hg compounds by affecting demethylation rates and recycling of MeHg in the enterohepatic cycle (Rowland et al. 1984).

Fecal excretions of Hg as Hg^{2+} were generally higher than in urine for both treatments (Fig. 2). Urine excretions were mainly as MeHg whose proportion was elevated by bean-MeHg treatments. The higher fecal excretions at week 5 were not consistent with increased demethylation expected in the saline-MeHg treatments if more Hg^{2+} were present due to gut microflora activity. Perhaps, under repeated MeHg treatment, the proportion of gut microflora population with the demethylating activity could have been destroyed and thus demethylating capacity was lost. Such differences in flora populations and sensitivities have been documented (Kozak and Forsberg 1979). This could be the reason for the sudden increase in MeHg in feces in week 4 and 5 (Fig. 2). The arguement against this is that in ruminant gut fluid, concentrations of up to 100 ug/ml for 48 hrs failed to eliminate the C-Hg breaking activity of bacteria. The time scale *in vivo* in ruminants would be too short (12-14 hrs) to damage gut flora. In contrast to this, the bean-MeHg treatments may be protected gut-microflora from toxic MeHg effects.

Antibiotic treatment of rodents has indicated that demethylation sites are not confined only to the gut microflora but also occur

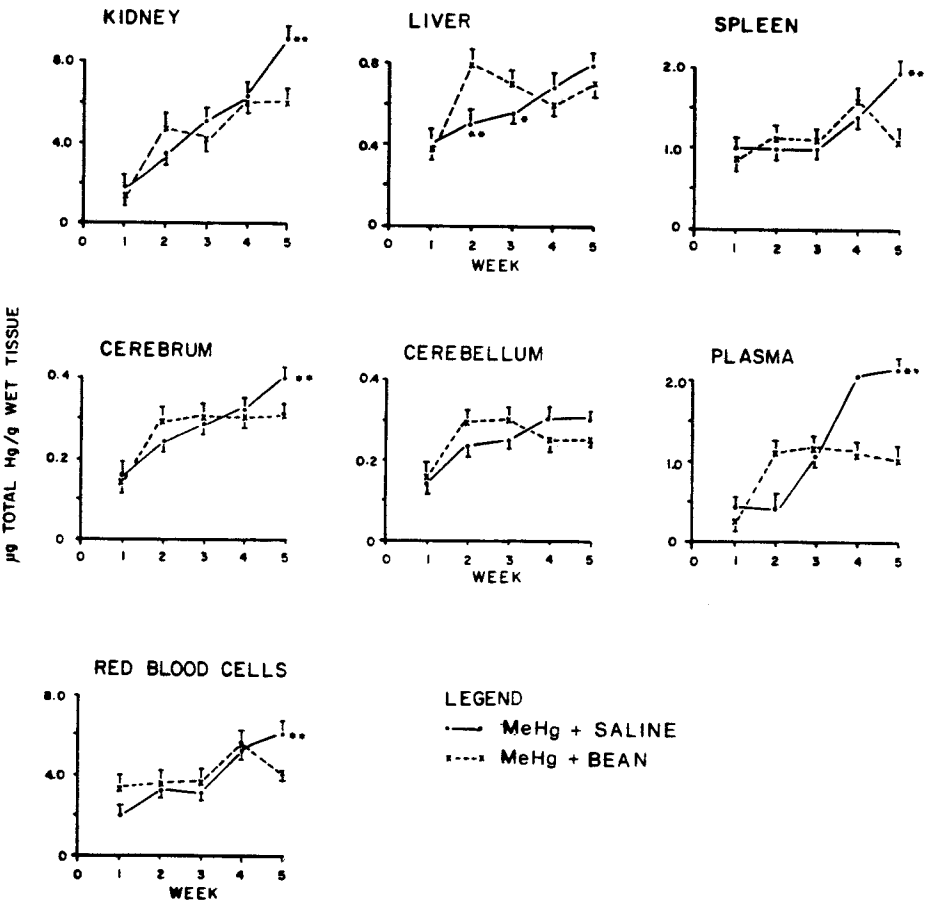

Figure 1. Comparison of patterns of accumulation and distribution of total Hg in some organs of rats dosed repeatedly with either saline-MeHg or bean-MeHg over a 5 week period.

in organs such as liver, kidney and others (Ishihara and Suzuki 1976). Diet changed the demethylation and excretion of MeHg in bile whether the animals were orally-dosed or intramuscularly injected by MeHg (Landry et al. unpublished results). In that study, the formation of gut bacteria metabolite complexes with MeHg which were or were not retained by tissues easily would be excreted at a different rate (Rowland et al. 1980). Perhaps this is the case with urine excretion which is mainly in the methyl-form (Fig. 2) whereas much of the demethylated Hg is retained by

