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Neurotoxicity of Organomercurial Compounds

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Mercury is a ubiquitous contaminant, and a range of chemical species is generated by human activity and natural environmental change. Elemental mercury and its inorganic and organic compounds have different toxic properties, but all them are considered hazardous in human exposure. In an equimolecular exposure basis, organomercurials with a short aliphatic chain are the most harmful compounds and they may cause irreversible damage to the nervous system. Methylmercury (CH_3Hg^+) is the most studied following the neurotoxic outbreaks identified as Minamata disease and the Iraq poisoning. The first description of the CNS pathology dates from 1954. Since then, the clinical neurology, the neuropathology and the mechanisms of neurotoxicity of organomercurials have been widely studied. The high thiol reactivity of CH_3Hg^+, as well as all mercury compounds, has been suggested to be the basis of their harmful biological effects. However, there is clear selectivity of CH_3Hg^+ for specific cell types and brain structures, which is not yet fully understood. The main mechanisms involved are inhibition of protein synthesis, microtubule disruption, increase of intracellular Ca^{2+} with disturbance of neurotransmitter function, oxidative stress and triggering of excitotoxicity mechanisms. The effects are more damaging during CNS development, leading to alterations of the structure and functionality of the nervous system. The major source of CH_3Hg^+ exposure is the consumption of fish and, therefore, its intake is practically unavoidable. The present concern is on the study of the effects of low level exposure to CH_3Hg^+ on human neurodevelopment, with a view to establishing a safe daily intake. Recommendations are 0.4 μg/kg body weight/day by the WHO and US FDA and, recently, 0.1 μg/kg body weight/day by the US EPA. Unfortunately, these levels are easily attained with few meals of fish per week, depending on the source of the fish and its position in the food chain.

Keywords: Intoxications with organomercurial compounds; Methylmercury; Neurotoxicity of organic mercury; Review; Risk assessment

INTRODUCTION

The use of mercury in manufacturing and medical purposes has been recorded since classical times in China, Egypt, Greece and Rome. Concomitantly, poisoning by this metal has also been reported since at least 2000 years ago, such as in Pliny the Elder's (23-79 AD) *Naturae Historiarum Libri,* which refers to cinnabar (HgS) poisoning among miners at Almaden, Spain (Rackman, 1952). Mercury as a poison has been documented for many centuries:

> *The leperous distilment; whose effect*
> *Holds such an enmity with blood of man*
> *That swift as quicksilver it courses through*
> *The natural gates and alleys of the body,*
> *And with a sudden vigour it doth posset*
> *And curd, like eager droppings into milk,*
> *The thin and wholesome blood: so did it mine;*
> [W. Shakespeare: Hamlet, *Prince of Denmark.*
> Act I, Scene 5 (1600)]

Historically, mercury poisoning has been mainly occupational and iatrogenic. In the 18th century Ramazinni described the occupational diseases developed by workers exposed to mercury (Goldwater, 1936). Elemental and inorganic mercury both continue to be widely used in industrial applications. In the 16th centu-

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ry, calomel (Hg_2Cl_2, mercurous chloride) was introduced as a treatment for syphilis (Laguna, 1555; Paracelsus, 1567 in Sigerest, 1996). Medical administration of mercury was largely practiced until the 20th century. It was present in cathartic, antisyphilitic, antihelminthic, diuretic, and many other preparations. It is still used in Chinese herbal medicines, in the form of calomel or even cinnabar, according to the traditional pharmacopeia (Ernst and Coon, 2001). Some of these preparations, which have become popular in Western countries, exceed the maximum concentrations permitted by regulatory bodies (WHO, 1991). Dental mercury amalgam, which releases low amounts of mercury (elemental mercury vapour and inorganic ions), was first recorded in China in 600 AD. The safety of mercury amalgam has long been a source of controversy (Dodes, 2001; Clarkson, 2002).

In contrast to the historical exposure to mercury, in the mid-20th century there appeared a new and unexpected form of mercury poisoning resulting from the environmental exposure to short-chain alkyl mercury compounds. Although organomercurials had been known since the 19th century, important poisoning outbreaks occurred in the 20th century. These organic forms of mercury were widely used as anti-fungicides for seed and cereal crop preservation and affected the general population mainly through contaminated food. Furthermore, organomercury compounds synthesised for various purposes have also exposed the population to these new agents (WHO, 1989; 1990; 1991).

This paper reviews the neurotoxicity of organomercurial compounds, focusing particularly on the effects of methylmercury (CH_3Hg^+), the most important form of mercury in terms of toxicity and adverse health effects from environmental exposure (Goyer and Clarkson, 2001). For the adverse effects of mercury vapour and inorganic mercury, the reader is referred to recent general reviews (WHO, 1991; Goyer and Clarkson, 2001; Klaassen, 2001; Clarkson, 2002). A great number of studies have been published on CH_3Hg^+ neurotoxicity, from the first toxic outbreaks to the present day. Because of the expansiveness of the subject, an overview is offered with greater detail in some of the issues and recent contributions. The first part of this review includes a chronological list of major epidemics of CH_3Hg^+ poisoning followed by a brief description of human toxic effects. The present criteria for risk assessment of CH_3Hg^+ exposure are fully discussed. Besides, a few other organomercurial compounds with some history of human harmful exposure are briefly revised. The second part of the review deals with the results of *in vivo* and *in vitro* experimental studies and examines the present knowledge of the neurotoxicity mechanisms of CH_3Hg^+ and related compounds. Finally, a short section about treatment of poisoning confirms the harmfulness of organomercurials.

HUMAN NEUROTOXICOLOGY

I. Epidemiology and Clinical Manifestations of CH_3Hg^+ Poisoning

Hunter-Russell Syndrome — Before the Minamata epidemic (see below) the human toxicity of CH_3Hg^+ had already been described as a rare neurological condition developed after occupational and laboratory exposures. The first cases recorded date from 1863, shortly after the synthesis of organic mercurials, and gravely affected three researchers working with these compounds (Marsh,1979). In 1940 Hunter *et al.* accurately described the clinical features of intoxication by CH_3Hg^+ in 4 workers after industrial exposure. They presented with paresthesias, dysarthria, sensory deficit, postlabyrinthine deafness, cerebellar ataxia, and progressive constriction of the visual field. This condition strikingly differed from the classic manifestations of mercury poisoning—both from exposure to elemental mercury vapour or to inorganic mercury salts—and became known as the Hunter-Russell syndrome (Pentschew, 1958).

Minamata Disease — In the mid 1950s CH_3Hg^+ began to be the subject of serious public health concern and initiated the recognition of a new toxicological phenomenon manifested in the incidence of epidemic outbreaks of mass poisoning produced by chemicals related to industrial pollution and affecting people through complex environmental pathways and food. An epidemic of a severe neurological disorder occurring among the residents around Minamata Bay (southwestern coast of Kyushu Island, Kumamoto Prefecture, Japan) was first reported in 1956. It was named Minamata disease given its unusual and baffling characteristics. The disease, affecting mainly the local fishermen and their families, was later identified as a Hunter-Russell syndrome caused by CH_3Hg^+ present in fish and shellfish from the Bay and consumed in the surrounding area (Takeuchi *et al.*, 1962; Igata, 1993; Harada, 1995; Kondo, 1997). Epidemiological observations that fish-eating animals in the area (cats and seagulls) had similar neurological signs, led to the identity of the agent. It took, however, several years to show that the inorganic mercury present in wastes discharged into the Bay by Chisso Hiryo Company, a large chemical factory in Minamata city, was methylated by microorganisms present in water and sediments and bioconcentrated through the aquatic food chain (Wood *et al.*, 1968; King *et al.*, 2000; Boening,

2000). The inorganic mercury present in the factory effluents, which contained also organic mercury, was used as a catalyst in the production of vinyl chloride, fertilizers and other chemicals since 1952 (Harada, 1995). A second epidemic outbreak of Minamata disease was also detected in Japan in 1965 along the Agano River (Niigata Prefecture, Honshu Island). The source of mercury was the effluent of a chemical plant located 50 km upstream of Niigata city, in the mouth of the river (Kondo, 2000).

The number of affected people in these two outbreaks of Minamata disease is still a matter of controversy due to the economical compensations from Chisso Co. and the Government involved in the official recognition as a victim. In 1990 there were 2920 cases certified (2230 from the Minamata region and 690 from Niigata), but the number of applications amounted to around 18000 (Kondo, 1997; 2000). It is considered that the total figure of officially recognized cases is only a modest estimate of the real number of patients (Miyai, 1997; Kondo, 2000). Epidemiological studies on chronic CH_3Hg^+ poisoning, relating Hg content in hair with careful neurological examination in exposed and non-exposed residents in Kumamoto Prefecture, suggest that the actual number of cases could be about 20000 if all people on the coastal area were taken into account (Ninomiya *et al.*, 1995; Watts, 2001). Until now more than 900 patients have died as a consequence of this disease (Watts, 2001).

