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Methylmercury-Cholinesterase Interactions in Rats

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The interaction of methylmercury hydroxide (MMH) and cholinesterases was studied in male and female rats. MMH administered subcutaneously in doses of 10 mg/kg for 2 days reduced the level of plasma cholinesterase (ButChE) by 68% in females and 47% in males while brain acetylcholinesterase (AChE) was unaffected. Normal females had higher but more variable ButChE levels than normal males. In a time-course experiment, a single dose of MMH (10 mg/kg) reduced ButChE levels when mercury levels reached 22 μg/ml in the blood. A 10% reduction in brain AChE was observed at 72 hours; however, mercury reached a concentration of only 2.0 μg/g in brain tissue. The determination of the Michaelis constant K_m and maximum velocity value V_{max} for butyrylcholine and ButChE in control and MMH-treated (1 mg/kg) animals indicated that MMH reduced V_{max} only. Since no loss in ButChE activity occurred when MMH and control plasma were incubated *in vitro*, MMH is not a direct inhibitor of ButChE. Because only the inactive monomeric form of ButChE contains free sulfhydryl groups, it is postulated that MMH combines covalently with the sulfur, preventing formation of active enzyme. By analogy, it is believed this is also the case with AChE.

Organophosphorus and carbamate insectides are known to inhibit butyrylcholinesterase (ButChE) and acetylcholinesterase (AChE) in warm-blooded animals and inhibition of these enzymes is considered to be the mode of action of these pesticides. Recent findings have demonstrated a lowering of the LD_{50} value of carbaryl to rats pretreated with methylmercury hydroxide (MMH) (*1*) that might be related to decreased hepatic mixed function oxidase activity (*2*). It is also possible that MMH has an effect on ButChE and AChE that might be related to decreased LD_{50} values. To investigate this possibility, we pretreated rats with MMH and compared the ButChE levels in the plasma and AChE levels in the brain to control levels. Preliminary investigations indicated that sex differences could be an important consideration in this study. Dose–response relationships and time-course effects were also investigated to understand further the interactions between these enzymes and MMH.

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Materials and Methods

Adult albino Charles River C-D male and female rats weighing 175–225 g were maintained in individual suspended cages, fed *ad libitum*, and weighed daily. Treated rats received subcutaneous (SC) injections of MMH (97% pure by NMR spectroscopy) in an aqueous solution, while control animals received injections of distilled water on the same schedule as the treated animals. The precautions for handling and administering the highly toxic MMH have been described (*3*). Mercury levels were determined by neutron activation analysis (*4*).

Vaginal smears were taken 2 or 4 days prior to sacrifice to establish the phase of the cycle on the day of ButChE determination. The maturation index was established by vaginal cytology measured by the relative cell count, including parabasal, small, intermediate, and superficial epithelial cells as well as leukocytes (*5*).

Methoxyflurane was used for inhalational anesthesia, and 4–7 ml of blood was removed by cardiac puncture and placed in 0.2 ml of heparin. Plasma was separated from erythrocytes by centrifuging at 3020 *g* for 10 min at 4°C. The plasma was held at 4°C until studied. All analyses were conducted within 6 hr of sacrifice.

Brain homogenates were prepared by decapitation and rapid removal of the entire brain with the exception of the medulla oblongata. The tissue was weighed, placed in distilled water, and homogenized to make a 10% mixture (w/v). To promote solubilization, two drops (or approximately 0.6%) of Triton X-100 were added prior to homogenation.

Cholinesterase levels were determined by using Brinkmann pH stats. Plasma studies were done with 40 ml of 4*mM* butyrylcholine iodide and 0.4 ml of plasma while brain studies were done with 40 ml of 2*mM* acetylcholine chloride and 0.2 ml of a 10% brain homogenate. Conditions were 25°C and pH 7.6, and no salts were added. The titrant was 5*mM* NaOH. Activities are expressed as μmole/min-ml for plasma and μmole/min-g for brain and all measurements were corrected for nonenzymatic hydrolysis. Enzyme kinetic constants were determined according to the method of Lineweaver and Burk (*6*). Linear regression analyses were performed according to Steel and Torrie (*7*).

