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The Effects of Methyl Mercury on Platelets

Induction of Aggregation and Release via Activation of the Prostaglandin Synthesis Pathway

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SUMMARY

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The effects of methyl mercury on human blood platelets was studied. It induced platelet shape change, platelet aggregation, the platelet release reaction, and the synthesis of malondialdehyde. All of these effects were inhibited by indomethacin, a cyclooxygenase inhibitor. The release reaction and malondialdehyde generation could be induced in the absence of aggregation, and prostaglandin E_1 inhibited release but not malondialdehyde production. It is concluded that the activation of arachidonate cycloperoxidation is a plausible and adequate explanation for these effects of methyl mercury, and speculated that the toxic effects of methyl mercury are due to an analogous disturbance of lipid metabolism in other tissue. Methyl mercury also inhibited the prostaglandin E_1-stimulated adenylate cyclase of platelets; influenced the rate of uptake of adenosine, adenine, and serotonin, and had minor effects on adenine nucleotide metabolism.

INTRODUCTION

Methyl mercury is a cumulative poison causing characteristic and largely irreversible neurological deficits which have caused a high mortality rate. Methyl mercury arises by the action of microorganisms in water polluted by inorganic mercury in industrial waste, and it is concentrated up the food chain to man. Methyl mercuric chloride is freely soluble in lipid and has a very high affinity for thiol groups. The properties and biological effects of mercurials were addressed in a published symposium (1).

Our interest in methyl mercury was aroused by its ability to induce platelet aggregation (2). We report here an investigation of the mechanism of this phenomenon from which we deduce that methyl mercury causes the hydrolysis of arachidonic acid from phospholipids which is then peroxidized to the aggregation-inducing endoperoxides and thromboxanes.

MATERIALS AND METHODS

Platelet-rich plasma. Platelet-rich plasma was prepared by the differential centrifugation of blood anticoagulated with 0.1 volume of 0.13 M Na_3 citrate. Normal donors were studied who had not taken aspirin during the previous week. The platelet-rich plasma was stored at room temperature for not more than 3 hr and was warmed to 37° before use. Platelets were counted by phase-contrast microscopy.

Platelet aggregation. Platelet aggregation and shape change were studied photometrically as previously described (3). The platelet release reaction was measured as follows. Platelet-rich plasma was incubated with 0.5 μM [^{14}C]serotonin for at least 30 min at 37°. EDTA was added to aliquots to give a final concentration of 10 mM, and the other reagents were added as indicated and briefly mixed. At the appropriate time an equal volume of 1% formalin in 154 mM NaCl was added (4) and the suspension was centrifuged. A portion (300 μl) of the supernatant was counted in 5 ml of a cocktail made by mixing 2 liters of toluene, 1 liter of Triton X-100, 100 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene, and 4 g of 2,5-diphenyloxazole. The results are expressed as the percentage of intracellular radioactivity released after subtraction of the supernatant radioactivity in control samples (5–20% of total). EDTA was used and stirring was avoided to prevent release induced by aggregation itself, which complicates interpretation of the results (5).

Generation of malondialdehyde. The generation of malondialdehyde was measured photometrically (6). Platelet-rich plasma was treated with EDTA and reagents as in the release experiments, but instead of

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terminating the reaction with formalin, 0.4 volume of 40% tricholoracetic acid in 1 N HCl was added and the precipitated proteins were centrifuged. A portion of the supernatant (1.0 ml) was incubated with 0.2 ml of 0.1 M sodium 2-thiobarbiturate at 70° for 30 min and absorbance at 507, 532, and 557 nm was determined in a Beckman ACTA III spectrophotometer. The mean of the values at 507 and 557 nm was subtracted from that at 532 nm to give the absorbance of the chromophore. A standard curve was prepared by adding to platelet-rich plasma appropriate amounts of malondialdehyde, prepared by hydrolyzing tetraethoxypropane.

Accumulation of cyclic AMP. The accumulation of cyclic AMP was measured in platelet-rich plasma which had been incubated for 90 min with 1 μM [^{14}C]adenine (7). After incubation of 0.5-ml aliquots with the reagents for the appropriate time, 0.8 ml of 0.6 M perchloric acid containing about 1500 cpm of ^{3}H-labeled cyclic AMP and 1 mM unlabeled cyclic AMP was added, and the mixture was centrifuged. The supernatant was applied to a Dowex 50-H^+ column (1-ml bed volume) followed by 2 ml of water. The cyclic AMP was eluted with an additional 2 ml of water, and this eluate was treated with 0.2 ml of 10% $ZnSO_4$ followed by 0.2 ml of equimolar $Ba(OH)_2$. After centrifugation, the precipitation was repeated and the final supernatant was counted in 10 ml of a cocktail prepared by mixing 85 g of napthalene and 8 g of (2,4″-*tert*-butylphenyl)-5-(4″-biphenyl-1,3,4-oxidazole) with 1 liter of dioxane. Results are expressed as the percentage of intracellular ^{14}C present as cyclic AMP after correction for recovery through the procedure as estimated by the added ^{3}H-labeled cyclic AMP.