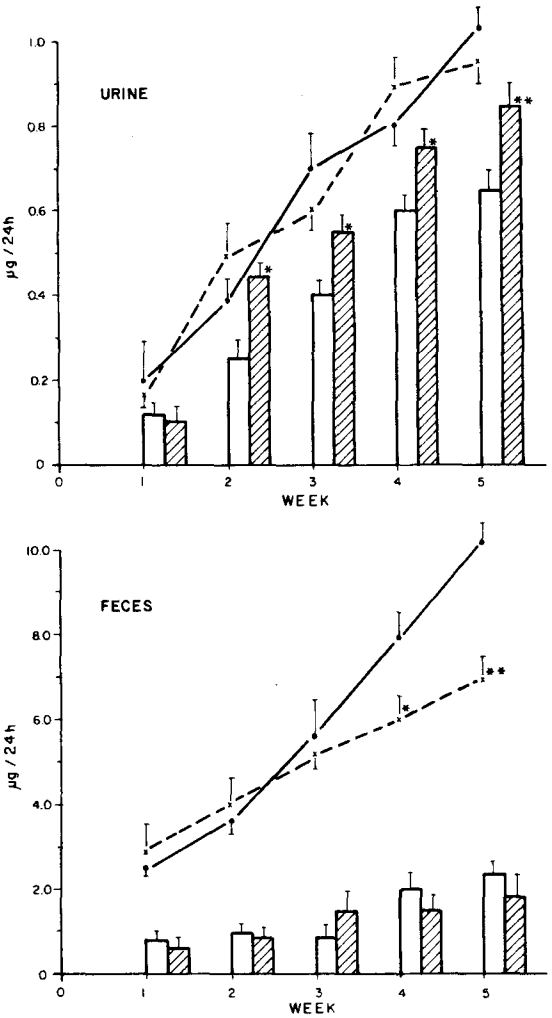

Figure 2. Comparison of excretion patterns of total (•—•, x---x) and MeHg (□,▨) (µg) in 24 hr after repeated doses with either saline-MeHg or bean-MeHg over a 5 week period. (I) represents standard deviation of samples and * and ** represent the Student's t-test at .05% and .01% respectively.

the kidneys (Fig. 3). Kidney was the organ that had the greatest accumulation and demethylation rate of all the organs tested. This is consistent with previous studies (Komsta-Szumska et al. 1983; Czuba et al. 1982). Plant constituents from the diet which play a role in detoxification mechanisms have been identified as various forms of secondary metabolites in cruciferous and leguminous plants which induce greater activity of enzymes such as

