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AN INTEGRATED STUDY OF THE MORPHOLOGICAL AND GROSS-ELEMENTAL CONSEQUENCES OF METHYL MERCURY INTOXICATION IN RATS, WITH PARTICULAR ATTENTION ON THE CEREBELLUM

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Abstract

Methylmercury accumulates in the kidney and liver of rats, but fairly selectively damages the cerebellum, resulting in the clinical symptoms of neurological ataxia after prolonged exposures. Within the cerebellum, morphological examination indicated that the small granule cells beneath the Purkinje layer are especially susceptible to the toxin, showing signs of pyknosis during the phase of locomotory disability, whilst the large Purkinje cells are relatively resistant to cytotoxic injury. Flame photometric and electron probe X-ray microanalysis (EPXMA) of digested samples of the major organs failed to detect any significant changes in the Na, K, Ca, Mg, S and P concentrations of the organs, including the cerebellum, at intervals after methylmercury administration by either gastric gavage or via the drinking water. It was suggested that if the lesion within the cerebellum is restricted, as the morphological evidence suggests, to a small cohort of functionally important granule cells, then it may be difficult to detect elemental changes within this subpopulation against the compositionally unaltered majority of cerebellar cells and their extracellular spaces. To identify and compositionally characterize the injured cells requires electron probe X-ray microanalysis of frozen sections, or fractured bulk samples. The deep-seated nature of the 'target cells' within the cerebellum presents formidable cryopreparative problems.

KEY WORDS: Methyl mercury, morphology, electrolytes, microdroplets, X-ray microanalysis.

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Introduction

Inorganic and organic mercurials appear to damage cells in different ways. Mercury has a high affinity for sulphydryl groups (the stability constant of $HgS \sim 10^{-50}$). Thus the massive K^+ leakage from yeast cells exposed to inorganic mercury was considered to reflect the general breakdown of the cell membrane as a physical permeability barrier, and not to the malfunction of a specific membrane function (Passow and Rothstein, 1960). But the mechanism of organomercurial cytotoxicity appears to be fundamentally different. Rothstein (1973) indicated that lipid soluble organomercurials, such as methylmercury, exert little direct effects on cell membranes, but accumulate within the cell and cause cell damage by inhibiting cellular metabolism.

Significant changes have been observed in the intracellular concentrations of physiologically active elements (Na, Mg, K, Ca, and Cl) in a variety of cell types that deviate from the 'normal' fully differentiated state. For example, Cameron and Smith (1980) recorded significantly higher Na and Cl concentrations and lower K and Mg concentrations in rapidly dividing and transformed cells. Dying thymocytes in experimentally diabetic rats have been shown (Warley, 1988; Warley and Morris, 1988) to lose elements, especially Mg and K, although the plasma membrane appears to be intact. Much evidence has also accumulated, suggesting that disturbances in intracellular Ca^{2+} homoeostasis, characterised by a sustained elevation of cytosolic Ca concentrations, play a crucial role in toxin-induced cell killing and pathological necrosis (Farber, 1981; Trump *et al.*, 1981; Jones *et al.*, 1989; Mc Conkey *et al.*, 1989).

The aim of the present study was to determine whether changes in the gross concentrations of essential elements (Na, K, Mg, Ca, P, Cl, and S) occur in some of the key target organs of rats exposed to methylmercury intoxication. The organs selected for analysis were: (i) the cerebellum, since the cerebellar granule neurones are selectively targeted by methylmercury, and the damage to this cohort of cells in reflected in the clinical symptoms of ataxia associated with organomercurial poisoning (Hunter and Russell 1954; Leyshon, 1988; Leyshon,

Jasani and Morgan, unpublished); (ii) the kidney, since it is the major site of mercury accumulation, irrespective of the chemical form in which the mercury is administered; (iii) the liver, a major depository of methylmercury that is relatively resistant to its cytotoxic effects. Chemical analysis was complimented by a study of morphological changes induced by methylmercury in the cerebellum, experiments that precede a proposed quantitative EPXMA study of elemental changes in cryosectioned susceptible (granule cells) and presumptive resistant target cells (Purkinje cells) in the cerebellum.

Materials and Methods

Two experiments were carried out, employing two different analytical techniques:-

(a) Experiment 1 - atomic absorption/emission spectrophotometry;
(b) Experiment 2 - EPXMA of sprayed microdroplets.

Experiment 1.

Animals and dosing. Male Wistar rats weighing 180-200g were taken for experimentation. Rats were dosed with 5 x 10.0 mg Hg/kg body weight (as methylmercuric chloride in 2.5 ml 0.9% sodium chloride) by gastric gavage over a 15 day time period. Control animals were administered a similar volume of the saline solution. Animals were killed 1, 14 and 28 days after the final dose. Control animals were taken 1 day after the final dose.

Light microscopy: cerebellum. Methyl mercury exposed animals were selected from the group killed at 28 days after the final dose, and fixed by whole-body perfusion with 4% glutaraldehyde in 0.1M cacodylate buffer, pH 7.4. The dissected cerebellae were than sliced before being dehydrated in a series of alcohols, and washed in acetone prior to embedding in methacrylate. Sections of nominal 3μm thickness were stained with Ehrlich's haematoxylin for 3.5 mins, differentiated in running tap water and counterstained in 1% aqueous eosin for 30 seconds.