The clinical manifestations of Minamata disease were found to be essentially those of Hunter-Russell syndrome. Nonetheless, the large number of cases showing sensory and motor disorders allowed a more precise and detailed study of the neurological features of CH_3Hg^+ intoxication. In Minamata it was confirmed that there is a latency period of weeks to months between exposure and the onset of symptoms, this interval depending on the level and duration of exposure as well as an individual's susceptibility. Mild cases tended to exhibit only sensory impairments, whereas severe cases manifested additional motor deficits (ataxia and dysarthria), peripheral scotoma and hearing loss. In nearly all cases paresthesias of the extremities (glove and stocking type) were the initial symptom reported by patients. This sometimes progressed to a fully developed syndrome which in the most severely affected patients included deterioration of cognitive functions, paralysis and eventually death, or survival with major neurological disabilities. Those moderately affected could greatly improve after exposure ended (WHO, 1990; Igata, 1993; Harada, 1995). A chronic form of Minamata disease has also been described for cases that steadily manifest several years after exposure (Uchino *et al.*, 1995).

Iraq Neuropathy — The 1972 outbreak of mass poisoning in Iraq illustrates the third aspect of public health concern—in addition to the industrial and environmental exposures mentioned—presented by CH_3Hg^+, that is, the direct contamination of food ensuing its use as pesticide in agriculture. This large epidemic appeared swiftly in rural Iraqi areas and was rapidly identified as being caused by CH_3Hg^+ poisoning, given the medical experience with organomercurial intoxications from previous lesser outbreaks in the country. Poisoning had resulted from consumption of home-baked bread prepared with flour from CH_3Hg^+-treated seed grain. That wheat grain had been imported treated with CH_3Hg^+ as a fungicide and was intended for planting and not for direct human consumption, but it was deviated to make bread. As a consequence there were 6,530 patients hospitalised and 459 died. However, field studies have suggested that as many as 40,000 to 50,000 people were actually affected (Bakir *et al.*, 1973; Myers *et al.*, 2000a; Clarkson, 2002).

The clinical features of Iraqi patients confirmed the neurological deficits reported for CH_3Hg^+ poisoning in Minamata. The differences observed—a lower rate of mortality and a higher degree of recovery in Iraq than in Minamata—probably resulted from the shorter time of exposure, and perhaps a lower dose, in Iraq, where the measures adopted by the government limited the outbreak to a few months. This provided the means to define a highly significant correlation between the amount of contaminated bread ingested, hair and blood mercury concentrations, and neurological manifestations (Bakir *et al.*, 1973; 1980; Greenwood, 1985; WHO, 1990; Myers *et al.*, 2000a; Klaasen, 2001; Clarkson, 2002).

II. Neuropathology

Since the first description of the CNS lesions reported by Hunter and Russell (1954) in a fatal case of CH_3Hg^+ intoxication, the neuropathology induced by this organomercurial has been extensively studied in the Minamata and Iraq epidemics, and in experimental animals. In victims of these outbreaks the neuropathological findings correspond, in general, fairly well to the clinical neurology observed.

Methylmercury poisoning produces histopathological damage in specific areas of the brain. The most marked changes observed at autopsy are focal atrophy in the cerebellum and in the cerebral calcarine cortex, with less severe disturbances in the precentral, postcentral and temporal cortices. A striking microscopical feature consists in an important loss of granule cells in the cerebellum with relative preservation of the adjacent Purkinje

cell layer. Therefore, ataxia results from this destruction of the granular layer (Takeuchi *et al.*, 1962; Shiraki, 1979; Eto, 1997; 2000). In dysarthria, however, the lesions in the precentral cortex may also be involved (Shiraki, 1979). Similarly, the selective and extensive neuronal loss found in the anterior area of the calcarine fissure of the occipital lobe provides the correlate for the peripheral constriction of the visual field, since this area receives the projection fibers from the peripheral zones of the retina; the caudal part of the calcarine cortex, representing the central zone of the retina, is much less damaged or even free of lesions (Shiraki, 1979; Eto, 1997; Fox and Boyes, 2001). Likewise, hearing impairment corresponds with the destruction of neurons observed in the auditory cortices, thus the deficit being of postlabyrinthic origin (Shiraki, 1979). The damaged areas also display glial proliferation and, in chronic cases, a secondary degeneration from primary lesions as well as a marked hemispheric demyelination (Shiraki, 1979; Eto, 1997). Moreover, astrocyte proliferation has been reported in the calcarine cortex and in the cerebellar granular layer (Takeuchi *et al.*, 1962; Shiraki, 1979). No pathological changes have been described in the optic nerves, retina, white matter, spinal cord or dorsal root ganglia in humans; lesions in peripheral nerves are unclear (Hunter and Russell, 1954; Shiraki, 1979; Eto, 1997).

III. Neurodevelopmental Toxicity

Both in the Minamata and Iraq epidemics there were pregnant women with low exposures and mild symptoms or even with no clinical manifestations at all who gave birth to infants with important neurological deficits.

In Minamata a high percentage of infants born between 1955 and 1960 presented with severe mental and motor retardation, ataxia, athetosis, deafness, blindness, spastic diplegia or quadriplegia and other symptoms similar to cerebral palsy (Takeuchi *et al.*, 1962; Marsh, 1979; Kondo, 2000). Neuropathological examination of these congenital cases showed diffuse and extensive damage in the brain, in contrast with the focal and selective lesions found in adult CNS. The main feature was an important disarray of the cortical organization of cerebral and cerebellar neural cells resulting from developmental alteration of the fetal brain. Neural migration during development had failed, and cells were in incorrect places, such as astrocytes in white matter or Purkinje cells outside their layer (Shiraki, 1979; Choi, 1989; WHO, 1990; Harada, 1995; Castoldi *et al.*, 2001).

In Iraq similar cases of severely affected children were also seen. In addition, infants with prenatal exposure and relatively mild neurological deficits were there first identified. This group manifested, in different degrees, mental retardation with delayed onset of speech and impaired motor, sensory and autonomic functions (Bakir *et al.*, 1980; Marsh *et al.*, 1987; WHO, 1990; Myers *et al.*, 2000a).

IV. Human Exposure and Risk Assessment of Neurotoxic Effects

The outbreaks cited above and other lesser episodes of CH_3Hg^+ poisoning raised an important public health issue: what levels of exposure to CH_3Hg^+ induce effects, and what is the threshold of safety for toxic hazards? The most crucial question was posed by the fetal neurovulnerability occurring at exposures from asymptomatic mothers. Retrospective analysis of the epidemics and new studies on specific populations have been conducted to approach these queries and attempt to establish concentration-effect relationships for the different types of exposure. As is detailed in the following sections, mercury content in hair was a very useful indicator or biomarker of present and past CH_3Hg^+ exposure as it accumulates in growing scalp hair at a concentration about 250 times that of blood levels (see Clarkson, 2002). As regard to other available biological media, levels of mercury in blood are used to monitor recent exposures, whereas the mercury excreted in urine represents only a small part of the body burden.

Concentration-Effect Relationships — Data from Japan showed a large range of hair mercury content—the only available indicator of exposure at that time—in patients of Minamata disease. Values up to 700 ppm were recorded and the mercury levels related qualitatively to the clinical severity. In Niigata a semiquantitative study indicated a good correlation between exposure (amount of Agano river fish ingested), total mercury in hair, and incidence of disease (Harada, 1995; Kondo, 1997; 2000). It was estimated from the Minamata studies that, at the onset of symptoms of CH_3Hg^+ intoxication, the lowest concentration of mercury in hair is about 50 ppm (WHO, 1990). This value, considered as a threshold level for neurotoxic effects in adults, has been found to be somewhat lower, about 30 ppm, when the neurological endpoints evaluated are more discriminative; the 'normal' upper limit of a non-toxic level of hair mercury was set at 10 ppm (WHO, 1990; Kondo, 2000).

Studies of the Iraq outbreak, where blood and hair samples were collected, permitted the establishment of concentration-effect relationships for adult and prenatal exposures to CH_3Hg^+. As concerns adults, blood and hair levels of mercury, which were used as biological markers of exposure, were associated with neurological endpoints (Hunter-Russell syndrome manifestations). The outbreak

characteristics, with high exposures — up to 674 ppm of mercury in hair — lasting only a few months, facilitated the dose-response analysis and definition (Bakir *et al.*, 1973; Marsh, 1979; Myers *et al.*, 2000a). Thus, it was observed that when the concentration of Hg in blood attain levels of 0.1-0.5 μg/ml (0.5-2.5 μM) paresthesias and dysarthria emerge as the first manifestations of CH_3Hg^+ poisoning (5% of cases); ataxia, and visual and hearing deficits appeared at 0.5-1.0 μg/g (2.5-5.0 μM), and as blood Hg levels rise increases the frequency of cases with these symptoms and signs along with death from 3-4 μg/ml (15-20 μM). From the concentration-effect study, *the lowest observed adverse effect level* (LOAEL) for paresthesias — the earliest symptom — was estimated to be at a blood Hg level of about 0.2 μg/ml, which correspond to a hair content of 50 μg/g (50 ppm) and is in good agreement with the threshold level reported for Minamata (Bakir *et al.*, 1973; 1980; Goyer and Clarkson, 2001; Klaasen 2001).

As to prenatal exposure to CH_3Hg^+, Hg concentration in maternal hair was associated with psychomotor retardation (late walking and talking), the critical endpoint in infants. Fetal exposure was assessed by analysing with X-ray fluorescence spectrometry contiguous segments of single strands of maternal hair, which can provide information on the duration and degree of exposure of previous periods of time (Marsh *et al.*, 1987). Here, the LOAEL for this neurological endpoint was calculated to be about 10 ppm in the mother's hair during pregnancy (Marsh *et al.*, 1987; Cox *et al.*, 1989; 1995). This hair content corresponds to a blood Hg level of 0.04 μg/ml (0.2 μM). The remarkable point of these results consists in that the LOAEL calculated for child effects occurs at maternal exposures well within the range of levels considered as "normal", a value that for adult effects was estimated as a *no observed adverse effect level* (NOAEL).