Results and Discussion

At 48 hr following two SC doses of MMH at 10 mg/kg the level of AChE as measured by acetylcholine hydrolysis was essentially unchanged for males (mean ± SE for controls 5.40 ± 0.09 μmole/min-g and for MMH-treated 5.36 ± 0.09 μmole/min-g. For females the level of AChE only decreased 4% (mean ± SE for controls 5.60 ± 0.20 μmole/min-g and for MMH-treated 5.36 ± 0.20 μmole/min-g). This may represent the lag period characteristic of short-chain alkyl mercurials which are bound to red blood cells prior to diffusing into the brain (*8*). Norseth and Clarkson (*9*) have demonstrated in rats that red blood cells preferentially accumulated methylmercury chloride which resulted in a cell to plasma ratio of 300:1. The time-course experiment which follows substantiates the slow movement of MMH into the brain.

In a similar experiment at the same MMH dosage rate, MMH reduced the ButChE levels by 68% in females as measured by butyrylcholine hydrolysis (mean ± SE for controls 0.743 ± 0.138 μmole/min-ml and for MMH-treated 0.237 ± 0.011 μmole/min-ml). For males the level of ButChE was reduced 47% (mean ± SE for controls 0.150 ± 0.014 μmole/min-ml and for MMH-treated 0.070 ± 0.009 μmole/min-ml). The amount of MMH administered (10 mg/kg on each of 2 days prior to sacrifice) was quite high since the dose-response curve showed near maximum reduction in the level of the enzyme at about 5 mg/kg (Fig. 1).

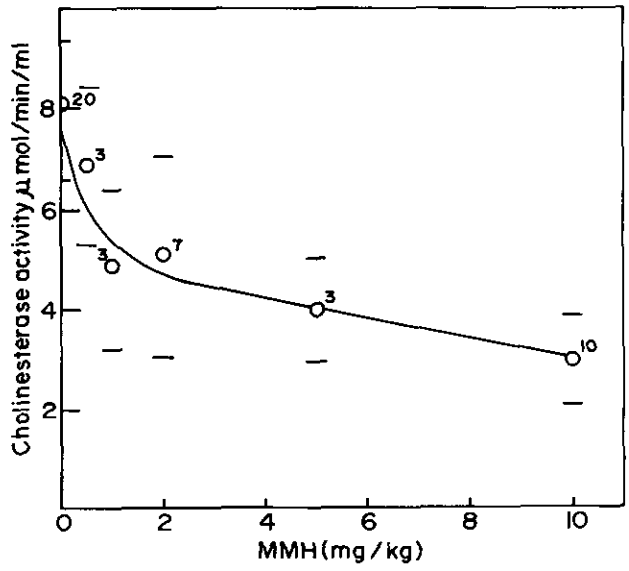

FIGURE 1. Effect of varying doses of methylmercury hydroxide (MMH) on the level on plasma cholinesterase in female rats. Each point is the mean value for the number of animals indicated near each point. The bars indicate the standard deviations of the mean values. Animals received 1 SC dose/day for 2 days prior to sacrifice.

However, this was the MMH dosage schedule used to decrease the LD_{50} value for carbaryl (*1*); on this schedule rats did not exhibit neurological symptoms, liver histopathology, or weight loss (*10*). There was a large variation between the individual control females. Male rats were not as variable nor as high in ButChE activity as were females.

It appeared that the high variability might be sex-associated and therefore related to the 4-day estrus cycle. We did observe a correlation between ButChE activity and the phase of the cycle, i.e., the

closer to estrus, the higher the titer of ButChE. In addition, MMH decreased ButChE activity in all phases of the estrus cycle.