Serotonin uptake. Serotonin uptake was measured by adding 2 μM [^{14}C]serotonin to platelets (2 ml) pretreated as indicated. Immediately and 90 and 180 sec thereafter, 500 μl of the suspension were added to 100 μl of 5% formalin in 154 mM NaCl. After centrifugation, 400 μl of the supernatant were counted. The uptake rate was calculated by the difference in counts in the timed samples, and was linear with time to within 2%. About 25% of the label was incorporated by control platelets in 180 sec.

Adenine uptake. Adenine uptake was measured by incubating platelets with 2 μM [^{14}C]adenine. After 3, 6, and 9 min, 200-μl samples were added to 500 μl of 1 M $HClO_4$ and centrifuged. A portion of the supernatant (500 μl) was placed on a Dowex 50-H^+ column (1-ml bed volume) and eluted with 2 ml of water. The eluted [^{14}C]ADP and [^{14}C]ATP were counted in 15 ml of dioxane-based scintillant. The uptake rate was calculated from the best line (by eye) joining the three points. During the 6-min observation, 11%–24% of the label was phosphorylated, and no point was more than 2% from the best line.

Adenosine uptake. Platelets were incubated with [^{14}C]adenosine diluted with unlabeled adenosine to give a final concentration of 2.5 μM and about 2×10^6 cpm/ml. After 5 and 10 min, 200-μl aliquots were added to 200 μl of 1.2 M $HClO_4$ containing chromatography standards. After centrifugation, the supernatant was neutralized with K_2CO_3, and 25 μl were electrophoresed on Whatman 3 MM paper using 50 mM sodium citrate, pH 4.05, for 75 min at 40 v/cm on a Shandon L 24 apparatus. The sum of the counts found in the ATP, ADP, IMP, and AMP spots was expressed as a fraction of the total counts in all spots (approximately 15% in control incubations after 10 min). Linearity of observed uptake did not deviate by more than 1%.

Adenine nucleotide metabolism. The influence of methyl mercury on adenine nucleotide metabolism was assessed in platelets prelabeled with [^{14}C]adenine (as described for the assay of cyclic AMP) after electrophoretic separation as described for adenosine uptake. The adenylate energy change was calculated as $AEC = (ATP + 1/2ADP)/(ATP + ADP + AMP)$.

Materials. All reagents to be added to platelets were diluted in 140 mM NaCl and 15 mM tris(hydroxymethyl)aminomethane HCl, pH 7.4, except where indicated. Methyl mercuric chloride was obtained both as a gift from Dr. J. J. Kocsis and from K & K Laboratories, Plainview, N. Y. Ethyl mercuric chloride and tetraethoxypropane were also obtained from K & K Laboratories. The latter was hydrolyzed to malondialdehyde in 0.1 N HCl for 1 hr for use as a standard. Triethyl tin bromide was obtained from CPL Inc., and 2-thiobarbituric acid from Eastman Kodak, Rochester, N. Y. The following were obtained from Sigma Chemical Company, St. Louis, Mo., thimerosal, *p*-mercuribenzene sulfonate (as chloride), L-cysteine HCl (freshly prepared), inosine, hypoxanthine, adenine, adenosine, IMP, AMP, cyclic AMP, ADP, ATP, and *N*-ethyl maleimide; from Aldrich Chemical Company, Milwaukee, Wisc., *N*-methyl maleimide; from Nutritional Biochemicals Corporation, Cleveland, Ohio, *N*-isopropyl maleimide, *N*-butyl maleimide, *N*-cyclohexyl maleimide, *N*-benzyl maleimide, and *N*-phenyl maleimide. The maleimides were dissolved in ethanol. Indomethacin was obtained from Sigma Chemical Company and was prepared and used in ethanol. PG[1] E_1 was a gift from Dr. J. E. Pike, The Upjohn Company, Kalamazoo, Mich.

The isotopes used were [*U*-^{14}C]adenine, specific activity 281 Ci/mole, Amersham. [*U*-^{14}C]adenosine, specific activity, 585 Ci/mole, Amersham; [2-*sidechain*-^{14}C]serotonin, specific activity 59 Ci/mole, Amersham; and [8-^{3}H] cyclic AMP, Schwarz-Mann.