Electron microscopy: cerebellum. Cerebellar material destined for electron microscopy was sliced into small cubes (approximately 1mm^3) after perfusion fixation and placed in free glutaraldehyde for 1hr. The tissue was washed overnight in cacodylate buffer, pH 7.4, post-fixed in Millonig's phosphate buffered osmium tetroxide for 1.5 hrs, and embedded in Araldite. Ultrathin tissue sections were viewed in a JEOL 100S TEM.

Atomic absorption/emission spectrophotometry: cerebellum, kidney, liver.

Tissue preparation. Fresh cerebellum, kidney and liver samples were dried at a temperature of 70°C for 48h and digested by boiling in an appropriate volume of nitric acid. After cooling to room temperature the digests were diluted with deionized water such that the strength of the acid matrix in the final solutions was 10%. Sample blanks were prepared in the same way and analysed.

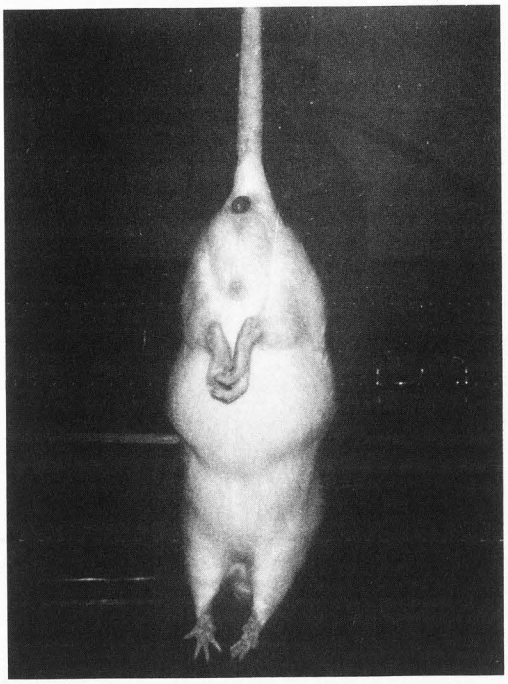

Figure 1: Hind-leg crossing reflex in a rat 28 days after completion of methylmercury administration via gastric gavage (Experiment 1).

Element analysis. Each sample solution was analysed for Ca, Na, Mg and K in a Varian AA 775 spectrophotometer. The Mg concentrations were determined by atomic absorption, while K, Ca, and Na levels were determined by flame emission. The analysis of each element is subject to interferences, consequently interference suppressants were added in the appropriate concentration as recommended by Varian (Varian Instruments, Walton-on-Thames, England).

Mercury Analysis. The total tissue mercury concentration was determined by atomic absorption spectrophotometry using the cold vapour technique (Hatch and Ott, 1968). Analysis was performed on a Varian Techtron Model 100 atomic absorption spectrophotometer with the Varian Model 64 analysis kit. For analysis the mercury must be in a readily reducible form (i.e. Hg^{2+}). Consequently, the organically bound mercury within the liver , kidney and cerbellar tissue was digested in concentrated sulphuric acid, according to the following modification of the method of Norheim and Froslie (1978).

Tissue slices were dried at 50°C for 48 hrs and then digested in 3 ml concentrated sulphuric for 6 hrs in a shaking water bath. After cooling, 5ml of 5% (w/v) potassium permanganate was added dropwise, and the samples were covered and stored overnight. To reduce all residual potassium permanganate and manganese dioxide in the samples, 20 ml of a sodium-chloride-hydroxylamine sulphate solution was added (60 ml of 30% sodium chloride and 50 ml of 25% hydroxylamine sulphate made up to 500 ml with deionized water) immediately prior to analysis. The now clear solution was transferred to a reaction vessel and 1 ml of 2% tin (II)

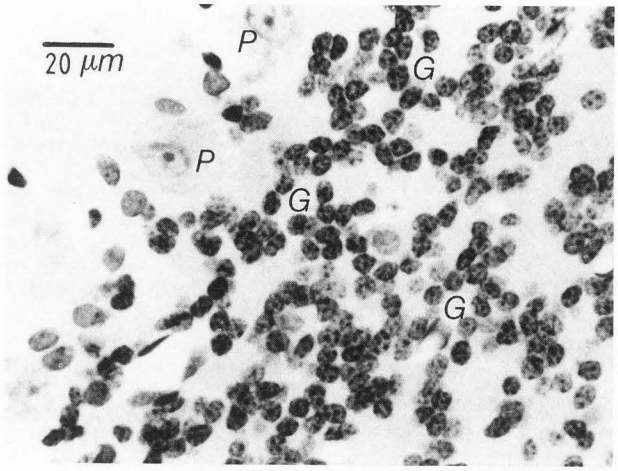

Figure 2: Histological micrograph of the cerebellum of a control rat (Experiment 1). Note the large Purkinje neurones (P) surrounded by the much smaller granule cells (G). There is no evidence of pyknotic nuclei in the granule cells within this field.