Figure 2 shows the time-course study for females receiving one dose of 10 mg/kg of MMH. ButChE levels decreased to 71% of normal at 24 hr when mercury levels reached 21.7 μg/g. Depression of ButChE levels continued during the 336-hr test period, although mercury levels gradually decreased to 7.3 μg/g. The highest MMH levels measured in brain tissue was 2.0 μg/g at 72 and 168 hr, and this caused about a 10% reduction in AChE levels (Fig. 3). The lack of AChE depression prior to 72 hr demonstrates the lag period described by Clarkson (*8*). This experiment was somewhat different from the carbaryl–MMH LD_{50} experiment (*1*), which consisted of two 10 mg/kg doses of MMH prior to dosing with carbaryl. However, the reduction in AChE and ButChE levels for our time-course studies are compatible with the toxicity associated with the LD_{50} experiments. The single SC dose and limited number of animals used in this study limit quantitative (*p* value) interpretation but suggest an inverse relationship between MMH levels and enzyme activity.

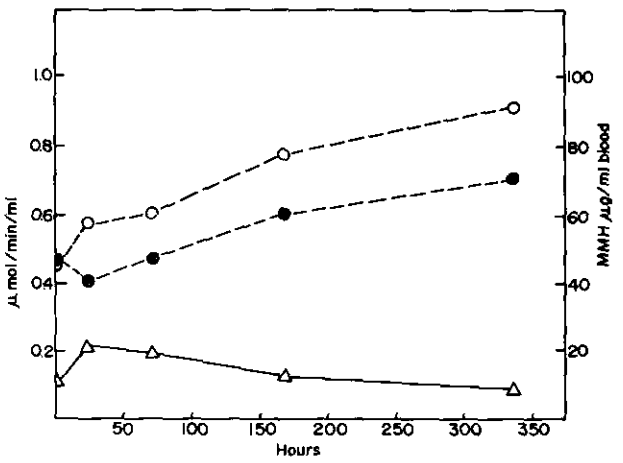

FIGURE 2. Time-course experiment showing the effect of a single SC dose of methylmercury hydroxide (MMH) (10 mg/kg) on the plasma cholinesterase of female rats. Differences between (o) control and (*) treated animals occurred at 24 (p=0.15), 72 (p0.31), 168 (p=0.08) and 336 hr (p=0.13; mercury levels (Δ), right margin. Each p value is based on three control and three treated animals.

To ascertain whether the reduction in ButChE by MMH was the result of direct inhibition or some other factor, the Michaelis constant K_m and maximum velocity value V_{max} for the substrate, butyrylcholine, were determined for control and

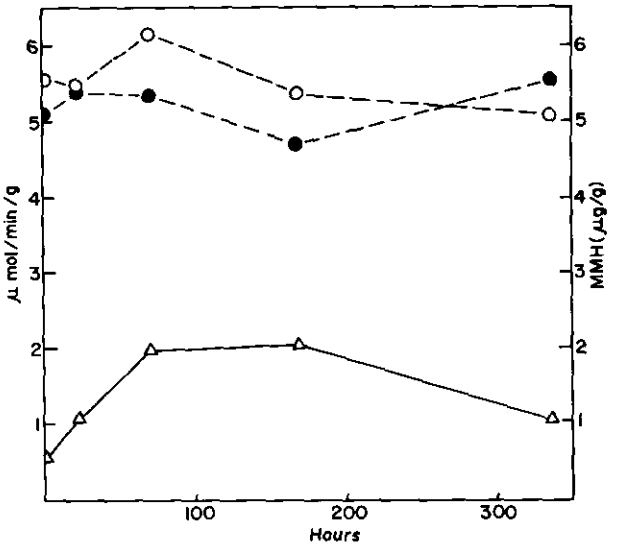

FIGURE 3. Time-course experiment showing the effect of a single SC dose of methylmercury hydroxide (MMH) (10 mg/kg) on the level of brain acetylcholinesterase in female rats. Differences between (o) control and (•) treated animals occurred at 2 (p=0.5), 72 (p=0.027) and 168 hr (p=0.13); mercury levels (Δ), right margin. Each p value is based on three control and three treated animals.

