

Mechanisms of methylmercury-induced neurotoxicity

WILLIAM D. ATCHISON¹ AND MICHAEL F. HARE

Department of Pharmacology and Toxicology, Neuroscience Program, and Institute for Environmental Toxicology,
Michigan State University, East Lansing, Michigan 48824-1317, USA

ABSTRACT Mercury in both organic and inorganic forms is neurotoxic. Methylmercury (MeHg) is a commonly encountered form of mercury in the environment. Early electrophysiological experiments revealed that MeHg potently affects the release of neurotransmitter from presynaptic nerve terminals. Recently, the hypothesis that these alterations may be mediated by changes in the intracellular concentration of Ca^{2+} ($[\text{Ca}^{2+}]_i$) has been supported. MeHg alters $[\text{Ca}^{2+}]_i$ by at least two mechanisms. First, it disrupts regulation of Ca^{2+} from an intracellular Ca^{2+} pool and second, it increases the permeability of the plasma membrane to Ca^{2+} . MeHg also blocks plasma membrane voltage-dependent Ca^{2+} and Na^+ channels in addition to activating a nonspecific transmembrane cation conductance. Chronic MeHg exposure results in ultrastructural changes and accumulation of MeHg within mitochondria. In vitro, MeHg inhibits several mitochondrial enzymes and depolarizes the mitochondrial membrane subsequently reducing ATP production and Ca^{2+} buffering capacity. Inhibition of protein synthesis is observed after in vivo or in vitro exposures of MeHg and may be an early effect of MeHg. Thus, the early cellular effects of exposure to MeHg are diverse and cell damage likely occurs by more than one mechanism, the effects of which may be additive or synergistic.—Atchison, W. D., Hare, M. F. Mechanisms of methylmercury-induced neurotoxicity. *FASEB J.* 8: 622-629; 1994.

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METHYLMERCURY (MeHg)² IS A COMMONLY encountered form of environmental mercury due both to its widespread use as well as to biomethylation by aquatic organisms. Exposure to MeHg in the food chain has led to catastrophic episodes of intoxication (1, 2), and exposure to inorganic and organomercurials still poses a significant toxicological problem (3, 4). Poisoning has occurred after both acute and chronic exposure to MeHg. Chronic poisoning with MeHg typically results in ataxia, disturbances of sensory and visual function, and extremity weakness (5). The underlying mechanism (or mechanisms) responsible for the effects of MeHg are still poorly understood, but they have been the object of much recent study and are the subject of this review. Because of space limitations, the entire spectrum of MeHg-induced neurotoxicity is not discussed. For other information on more general aspects of mercury toxicity, the reader is referred to other excellent reviews (6-8).

MeHg accumulates in the brain and becomes associated with mitochondria, endoplasmic reticulum, golgi complex, nuclear envelopes, and lysosomes. In nerve fibers, MeHg is localized primarily in myelin sheaths and mitochondria (6).

Pathological examination of patients with MeHg poisoning indicates that the cerebellar cortex is prominently affected, with granule cells being more susceptible than Purkinje cells. Typically, glia are spared direct damage, although reactive gliosis may occur. Pathological observations from cats, dogs, and birds poisoned at Minamata Bay, Japan, were similar to those of human victims (6). Rats treated chronically with MeHg exhibit a similar pattern of changes in the cerebellum, but damage to cerebral cortical neurons is not observed. Instead, peripheral neuropathy and dorsal root ganglion damage are more prevalent (9).

Studies of MeHg neurotoxicity have traditionally involved either behavioral or pathological observations in animals treated for days or weeks with MeHg, or observations of acute or semiacute effects of MeHg primarily using in vitro systems. There have been few mechanistic studies using animals treated chronically with MeHg. Toxicity with MeHg probably does not result from action on a single target. Instead, because of its highly reactive nature, a complex series of many unrelated effects may occur more or less simultaneously, initiating a sequence of additional events that may ultimately lead to cell death.

EFFECTS OF MeHg ON SYNAPTIC TRANSMISSION AND MEMBRANE EXCITABILITY

Mechanistic studies designed to examine early effects of MeHg have focused on several areas; one of these is the effect of MeHg on synaptic transmission. In part because of its well-characterized physiology, microscopic anatomy, and biochemistry, and in part because of increased incidences of neuromuscular weakness in the Iraqi poisoning episode (10), early studies focused on the vertebrate neuromuscular junction. Acute exposure to MeHg causes a time-dependent block of nerve-evoked twitches (11). Responses evoked by direct stimulation of the muscle are less affected. Thus, MeHg either impairs conduction of the nerve action poten-

¹To whom correspondence and reprint requests should be sent, at: Department of Pharmacology and Toxicology, Michigan State University, B-331 Life Sciences Building, East Lansing, MI 48824-1317, USA.