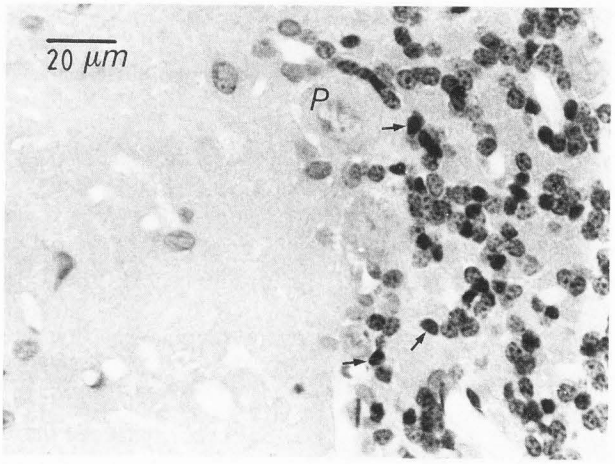

Figure 3: Histological micrograph of the cerebellum of a rat 28 days after the completion of methylmercury administration via gastric gavage (Experiment 1). Note the propensity of pyknotic granule cells (arrows) adjacent to the Purkinje layer; there are no obvious signs of damage in the Purkinje cells.

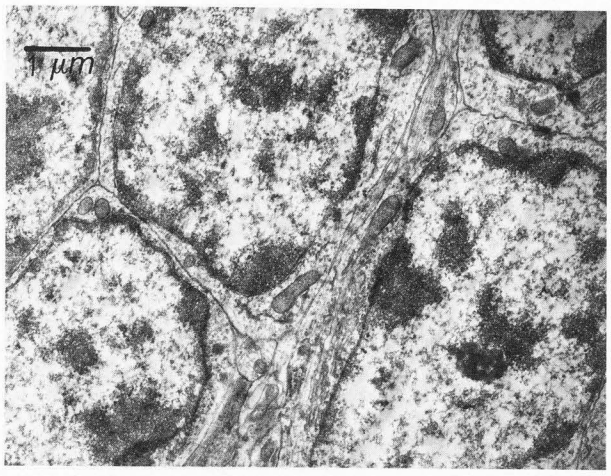

Figure 4: Electron micrograph of a granule cell in the cerebellum of a control rat. Note the very high nucleus: cytoplasm ratio.

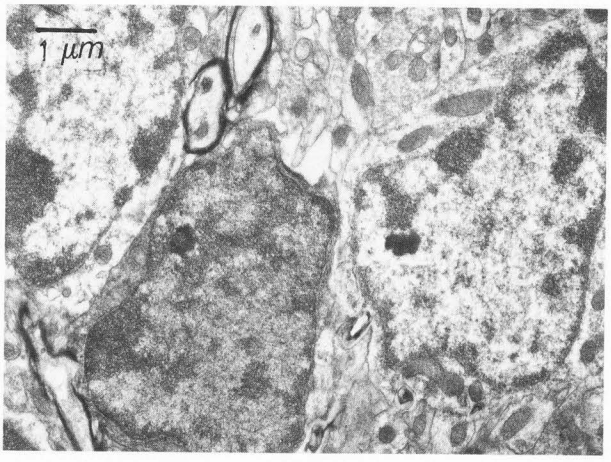

Figure 5: Electron micrograph of a pyknotic granule cell in the cerebellum of a rat after 28 days of methylmercury intoxication. (Experiment 1). Note the highly condensed cytoplasm of the cell making it difficult to discern individual organelles.

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chloride in concentrated hydrochloric acid was added. The vessel was closed immediately and the reaction mixture stirred vigorously for 60 seconds. After 60 seconds the air supply was connected to purge the mercury vapour from the reaction vessel into the quartz absorption cell. A flow rate of 2.5 l/min was maintained and mercury was recorded at 253.7 nm as a sharp analytical peak.

Experiment 2.

Animals and Dosing.

Male Wistar rats of 180-200g were selected for experimentation. Rats were exposed to methylmercuric chloride in their drinking water (20 mg/l). Control animals were maintained on mercury free water, available *ad libitum*. Groups of rats were killed after 14 and 42 days exposure. Total mercury concentration in the 3 target tissues was determined as described above.

EPXMA of sprayed microdroplets.

Sample preparation. Tissue samples (0.05g dry weight) were dried at 70°C for 48h, and digested in 3 ml boiling nitric acid. The digest were evaporated to dryness and allowed to cool to room temperature. Element extraction was carried out by the addition of 1ml 10% nitric acid, containing a known quantity of cobalt as a reference element (0.59g $Co(NO_3)_2$. $6H_2O$/250ml). The samples were sealed and stored for 72 hrs to

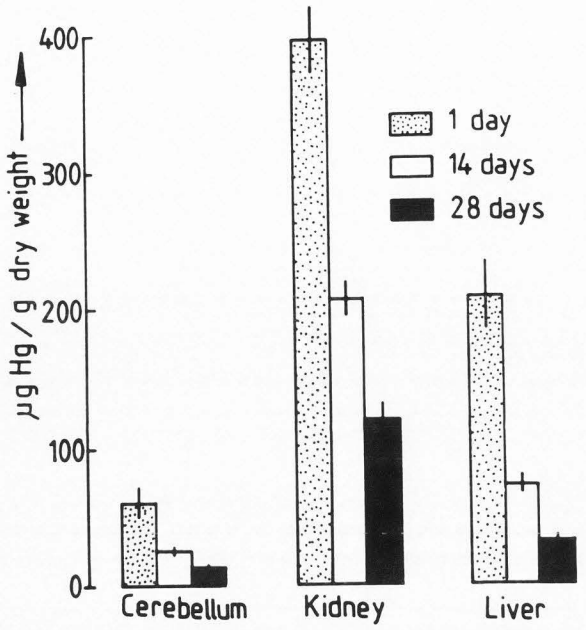

Figure 6: Concentration (µg/g dry weight) of total mercury in the organs of rats 1 (n=5), 14 (n=6) and 28 (n=5) days after the final dose of methylmercury adminstered via gastric gavage (Experiment 1). The data are expressed as mean ± 1 S.E. [Mercury was non-detectable in the organs of unexposed 'control' rats].

permit complete extraction to take place. Microdroplets of the tissue fluid were subsequently produced by spraying on coated titanium grids as described by Morgan (1983).