RESULTS

The addition of methyl mercury to stirred, citrated platelet-rich plasma induced aggregation after a delay of 15–60 seconds. During this delay an increase in optical density occurred together with loss of fine oscillations, indicating that platelets lost their disc shape. The aggregation always occurred in a single wave, and at low concentrations aggregation reversed. The degree of aggregation was dependent on the amount of added methyl mercury in the range 10–200 μM, but there was considerable variation between different donors. A typical result is shown in Fig. 1. As reported (2), higher concentrations of methyl mercury (i.e., greater than 500 μM) resulted in reduced aggregation, and platelets so treated did not subsequently aggregate to ADP (data not shown). Pretreatment of the platelets with the cyclooxygenase

[1] The abbreviation used is: PG, prostaglandin.

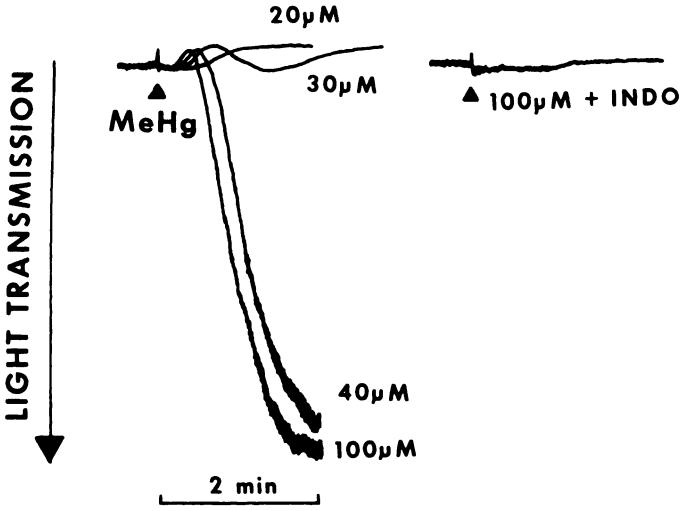

FIG. 1. *Superimposed aggregometer traces showing the induction of platelet aggregation by methyl mercury added at ▲ to give the concentration shown*

The plasma contained ethanol, 0.5 μl/ml. The tracing on the *right* shows the inhibition of aggregation by 5 μM indomethacin (INDO) in ethanol, 0.5 μl/ml, added 30 min before methyl mercury. In this and all subsequent figures final concentrations are given.

inhibitor indomethacin (5 μM) completely inhibited the induction of aggregation even by a high concentration of methyl mercury, suggesting that the cycloperoxidation of arachidonate to endoperoxides and thromboxanes (8) is essential for methyl mercury to induce aggregation.

Figure 2 shows the effect of adding methyl mercury to platelet-rich plasma after 10 mM EDTA to prevent aggregation. Methyl mercury induced a typical shape change after a brief delay, and indomethacin inhibited this effect almost completely. Plasma contains about 400 μM free thiol, mainly as mercaptoalbumin (9), which reacts with a variety of thiol agents, including mercurials. Thus it is likely these thiols compete with platelets for methyl mercury. To assess the influence of this competition, the free thiol concentration was increased by adding cysteine (Fig. 3A) and reduced by adding the non-

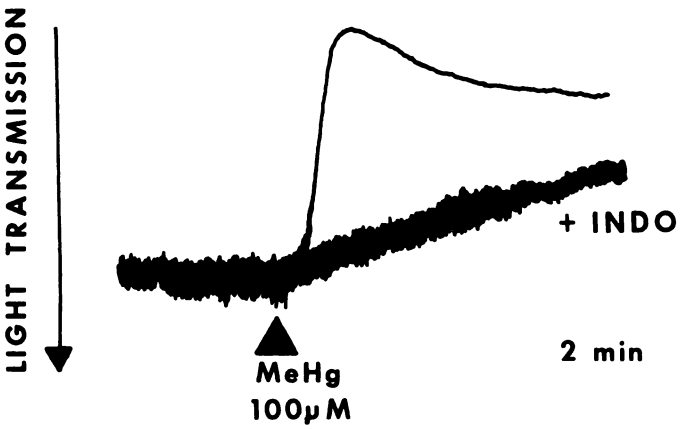

FIG. 2. *Superimposed aggregometer traces showing the induction of platelet shape change by methyl mercury*

EDTA (6.25 mM) had been added 2 min before methyl mercury. The *upper tracing* is the ethanol control and the *lower tracing* is that obtained with plasma preincubated with indomethacin (INDO) as in Fig. 1. The instrument gain was 10-fold higher than in Fig. 1.

cell-penetrating mercurial *p*-chloromercuribenzene sulfonate (Fig. 3B). A 12.5-fold excess of cysteine over methyl mercury had little effect, and even a 25-fold excess did not abolish aggregation completely. Thus the platelet competes very successfully with both plasma thiols and cysteine for methyl mercury. Conversely, pretreatment of platelet-rich plasma with *p*-chloromercuribenzene sulfonate at 0.4 mM potentiated the effect of methyl mercury. As with other aggregating agents (10), higher concentrations partially inhibited aggregation by methyl mercury.