From these studies on 81 prenatally exposed Iraqi children have been derived various safety values of reference for health protection, as the most recently established by the US Environmental Protection Agency (US EPA, 1997; 2001), which has set as the maximum daily intake of CH_3Hg^+ a dose of reference (RfD) of 0.1 μg/kg body weight/day. To establish a dose as low as that, the risk assessment of CH_3Hg^+ has taken into account the specific toxicokinetic characteristics of this agent.

Toxicokinetics of CH_3Hg^+ — The biological markers of exposure — i.e., in the present case methylmercury content in hair, blood, urine and cord blood — are used in the concentration-effect analyses, which constitute the basis for health risk and safety assessment and for regulatory policies. These exercises require knowledge of the biokinetics of the agent. In brief, in humans CH_3Hg^+ — unlike the inorganic mercurials — is absorbed ~90–95% from the gastrointestinal tract. In blood it is accumulated in erythrocytes (ratio red cells/plasma ~20:1) —measurements should be made in red cells, since most inorganic Hg is in plasma — and distributed to all tissues, crossing easily the blood-brain barrier and the placenta. The half-life ($t_{1/2}$) in human blood is between 40 and 105 days, which implies that for an average $t_{1/2}$ of 70 days the steady-state body burden is reached in about 1 year (five $t_{1/2}$). Methylmercury is secreted into bile conjugated with glutathione but Hg is largely reabsorbed through an extensive enterohepatic recirculation, the small amount remaining in the intestinal tract being eliminated in the feces. Excretion in urine represents less than 10% of the total load excreted. The kinetic properties of CH_3Hg^+ determine that in steady state only about 1% of the body burden is eliminated per day (US EPA, 1997; Goyer and Clarkson, 2001; Klaasen, 2001; Clarkson, 2002). In the organism, about 5% of the total CH_3Hg^+ body burden is in the blood compartment, and ~10% in the brain. The ratio of brain/blood concentrations is ~7 and the most useful hair/blood is ~250; levels in fetal brain are ~5-7 times higher than in maternal blood (Klaasen, 2001; Clarkson, 2002). In addition, other frequently applied ratios relate blood concentration and daily intake and it has been estimated to be ~0.95 μg Hg/L (4.75 nM) in blood per 1 μg day of CH_3Hg^+ intake (WHO, 1990; US EPA, 1997). It should be noted that these figures are averages and that their use in concentration-effect modelling, in risk assessment or as biomarker ratios has to take into account the large variability of CH_3Hg^+ toxicokinetics. Thus, for example, the much used hair/blood ratio shows an interindividual variation from 140 to 370 (WHO, 1990; Bartell *et al.*, 2000).

Reference Concentration and Daily Intake — The parameters mentioned — using various complex mathematical models and other factors — allows for estimation of the maximum daily intake of CH_3Hg^+ to avoid attaining the toxic threshold. Thus, in a simplified form, considering that 10 μg/g (10 ppm) of Hg in hair is the NOAEL, and assuming steady-state kinetics, from the average hair/blood ratio is calculated a blood Hg level of 0.04-0.08 μg/ml (0.2-0.4 μM), value confirmed by direct determination of CH_3Hg^+ in blood. From the distribution parameters (~5% of total amount in blood compartment) a body burden of 4-8 mg is calculated. Since ~1% of the body burden is eliminated in steady-state, that means 40-80 μg day, which would correspond to the maximum intake to maintain the no effect level. Applying the usual safety factor of 1/10, to account for population variability and sensibility, a RfD of 4-8 μg day or 0.06-0.12 μg CH_3Hg^+/kg body weight per day is

obtained as maximum intake. A similar value is obtained applying the ratio of 0.95 μg/L per 1 μg of CH_3Hg^+ daily intake. In 1997 the US EPA (1997; 2001), after consideration of many factors and especially the fetal neurovulnerability, recommended the mentioned RfD of 0.1 μg/kg body weight/day, reducing by five the previous estimate of safe intake. WHO had recommended previously 0.4 μg/kg/day as well as the US FDA, which still maintains this value (WHO, 1990; US EPA, 2001).

Effects of Low-Level Exposures — There exist at present many anthropogenic sources of mercury. The main emissions are originated in fossil fuel combustion, waste disposal, iron and steel production, gold mining (Amazonian basin) and the mercury cell method of industrial chlorine production. It is estimated that by the mid 1990s the world atmospheric emissions of mercury amounted to about 2235 tons per year (McGinn, 2002). This mercury is added to the natural sources (volcanoes, oceanic emissions, etc.) and represents probably a larger amount of emissions. There exists a global environmental transport and distribution of mercury as well as a complex ecological cycle (reviewed in Boening, 2000). As mentioned above, mercury in river and sea water and in sediments can be converted to CH_3Hg^+ by microbial action (Wood *et al.*, 1968; King *et al.*, 2000), which then is incorporated into the food chain and progressively concentrated in fish (Boening, 2000). Content of CH_3Hg^+ in fish, which is mainly present in soft tissues (edible muscle), varies largely according to the areas and the local contamination. Thus, ocean fish has 0.05-0.25 ppm on average, freshwater fish in the Amazon river 0.5 ppm, freshwater fish in Northwest USA 0.5-1 ppm, pilot whale up to 3 ppm, etc. (Myers *et al.*, 2000a; Castoldi *et al.*, 2001; Love *et al.*, 2003). Moreover, fish resident in the Mediterranean Sea contains 3-4 times more CH_3Hg^+ than the same Atlantic species (Renzoni *et al.*, 1998). In Minamata Bay levels of CH_3Hg^+ reached 50 ppm in fish and 85 ppm in shellfish (Harada, 1995).

Human exposure to CH_3Hg^+ is primarily from fish consumption. Neurodevelopmental effects observed in Iraq at exposures of CH_3Hg^+ theretofore considered to be safe, led to the EPA lowering the recommended maximum daily intake. This has prompted different studies on the possible effects in children, related to the maternal exposure to CH_3Hg^+ from fish ingestion. In fact, the lowest exposure to CH_3Hg^+ that can affect neurodevelopment is still unknown. Most human studies have focused on specific populations consuming large amounts of fish with high content of CH_3Hg^+, and measuring indicators of exposure along with neurological endpoints, especially in children. These studies have produced contradictory and controversial results. In a prospective longitudinal cohort study carried out in the Seychelles Islands — the Seychelles Child Development Study — on a large number of mother-child pairs (n=711), no association between prenatal CH_3Hg^+ exposure and any endpoint was observed. This population consumes ocean fish with CH_3Hg^+ concentrations of 0.05-0.25 ppm. It was measured a maternal hair Hg content of ~6.8 ppm and 6.5 ppm in 66-month-old children (Davidson *et al.*, 1999; 2000; 2001; Myers *et al.*, 2000a; 2000b).

Different results have been reported from another large cohort (n=917) studied in the Faroe Islands, where seafood diet includes pilot whale with average concentrations of CH_3Hg^+ of about 1.6 ppm and a maximum of 3 ppm. Maternal hair Hg content of 4.08 ppm at parturition was associated with a cord blood Hg level of 0.02 μg/ml. Children were examined with a wide-ranging battery of neurological and psychological tests. The Faroe Islands epidemiological study found a statistically significant association between prenatal CH_3Hg^+ exposure and deficits in memory, language, attention, motor function, and visual spatial perception in 7-year-old children (Grandjean *et al.*, 1997; 1998; 1999; 2003; Grandjean and White, 1999; Myers *et al.*, 2000a; Steurwald *et al.*, 2000). The differences between these two studies on large cohorts have been discussed in relation to the different ethnic composition of the populations investigated, the source and content of CH_3Hg^+ exposure (oceanic fish *vs* pilot whale with higher CH_3Hg^+), the nature of endpoints, criteria for selection of neurobehavioral tests, presence of polychlorinated byphenyls (PCBs) — children with high levels were excluded —, the use of cord-blood concentration as a better indicator of fetal exposure than maternal hair content, etc. (Grandjean and White 1999; Myers *et al.*, 2000a). As concerns the Faroe Islands study, it is of interest to note that a panel of the US National Academy of Sciences (NAS, 2000) endorsed the EPA safety levels of 1997 relying on its assessment of the Faroe Islands study findings of effects at low-level exposures (Kaiser, 2000).