Quantitation of electrolytes in microdroplets. Analysis was performed in a Philips EM300 TEM equipped with a LINK 30mm^2 energy dispersive X-ray spectrometer interfaced with a LINK 860 Series 2 microprogrammed computer (Link Systems Ltd., London, England). The operating conditions of the microscope were: accelerating voltage of 80kV; magnification of x 6,300; a specimen tilt angle of 21°; and beam current of about 1nA. Microdroplet (5 per grid) of approximately 3.5µm in diameter were selected for analysis (100 seconds live time). The output count rate was standardized at approximately 1000 counts per second, the objective aperture was withdrawn, and a probe diameter sufficient to enclose entire microdroplets was selected.

The spectra obtained were initially processed by the Quantem Filtered Least Squares Programme produced by Link Systems. However, the actual quantitation was based on the Russ Ratio Model (Morgan *et al.*, 1975; Nott and Mavin, 1986). Quantitation was achieved using the known concentration of cobalt and the relative analytical sensitivities ("FST values" according to the LINK nomenclature of the elements compared to cobalt, previously determined from binary standards (Morgan and Winters, 1988).

Statistical analysis. Differences between the element compositions of the tissue from different time points were tested by the non-parametric Mann-Whitney U Test.

RESULTS

Experiment 1.

Clinical symptoms and morphological evidence of cerbellar intoxication. A characteristic latent period is associated with methylmercury induced central nervous system damage, such that it may be several weeks after exposure before the onset of symptoms. In the rat model described by Magos *et al.*, (1978) the so-called "flailing reflex" was found to be an early indicator of methylmercury poisoning. This is characterized by the swinging of the lower body when the intoxicated animal is held loosely under the forelimbs. Several days later a second functional abnormality, 'hind-leg crossing', is observed when the rats are suspended by their tails (Fig. 1). Figure 1 was taken 28 days after the completion of methylmercury administration, at which stage the disease process is judged to be irreversible (Magos *et al.*, 1978). The pathology of methylmercury poisoning is revealed at histological level as a selective cerebellar cortical atrophy of the granule cell layer. The control rat cerebellum is illustrated in Fig. 2; the large Purkinje neurones lie at the boundary of the molecular and granular layers, and are easily recognized by the deeply staining nucleolus. The adjacent granule cells are found tightly packed with nuclei characterized by an abundance of heterochromatin dots. After exposure to methylmercury (Fig. 3) a large proportion of the granule neurones appear heavily stained and shrunken. No histological evidence of damage could be detected in the Purkinje cells.

In the electron microscope, the normal rat granule cell body is seen to be occupied almost entirely by the nucleus (Fig. 4). The cytoplasm is confined to a thin peripheral shell, with the cytoplasmic organelles arranged in pools enclosed by the dimpled nucleus. Within the nucleus chromatin is distributed in large condensed blocks. In methyl mercury treated animals, some of the granule neurones had a condensed form (Fig. 5). The dense cytoplasm of these damaged cells made the organelles barely visible. Furthermore, the chromatin blocks were only just discerned within the nucleus.

Mercury concentrations in the cerebellum, kidney and liver. Total mercury concentrations of the three targeted organs at different times after the final methylmercury dose are presented in Fig. 6. Despite the cerebellum being the principal target organ of methylmercury toxicity, the toxin was found in greater concentrations in the parenchymatous kidney and liver, such that the distribution follows the order:- kidney liver cerebellum. Total mercury levels declined rapidly from days 1 to 28, with the liver concentration decreasing by approximately 85%, cerebellum by 75% and kidney by 50%.

Atomic absorption/emission spectrophotometry of electrolytes. Ca, Mg, Na and K concentrations in organs from the five experimental groups are shown in Fig. 7. Few significant differences were detected (Table 1), and no general trends arising from methylmercury exposure could be deduced.

Organ differences within the experimental time periods were detected. Both Ca and Na levels