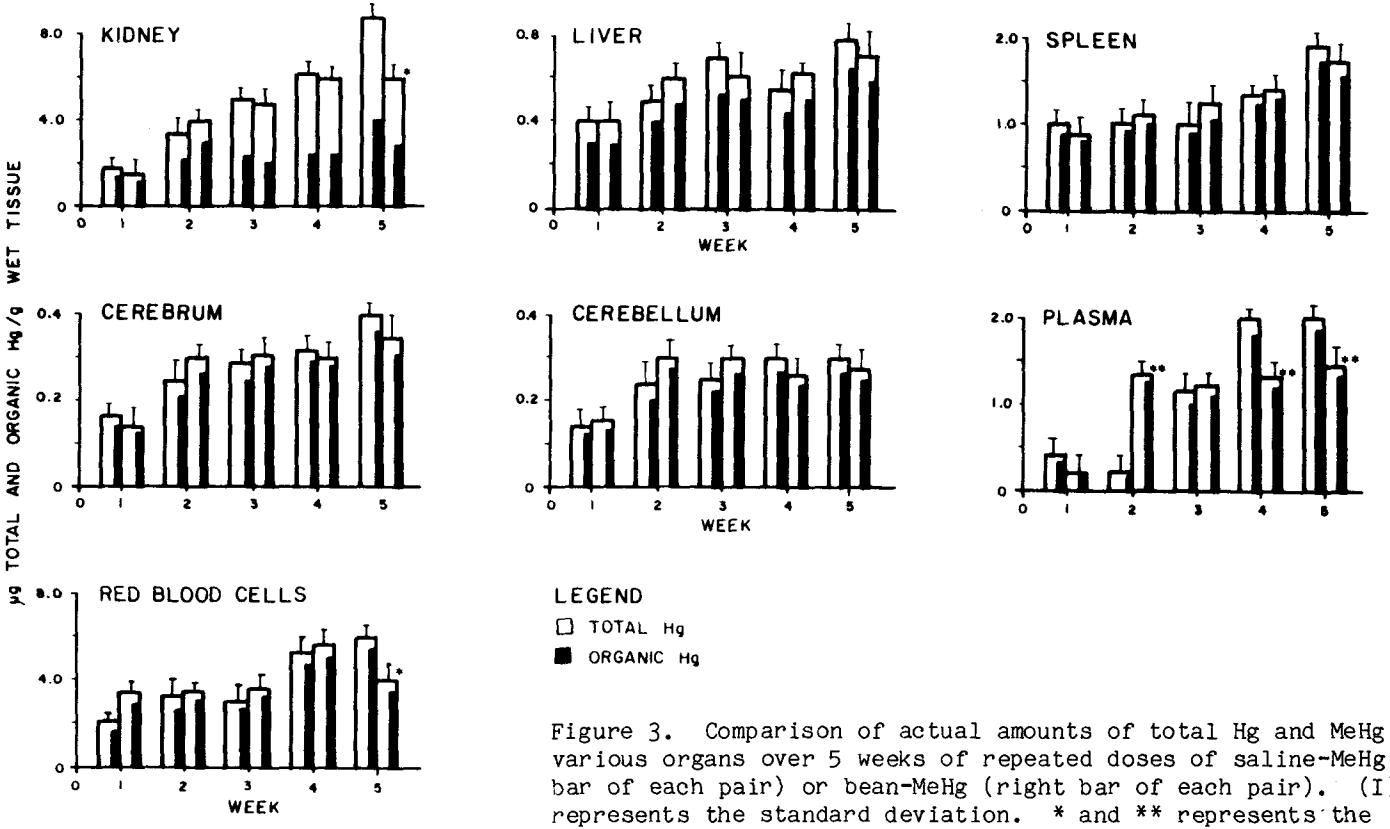

Figure 3. Comparison of actual amounts of total Hg and MeHg in various organs over 5 weeks of repeated doses of saline-MeHg (left bar of each pair) or bean-MeHg (right bar of each pair). (I) represents the standard deviation. * and ** represents the Student's t-test at .05% and .01% respectively.

glutathione-S- transferase in animals ingesting these plants (Wattenberg 1983). Also, the presence of large amounts of thiols (as found in crucifers) may influence demethylation non-enzymatically (Rowland et al. 1984). A similar effect may be operating in the present study to cause the differences observed after the bean-MeHg ingestion.

Blood parameters such as haematocrit and sedimentation rate did not change among treatments, indicating that within this time (5 weeks) no discernible anemia nor inflammatory-like process had been initiated (data not shown). The osmotic fragility did not change much during the 3 weeks but did drop slightly in week 4 and 5 in the saline-MeHg treated cells (data not shown). If the experiment had been continued, perhaps membrane damage in RBC would have been significant. Creatine values, on the other hand, were strikingly different among treatments (Table I); changes

Table 1. Changes in creatine concentration in red blood cells in the presence of methylmercury.

	mg/100 mL packed red blood cells	
	week 4	week 5
Control		
Saline	5.3 ± .3*	4.6 ± .5*
Bean	5.2 ± .5*	5.0 ± .6*
Bean-MeHg	3.6 ± .3**	3.8 ± .4**
Saline-MeHg	2.7 ± .3**	2.9 ± .3**

*n = 7, **n = 4
Normal physiological blood levels for creatine:
4.8 to 5.0 mg/100 mL (Griffiths 1968).

occurred after 4 and 5 weeks of repeated dosing. In saline-MeHg treated animals, creatine levels dropped by about 50% in RBC, but only about 35% in bean-MeHg treatments indicating a protective effect of the bean.

Methylmercury effects in the blood, plasma, liver and kidney may interfere with some step in creatine formation or utilization (glycine + arginine (kidney) → ornithine + guanidoacetic acid (liver) via S-adenosylmethionine → creatine (Swenson 1982). In relation to this, other studies in our laboratory have indicated that MeHg toxicity results in a dramatic wasting of muscle tissue

and a noticeable dilation of blood vessels in the ears of guinea pigs (Komsta-Szumska personal communication).

The results suggest that repeated dosing with MeHg may be affecting membrane integrity in RBC and also the phosphogen pool by interfering with creatine metabolism. Disruptions concurrent with or at a later time than the changes in the kidney may occur in blood and blood compartments (ie. spleen, liver) and are also strongly influenced by the action of the gut flora and/or their metabolites on plant products containing MeHg. The resulting decline in creatine levels could be part of the "latent or silent" period which has been described previously as characteristic of MeHg intoxication. A bean-incorporated MeHg protects against these effects. Further studies would have to involve a detailed analysis of the plant constituents and an investigation of enzymatic and hormonal changes as they are affected by plant secondary products in addition to how these products affect gut microflora *in vivo*.

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