V. Neurotoxicity of Other Organomercurials

Outbreaks — Organomercurials, especially the alkylmercury compounds, have been widely used as disinfectants and fungicides since 1914. Their main application has been in seed grains (cereal crops, legumes, vegetables, etc.) and timber. Outbreaks of poisoning similar to the Iraq epidemic, but less extensive, have been reported in many countries since the mid 1950s (Pakistan, Guatemala, Iraq on three separate occasions, USA, Ghana, etc.). It is very probable that intoxications of this type had occurred before but had gone undetected. Most

of the incidents followed the Iraqi pattern: organomercurial-treated seed grain used as edible grain. Organomercurial compounds other than CH_3Hg^+ have also been implicated in these outbreaks. This was the case of over 100 people in Iraq poisoned with wheat seeds treated with ethylmercury-*p*-toluene sulfonalide in 1956; a second Iraqi episode occurred in 1961 when about 100 people were poisoned by flour from grain treated with ethylmercury chloride and phenylmercury acetate. In addition to neurological signs they suffered renal and gastrointestinal disorders, diaphoresis, muscular pain, pruritus of the extremities and other clinical ailments. It should be noted that many of these manifestations are reminiscent of inorganic mercury effects. In fact, in most organomercurials the carbon-mercury bond is labile — case or aryl mercurial — or cleaved after absorption, as occurs extensively with alkylmercurials other than CH_3Hg^+. Hence, toxicity results from the inorganic mercury ions formed in the body. A case in point was the acrodynia episode in Buenos Aires in 1980, in which infants absorbed phenylmercury through the skin from diapers treated with this fungicide agent (Gotelli *et al.*, 1985; Klaasen, 2001). Acrodynia, or *pink disease*, is a disorder (erythema and itching of the extremities, irritability, anorexia, diaphoresis, photophobia, etc) that manifests itself in infants and young children after long-term ingestion of inorganic mercury (such as calomel, a cathartic). The use of organomercurials is at present banned in most countries (Marsh, 1979; WHO, 1989, 1990; 1991; Klaasen, 2001; Clarkson, 2002).

Thimerosal — Thimerosal, or thiomersal, is a thiolate ethylmercury derivative, which is metabolised to ethylmercury. It was previously used as a topical bacteriostatic and fungistatic mercurial antiseptic at 0.1%, but its use was restricted due to the high toxicity levels of organic mercurials. There are numerous reports on sensitisation caused by the ethylmercury radical in this compound, but it is still used in several countries. As a preservative in biological and pharmaceutical products, concentrations of 0.005 to 0.02% are usual. Thimerosal is now being withdrawn from vaccines because of widespread concern about potentially long-term deleterious effects on children (Frankish, 2001; Bernard *et al.*, 2001). According to the vaccination schedule, some infants could be exposed to cumulative levels of up to 200 μg of mercury during the first 6 months of life, exceeding EPA recommendations but not those of other regulatory bodies (for reviews see Ball *et al.*, 2001; Veen, 2001; Pichichero *et al.*, 2002). However this exposure is in an ethylmercury form, with its risk of neurodevelopmental disorders not equal to that of CH_3Hg^+. In experimental rat studies, an equimolar dose of ethylmercury was less neurotoxic than CH_3Hg^+ but with little difference (Magos *et al.*, 1985). In the biomedical laboratory, thimerosal is used for its properties as a sulphydryl reagent and as a Ca^{2+} mobilising agent (Elferink, 1999).

Dimethylmercury — Because dimethylmercury is only used in a small number of chemistry laboratories, poisoning is very rare. It is extremely toxic, however, and a few fatal poisonings have occurred as a consequence of small accidental spills. The most recent case was that of a 48-year-old chemistry professor who died nine months after accidentally spilling a few drops of dimethylmercury on her latex glove (Siegler *et al.*, 1999). Five months after the accident she had dysmetria, dystaxic handwriting, dysarthria, and a broad-based gait. Her gait, vision, speech and hearing worsened in subsequent days and she entered a vegetative state within 22 days. Chelation therapy mobilised mercury into her blood (Toribara, 2001) but this treatment and medical support did not prevent her death 4-months later. At the onset of clinical symptoms she had a mercury blood level of 4 μg/ml (normal 1 to 10 μg/L). The main autopsy findings were consistent with a previously reported case of a 28-year-old chemist (Pazderová *et al.*, 1974). The areas most profoundly affected were the cerebellum and the visual cortex, in line with reports on CH_3Hg^+ poisoning. However, additional findings not observed for CH_3Hg^+ included an affectation of the temporal lobe and a massive Purkinje cell loss in the cerebellum (Pazderová *et al.*, 1974; Siegler *et al.*, 1999).

Merbromin — Merbromin, known also as mercurochrome, is a fluorescein mercuric compound that was previously used widely as an antiseptic mainly for children. Merbromin absorption after topical application led to a few cases of mercury poisoning (Beasley and Jones, 1986; Mullins and Horowitz, 1999). Although still in use in some countries, the problems associated with it, together with allergic reactions, have meant its progressive substitution by less toxic antiseptics.

NEUROTOXICITY MECHANISMS

Major mechanisms leading to neuronal cell damage by organomercury compounds are discussed below. The following classification is rather arbitrary, as all the mechanisms could probably be reduced to a few pathologic pathways. We cannot define these pathways with our limited knowledge of the sequence of changes activated by the toxic injury and their relative weigh. However, we can consider with the basic importance of thiol binding that will lead to different consequences depending on the cell

compartment affected. Namely, damaged cell membranes will lead to increased Ca^{2+} levels with disruption of neurotransmitter signalling. Probably in parallel, damaged mitochondria will increase oxidative stress, leading to excitotoxic damage and to a lessening of defense mechanisms such as reduced glutathione (GSH) content. Both triggered chains of events will interact with an amplification of their disruptive consequences. Besides, inhibition of general mechanisms such as protein synthesis and microtubule assembly will add wide consequences in neurotransmission and neural cell development.

I. Target Cells and Experimental Models

Most of the neuropathological changes caused by CH_3Hg^+ in humans have been reproduced in experimental animals (Shiraki, 1979; Eto, 1997; 2000). Nevertheless, some differences exist. Thus, even if cerebral lesions similar to humans are produced in exposed monkeys, CH_3Hg^+ accumulates in the retina and induces selective alterations (DuVal *et al.*, 1987; Fox and Boyes, 2001). Conversely, rats are considered a good model of organic mercury intoxication, with the selective death of cerebellar granule cells identical to that in the human form of the disease (Nagashima, 1997). However, the selective degeneration of the visual cortex is not reproduced. Also, the sensory neurons in the dorsal root ganglia of the spinal cord are those most sensitive to CH_3Hg^+ toxicity in rats (Schionning *et al.*, 1998). A 7-day subcutaneous administration of 10 mg/kg of CH_3Hg^+ in adult rats led to the development of clinical signs of spastic gait and hind-leg crossing when suspended by the tails some 10 days after dosage. Lower dosages resulted in a delayed onset of the disease, which appeared when CH_3Hg^+ concentrations in the brain exceeded approximately 20 μg/g (Nagashima, 1997). Pathological changes in the cerebellum occurred later than those in the peripheral nervous system. The granular cell layer of the cerebellum is also considered one of the main organic mercury targets in adult animals (reviewed by Chang, 1977; Nagashima, 1997). Abnormalities in the developing brain vary from gross organ defects to subtle histological changes and the subsequent appearance of altered neurobehaviour. Areas affected include the cerebral neocortex, neostriatum, thalamus, cerebral basal ganglia, limbic system, cerebellum, brain stem and spinal (dorsal root) ganglia. The most vulnerable developmental periods with associated lesion patterns were characterised by the administration of CH_3Hg^+ to pregnant animals and pups during different developmental phases (Choi, 1989; Sakamoto *et al.*, 2002; Goulet *et al.*, 2003).

Species differences for the organic mercury damage may be partially mediated by the different pharmacokinetics of CH_3Hg^+ in those animals. In the case of rats, one of the striking differences is that their red blood cells have exceptionally high affinity for CH_3Hg^+ leading to a ratio of red cells/plasma ~300:1 in front of the above cited 20:1 for humans. The rat hemoglobin molecule has a higher number of cysteinyl residues that act as binding sites for CH_3Hg^+ (Doi and Tagawa, 1983). However the resultant affinity difference does not fully account for the extremely low brain/blood ratio of ~0.07 in rat in front of ~7 to 10 for monkey and man. Additional differences such as high permeability of the blood-brain barrier in primates may be involved. Therefore, an equivalent dosage of CH_3Hg^+ will lead to higher brain levels in monkeys than in rats. On the other hand, brain area sensitivity differences will be difficult to fully understand while we do not have complete knowledge of the toxicity mechanisms. An important factor may be that some specific neuron types have a lower burden of defense mechanisms against organic mercury injury. That could be the case of cerebellar granule cells with low antioxidant efficiency as discussed below. A diminished blood-brain barrier or a higher accessibility leading to the toxic agent accumulation may be other crucial factors. That seems to be the case of dorsal root ganglion cells and dorsal nerve roots in the rat, where the endoneurial blood vessels are permeable to plasma constituents in this particular region of the nervous system (for discussion see Schionning *et al.*, 1998)

In vitro studies have permitted a better characterisation of the cellular effects of organomercuric compounds. Sensitivity to organic mercury is different depending on the nervous system cell types. Immature neurons have a demonstrated sensitivity to CH_3Hg^+— higher than for neurons cultured for enough days to be considered mature, with the sensitivity difference being more marked in cortical neurons than in cerebellar granule cells (Mundy and Freudenrich, 2000). Mature cerebral cortical neurons vs cerebellar granule cells are less sensitive to CH_3Hg^+ exposure *in vitro*, and astrocytes are less sensitive than neurons (Gassó *et al.*, 2001; Sanfeliu *et al.*, 2001). In addition, the fine interplay between the different cell types in the nervous system may be altered at concentrations lower than those causing cell deaths, with similar deleterious consequences. However, neuroprotective mechanisms may also be present. In particular, astrocytes may protect neurons from low doses of organic mercury by upregulation of metallothionein (MT) synthesis, the sulfhydryl-containing proteins that maintain metal homeostasis in the CNS (Aschner *et al.*, 1997). Other cell interactions reported after CH_3Hg^+ exposure are more complex. Namely, interaction between astrocytes and microglia activated by noncytotoxic concentrations of CH_3Hg^+ may increase local interleukin-6 release that