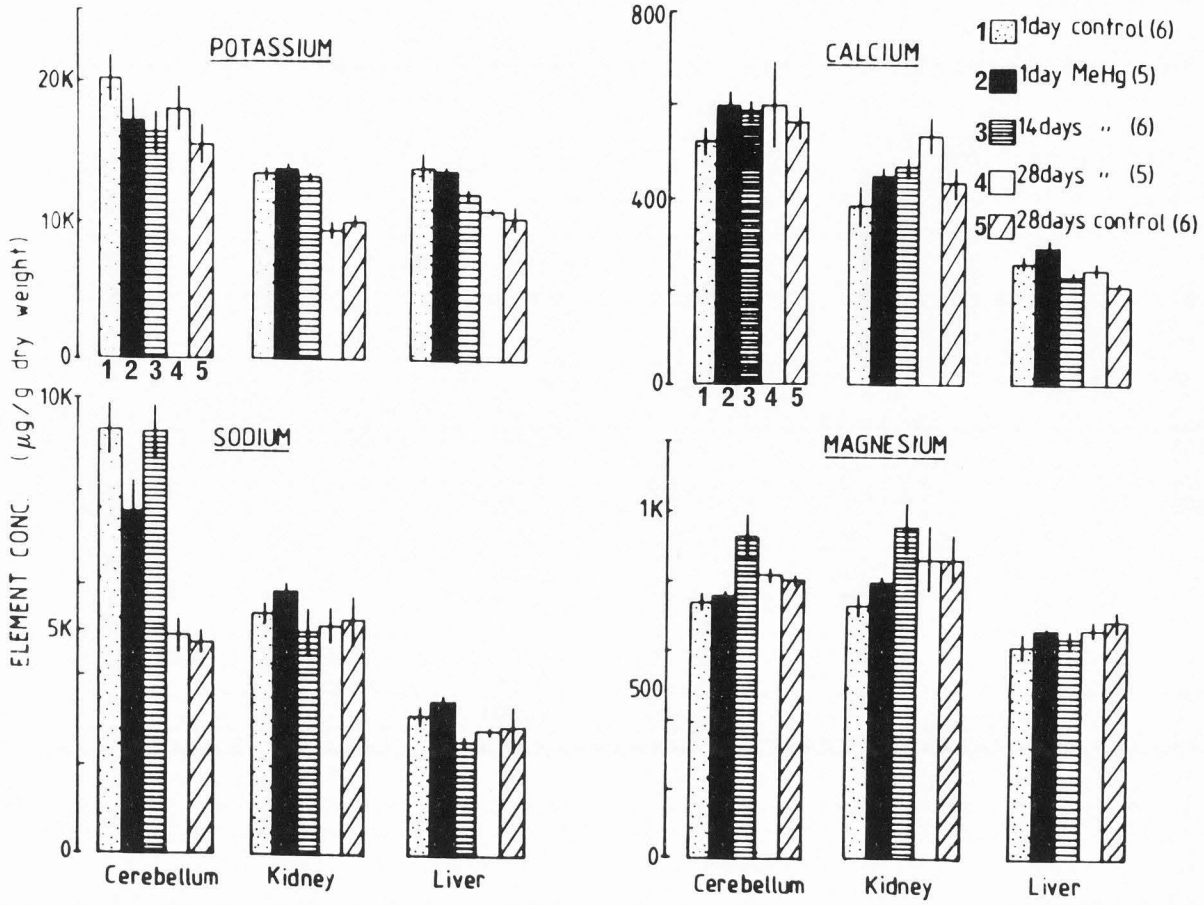

Figure 7: Na, Ca, K and Mg concentations (μg/g dry weight) in the organs of control rats and rats 1,14, and 28 days after the final dose of methylmercury administered via gastric gavage (Experiment 1). The data are expressed as mean ± 1S.E. [For statistical analysis of the data, see Table 1].

were of the order: cerebellum kidney liver. The K concentrations were found to be greatest in the cerebellum, with lower and comparable values in the liver and kidney tissues. The liver contained the lowest Mg concentrations.

Experiment 2.

Clinical symptoms. The appearance and behaviour of the dosed animals were closely observed for the onset of clinical symptoms of neurotoxicity. Activity was much reduced in the final week of the experiment and hind-leg crossing (see Fig. 1) was recorded in the greater proportion of the test group.

Mercury concentrations in the target organs. The total mercury concentrations of cerebellum, kidney and liver after different exposure periods to methylmercury are illustrated in Fig. 8. After 14 days exposure to the toxin, the gross organ distribution was as follows:- kidney liver cerebellum. At 28 days, the total mercury content of the cerebellum had increased by approximately 100%, while that of the kidney has increased by

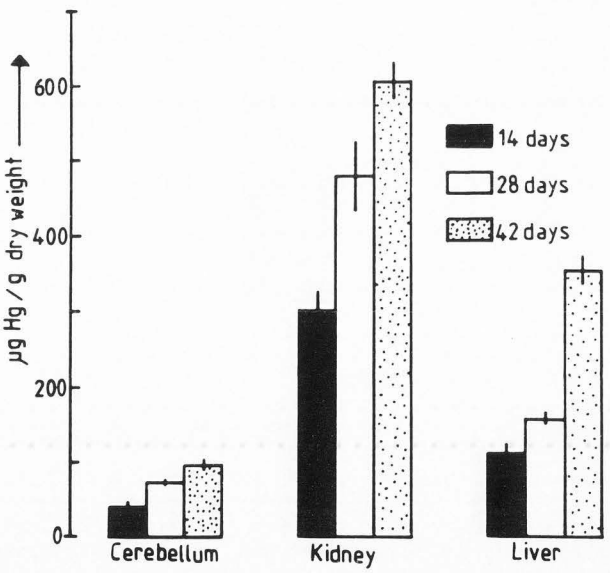

Figure 8: Concentration (μg/g dry weight) of total mercury in the organs of rats 14, (n=5) 28 (n=5), and 42 (n=5) days after the administration of methylmercury via their drinking water (Experiment 2). [Mercury was non-detectable in the organs of unexposed 'control' rats].