²Abbreviations: MeHg, methylmercury; EtHg⁺, ethylmercury; Me₂Hg, dimethylmercury; PCMB, *p*-chloromercuribenzoate; PCMBs, *p*-chloromercuriphenylsulfonate; ACh, acetylcholine; EPP, end-plate potential; MEPP, miniature end-plate potential; *m*, mean quantal content; *n*, immediately available store of neurotransmitter; *p*, probability of neurotransmitter release; $\text{Ca}^{2+}_{i(e)}$, intracellular (extracellular) Ca^{2+} ; $[\text{Ca}^{2+}]_{i(e)}$, intracellular (extracellular) concentration of Ca^{2+} ; PS, population spike; EPSP, excitatory postsynaptic potential; GABA, γ -aminobutyric acid; PC12, rat pheochromocytoma; DHP, dihydropyridine; NG108-15, mouse neuroblastoma \times rat glioma hybrid; IP₃, inositol trisphosphate; Ψ_m , mitochondria membrane potential; GSH, glutathione; ROS, reactive oxygen species.

tial, disrupts synaptic transmission directly, or both. Twitch and tetanic tension measured in situ, from the gastrocnemius of rats treated acutely or subchronically with MeHg, were diminished compared with controls (12). Twitches in response to direct stimulation of the gastrocnemius-soleus were not measured, so the reductions could be due to direct effects of MeHg on muscle or effects on the motor nerve impulse or neuromuscular junction. These results signify that effects of MeHg on muscle contractility occur not only with direct application, but also after systemic application.

Alterations in evoked release of neurotransmitter by MeHg

Studies using conventional intracellular microelectrode recording techniques have clarified the effects of MeHg on neuromuscular transmission. The primary effect of MeHg is to decrease nerve-evoked release of acetylcholine (ACh) and to increase, then decrease, spontaneous quantal release (11, 13). Decreased nerve-evoked release appears as decreased amplitude of the postsynaptic potential, known as the neuromuscular junction as the end-plate potential (EPP), whereas effects on spontaneous release appear as alterations in frequency of occurrence of miniature EPPs (MEPPs). MeHg transiently increases the amplitude, then blocks EPPs (11). Similar stimulatory effects of MeHg have been observed on transmission at autonomic ganglia (14) and in hippocampal slices (15).

Effects of MeHg on nerve-evoked transmitter release are time dependent, but not strictly concentration dependent. Higher concentrations reduce the time required to produce an effect (11, 13, 16, 17). The decline in EPP amplitude is progressive, proceeding to complete block. Thus, block of the EPP does not attain a steady-state short of complete block. For this reason, a strict concentration dependence does not occur. At low concentrations, block of evoked release has not been observed (13). However, this may reflect the fact that the latent period was longer than the measurement period and the effect was missed. Thus, it is not possible to state unequivocally that there is a threshold concentration of MeHg needed to block evoked transmitter release.

The effects of MeHg on mean EPP amplitude parallel those on mean quantal content (m) (11, 13), indicating that block of synaptic transmission results primarily from presynaptic effects. Analysis of neurotransmitter release statistical parameters indicates that the depression of m results primarily from depression of the immediately available store of neurotransmitter (n) (13), indicative of an intracellular action of MeHg on the release process. The probability of transmitter release (p) is actually increased by MeHg (13).

Block of EPP by MeHg appears initially to be independent of external Ca^{2+} concentration ($[\text{Ca}^{2+}]_e$). Increasing $[\text{Ca}^{2+}]_e$ from 2 mM to 4 or 8 mM does not prolong the time to block or decrease the degree of block of the EPP by MeHg (14). However, the effect is complicated by the fact that MeHg impairs axonal conduction, albeit at high concentrations (17–19). Nonetheless, once an increase in membrane excitability is induced, raising $[\text{Ca}^{2+}]_e$ causes partial reversal of effects of MeHg on EPP amplitude (17), suggesting complex effects of MeHg on both membrane excitability and Ca^{2+} -dependent ACh release.

At the time of EPP block, MEPPs of normal amplitude and duration still occur (13), which suggests that postsynaptic sensitivity to ACh is not diminished. Block of EPP by MeHg is not reversed by washing the preparation with MeHg-free solutions (13, 17).

Effects of MeHg on synaptic function are not limited to peripheral transmission. In isolated hippocampal slices, MeHg increases population spike (PS) amplitude by ~ 20% at 20, 100, and 500 μM , then reduces and ultimately blocks the PS; time to increase and time to block are concentration dependent (15). MeHg (100 and 500 μM) also increases mean amplitude and then blocks field excitatory postsynaptic potential (EPSP) amplitude in a concentration-dependent manner. The PS induced by antidromic activation of CA1 cells is increased only by 4 μM MeHg. Higher concentrations only reduce PS amplitude; time to decrease is concentration dependent. Thus, acute exposure to MeHg causes a biphasic effect on central synaptic transmission, with concentration range and time course similar to those at neuromuscular synapses.

The only biochemical functional test of MeHg effects on evoked release of neurotransmitter was by Minnema et al. (20). In rat brain synaptosomes, MeHg had no effect on evoked release of dopamine even though spontaneous release had been increased.