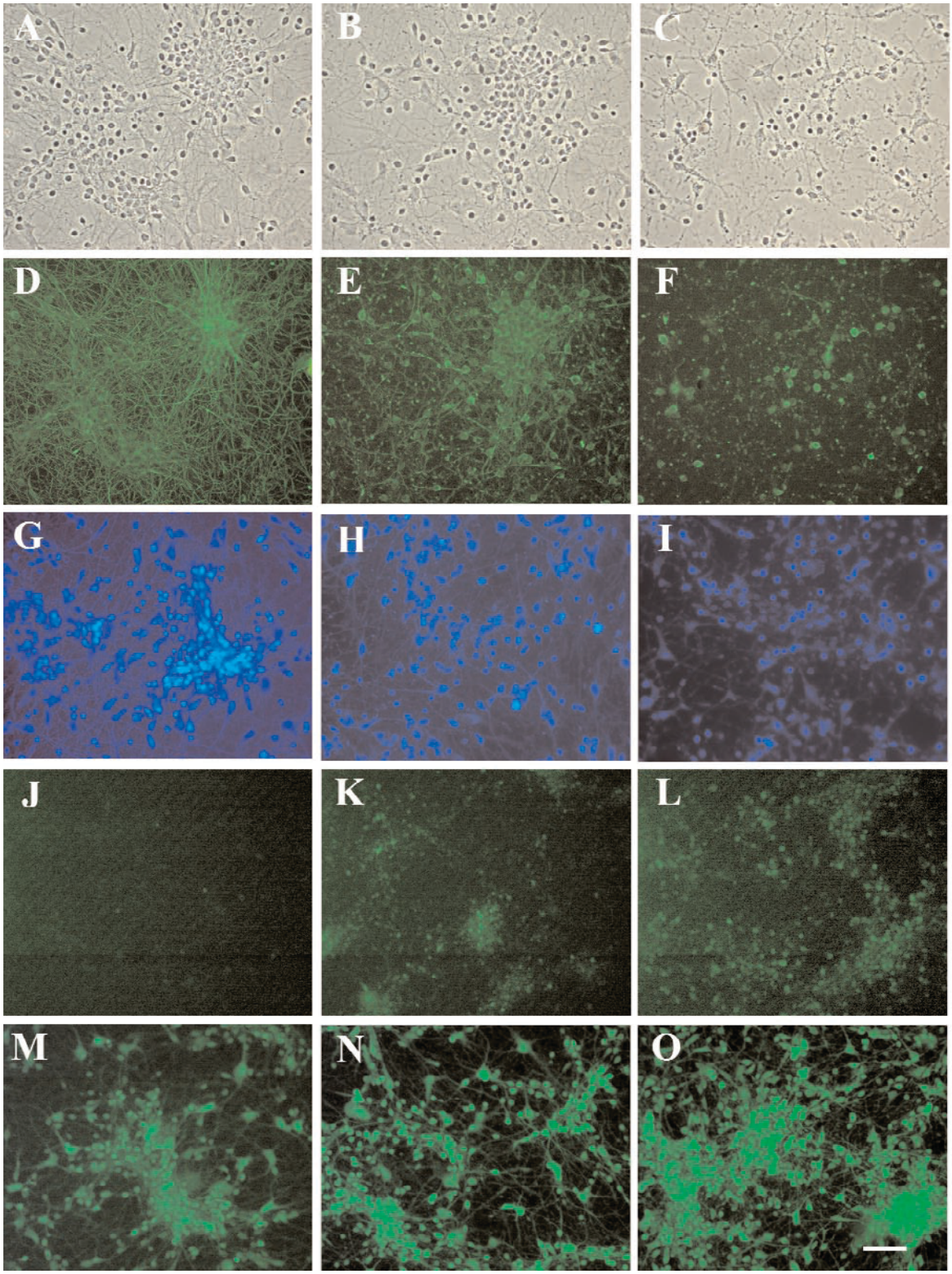

FIGURE 1 Cultures of human central nervous system, enriched in neurons, obtained from cerebral cortical fetal tissue and maintained for 12 days *in vitro*. Control cultures are displayed in A, D, G, J and M. Methylmercury exposure was performed at 2 µM (B, E and H) or 5 µM (C, F and I) for 24 hours, or at 10 µM (K and N) or 20 µM (L and O) for shorter periods (see below). Images of phase contrast of paraformaldehyde-fixed cultures are presented in A-C and of β-tubulin immunostaining in D-F, after 24 hours of vehicle or methylmercury treatment. Cells stained with 7-amino-4-chloromethylcoumarin (CMAC) to visualize GSH content after a 24-hour exposure to vehicle or methylmercury are shown in G-I. Fresh cells incubated with 2',7'-dichlorofluorescein diacetate (DCFH-DA) to show ROS generation after 1 hour exposure to methylmercury or vehicle are in J-L. Those incubated with Fluo-3 AM to label intracellular Ca^{2+} after 10 minutes exposure to vehicle or methylmercury are shown in M-O. High toxicity of methylmercury is demonstrated in C, F and I, whereas an important reduction of neurite microtubules and also a lower GSH content are already evidenced in E and H, respectively, as related to control cultures (D and G). A 1-hour exposure to methylmercury causes a clear increase in the presence of cellular ROS (K and L) over the background ROS generated by control cultures during this period (J). On the other hand, $[Ca^{2+}]_i$ is visibly higher after 10 minutes exposure to methylmercury (N and O) vs control cells

would lead to neuroprotection in 3 D brain cell cultures (Eskes *et al.*, 2002).

II. Thiol binding

The great affinity of CH_3Hg^+ and all mercury compounds for the anionic form of –SH groups has long been documented, and it was this notion that led to the idea that reaction with thiols was the basis for the biological effects of CH_3Hg^+ (Hughes, 1957). Both the association and dissociation reactions are fast, and mercury may move from one protein or thiol-containing molecule (i.e. glutathione and cysteine) to another. Indeed, mercurials inhibit a number of cell enzymes and inactivation may be related to their binding to sulfhydryl groups as it can be reversed by thiol compounds. For instance, inhibition of alkaline phosphatase and of brain neurotransmitter enzymes in rats treated with CH_3Hg^+ was prevented by *N*-acetyl-DL-homocysteine thiolactone (Sood and Unnikumar, 1987) and DDT and sodium thioglycolate (Kung *et al.*, 1987), respectively. Conversely, the inhibition of protein kinase C by CH_3Hg^+, phenyl mercury and *p*-chloromercuribenzoic acid *in vitro* was mimicked by other sulfhydryl blocking reagents such as 5,5'-dithiobis-2-nitrobenzoic acid and *N*-ethylmaleimide (Inoue *et al.*, 1988). Also, blockage or conformational changes of cellular thiol molecules by organomercuric compounds may explain several of the neurotoxicity mechanisms (to be described below). An initial dispersion of these chemical interactions would explain the triggering of a number of independent pathological mechanisms. However, despite several decades of experimental studies, the pathways implicated and their relative weight in impairing cell survival and nervous system functions have not yet been clearly defined. There is, besides, another missing link between the initial chemical reaction of mercurial compounds with cell thiol molecules and the selective damage to some neuron cell types and brain structures. As regards access to the brain by organic mercury compounds, these are capable of crossing the blood-brain barrier, and their conjugation with endogenous sulfhydryls (mainly cysteine) facilitates their entry into the brain through the L-neutral amino acid transport system (Aschner and Clarkson, 1988; Aschner, 1989). Through the same carrier system, the CH_3Hg^+-cysteine complex also crosses the placenta and reaches the fetus where the developing nervous system is especially sensitive to its effects (Kajiwara *et al.*, 1996).

Research into the neurotoxicity mechanisms or other systemic effects of CH_3Hg^+ is still an active research field after many years, and new insights are continuously obtained. A recent report showed that a gene encoding the Cdc34 ubiquitin-conjugating enzyme is highly resistant to CH_3Hg^+ in both saccharomyces cerevisiae and a human cell line. Therefore, the ubiquitin-proteasome system would degrade a toxic protein produced by CH_3Hg^+ modification or accelerated synthesis (Hwang *et al.*, 2002). The implications of this new finding for nervous system poisoning and neuroprotection still remain to be uncovered.

III. Microtubule Disruption

Although less sensitive to inorganic mercury, cell microtubules are a primary CH_3Hg^+ target. Methylmercury was observed to inhibit *in vitro* glioma cell mitosis by blocking tubulin polymerisation into microtubules, after a 4-hour exposure to 5 μM CH_3Hg^+ (Miura *et al.*, 1978). Depressed tubulin synthesis, also present, was mainly attributed to an auto-regulatory repression of post-transcriptional processes rather than to a failure of protein synthesis (Miura *et al.*, 1998). *In vivo* and *in vitro* depolymeration of microtubules by CH_3Hg^+ was reported for different cell types, with neuronal cells equally or more sensitive, and fibroblasts less sensitive, than glial cells (Sager and Syversen, 1984; Cadrin *et al.*, 1988). Cytoskeletal breakdown of nerve cells by a reduced microtubule mass may play a role (not as yet fully clarified) in depressed nerve fibre growth and the apoptosis caused by CH_3Hg^+ (Miura *et al.*, 1999; 2000; Castoldi *et al.*, 2000; Heidemann *et al.*, 2001). The most damaging microtubule disruption occurs during CNS development, where inhibition of cell division and migration impairs the architectonic makeup of the different brain structures (Choi, 1991). In more sensitive areas, such as the cerebellum, architectural disruption of neuronal elements may be complete. It is thought that CH_3Hg^+ binding to microtubular -SH groups is the cause of depolymerization or the lack of assembly of microtubules (Han *et al.*, 1987). Although immature tyrosinated microtubules are very sensitive, more mature acetylated microtubules are resistant to low doses of CH_3Hg^+. Therefore, the most critical period of microtubule susceptibility occurs very early on in *in vivo* development (Graff *et al.*, 1997). See images of disrupted microtubules in cerebellar granule cells immunostained for β-tubulin after being exposed to CH_3Hg^+ *in vitro* (FIG. 1D-1F).