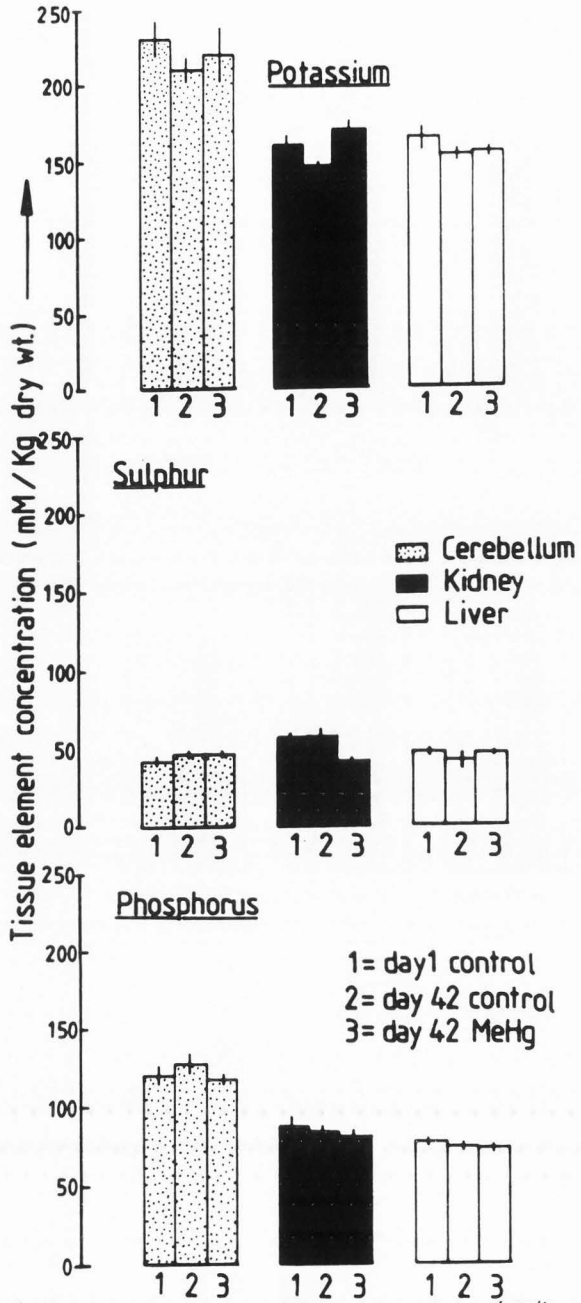

Figure 9: K, S, and P concentrations (mM/kg dry weight) in the organs of rats and 'experimental' rats 42 days after the administration of methylmercury via their drinking water (Experiment 2). Analyses performed by EPXMA. [For statistical analysis of the data, see Table 2].

60% and the liver by 45%. After 42 days methylmercury exposure, the overall gross distribution of mercury remained unaltered, yet a substantial increase of 80% was noted in the liver value.

Thus, changes in the concentrations of mercury show a progressive decline from high values in Experiment 1 (gastric gavage), but a progressive increase throughout Experiment 2 (via drinking water). This can be explained by differences in the application procols and the durations of the exposures; in Experiment 1, the rats were maintained for 14 or 28 days on a Hg-free diet after the completion of dosage; in Experiment 2, the rats were exposed continuously to Hg.

EPXMA of sprayed microdroplets of solubilized tissues. EPXMA was employed for the elemental analysis of tissue collected from rats closed according to the Expt. 2 protocol (i.e. methyl mercury via drinking water, not by gastric gavage). Three elements only were analysed: (P, S, and K) because (a) they occur at relatively high concentrations in most healthy tissues; (b) their analytical sensitivities are good; (c) they present few analytical difficulties due to peak overlaps; (d) P concentrations may provide a good 'marker' of changes in the content of phosphate-containing molecules, but EPXMA cannot of course identify the molecules; (e) S concentrations may reflect the mercury-induced synthesis of sulphydryl-containing sequestration proteins, such as metallothionein (Hamer, 1986; Rugstad, 1984); (f) K concentrations may indicate changes in membrane permeability.

The results are presented in Fig. 9. No significant differences were recorded between test and control tissues (Table 2) thus invalidating the hypothesis that methylmercury exposure causes the redistribution of these elements, and is eventually manifest as neurological disorder. However, organ differences within the test group were apparent. The cerebellum contained higher P and K concentrations compared with kidney and liver; S concentrations were highest in the kidney.

The element concentrations for Expt. 2 tissues are presented as mM/kg dry weight to permit comparisons with published observations on cryosectioned tissues. In general, the K concentrations (Fig. 9) are lower than these observed in cells (for example, normal thymocytes (Warley, 1988; Warley and Morris, 1988), which reflects the inclusion of the K-poor extracellular spaces in the bulk specimens. A disparity was found between our EPXMA and flame photometry K values (cf. Figs 9 and 7). The reasons for this are not absolutely clear, especially since the microdroplet method has been unequivocally validated (Morgan, 1983). It may be that the interference suppressants that were added to the flame emission samples were less than efficient. However, since no direct comparison between the findings of Expts. 1 and 2 was intended, the disparity is of no biological consequence in the present circumstances.

Discussion

The hind-leg crossing reflex that characterises irreversible methylmercury poisoning in the rat was observed in both the drinking-water and gastric-gavage dosed groups. The clinical lesion producing this neurological disturbance is located in the cerebellum. Within the cerebellum, the cytotoxicity of methylmercury appears to be fairly selectively targeted to the small granule neurones. These sensitive cells showed early signs of pyknosis in response to the toxin (Leyshon,

TABLE 1: Statistical comparison (Mann-Whitney U Test) of organ Ca, Na, Mg and K concentrations within the five experimental groups (see Fig. 7): Experiment 1, gastric gavage administration.