Alterations in the spontaneous release of neurotransmitter by MeHg

The effect of MeHg on spontaneous transmitter release is biphasic. The time course for effects on MEPP frequency differs from those on EPP amplitude. Effects of MeHg on spontaneous release occur after a latent period of 15–70 min depending on the concentration of MeHg (13). Other peripheral synapses including autonomic ganglia are similarly affected by MeHg. The latent period preceding an increase in MEPP frequency is shortened by facilitation of Ca^{2+} entry into the terminal using a Ca^{2+} ionophore, a Ca^{2+} channel agonist and depolarization (21, 22), or is prolonged when $[\text{Ca}^{2+}]_e$ is lowered (21). The increase in spontaneous release by MeHg is not strictly dependent on extracellular (Ca^{2+}_e), because increases in MEPP frequency still occur when $[\text{Ca}^{2+}]_e$ is lowered (21). Results in low Ca^{2+} solutions suggest that intracellular Ca^{2+} (Ca^{2+}_i) stores may be the source of Ca^{2+} responsible for the increased MEPP frequency. Similarly, increased MEPP frequency by MeHg is not prevented by blocking either the axon membrane Na^+ channels with tetrodotoxin (13, 23) or membrane Ca^{2+} channels with Mg^{2+} (13) or Co^{2+} (23). The implication is that MeHg may enter the cell not only through the channels but also directly through the membrane because of its increased lipophilicity.

The mechanism responsible for the ultimate block of transmitter release by MeHg is unknown, but it is not depletion of releasable stores of ACh, as treatment of the preparation when MEPPs disappeared with LaCl_3 , a stimulator of spontaneous release, induced high frequencies of MEPPs (21).

MeHg also alters the spontaneous release of neurotransmitters, as measured biochemically. Incubations of brain homogenates with MeHg increase the amount of various [^3H]neurotransmitters in the supernatant (24). With the exception of [^3H]dopamine, MeHg increases the release of these neurotransmitters with similar potency. This action of MeHg is not significantly altered by reduction of Ca^{2+}_e . However, in these experiments it is not clear whether these increases result from MeHg-induced facilitation of neurotransmitter release or inhibition of reuptake of released neurotransmitter into the terminals.

Using synaptosomes from specific regions of rat brain, Minnema et al. (20) observed a concentration-dependent increase in release of [^3H]neurotransmitter by MeHg. The time to onset was also hastened in a concentration-

dependent manner. Enhanced release of [^3H]dopamine and [^3H]GABA occurred in the absence of Ca^{2+}_e whereas increased release of [^3H]ACh occurred in the presence of Ca^{2+}_e . However, lowering [Ca^{2+}_e] attenuates the enhancement of spontaneous release of [^3H]ACh from synaptosomes by MeHg (25). The persistence of facilitated neurotransmitter release upon removal of Ca^{2+}_e suggests a possible intracellular source for Ca^{2+} . Removal of Na^+_e does not reduce the effects of MeHg on spontaneous neurotransmitter release from rat brain synaptosomes (20). Because the effects do not seem to be specific for certain types of neurotransmitter-containing nerve terminals, MeHg may alter transmitter release via a common mechanism.

Effects of MeHg on transmitter precursor and uptake

Alterations in the reuptake or metabolism of released neurotransmitter can produce effects that mimic increases in spontaneous transmitter release. MeHg reduces choline uptake and increases the uptake of the neurotransmitters glycine and glutamate into mouse neuroblastoma cells in culture (26). MeHg inhibits the uptake of monoamines into the crude mitochondrial fraction of selected areas from rat brain (27) and choline into synaptosomes from rat brain (25). The uptake of several neurotransmitters and choline into homogenates of mouse brain is reduced by MeHg (24). The inhibitory potency roughly correlates with the Na^+ dependence of the various uptake systems. Similarly, the potency of MeHg-induced inhibition of [^3H]dopamine and [^3H]norepinephrine uptake into rat brain synaptosomes matches the potency with which it inhibits plasma membrane Na^+/K^+ -ATPase (28). Thus, MeHg may alter uptake by a common mechanism involving inhibition of plasma membrane Na^+/K^+ -ATPase and subsequent perturbation of intracellular Na^+ concentrations. Because Na^+ manipulations have little effect on MeHg-induced alterations in spontaneous release, it is likely that these effects of MeHg are mediated by different mechanisms.

Preparations from rats given MeHg *in vivo* have also demonstrated deficits in neurotransmitter uptake *in vitro*. O'Kusky and McGeer (29) observed significant decreases in choline and GABA uptake in homogenates from cerebral cortex, but only GABA uptake was reduced from caudate putamen before or at the onset of neurological impairment. This is consistent with the report of a rather selective degeneration of GABA-ergic neurons in the cerebral cortex resulting from MeHg exposure *in vivo* (30). Chronic exposure of rats to MeHg also alters catecholamine turnover in synaptosomes from whole brain (31) and ACh from homogenates of cerebellum, striatum, and cerebral cortex (32). Activities of enzymes associated with synthesis and/or degradation of several transmitters are reduced by chronic exposure to MeHg (33, 34). Thus, chronic MeHg alters the activity of a number of neurotransmitter systems in the central nervous system.

NERVE TERMINAL MEMBRANE DEPOLARIZATION BY MeHg

An explanation compatible with both the decreased evoked release and increased spontaneous release of transmitter by MeHg is nerve terminal depolarization. Depolarization decreases the effectiveness of the presynaptic action potential in eliciting neurotransmitter release. Moreover, depolarization opens membrane Na^+ and Ca^{2+} channels, increasing cation influx, ultimately leading to increases in spontaneous neurotransmitter release. MeHg depolarizes the plasma

membrane of guinea pig (35) and rat brain synaptosomes (36). Relatively low concentrations of MeHg increase synaptosomal membrane permeability to small molecules (20) and probably to cations such as Na^+ and Ca^{2+} . The increased Ca^{2+} and Na^+ permeability may be through voltage-independent, nonspecific cation channels. Such a conductance has been described to occur for high concentrations of MeHg (37). Whether this effect is accompanied by membrane depolarization sufficient to open voltage-dependent Na^+ and/or Ca^{2+} channels is unknown.

