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Methylmercury and the skeletal muscle receptor

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Methylmercury at a bath concentration of 2×10^{-5} M was capable of inhibiting muscular contractions of the isolated rat phrenic-nerve hemidiaphragm preparation. At the height of inhibition, nerve action potential could still be recorded and the muscles continued to respond to direct stimulation. The inhibition was not reversible with L-cysteine or D-penicillamine but limited protection was possible by prior treatment with (+)-tubocurarine. Treatment of frog rectus muscles with methylmercury (0·2 mM for 15 min) resulted in a shift to the right of 1 log unit in the dose response curve to acetylcholine and a reduction in the maximum response of the tissue. The observed inhibitory action of methylmercury on neuromuscular transmission may be explained by an action on the disulphide bond believed to be present on a cholinergic receptor.

Muscular weakness associated with methylmercury toxicity in animals and humans has been reported (Hunter, Bomford & Russell, 1940; Somjen, Herman & Klein, 1973; Von Burg & Rustam, 1974). However since instances of methylmercury poisoning exhibit prominent central nervous system signs and symptoms (Bakir, Damluji & others, 1973) this aspect of the intoxication syndrome has been neglected. A greater incidence of motor involvement was noted in the Iraqi methylmercury epidemic than previously reported in the Japanese episodes (Rustam & Hamdi, 1974) and electrophysiological studies on these Iraqi patients uncovered a disorder that suggested a failure at some point in the neuromuscular chain (Von Burg & Rustam, 1974; Rustam, Von Burg & others, 1975). These workers found three patients who exhibited a myasthenia-like syndrome that was responsive to neostigmine therapy at the clinical level while three other patients gave an indication of a myotonic syndrome. A subsequent study conducted nine months after normal blood mercury concentrations were established did not reveal these abnormalities in these groups.

In rats, Somjen & others (1973) noted a muscular weakness that could not be accounted for by the nutritional deficiency of methylmercury intoxication. In contrast to the human findings (Von Burg & Rustam, 1974), indirect high frequency stimulation showed little evidence for myoneural transmission failure. However the possibility of a cholinergic involvement in methylmercury intoxication has also been suggested by *in vitro* observations in rats where crude extracts of choline acetylase have been inhibited by methylmercury chloride (reported in Rustam & others, 1975). Therefore the aspect of neuromuscular transmission failure was investigated in greater detail in isolated tissue preparations.

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MATERIALS AND METHODS

Male Sprague-Dawley rats, 180–250 g (Holtzman Farms, Madison, Wisc.) were decapitated and the left phrenic-nerve hemidiaphragms were placed in 50 ml cups in an organ bath (Phipps-Bird, Richmond Va.) maintained at 34° and aerated with 5% CO_2 in oxygen. McEwen solution was used as the physiological bathing medium (McEwen, 1956). A Grass stimulator model SD-4 (Quincy, Mass.) delivered supramaximal, biphasic electrical pulses of 0·1 ms duration through platinum wire or stainless steel electrodes. Contractions of the muscles were recorded by a Grass isometric force transducer (FT.03) and a Beckman Dynagraph.

Male frogs (*Rana pipiens*, Ward's Natural Science, Rochester, N.Y.) were purchased in August (Maeno, 1969). The abdominal rectus muscles were excised, separated and mounted in 50 ml cups in the organ bath at room temperature (22–24°). Frog Ringer solution was used as the bathing medium (pH 7·0–7·4). Details for these techniques can be found in Edinburgh University (1970).

The chloride salts of methylmercury, ethylmercury and phenylmercury were obtained from K & K Labs (Plainview, N.Y.). Acetylcholine chloride, choline chloride, *p*-chloromercuribenzoate, L-cysteine and D-penicillamine from Sigma Chemical Co. (St. Louis, Mo.), (+)-tubocurarine HCl from Squibb and Sons (Princeton, N.J.) and 2-mercapto-

ethanol (99% pure) from Cole Matheson Co. (East Rutherford, N.J.). All chemicals were injected directly into the organ bath and the molar concentrations are reported in terms of the final bath concentrations unless noted otherwise.

RESULTS

Methylmercury chloride at a bath concentration of 2×10^{-5} M consistently blocked muscular contractions of the indirectly stimulated muscles. The time course for the production of the block was independent of the stimulation frequency in the range of 1–0·1 s^{-1}. The block was usually completed within 15–20 min. The form of the blockade could be biphasic (Fig. 1A) particularly when concentrations below 2×10^{-5} M were used. At higher concentrations the production of the block was more rapid and the biphasic pattern was not evident (Fig. 2). At the concentrations of exposure investigated, the muscles continued to respond to direct stimulation with no evidence of interference with contractile processes that could be attributed to methylmercury. Nerve action potentials simultaneously recorded on an oscilloscope did not demonstrate any obvious changes in amplitude or duration.