Organ	Treatment comparisons (group numbers)	Ca	Na	Mg	K
Cerebellum	1 : 2	NS	NS	NS	NS
	2 : 3	NS	NS	NS	NS
	3 : 4	NS	NS	NS	NS
	4 : 5	NS	NS	NS	NS
	1 : 5	NS	***	NS	NS
Kidney	1 : 2	NS	NS	NS	NS
	2 : 3	NS	NS	*	NS
	3 : 4	NS	NS	NS	**
	4 : 5	NS	NS	NS	NS
	1 : 5	NS	NS	NS	***
Liver	1 : 2	NS	NS	NS	NS
	2 : 3	**	*	NS	NS
	3 : 4	NS	NS	NS	NS
	4 : 5	NS	NS	NS	NS
	1 : 5	NS	NS	NS	NS

NS = not significantly different; * = $P<0.02$;
** = $P<0.01$; *** = $P<0.005$

TABLE 2: Statistical comparison (Mann-Whitney U Test) of organ P, S, and K, concentrations (mM/kg dry weight) of control and methylmercury dosed rats (Experiment 2) - see Fig. 9 for concentration values.

Treatment comparisons (group numbers)	Phosphorus	Sulphur	Potassium
1:2	NS	NS	NS
2:3	NS	NS	NS
1:3	NS	NS	*
2:3	NS	NS	***
1:2	NS	NS	NS
2:3	NS	NS	NS

NS = not significantly different; * $P<0.05$; *** = $P<0.05$.

Jasani and Morgan unpublished), whilst the much larger Purkinje neurones adjacent to them displayed much less easily discernible morphological signs of damage (Syversen *et al.*, 1981).

A knowledge of ion shifts following cellular injury provides a better understanding of the pathophysiology of disease processes. Since the kidney and liver are the major depositories of accumulated mercury in methylmercury intoxicated rats, these organs were analysed in addition to cerebellum.

The participation of Ca in cell injury caused by several diverse factors has been firmly established (Farber, 1981; Trump *et al.*, 1981; Orrenius *et al.*, 1989). Accumulating evidence suggests that sustained increases in cytosolic Ca^{2+} concentrations give rise to the following cytotoxic responses that can lead to cell death: activation of Ca^{2+} - dependant phospholipases (Chien *et al.*, 1977) and neutral proteases neutral (Nicotera *et al.*, 1989); stimulation of Ca^{2+} dependent endonuclease, resulting in DNA fragmentation (Jones *et al.*, 1989; McConkey *et al.*, 1989). However, the cytotoxicity of methylmercury may not be mediated via elevated cytosolic Ca^{2+} levels. Komsta-Szumska and Miller (1984) observed that Ca concentrations were markedly reduced in guinea pig cerebellum after exposure to methylmercury. These authors

proposed that the deficiency of Ca, a known neurotransmitter, can induce the neurological abnormalities of ataxia associated with this toxin. In the present study, however, we observed the clinical symptoms of intoxication but did not detect a significant change in the gross Ca concentrations in the intoxicated cerebellae. It is possible that the cytotoxicity is caused by a shift in Ca homoeostasis, involving a redistribution of Ca within certain cells and/or a change in the physiologically active Ca^{2+} ion pool. It may be significant that an ATP- and calmodulin-dependent Ca^{2+} uptake system was recently described in hepatocyte nuclei (Nicotera *et al.*, 1989). Whether or not such an intranuclear Ca^{2+} regulatory system exists in other cell types, and whether or not it is inhibited by methylmercury, is not presently known. Clearly measuring total Ca concentrations in whole tissue samples does not address these fundamental questions.

Trump *et al.* (1981), in a study of ischaemic kidney cortex, recorded movements of Na, K, Ca and Mg down their respective concentration gradients as early as 5 minutes after the onset of ischaemia. These ion fluxes reflected the disruption of ATPase activity. The action of mercury on the cell membrane is not clearly understood in these terms, although the aetiology of mercurial cytotoxicity appears to be dependent on the chemical form of the toxin. Miyamoto (1983) proposed two independent transcellular pathways for inorganic and organic mercurials. It was suggested that inorganic forms act at intracellular sites by entering through discrete membrane channels; lipid-soluble organomercurials, on the other hand, enter the cytosol by membrane diffusion. This model is supported by work on the effect of methylmercury on the lenses of humans living in contaminated environments (Takehara *et al.*, 1984). These workers found no differences in the Na, K, and Ca concentrations in the mercury-containing lenses of exposed patients compared with uncontaminated controls. These observations are similar to ours on the electrolyte concentrations of experimentally intoxicated rat tissue. It is also pertinent to note that Warley and Morris (1988) found that there was no loss of elements from thymocytes that had undergone the early morphological changes associated with pyknosis induced by experimental diabetes. Even when the pyknosis progressed, with a loss of Mg and K, this appeared to take place without a gross disruption of the plasma membrane.

Conclusion

The data obtained in the present study suggest that significant changes in the "whole-organ" element concentrations of the cerebellum, kidney and liver do not occur in rats exposed to methylmercury either via their drinking water or gastric gavage. These findings were contrary to expectations, given the level of total mercury accumulated in each organ and the progressive appearance of neurological symptoms. One possible explanation is that, although gross elemental concentrations did not change appreciably in the organ, there may have been a significant exchange of electrolytes between cells and their interstital fluid. For example, cells may have lost K to the extracellular compartment, and gained Na and Cl from it. Redistributions such as these, evoking considerable functional changes, could not be revealed by the analytical procedures adopted in the present study.On the other hand, morphological studies indicated that the lesion within the cerebellum was confined to a local subpopulation of granule neurones adjacent to the Purkinje layer (Leyshon Jasani and Morgan, unpublished observations). Thus, the disturbances in elemental composition, if they exist, very likely occur within this small, yet clinically important, cohort. It follows that a localized pathological injury can easily evade detection, due to the poor "signal: noise ratio" in a whole organ analysis where the majority of cells may be unaffected.