At very high concentrations (500 μM) MeHg depolarizes axonal membranes, whereas at 25–100 μM no depolarization occurs (18). In neuroblastoma cells, MeHg has only a slight depolarizing action at 30 μM (38).

EFFECTS OF MeHg ON MEMBRANE ION CHANNELS

Effects of MeHg on nerve Ca^{2+} channels

Block of the EPP by MeHg appears to be noncompetitive and irreversible with respect to [Ca^{2+}_e], as the time required for cessation of the EPP is identical irrespective of [Ca^{2+}_e] (16). Thus, MeHg could interfere with evoked release of neurotransmitter by suppressing Ca^{2+} entry into the nerve terminal. This problem has been examined in detail using isolated nerve terminals from the brain.

MeHg decreases total K^+ -stimulated $^{45}\text{Ca}^{2+}$ uptake into synaptosomes in a noncompetitive, irreversible manner (16). Block of the "fast phase" of $^{45}\text{Ca}^{2+}$ uptake occurs at lower concentrations but block of the "slow phase" is more efficacious. Ethylmercury (EtHg^+) and Hg^{2+} also block depolarization-dependent uptake of $^{45}\text{Ca}^{2+}$ in a concentration-dependent manner, whereas the nonpositively charged mercurials dimethylmercury (Me_2Hg), *p*-chloromercuribenzoate (PCMB), and *p*-chloromercuriphenylsulfonate (PCMBs) cause no appreciable reduction in K^+ -induced $^{45}\text{Ca}^{2+}$ uptake (39). Concentrations of MeHg that block fast-phase $^{45}\text{Ca}^{2+}$ uptake do not alter depolarization-independent uptake of $^{45}\text{Ca}^{2+}$ (40). Very high concentrations of MeHg^+ , Hg^{2+} , and EtHg^+ reduce depolarization-independent uptake of $^{45}\text{Ca}^{2+}$, whereas no consistent inhibitory effect is seen for PCMB, PCMBs, or Me_2Hg . Thus, mercurials of dissimilar charge and lipophilicity affect synaptosomal Ca^{2+} uptake differentially, suggesting that both charge and lipophilicity may be important in determining the characteristics of Ca^{2+} channel block by mercurials.

Increasing [Ca^{2+}_e] partially reverses the fast-phase block by MeHg and completely reverses the slow-phase block of $^{45}\text{Ca}^{2+}$ uptake into synaptosomes (40). Removal of Na^+_e , which inactivates plasma membrane $\text{Na}^+/\text{Ca}^{2+}$ exchange, exposes an Na^+ -independent component of slow-phase $^{45}\text{Ca}^{2+}$ uptake, which appears to be irreversibly and noncompetitively blocked by MeHg. Thus, each phase has a component that is irreversibly blocked by MeHg. The mechanism of block differs from that produced by divalent inorganic cations such as Co^{2+} , Cd^{2+} , Ni^{2+} , Mn^{2+} , and Pb^{2+} , for which block is reversible and competitive (41).

The magnitude of block of $^{45}\text{Ca}^{2+}$ uptake by MeHg increases as [K^+]_i increases, suggesting block to be voltage-dependent. Furthermore, the blocking actions are equally avid whether MeHg has access to the channels during activation, or at rest, which suggests that block is not state dependent. The $t_{1/2}$ for inactivation of $^{45}\text{Ca}^{2+}$ influx is not altered by MeHg although a greater proportion of channels is inactivated. Thus, MeHg does not reduce Ca^{2+} entry by hastening

inactivation of Ca^{2+} channels. MeHg affects the relative influx of $^{45}\text{Ca}^{2+}$, $^{85}\text{Sr}^{2+}$, and $^{133}\text{Ba}^{2+}$, indicating that ionic selectivity of synaptosomal Ca^{2+} channels is altered (42).

Does MeHg affect one or another of the subtypes of Ca^{2+} channels preferentially? In rat pheochromocytoma (PC12) cells, which express dihydropyridine (DHP)-sensitive L-type Ca^{2+} channels, MeHg blocks $^{45}\text{Ca}^{2+}$ uptake with similar potency as observed in synaptosomes (42). Moreover, MeHg appears to block both DHP-sensitive and -insensitive (N-type) Ca^{2+} uptake. Both of these channel types are associated with neurotransmitter release in several types of cells. This is consistent with the idea that MeHg blocks evoked release of neurotransmitter by blocking voltage-dependent Ca^{2+} channels. Effects of MeHg on P-type, or other recently identified Ca^{2+} channel types, have not yet been examined.