IV. Inhibition of Protein Synthesis and Alteration of Protein Phosphorylation

Organomercury compounds inhibit protein synthesis *in vivo* at a very early stage and prior to the development of neurological symptoms (Yoshino *et al.*, 1966). Nearly 40% of protein synthesis inhibition was obtained after intraperitoneal administration of 40 nmol/g (10 mg/kg) body weight of CH_3Hg^+ in weanling rats (Cheung and Verity, 1985). It was concluded from *in vivo* and *in vitro* studies that the failure was not with synthesis initiation – where mercury-cysteine complexes could have interacted with the structurally similar methionine – but rather with the reactions required for peptide elongation. This upset to brain cell-free amino acid incorporation was caused by an inhibition of the activity of aminoacyl-tRNA synthetase enzymes, where functional -SH groups are required (Cheung and Verity, 1985; Hasegawa *et al.*, 1988).

Changes in protein phosphorylation patterns by CH_3Hg^+ were also described, mainly leading to both *in vivo* and *in vitro* elevated phosphorylation (Sarafian and Verity, 1990; Yagame *et al.*, 1994). Tubulin subunits were between proteins with excess phosphorylation. Such modifications to the functional proteins may lead to impairment of cellular processes, with important deleterious consequences on the nervous system.

V. Increase of Intracellular Ca^{2+}

It has long been acknowledged that mercurial compounds interfere with calcium regulation in neuronal systems, leading to an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (reviewed by Denny and Atchison, 1996). This Ca^{2+} accumulation leads to neuronal death through several reactions such as derangement of Ca^{2+}-signalling, activation of degradative enzymes, mitochondrial disfunction, cytoskeletal alterations and generation of free radicals (Bondy, 1989; Nicotera *et al.*, 1992). Intensity of the stimulus and influx pathways both determine the neuronal vulnerability and type of cell death, whether necrosis or apoptosis (Sattler *et al.*, 1998; Toescu, 1998). Although inorganic mercury is more powerful than CH_3Hg^+ in increasing $[Ca^{2+}]_i$ in cerebellar granule cells (Gassó *et al.*, 2001), the key role played by increases in $[Ca^{2+}]_i$ in CH_3Hg^+ neurotoxicity has received a great deal of experimental support. A number of $[Ca^{2+}]_i$-mediated mechanisms and Ca^{2+} pool fluxes are affected by CH_3Hg^+ but their relative contribution to cell death has not yet been clearly elucidated. See the increased Fluo 3/AM fluorescence in cerebellar granule cells exposed to CH_3Hg^+ (FIG.1M-1O).

At the cell membrane level, micromolar concentrations of mercury inhibit Ca^{2+}-pump activity in brain microsomes and in synaptosomes (Freitas *et al.*, 1996; Yallapragada *et al.*, 1996). This would contribute to the increase in $[Ca^{2+}]_i$. In synaptosomes, there was an increase in cytosolic $[Ca^{2+}]$ due to inhibition of ATP production at 30 μM CH_3Hg^+, whereas an increased ionic permeability of the plasma membrane caused a large increase in cytosolic $[Ca^{2+}]$ at 100 μM CH_3Hg^+ (Kauppinen *et al.*, 1989). In cerebellar granule cells there was a latency period followed by a biphasic rise in $[Ca^{2+}]_i$ following exposure to low concentrations of CH_3Hg^+ (Denny *et al.*, 1993; Marty and Atchison, 1997). The latency period was about 10 minutes after 0.5 μM CH_3Hg^+, with this period shortening in line with increased concentrations. In this system, the first elevation of $[Ca^{2+}]_i$ was moderate and independent of extracellular Ca^{2+}. The intracellular stores involved were an inositol 1,4,5-triphosphate (IP_3)-sensitive Ca^{2+} pool in the NG108-15 neuroblastoma cells (Hare and Atchison, 1995), whereas they were not so positively identified in cerebellar granule cells. However, recent works have described that in these cells CH_3Hg^+ (0.2-0.5 μM) induces an initial Ca^{2+} release from the smooth endoplasmic reticulum, which is buffered by the mitochondria, and followed by release of mitochondrial Ca^{2+} through the mitochondrial permeability transition pore (MTP) (Limke and Atchison, 2002; Limke *et al.*, 2003). In the second phase, in which onset was delayed a further 10 minutes approximately, there was a great amount of Ca^{2+} entry. This influx occurred partially via voltage-dependent calcium channels (VDCC), since it was not inhibited by but its latency was increased by nifedipine and ω-conotoxin-MVIIC, blockers of L-type and Q-type Ca^{2+} channels respectively (Marty and Atchison, 1997). However, VDCC blockage also delayed the onset of the first phase. These authors therefore raise the possibility that the VDCC blockage simply delays CH_3Hg^+ interaction with its target site or its cell entry. Na^+ channels were not involved, as tetrodotoxin did not block Ca^{2+} entry, neither in cerebellar granule cells nor in synaptosomes (Komulainen and Bondy, 1987; Marty and Atchison, 1997). Ca^{2+} entry was neither through Ca^{2+} channels operated by the excitatory amino acid receptors (Marty and Atchison, 1997). Conversely, the rise in $[Ca^{2+}]_i$ in cerebellar granule cells caused by a 10 μM exposure to

CH_3Hg^+ was partially reduced by the T-type channel blocker flunarizine, the Na^+/Ca^{2+} channel blocker benzamil and, at the intracellular level, the inhibitor of endoplasmic reticulum Ca^{2+}-ATPase thapsigargin (Gassó *et al.*, 2001). Moreover, this research indicated a lower increase in $[Ca^{2+}]_i$ by CH_3Hg^+ in the presence of the antioxidants propyl gallate and probucol.

The involvement of the different Ca^{2+} fluxes in CH_3Hg^+ toxicity may be suggested by the protective effect of the above-mentioned Ca^{2+} blockers against mercury toxic effects. Only one study *in vivo* performed so far has demonstrated an amelioration of CH_3Hg^+-toxicity in rats with several VDCC blockers, especially flunarizine (Sakamoto *et al.*, 1996). Flunarizine protection was also demonstrated in cultures of cerebellar granule cells (Sakamoto *et al.*, 1996; Gassó *et al.*, 2001). In the same neuronal preparation, partial protection against CH_3Hg^+ was also obtained with nifedipine, ω-conotoxin MVIIC, benzamil and thapsigargin (Marty and Atchison, 1998; Gassó *et al.*, 2001). Along the same lines, cytotoxicity was delayed for a few hours with the Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetrakis (acetoxymethyl)ester (BAPTA) (Marty and Atchison, 1998). Interestingly, pre-treatment with the MTP inhibitor cyclosporin A also provided partial protection against low concentrations of CH_3Hg^+ (Limke and Atchison, 2002).

VI. Increase of Intracellular Zn^{2+}

In vitro treatment with CH_3Hg^+ was found to elevate the intracellular/intrasynaptosomal concentrations of Zn^{2+}, independently of the $[Ca^{2+}]_i$ increase and prior to the extracellular Ca^{2+}-dependent phase (see above) (Denny *et al.*, 1993; Denny and Atchison, 1994; Marty and Atchison, 1997). It is possible that CH_3Hg^+ displaces endogenous Zn^{2+} bound to proteins such as the metallothioneins. Zinc, abundant in the brain, carries out physiological functions, but an overload or excessive release is implicated in several cytotoxic mechanisms and pathological conditions (Noh and Koh, 2000; Koh, 2001).

VII. Alteration of Neurotransmitter Function

Disruption of synaptic transmission caused by mercury compounds may be the cause of muscular weakness and many CNS alterations. Methylmercury and other mercury compounds have generalised effects on neurotransmitter release, stimulating spontaneous transmitter release and attenuating depolarization-evoked release (see Gassó *et al.*, 2000). Increases in $[Ca^{2+}]_i$, as discussed above, may augment spontaneous neurotransmitter release. Conversely, VDCC blockage may mediate the observed reductions in depolarization-evoked neurotransmitter release in nerve terminals (Denny and Atchison, 1996). Indeed, detailed biochemical and electrophysiological analysis subsequent to ^{45}Ca uptake inhibition have demonstrated VDCC blockage both in synaptosomes and in intact neuronal cells (Shafer and Atchison, 1991; Hewett and Atchison 1992; Szucs *et al.*, 1997). The charge and lipophilicity of mercury compounds both affected the characteristics of Ca^{2+} channel blockage since CH_3Hg^+ and ethylmercury, both positively charged, were less potent than inorganic mercury while dimethylmercury caused no appreciable reduction in ^{45}Ca uptake (Hewett and Atchison 1992). Therefore, Ca^{2+} channels may be a main target for CH_3Hg^+ neurotoxicity as suggested by Sirois and Atchison (2000). These authors obtained a blockage effect at low concentrations of CH_3Hg^+ (0.25-1 μM) in cerebellar granule cells. The sensitivity of this effect means that its contribution to CH_3Hg^+-induced cell death in the cerebellum of intoxicated individuals, through an initial disruption of neurotransmitter release, is a highly feasible hypothesis. The exact mechanism of the channel blockage is not known and is probably complex because, as we have discussed above, the $[Ca^{2+}]_i$ increase induced by CH_3Hg^+ is partially mediated by extracellular Ca^{2+} entering through VDCC.