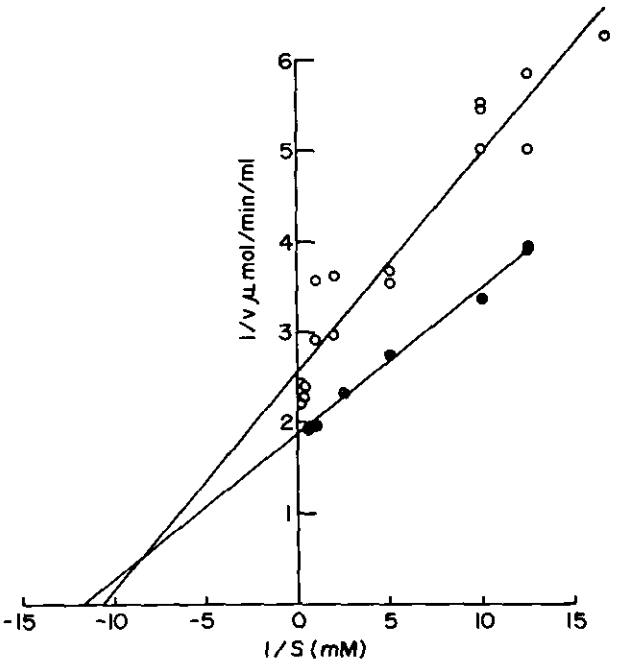

FIGURE 4. Lineweaver-Burk plots for butyrylcholine and plasma cholinesterase from pooled plasma of (•) control and (o) methylmercury hydroxide-treated females (1 mg/kg/2 days sc). Each point represents two determinations on separate pH stats. For treated animals K_m was 9.35 x $10^{-5}M$ and V_{max} was 0.386 μmole/min-ml; control animals had a K_m of 8.53 x $10^{-5}M$ while V_{max} was 0.533 μmole/min-ml.

MMH-treated (1.0 mg/kg for 2 days) females. Figure 4 indicates that MMH effectively reduced V_{max} (control, 0.533; treated, 0.386 μmole/min-ml) while not affecting K_m (control, $853 \times 10^{-5} M$; treated, $9.35 \times 10^{-5} M$). Finally, an *in vitro* experiment was conducted by incubating $4.3 \times 10^{-4} M$ MMH with control ButChE for 0, 3, 20, and 120 min at 25°C and pH 7.0. No loss in ability to hydrolyze butyrylcholine was observed. Obviously, the effect of MMH is not direct inhibition.

It was recently shown that ButChE is composed of four nonactive subunits or monomers (molecular weight MW 77,300), two active subunits or dimers (MW 143,000), and one active tetrameric form (MW 315,000) (*11*). Sodium dodecyl sulfate (SDS) (which breaks noncovalent bonds) converts the tetramer to the dimer, but SDS and 2-mercaptoethanol (which break disulfide bonds) are required to convert the dimer to the monomer. Thus, it seems evident that the inactive monomeric form of ButChE has a free sulfhydryl which would be susceptible to covalent bonding by MMH. On assuming the formation of the active enzyme depends upon agglomeration of the nonactive monomers, our data suggest MMH prevents formation of active enzyme. Clearly the *in vitro* study supports this contention since the active enzyme does not contain free sulfhydryl groups and would not be affected by MMH. The structural similarity of AChE (*12,13*) and ButChE suggests a similar mechanism of reaction for MMH and the monomeric form of AChE; however, the slow rate of MMH accumulation in the brain does afford some protection.

The combination of reducing the formation of ButChE and AChE and increasing the degradation of hepatic cytochrome P-450 (*2*) would explain the lower LD_{50} value of carbaryl to rats pretreated with MMH. This should be true for MMH and any cholinesterase-inhibiting pesticide which is primarily detoxified by the mixed function oxidase system.

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