Electrophysiological measurements of effects of MeHg on Ca^{2+} channels have been made in cell soma using differentiated PC12 cells (43) and whole cell patch voltage-clamp, and at intact motor nerve terminals using perineural recording techniques (19). Differentiated PC12 cells exhibit voltage-dependent currents containing inactivating and noninactivating components that correspond pharmacologically to both L- and N-type Ca^{2+} channels. MeHg causes rapid and complete block of I_{Ba} (currents carried by Ba^{2+} , a Ca^{2+} substitute), without altering leak or capacitance currents. MeHg blocks I_{Ba} in a concentration-dependent manner between 1 and 20 μM . Increasing the frequency of stimulation facilitates block of I_{Ba} , but channel use is not required for block which indicates that block is not state dependent. Also, MeHg blocks I_{Ba} when the membrane-holding potential is -40, -70, and -90 mV, indicating that block is not holding potential dependent, and that both N- and L-type Ca^{2+} channels are blocked. Block is antagonized slightly by increasing $[\text{Ba}^{2+}]$, but not by washing with MeHg-free solution. Conversely, block of I_{Ba} by 1 μM Cd^{2+} is reversed by washing in Cd^{2+} -free solutions or increasing $[\text{Ba}^{2+}]$. As is observed in synaptosomes, the ionic selectivity of PC12 cell Ca^{2+} channels is altered by MeHg.

Block by MeHg of nerve terminal Na^{+} and Ca^{2+} channels in intact neuromuscular junctions was examined in mouse *triangularis sterni* motor nerves (19). Potential changes arising from Na^{+} and Ca^{2+} channel function were recorded from the perineural sheath surrounding motor nerves; when K^{+} channels were blocked two components of the Ca^{2+} -dependent potential were observed. MeHg (100 μM) rapidly blocked both Ca^{2+} components as well as the Na^{+} component. Occasionally, at 50 μM MeHg, the Ca^{2+} components were blocked before the Na^{+} component. Block of Na^{+} or Ca^{2+} components by MeHg was not reversed by perfusing with MeHg-free solutions or by increasing the intensity and/or duration of intercostal nerve stimulation. Thus, MeHg blocks nerve terminal Na^{+} and Ca^{2+} channels in intact neuromuscular preparations irreversibly at concentrations that block the EPP.

Effects of MeHg on other voltage-gated and receptor-operated ion channels

Measurements of MEPP amplitude at the neuromuscular junction (see above) suggest that MeHg does not affect nicotinic-type ACh receptor-operated channels. Ionophoretic application of ACh onto the motor end-plate of MeHg-poisoned preparations was used to test unequivocally for direct effects of MeHg on the ACh receptor (13). End-plate depolarizations due to ACh are not decreased in amplitude by concentrations of MeHg as high as 100 μM or for periods of exposure of up to 60 min, by which time MEPPs

are blocked. Thus, at the motor end-plate, MeHg has few postsynaptic effects. Postsynaptic actions of MeHg on single nicotinic, ACh receptor-activated ion channels were examined in the G8 clonal myoblast cell line using patch voltage clamp techniques. Single channel conductance, and mean channel open and closed time, were examined in the absence and presence of MeHg (10 and 100 μM) (W. D. Atchison, unpublished observations). MeHg did not affect either the mean amplitude of single channel events or the distribution of open and closed times. Thus, there was no apparent effect of MeHg on muscle nicotinic receptors or receptor-activated ion channels. In contrast, biochemical studies indicate that MeHg decreases the binding of cholinergic agonists to nicotinic receptors (44). The apparent lack of effect of MeHg on the postsynaptic membrane at the neuromuscular junction is puzzling given the well-known affinity of MeHg for sulfhydryl groups. The ACh receptor is known to contain sulfhydryl groups, whose modification leads to decreased affinity to cholinergic agonists. Presumably, MeHg should interact with the sulfhydryl groups to modify the ACh receptor and decrease the postsynaptic response to ACh.

Studies of the effects of MeHg on neuronal nicotinic and muscarinic receptor-mediated responses in N1E-115 neuroblastoma cells reveal that MeHg decreases depolarizing and hyperpolarizing responses to activation of both types of ACh receptors (38). Conversely, responses in the same cell to dopamine were unaffected. In dorsal root ganglion cells, MeHg at 100 μM decreases GABA-induced chloride currents (37).

In addition to its effects on synaptic transmission, MeHg directly affects conduction of nerve impulses by effects on axonal membrane properties. For example, Na^{+} and K^{+} conductances associated with step depolarizing pulses are suppressed by 25 μM MeHg, whereas conductance through leakage channels is increased (18). A similar inhibitory effect of mercury on Na^{+} conductance in isolated lobster axons has been reported (45), and MeHg (20–60 μM) reduces action potential amplitude, secondary to decreased peak Na^{+} and steady-state K^{+} currents in mouse neuroblastoma cells (38). Acute and subchronic intoxication of rats with MeHg impairs dorsal root conduction velocity and prolongs action potential duration from dorsal root ganglion cells (12). Skeletal muscle membranes of crab and barnacle are depolarized by mercury (26–370 μM) and MeHg (100 μM) (46, 47). Thus, nerve and muscle membranes are also sensitive to block by mercurials, with both Na^{+} and K^{+} channels being affected.

EFFECTS OF MeHg ON INTRACELLULAR CALCIUM HOMEOSTASIS

MeHg-induced increases in neurotransmitter release may be mediated by increases in $[\text{Ca}^{2+}]_i$ (21). Concentrations of MeHg as low as 2.5 μM increase the apparent $[\text{Ca}^{2+}]_i$ of rat brain (48) and guinea pig cerebrocortical synaptosomes (35). This increase is mediated in part by the influx of Ca^{2+} , as lowering Ca^{2+}_e reduces the effect. Apparently the Ca^{2+} influx is not through verapamil-sensitive pathways.