Organic mercury directly affects specific neurotransmitter receptor functions. In different experimental systems, CH_3Hg^+ inhibited both the muscarinic (Castoldi *et al.*, 1996) and nicotinic (Eldefrawi *et al.*, 1977) types of acetylcholine receptors, *N*-methyl-D-aspartate (NMDA) (Rajanna *et al.*, 1997), dopamine (Scheuhammer and Cherian, 1985) and $GABA_A$ receptors (Yuan and Atchison, 1997). In a recent work, Fonfría *et al.* (2001) characterised the interaction of CH_3Hg^+ with $GABA_A$ receptors in cerebellar granule cells, demonstrating an increase in benzodiacepine binding with an EC_{50} of 15 μM CH_3Hg^+ after 30 minutes exposure. These authors concluded that the basis for the $GABA_A$ receptor function alterations would be a cysteinyl oxidation of the receptor subunits leading to an abnormal conformational state of this receptor. Similarly, an interaction of mercury with –SH groups adjacent to the active site may be the reason for the binding site occlusion in other receptors (Scheuhammer and Cherian, 1985). Blockage of receptor binding by CH_3Hg^+ occurs at micromolar concentrations. Therefore, subsequent interference with postsynaptic events may contribute to the neurological consequences of mercury poisoning (Bondy and Agrawal, 1980). Interestingly, low-level chronic dosages of

CH_3Hg^+ (0.5-2 mg/kg for 16 days) in rats lead to an upregulation of muscarinic cholinergic receptors in the hippocampus and cerebellum (Coccini *et al.*, 2000).

An excitotoxic mechanism is involved in CH_3Hg^+-induced neurotoxicity through an alteration of astrocyte excitatory amino-acid homeostasis. The role of astrocytes in CH_3Hg^+ neuronal damage has been extensively studied by Aschner and coworkers (reviewed by Aschner *et al.*, 2000). Toxic effects on astrocytes were observed at concentrations of 5-10 μM CH_3Hg^+. Regarding excitotoxicity, these authors concluded that an inhibition of glutamate uptake and an increase in endogenous excitatory amino acid release by astrocytes both cause secondary excitotoxic damage to neurons. They demonstrated that inhibition of glutamate transport by CH_3Hg^+ is mediated through an excess of ROS generation (Allen *et al.*, 2001a). Accordingly, neuroprotection against CH_3Hg^+ by antioxidants and NMDA excitatory amino acid receptor antagonists was reported for neuronal cultures (Park *et al.*, 1996). The implication of NMDA receptors may contribute to high vulnerability in immature nervous system to CH_3Hg^+ (Miyamoto *et al.*, 2001).

Conversely, the inhibition of glutamate metabolism observed in cerebellar astrocytes by CH_3Hg^+ may have consequences for neurotransmitter synthesis in neurons (Qu *et al.*, 2003).

VIII. Generation of Oxidative Stress

Disruption of redox cellular homeostasis by an excess of reactive oxygen species (ROS) formation leading to cumulative oxidative stress appears to be an important contributor to organomercuric neurotoxicity. Several mechanisms and cell targets are involved (reviewed by Sarafian, 1999). It is well known that the nervous system is extremely sensitive to oxidative stress mainly because of its low antioxidant defense content in contrast to its high metabolic activity and elevated highly oxidizable compound content (Halliwell and Gutteridge, 1985; LeBel and Bondy, 1991). As a matter of fact, granule cerebellar cells are sensitive to a number of toxic substances where a deficiency of antioxidants, such as GSH, may be an important toxicity factor (Fonnum and Lock, 2000).

An induction of oxidative stress occurs in the brain of CH_3Hg^+-exposed animals and in neuronal and glial cell cultures, as evidenced by membrane peroxidation and generation of ROS (Taylor *et al.*, 1973; LeBel *et al.*, 1990; Sarafian and Verity, 1991; Ali *et al.*, 1992; Yee and Choi, 1994; Shanker and Aschner, 2003). Several studies have demonstrated partial amelioration of mercury toxicity in the presence of the selenite and vitamin E antioxidants (see Sanfeliu *et al.*, 2001). The beneficial effect of selenite is complex, as it may directly detoxify mercury, as discussed below. A reduction in ROS generation in cerebellar granule cells by the probucol and propyl gallate antioxidants (Gassó *et al.*, 2001) or by bcl-2 gene expression in the GT1-7 neural cell line (Sarafian *et al.*, 1994) correlated with the preservation of viability following CH_3Hg^+ exposure. *In vivo*, Trolox, the water-soluble derivative from vitamin E, protected rat cerebellum from CH_3Hg^+-induced apoptosis (with doses of 2.5 mg/kg body weight of Trolox and an approximate intake of 2 mg/kg of mercury over 28 days) (Usuki *et al.*, 2001). Of note is the fact that cell death protection obtained by antioxidants is much higher than that reported for Ca^{2+}-related agents (as discussed above). Indeed, the radical scavenger propyl gallate reduced both ROS generated during 1-hour exposure at 20 μM CH_3Hg^+ and cytotoxicity after 24 hours at 5 μM CH_3Hg^+ to practically undetectable levels. The same treatment also maintained cytotoxicity at an extremely low level in human cortical cultures for up to 3 days (Gassó *et al.*, 2001). Similarly, the 17β-estradiol antioxidant reduced apoptosis in cerebellar granule cells to basal levels after 24 hours at 1 μM CH_3Hg^+; the protection was total after 48 hours CH_3Hg^+ exposure with the presence of the J811 estradiol derivative (Daré *et al.*, 2000). The Ca^{2+}-mobilization blockers thapsigargin, flunarizine and benzamil, even though they are partially protective against CH_3Hg^+ cell death as mentioned above, did not inhibit CH_3Hg^+ ROS generation (Gassó *et al.*, 2001). Therefore, intracellular generation of ROS may be an upstream mechanism in the series of events leading to CH_3Hg^+ neurotoxicity. See the increased hydroperoxide formation in cerebellar granule cells exposed to CH_3Hg^+ as indicated by 2',7'-dichlorofluorescein fluorescence (FIG.1J-1L).

A major source of CH_3Hg^+-increased ROS generation may be the mitochondrial electron transport chain, which is the main source of intracellular ROS production under normal metabolic conditions. As a reference, isolated mitochondria convert 1-2% of consumed oxygen molecules into ROS (Boveris and Chance, 1973; Finkel and Holbrook, 2000). Methylmercury was reported to directly interact with respiratory enzyme complexes and cause oxidative damage in the mitochondria (Verity *et al.*, 1975; Yee and Choi, 1996). Specifically, CH_3Hg^+ caused increased ROS generation after stimulation of the ubiquinol:cytochrome *c* oxidoreductase complex in isolated mitochondria (Yee and Choi, 1996). Furthermore, blockage of the MTP by cyclosporin A in brain synaptosomes lowered methylmercurial ROS generation (Myhre and Fonnum, 2001). Loss of the mitochondrial respiratory function after exposure to organomercury has long been observed *in vivo* and *in vitro* and had led to the suggestion of the mitochondria as one of the CH_3Hg^+ target sites.

Nitric oxide (NO) is increasingly generated *in vivo* after neuronal NO synthase induction due to CH_3Hg^+ damage (Shinyashiki *et al.*, 1998; Ikeda *et al.*, 1999). Overproduction of this messenger molecule may lead to oxidative brain damage. However, it has not been conclusively demonstrated that NO does, in fact, significantly contribute to organic mercury neurotoxicity.

The glutathione redox cycle is one of the principal endogenous antioxidant mechanisms. Detoxification of organomercury compounds (see below) will cause a depletion in GSH, leading to a decline in ROS-cell defenses. Decreased GSH levels usually parallel increased oxidative stress in CH_3Hg^+ intoxication experimental systems (Sarafian and Verity, 1991; Sarafian *et al.*, 1994; Vijayalakshmi and Sood, 1994). GSH depletion is not the predominant cause of organomercury-induced neurotoxicity, but its upregulation in certain cell types (Li *et al.*, 1996) or the induction of an increased synthesis (Choi *et al.*, 1996) is neuroprotective. Further, CH_3Hg^+ inhibits both cysteine (Shanker *et al.*, 2001) and cystine (Allen *et al.*, 2001b) uptake into astrocytes. Cysteine is the rate-limiting substrate for the biosynthesis of GSH, but astrocytes prefer cystine for GSH synthesis (Kranich *et al.*, 1998). Thus, the impairment of GSH astrocyte synthesis by CH_3Hg^+ will undermine GSH synthesis in neurons, which rely on an astrocyte supply of GSH precursors (Dringen *et al.*, 1999; Shanker and Aschner, 2001).