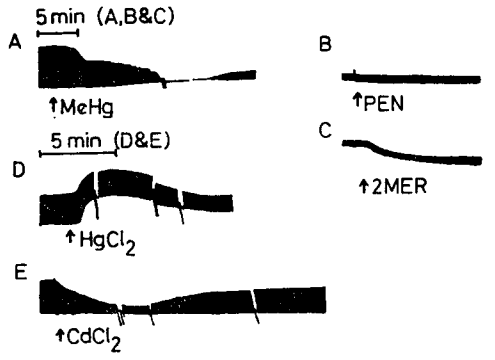

FIG. 1. A. Biphasic pattern of inhibition of muscular contractions observed with 1·5–2·0 $\times 10^{-5}$ M methylmercury chloride. B. Failure of D-penicillamine (2×10^{-3} M) to produce a noticeable recovery. C. Response of partially inhibited muscle to 2 mercaptoethanol (3×10^{-4} M). D. Muscle response to 2×10^{-4} M $HgCl_2$. E. Muscle response to 2×10^{-4} M $CdCl_2$. Downward deflections and spaces indicate washings.

When the contractions were completely inhibited, the preparations were washed. Following 4–5 additional washings extended over a period of approximately 20 min, the preparations showed

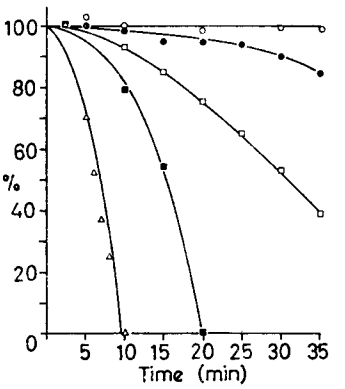

FIG. 2. Time course of contraction inhibition at the indicated bath concentrations of methylmercury. ○, 1; ●, 10; ■, 20; △, 29 μM Me Hg.y axis—% of critical amplitude.

evidence of recovery. This recovery usually amounted to 10–15% of the initial contraction amplitude but on occasion reached 30%. Attempts to hasten this recovery by the addition of choline, L-cysteine or D-penicillamine (10^{-3}–10^{-5} M) were unsuccessful (Fig. 1B). The enhancement of contractile height produced by 2-mercaptoethanol is also seen in muscles not exposed to methylmercury. The prompt lowering of the base line (Fig. 1C) in this instance was observed only when methylmercury caused an elevation. This elevation was observed mainly at the higher concentrations of exposure and may be similar to the action of $HgCl_2$ where the elevation was always observed. $HgCl_2$ 4×10^{-5} M, could produce a change in the contraction pattern of the muscle preparations but the effect was distinctly different from that produced by methylmercury (Fig. 1D). In such an instance, there was an initial enhancement of the contractions followed by an almost immediate rise in the base line and a subsequent, gradual decline in the amplitude. $CdCl_2$ also inhibited muscular contractions (Fig. 1E), but the necessary exposure (2×10^{-4} M) was considerably greater than that needed for methylmercury. $ZnCl_2$ up to 10^{-3} M was without any noticeable effect on the contraction amplitude. Ethylmercury, but not phenylmercury, was as effective as methylmercury.

When the muscle preparations were exposed to equimolar concentrations of (+)-tubocurarine before methylmercury exposure, a greater degree of recovery was observed when both drugs were washed out. This recovery amounted to 60–90% of the pre-drug control (Fig. 3).

Six frog rectus muscle preparations were tested for reduced sensitivity to acetylcholine and carbachol

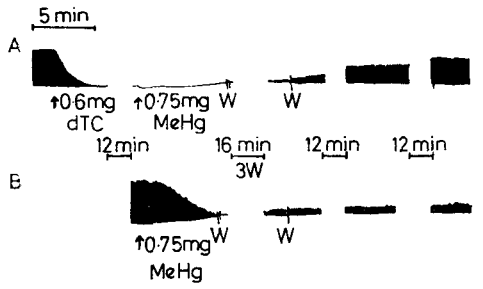

FIG. 3. Recordings of inhibition and recovery in paired simultaneous preparations. A. Protection against methylmercury inhibition by prior treatment with $1{\cdot}7 \times 10^{-5}$ M (+)-tubocurarine. This preparation was exposed to methylmercury for the same time period needed to produce complete muscular blockade in B.

after methylmercury exposure. Four similar preparations were tested for reduced sensitivity after treatment with dithiothreitol. In a direct comparison of these preparations, methylmercury caused comparable or greater interference with the muscle response. Fig. 4 illustrates this interference where the muscle was treated with methylmercury chloride (2×10^{-4} M) for 15 min. The shift to the right in the dose response curve was slightly greater than 1 log unit and the maximum response of the tissue was greatly reduced. When these preparations were tested by direct electrical stimulation, the degree of contraction elicited by increasing voltages was not significantly changed by the methylmercury exposure. No attempt was made to check the irreversibility of the observed interference with L-cysteine or D-penicillamine.