MeHg increases $[\text{Ca}^{2+}]_i$ in NG108-15 cells (49, 50). Using digital imaging combined with fluorescence microscopy of single cells, 2 and 5 μM MeHg cause multiphasic alterations in fura-2 fluorescence. Initially MeHg increases $[\text{Ca}^{2+}]_i$ (first Ca^{2+} phase), followed by an increase in the concentration of another endogenous polyvalent cation (non- Ca^{2+} phase). Finally, there is a massive increase in $[\text{Ca}^{2+}]_i$ (second Ca^{2+} phase) due to influx of Ca^{2+} . This influx of Ca^{2+} is nifedipine

sensitive but does not appear to be the voltage-dependent, L-type Ca^{2+} channel (unpublished results). The first Ca^{2+} phase is essentially unaltered by lowering Ca^{2+}_e or prepolarizing the mitochondria. However, depletion of the IP_3 -sensitive Ca^{2+} pool reduces, but does not block, this effect, which suggests that the first Ca^{2+} phase is due, in part, to release of Ca^{2+} from an IP_3 -sensitive pool (50).

MeHg increases the intracellular ionic concentration of a non- Ca^{2+} cation (or cations) in NG108-15 cells (49) and synaptosomes (51). The identity of the cation (or cations) in NG108-15 cells is presently unknown, but in synaptosomes it has been determined to be Zn^{2+} (52). Of the endogenous cations at highest concentration within cells, Zn^{2+} , as opposed to Cu^{2+} and Fe^{3+} , not only binds to fura-2 with approximately 100-fold greater affinity than does Ca^{2+} , but of these three cations it is the only one that produces effects on fura-2 fluorescence consistent with those observed *in situ* (i.e., Cu^{2+} and Fe^{3+} quench fura-2 fluorescence rather than increase it). The relevance of this finding to the neurotoxicity of MeHg is unknown.

MITOCHONDRIA AS A SITE OF MeHg NEUROTOXICITY

The idea that mitochondria may be a site of action of MeHg developed from both *in vivo* and *in vitro* studies. Exposure to MeHg *in vivo* results in accumulation of the mercurial into mitochondria followed by biochemical and ultrastructural changes in this organelle (52, 53). These effects are similar to those observed upon inhibition of respiration.

Rats exposed to MeHg *in vivo* display neurological symptoms after a latent period. Mitochondrial function (as measured by oxygen consumption of brain slices) is impaired during the symptomatic phase but not during the latent phase (54). Although MeHg concentrations are maximal during the latent phase, the effects of MeHg on mitochondria may be indirect as they are preceded by inhibition of protein synthesis (53, 54).

Synaptosomes from rats treated with MeHg and from naive rats exposed to MeHg *in vitro* have reduced rates of respiration (55). This effect is blocked by removal of K^+ , suggesting that there is an increase in the K^+ permeability of the inner mitochondrial membrane. Alterations in respiration are also observed in guinea pig brain slices at slightly higher concentrations of MeHg (56). The decrease in respiratory rates may be due to MeHg-induced inhibition of the tricarboxylic acid cycle. This is consistent with earlier work in which *in vivo* MeHg exposure decreases succinate dehydrogenase activity (54).

These effects of MeHg are not unique to brain mitochondria. *In vitro* exposure of isolated mitochondria from rat liver to MeHg inhibits electron transport and phosphorylation, increases K^+ permeability, and dissipates the mitochondrial membrane potential (Ψ_m) (57). The increase in permeability of the inner mitochondrial membrane may occur secondary to release of membrane-bound Mg^{2+} from mitochondria upon depolarization (58).

Loss of Ψ_m results in efflux of mitochondrial Ca^{2+} and an inhibition of mitochondrial Ca^{2+} uptake (5, but see refs 48, 49, 51). But could mitochondrial depolarization by MeHg release sufficient amounts of Ca^{2+} to explain the increase in Ca^{2+}_e -independent release of neurotransmitter? Addition of MeHg to isolated rat brain mitochondria causes ATP-dependent and -independent decreases in $^{45}\text{Ca}^{2+}$ uptake and increases efflux from preloaded mitochondria (59). This ac-

tion is blocked by ruthenium red. These effects are thought to result from a specific action of MeHg on the Ca^{2+} uniporter in the inner mitochondrial membrane, as ruthenium red is thought to act at this site. Moreover, ruthenium red blocks MeHg-induced increases in ACh release from synaptosomes in low $[\text{Ca}^{2+}]_e$ solutions (25).

Although there is little doubt that mitochondria participate in Ca^{2+}_i buffering at relatively elevated $[\text{Ca}^{2+}]_i$, the affinity of the uniport carrier for Ca^{2+} is low, and mitochondria may play only a minor role in buffering $[\text{Ca}^{2+}]_i$ under normal conditions (60). Increases in $[\text{Ca}^{2+}]_i$ upon depolarization of Ψ_m , are not observed universally (48, 51) or are relatively small (49). Moreover, prepolarization of Ψ_m in low $[\text{Ca}^{2+}]_e$ medium does not influence the subsequent action of MeHg in synaptosomes (51) or NG108-15 cells (49). Apparently, under normal conditions mitochondria do not contain sufficient Ca^{2+} to alter $[\text{Ca}^{2+}]_i$ significantly upon release.