IX. Necrosis and Apoptosis

Cell death occurs after irreversible damage produced via some of the pathways listed above. The processes are not fully understood, but as happens with other xenobiotics, the outcome depends on the extent of injury. High concentrations of CH_3Hg^+ (5-10 μM)cause widespread damage leading to necrotic death, whereas low concentrations of CH_3Hg^+ (0.5-1 μM) cause specific damage that will trigger an apoptotic pathway, both *in vivo* and *in vitro* (Castoldi *et al.*, 2000; Belletti *et al.*, 2002). The processes involved in apoptosis include microtubular disruption, development of mitochondrial permeability transition, loss of mitochondrial membrane potential, generation of ROS and depletion of GSH. Detailed studies on apoptosis caused by CH_3Hg^+ in nerve cells have shown that apoptosis occurs via a p53-independent pathway in the PC12 cell line (Miura *et al.*, 1999) involving a caspase-3-like protease in cultured rat microglia (Nishioku *et al.*, 2000), and by means of an activation, mainly of calpain and to a lesser extent of caspase-3, and by translocation of the apoptosis-inducing factor, in cerebellar granule cells (Daré *et al.*, 2000; Fonfría *et al.*, 2002).

The extent of cellular damage will be crucial for the organism's survival and maintenance of CNS function. The consequences of organomercury poisoning will be much more deleterious during development, given that the processes described above, –mainly those deriving from a lack of microtubules–, interfere with the structural and functional construction of the nervous system.

X. Cellular Detoxification Mechanisms

Glutathione — GSH is the first cellular defense against mercury compounds as it is involved in detoxification of the thiol-reactive xenobiotics (Eriksson and Svenson, 1978; Parkinson, 2001). *In vivo*, a major route for CH_3Hg^+ excretion is dependent upon intracellular GSH, and a glutathione- CH_3Hg^+ complex (CH_3Hg-SG) has been detected in liver tissues and bile. Characterization of the transport system for CH_3Hg-SG in isolated rat liver canalicular plasma membrane vesicles have shown that this complex is a substrate for carriers that also transport GSH (Dutczak and Ballatori, 1994). In urine, degradation of complexed glutathione by tubular enzymes led to the finding of CH_3Hg^+ as a cysteine conjugate (Yasutake *et al.*, 1989). Efflux systems for GSH are found in all mammalian cells and transportation of glutathione-metal complexes by such carriers may be a common mechanism for CH_3Hg^+ removal. However, little information is available on the elimination process for mercury organic compounds from neural tissues. Astrocyte studies suggest an efflux as CH_3Hg^+-L-cysteine conjugated through the same neutral system L amino acid that facilitates its uptake (Aschner *et al.*, 1991) or as a GSH conjugate (Fujiyama *et al.*, 1994). Confirmation of the important role of GSH comes from the fact that treatments leading to an intracellular increase in GSH level are neuroprotective against CH_3Hg^+ poisoning, either by increase of conjugation of CH_3Hg^+, scavenging of free radicals, restoration of reactive thiol groups, or enhancement of CH_3Hg^+ efflux (Choi *et al.*, 1996). Besides, some increase in GSH synthesis was reported in the brain of mice chronically exposed to CH_3Hg^+(Thompson *et al.*, 1999), whereas some neurons with higher GSH synthesis are those less sensitive to CH_3Hg^+ injury (Li *et al.*, 1996). See a decreased content of glutathione in cerebellar granule cells after exposure to CH_3Hg^+, as indicate by a lower 7-amino-4-chloromethylcoumarin fluorescence (FIG. 1G-1I).

Selenium — Selenium is an essential metal that exerts multiple actions in the brain (Whanger, 2001). In combating toxic xenobiotics, its lipid peroxidation reduction action is less significant than that of vitamin E, another natural antioxidant. Its efficacy against mercury resides, for the most part, in a decrease in its bioavailability due to the formation of equimolecular mercury and selenium

complexes that bind with a high affinity to a very specific plasma protein called Selenoprotein P (Yoneda and Suzuki, 1997). In selenium-deficient animals this protein is taken up in significant amounts by the brain but not by other organs, and this would therefore suggest a critical function for this selenoprotein in the brain (Whanger, 2001). Indeed, selenium causes mercury accumulation in brain (Glynn *et al.*, 1993). A near equimolar concentration of mercury and selenium was observed in the hair of populations exposed to mercury, in relation to the importance of the formation of this presumably non-toxic complex $[(Hg\text{-}Se)_n]_m$-Seleprotein P, in inorganic mercury and CH_3Hg^+ detoxification (Soares *et al.*, 2002). However, the toxicologically significant interaction of CH_3Hg^+ and selenite is not mimicked by organic acid mercurials (merbromine, mercuribenzene sulfonic acid and mercuribenzoic acid) (Gregus *et al.*, 2001).

***Metallothioneins* (MTs)** — The MT family consists of a group of low molecular weight metal-binding proteins with high cystine content. MTs have an affinity for both essential (zinc and cooper) and non-essential (cadmium and mercury) metals. In the CNS, MTs are considered to have important neurophysiological, neuromodulatory and neuroprotective functions (Hidalgo *et al.*, 2001). As regards neuroprotection, MT is a stress protein that plays an important role in the toxicokinetics of toxic metals. The first three of its four forms, MT-I, MT-II, MT-III and MT-IV, are present in the brain. The neuronal MT-III is specific to this tissue, but it is less responsive to induction. In general, MT chemical induction is lower in the brain than in other organs. Additionally, CH_3Hg^+ appears to be a weaker MT inducer than mercury vapour and inorganic mercury, and this fact may account for the somewhat contradictory results reported. Methylmercury was reported to be non-effective in inducing MT expression in the brain after systematic administration whereas vapour mercury induced MT-I and MT-II, and to a lesser extent MT-III (Yasutake *et al.*, 1998). Similar results were obtained by Kramer *et al.* (1996) with cultured astrocytes, with inorganic mercury acting as the positive inducer in this case. However, Rising *et al.* (1995) found MT-I and MT-II induction for astrocytes exposed to CH_3Hg^+. Interestingly, zinc induction of the astrocyte-specific isoform MT-I (Aschner *et al.*, 1998) or its overexpression (Yao *et al.*, 1999) in astrocyte cultures attenuated the cytotoxic effects of CH_3Hg^+. As suggested by Aschner *et al.* (1997), MT may afford protection against CH_3Hg^+ by forming high-affinity thiolate clusters. Thus, within the astrocytes, where mercury is known to accumulate, MT keeps mercury molecules in a relatively non-toxic form.

Other Defence Mechanisms — In addition to modulation of the GSH and MT levels, the general cell defense response to organic mercury and other heavy metals and pro-oxidant insults, includes other inducible responses such as synthesis of stress and anti-apoptotic proteins, and increased antioxidant enzymatic activities. Unfortunately, in comparison to other cell types, neurons are revealed to be rather deficient in these mechanisms, and its selective vulnerability to CH_3Hg^+ may be a consequence of this (see Sarafian *et al.*, 1996).

TREATMENT OF POISONING

Organic mercury lesions are irreversible, but the latency period of weeks or months between exposure and the onset of symptoms permits limited therapy in the form of a reduction in the mercury body burden. An excess of thiol-containing complexing agents is administered in order to remove mercury compounds from the thiol-containing molecules and to promote its elimination from the body (Klaassen, 2001). The short-chain organic mercurials, particularly CH_3Hg^+, are the most difficult forms of mercury to mobilise from the body. The soluble chelating agents penicillamine and *N*-acetyl-penicillamine (oral) and 2,3-dimercaptopropane-1-sulfonate (intramuscular) were shown to produce a significant reduction in mercury blood levels and increased urinary excretion when tested during the Iraq outbreak. A thiolated resin given orally to trap the CH_3Hg^+ secreted in bile and subsequently reabsorbed from the intestinal tract was simultaneously tested with positive results (Clarkson *et al.*, 1981). More recent animal studies with *N*-acetyl-cysteine have demonstrated relative selectivity for CH_3Hg^+ and a better response than with other oral chelating agents in accelerating the elimination of CH_3Hg^+, which is mainly excreted in the urine (Ballatori *et al.*, 1998). *N*-acetyl-cysteine also protected against CH_3Hg^+ embryotoxicity in mice (Ornaghi *et al.*, 1993) and was demonstrated to be useful in human treatment (Lund *et al.*, 1984). Selenium and vitamin E dietary supplements or GSH-elevating agents would be useful for both prophylaxis and as a therapy for poisoning resulting from low-level exposure (Fredriksson *et al.*, 1993; Vijayalakshmi and Sood, 1994; Choi *et al.*, 1996; Usuki *et al.*, 2001; Bapu *et al.*, 2003).

CONCLUSIONS

The challenges posed by CH_3Hg^+ poisoning nowadays can be summarized in the following points:

The need for a definition and description of the effects of low-level exposure to CH_3Hg^+ through seafood and fresh water fish, particularly in relation to neurodevelopment, i.e. *in utero* and perinatal exposure. This would require a definition of the non-effect range (LOAEL) for CH_3Hg^+ concentrations in the most vulnerable populations. Furthermore, even if the fetal brain has been identified as the most vulnerable target, many sensitivity factors still remain to be investigated.

The need for an improved understanding of CH_3Hg^+ neurotoxicity mechanisms. Although a wealth of information is available on the subject, what is required is a clear explanation that takes the phenomena underlying specific clinical and neuropathological toxicity manifestations fully into account.

A better knowledge of the above mentioned points will undoubtedly lead to more effective preventative public health measures.

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