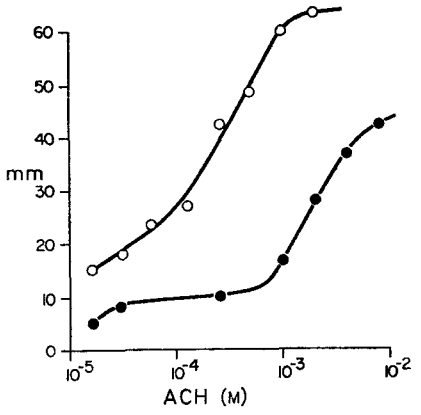

FIG. 4. Dose response curve of frog rectus muscle to acetylcholine before (open circles) and after (closed circles) treatment with 2×10^{-4} M methylmercury chloride. y axis—contraction amplitude (mm).

DISCUSSION

The change in muscular contraction of the rat phrenic-nerve diaphragm preparations produced by methylmercury in these experiments differs from that produced by the inorganic heavy metals tested. The mercuric ion produced a prompt response that involved a sustained contraction and caused elevation of the base line. $CdCl_2$ produced a blockade as did methylmercury, but the necessary concentration of cadmium ions was an order of magnitude greater than that needed for methylmercury. In instances of inorganic metal blockade of end plate potentials, the action can easily be reversed by washing and completely reversed by the use of thiols such as cysteine and glutathione (del Castillo-Nicholau & Hufschmidt, 1951). This was not observed with methylmercury for L-cysteine and D-penicillamine were ineffectual. The apparent limited reversal observed with 2-mercaptoethanol can be attributed to (a) the ability of this thiol to cleave cholinesterase (Leuzinger & Goldberg, 1969) and (b) a reversal of a direct muscle action of methylmercury similar to that seen with $HgCl_2$. Our observations on unpoisoned preparations treated with 2-mercaptoethanol support the former explanation. In addition, del Castillo, Escobar & Gijon (1971) report that 2-mercapto-ethanol by itself reduces the sensitivity of skeletal muscle receptors. Thus, in the present instance, the limited restoration of neuromuscular function by this compound is of little significance.

The results confirm the possibility suggested earlier (Rustam & others, 1975) that methylmercury has the potential of inhibiting transmission through neuromuscular junctions. Of the specific substances known to be present at such junctions, namely, cholinesterase, acetylcholine and cholineacetylase (MacIntosh, 1959), interference with any one of these could be manifested by a neuromuscular failure. However, the inability to demonstrate an *in vitro* inhibition of cholinesterase by methylmercury (Rustam & others, 1975) indicates that this enzyme is not directly associated with the observed inhibition.

The sequence of events from acetylcholine synthesis to depolarization of the post-synaptic membrane makes the distinction between the two remaining substances more difficult (Katz, 1962). Martin (1971) has reported an essential involvement of the sulphhydryl groups in choline transport and Rustam & others (1975) has demonstrated that methylmercury can inhibit choline-acetylase. In the present experiments, the lack of a significant frequency-dependent blockade suggests that the

immediate mode of action of methylmercury does not mimic the presynaptic action of hemicholinium or its analogues (Bowman, Hemsworth & Rand, 1967; Elmquist & Quastel, 1965). The time course of the blockade eliminates the possibility of acetylcholine depletion. Consequently, the neuromuscular failure appears to be confined within the immediate limits of the synaptic space.

Further support for this concept comes from the observation that curare had an ability to protect the muscle preparations against the methylmercury-induced inhibition. This action can best be explained by a simple mass action competition of the two substances for a common site, namely the receptor. However, since curare can bind to the presynaptic membrane, as well as the post-synaptic membrane, and there have been suggestions that receptors are located on the presynaptic surface (Webb, 1971) a further delineation of the mechanism of action of methylmercury is not possible from these experiments.

Karlin (1969) has postulated that the cholinergic receptor of the electroplax contains a disulphide bond that can be reduced by the action of dithiothreitol. The effect of this reduction is a decreased sensitivity to acetylcholine and carbachol. Mittag & Tormay (1970) demonstrated a similar action on frog rectus muscle preparations using 2 mM dithiothreitol for 20 min. This treatment resulted in a shift to the right of 1–1·5 log units in the dose response curve to acetylcholine. The present results indicate that methylmercury at 0·2 mM for 15 min produces an equivalent antagonism to the action of acetylcholine on the receptor of the frog muscle. A similar finding has recently been reported by Shamoo, MacLennan & Eldefraw, (1976) where methylmercury interferred with the binding of acetylcholine to purified electroplax receptors. Consequently, it may be speculated that methylmercury can disrupt the disulphide bridge within the cholinergic receptor and form a methylmercury-sulphur combination which blocks acetylcholine. Due to the known strong affinity of methylmercury for the sulphhydryl grouping and the finding that the maximum amplitude of the pharmacologically evoked response of the frog muscle was considerably reduced, the mode of inhibition by methylmercury can be considered to be irreversible and non competitive (Kirschner & Stone, 1951). The inhibition constant for methylmercury reported by Shamoo & others (1976) was approximately 10^{-6} M. The Ki that can be calculated from the present results is approximately 10^{-5}M. This lower inhibition constant may be attributed to non specific sulphhydryl binding to surface proteins of the muscle thereby reducing the effective methylmercury concentration.

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