Recent evidence suggests that local concentrations of Ca^{2+} in excess of 100 μM may be required for activation of neurotransmitter release. Thus, the relatively small changes in $[\text{Ca}^{2+}]_i$ observed upon depolarization of mitochondria are likely insufficient to alter the release of neurotransmitter via normal physiological mechanisms.

OXIDATION AS A MECHANISM OF MeHg NEUROTOXICITY

Reactive oxygen species (ROS), such as the free radicals superoxide anion and hydroxyl radical, and the radical generator, hydrogen peroxide, are thought to initiate peroxidative cell damage. MeHg causes membrane lipoperoxidation in nerve cells (61). Because the brain is exceptionally sensitive to oxidative, free radical-mediated injury and the antioxidants vitamin E and selenium provide some degree of protection against MeHg neurotoxicity *in vivo* (62), free radical-induced lipid peroxidation may be involved in MeHg-induced cell damage (63, 64). This hypothesis is supported by findings in which MeHg exposure *in vivo* elevates ROS in brain regions sensitive to MeHg but not in regions less sensitive to MeHg (65, 66). These increases may be secondary to inhibition of glutathione (GSH) -S-transferase and GSH peroxidase, necessary for the metabolism of ROS (67). In cultured mouse neuroblastoma cells, MeHg decreases GSH peroxidase activity (68). Moreover, levels of the endogenous antioxidant GSH are both decreased (61) and increased (68) by *in vitro* MeHg exposure.

Vitamin E protects against the growth inhibitory effects of MeHg on rat glioma cells in culture but not on mouse neuroblastoma cells (69). However, the protective effect of vitamin E may not be due to its antioxidant properties, as other antioxidants are not protective. In cerebellar granule cells, vitamin E is effective in reversing MeHg-induced elevations in malonaldehyde, but only partially effective in reversing the loss in cell viability (61). Indeed, of the agents tested, only GSH and the specific iron chelator, deferoxamine, reverse MeHg-induced loss in cell viability. Deferoxamine also inhibits the formation of ROS by MeHg in crude synaptosomal suspensions (66). EDTA and EGTA are marginally effective and the hydroxyl radical scavenger, mannitol, as well as catalase and superoxide dismutase, are ineffective in reversing any aspect of MeHg toxicity. However, this could reflect their inaccessibility to the intracellular sites of ROS generation. The protective effects of deferoxamine against MeHg-induced lipoperoxidation and cell death in cerebellar granule cells implicate iron-mediated hydroxyl radical formation in the acute toxicity of MeHg in these cells.

Despite clear evidence of MeHg-induced lipoperoxidation, apparently it is not the critical target for lethal cell injury. First, cerebellar granule cells in culture exposed to no MeHg display greater than 90% viability for 3 h of incubation but have greater than 100% increases in malonaldehyde content (61). Second, vitamin E blocks MeHg-mediated increases in lipoperoxidation without substantially improving cell viability. Third, EDTA and EGTA also effectively block control and MeHg-mediated lipoperoxidation without protecting against cell death (61). MeHg appears to accelerate the production of highly reactive free radicals that produce peroxidative damage to biological membranes. Although this mechanism may contribute to the development of neurotoxicity caused by MeHg, it does not appear to be critical to the neurotoxicity of MeHg.

INHIBITION OF PROTEIN SYNTHESIS

Disruption of protein synthesis may be an early manifestation of MeHg toxicity in vitro and in vivo, and has been proposed to be the proximal event and primary mechanism of action of MeHg in the nervous system (54, 70). However, no direct relationship between the inhibition of protein synthesis and neuropathologic changes in MeHg poisoning has been established.

Brown and Yoshida (71), using young chicks exposed to MeHg in vivo, proposed that MeHg-induced alterations in membrane structure may be due to interference by MeHg of protein production in cerebellar neurons. This hypothesis was supported by several observations. For example, [^{14}C]leucine incorporation into rat brain cortex slices after MeHg exposure in vivo is decreased (54). This effect occurred before the appearance of neurological symptoms. Rats fed MeHg also had decreased [^{14}C]leucine incorporation into cerebellar slices in both the latent and neurotoxic phases (70). Finally, brain slices and synaptosomes exposed to MeHg in vitro had decreased [^{14}C]leucine levels, and this inhibition was not likely to be secondary to mitochondrial perturbations as it was ATP and ADP insensitive (55).

In cultured astrocytes, inhibition of protein synthesis is apparently not the most sensitive action of MeHg, as concentrations twofold greater than those that produce gross morphological alterations in the form of "bleb"like swellings are required (72). Examination of protein synthesis after different injection protocols for MeHg indicated either no change (73) or a decrease (74). Stimulation of protein synthesis in MeHg-treated rats has also been reported (75). The apparent discrepancy may result from differences in dose or concentration of MeHg, age, and/or weight of the animals.

MeHg alters protein phosphorylation, although the patterns of alteration differ somewhat from study to study. Sarafian and Verity (76) report stimulation of protein phospholabeling in primary cultures of cerebellar granule cells exposed to low concentrations of MeHg for 24 h, whereas cerebellar glial cells had decreases in protein phosphorylation under identical exposure conditions.