To obtain a better understanding of target cell responses to methylmercury intoxication in the cerebellum it is necessary to perform quantitative EPXMA analyses on individual cells. However, the cerebellum presents certain formidable preparative problems. The Purkinje layer, and the associated susceptible granule cells, lie approximately 200-250μm below the pial surface of the organ (Palay and Chang-Palay, 1974). Given that with quench-freezing rates of about 10,000°C/second, at normobaric pressures, ice-crystal free tissue is confined to a layer about 10-20μm deep adjacent to the cryogen/specimen boundary (Escaig, 1982), ice-crystal damage would preclude the EPXMA analysis in thin cryosections of such deep-seated cells. There are three alternatives that could be considered. First, to adopt a projectile or ballistic freezing technique for simultaneously freezing and excising a core of tissue (von Zglinicki *et al.*, 1986). Second, to prepare much thicker freeze-dried cryostat (alternatively, resin sections of freeze - dried or freeze-substituted samples), and to restrict the spatial resolution of the analysis to a whole cell or cytoplasm: nucleus level (Wroblewski *et al.*, 1978; Wroblewski and Wroblewski, 1986; Wroblewski, 1989). Third, the analysis could be performed on bulk specimens (Pieri *et al.*, 1977; Zs. Nagy *et al.*, 1977; Lustyik and Zs. Nagy, 1985).

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Discussion with Reviewers

D. Sigee: What proportion of the granule neurones had a condensed form at 28 days after Hg administration, and how did this level vary with time?

Authors: We have not conducted quantitative histological observations that enable us to answer this important question. However, it may be pertinent to outline some of the factors that we consider to affect the viability of granule cells. All of these factors are inextricably linked to time. First, the granule cells may, of course, be inordinately susceptible to Hg at concentrations below the detectable level of the physical development histochemical method that we have employed (Leyshon et al., unpublished). Second our observations indicate that the Purkinje neurones have the capacity to de-methylate mHg, and that the released (histochemically visible) inorganic Hg induces the synthesis of the cysteine-rich protein, metallothionein. Metallothionein has been shown to possess free-radical scavenging properties (Thornalley, P.J. and Vasak, M, 1985 - Possible role for metallothionein in protection against radiation-induced stress: kinetics and mechanisms of its reaction with superoxide and hydroxyl radicals. Biochim. Biophys. Acta 827, 36-44), and can thus play an important general cytoprotective role in inflammatory pathologies. However, it is conceivable that 'untrapped' free radicals escape from the Purkinje cells into the surrounding extracellular spaces and damage the small granule cells, whose endogenous cytoprotective capacity may be low. If this hypothesis has any merit, then we would predict that not only would the incidence of granule cell pyknosis vary with time, but also with space. The proportion of pyknotic cells would decrease along a perpendicular transect away from the focus of mHg accumulation and demethylation i.e. the Purkinje layer.

D.Sigee: Administration of Hg by gastric gavage (experiment 1) lead to a declining profile (Fig. 6) in mercury levels in the different organs, while administration via drinking water (experiment 2) lead to an increasing profile (Fig. 8). Why did this difference occur between the two experiments?

Authors: In experiments 1 and 2, not only were the routes of mHg administration different, but the entire dosing and sampling protocols were also very different. Specifically, the rats dosed by gastric gavage were not killed and analysed until 28 days after the final administration. Rats dosed via their drinking water were continuously exposed to low concentrations of mHg, and killed 14 and 42 days, respectively, into the exposure period. This accounts for the different mercury 'profiles' in the organs

R. Wroblewski: Could you please comment on the problem of analysis of Hg in the electron microscope (organic and inorganic)?

Authors: Mercury (boiling point = 356.6°C), in common with the halide elements (Roinel, N. et al., 1980 - Accuracy of electron microprobe analysis of biological fluids: choice of standard solutions, and range of linearity of the calibration curves. National Bureau of Standards Special Publication, 533, 101-130; Morgan, A.J. and Davies, T.W., 1982 - An electron microprobe study of the influence of beam current density on the stability of detectable elements in mixed-salts (isoatomic) microdroplets. J. Microsc. 125, 103-116), is very unstable under electron irradiation (Hodges, G.M. and Muir, M.D., 1974- X-ray spectroscopy in the scanning electron microscope study of cell and tissue culture material. In: Microprobe Analysis as Applied to Cells and Tissues ed. by T. Hall, P. Echlin, R. Kaufmann, 277-291, Academic Press, London; Bistricki, T., 1978 - Quantification of losses during X-ray microanalysis of mercury. In: Electron Microscopy 1978, ed, by J.M. Sturgess, Vol. II, p.12, Microscopical Society of Canada, Toronto). It is assumed that the strategies adopted by Roinel et al., for stabilizing chlorine in dried microdroplets (analysis at low temperature, surrounding with an organic matrix, and coating with a conductive film) would enhance the stability of Hg under electron irradiation. However, the fate of the volatilized Hg within the microscope column is a serious source of concern.