SUMMARY

The effects of MeHg on the normal functioning of the nervous system are numerous. It is unlikely that any single event is responsible for the neurotoxicity of MeHg. Rather, MeHg likely causes disruptions in cellular processes including synaptic function, excitability, ion regulation, and protein synthesis. We have hypothesized that the disruption of

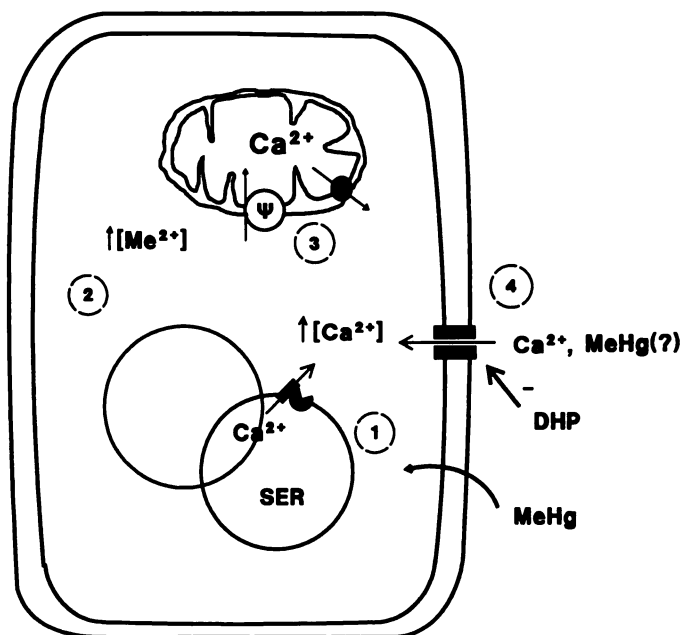


Figure 1. Sequence by which MeHg alters intracellular cation homeostasis. The sites of MeHg actions are denoted by the circled numbers. 1) The initial site of MeHg action appears to be on the IP_3 -sensitive stores of Ca^{2+} in the smooth endoplasmic reticulum (SER). 2) After the initial increase in $[\text{Ca}^{2+}]_i$ by depletion of one or more Ca^{2+} pools of the SER, there is an increase in the concentration of an endogenous polyvalent cation (Me^{2+}), possibly Zn^{2+} . The origin of this cation is unknown. 3) MeHg then depolarizes the mitochondrial membrane. 4) Subsequently, there is a large increase in $[\text{Ca}^{2+}]_i$ resulting from Ca^{2+} influx probably through a nifedipine-sensitive pathway. This pathway does not appear to be the L-type, voltage-dependent Ca^{2+} channel (unpublished results). Although not shown here, several minutes after the second Ca^{2+} phase there is a Ca^{2+} -dependent loss of the intracellular entrapped dyes fura-2 and rhodamine 123 (and presumably endogenous intracellular components), ultimately leading to cell death.

ionic (in particular, Ca^{2+}) homeostasis may contribute to the etiology of MeHg neurotoxicity. **Figure 1** depicts several sites and mechanisms by which MeHg may perturb cellular ion homeostasis. First, MeHg increases $[\text{Ca}^{2+}]_i$ via release of Ca^{2+} from an IP_3 -sensitive pool. Second, MeHg alters the concentration of another polyvalent cation, possibly Zn^{2+} . This effect has been observed in both NG108-15 cells and in synaptosomes where it has been identified, by using ^{19}F -NMR spectroscopy, to be Zn^{2+} . The change in $[\text{Zn}^{2+}]_i$ occurs simultaneously with or slightly before depolarization of Ψ_m . Apparently the change in $[\text{Zn}^{2+}]_i$ is not causative in the depolarization of Ψ_m nor in the increase in plasma membrane permeability to Ca^{2+} , as these two events occur in the presence of TPEN. The exact source of the Zn^{2+} has not been determined but is intracellular. Third, MeHg depolarizes mitochondria. This depolarization is not altered by manipulations of $[\text{Ca}^{2+}]_i$ or $[\text{Zn}^{2+}]_i$. It is not known if this is a direct or indirect effect of MeHg on mitochondria. Because the first Ca^{2+} phase occurs before depolarization of Ψ_m (although MeHg could release Ca^{2+} from mitochondria without depolarization of Ψ_m), and depolarization of Ψ_m with known mitochondrial inhibitors causes only a modest increase in $[\text{Ca}^{2+}]_i$, it is questionable whether mitochondria contribute significantly to elevated $[\text{Ca}^{2+}]_i$. Fourth, MeHg causes an extracellular Ca^{2+} -dependent increase in $[\text{Ca}^{2+}]_i$. The Ca^{2+} may enter via pathways that are sensitive to nifedipine but

probably not the voltage-dependent, L-type Ca^{2+} channel. However, we cannot determine whether the nifedipine-induced delay in the second Ca^{2+} phase results from block of Ca^{2+} influx, or from block of MeHg influx and the subsequent delay of MeHg access to critical sites. The evidence in favor of the latter is that high concentrations of nifedipine delay the onset of all three phases. Ultimately, the mosaic of disruption caused by MeHg leads to cellular dysfunction and cell death. Whether there is a proximal or more sensitive target for MeHg remains unclear.

[F]

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