The release reaction of platelets can be triggered by their aggregation (11), especially when the free Ca^{2+} ion concentration is reduced (12). This release is accompa-

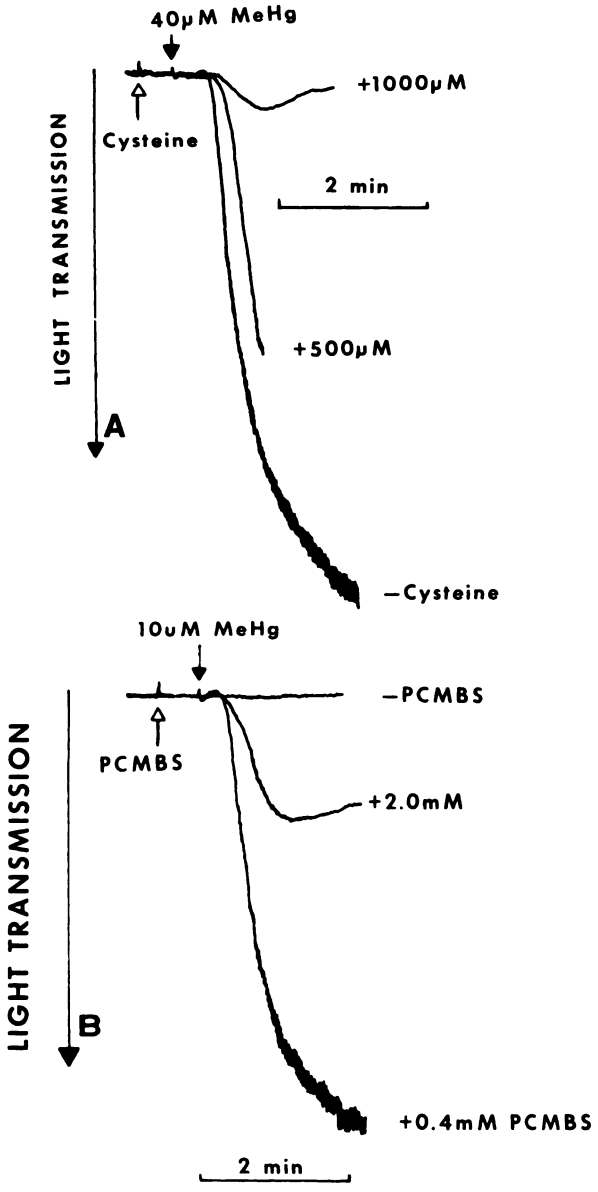

FIG. 3. *The effect of cysteine on methyl mercury-induced aggregation*

A. Cysteine was added to give the concentrations shown at the end of the superimposed traces, followed by 40 μM methyl mercury at the times indicated by the *arrow*.

B. p-mercuribenzene sulfonate (*PCMBS*) was added before methyl mercury (10 μM) to give the concentrations shown.

nied by the cycloperoxidation of arachidonic acid to generate endoperoxides and thromboxane A_2 which themselves induce aggregation and release (13), and also the cleavage product malondialdehyde. This cylcoperoxidation is blocked by indomethacin. We measured malondialdehyde production by platelets exposed to methyl mercury under conditions in which aggregation was blocked by the absence of stirring and EDTA. A burst of malondialdehyde synthesis occurred (Fig. 4). When release and malondialdehyde production were measured in the same sample, the use of PGE_1 as a release inhibitor and indomethacin as a cyclooxygenase inhibitor demonstrated that malondialdehyde was not produced as a consequence of release, since PGE_1 inhibited the latter but not the former, and that release was dependent on the activity of the cyclooxygenase, since indomethacin inhibited both (Fig. 5).

It has been known for some time that *N*-ethyl maleimide induces malondialdehyde formation by platelets (14) and that this effect is blocked by aspirin for the life-span of the platelet (15). *N*-Ethyl maleimide induces dramatic changes in platelet morphology, but these do not result in platelet aggregation (16, 17). We assessed the ability of several *N*-substituted maleimides to induce malondialdehyde production (Fig. 6). A clear order of potency was found such that the smaller the substituent the more efficacious the agent. It is tempting to conclude that this is caused by steric hindrance because of the bulk of the substituent, but other factors such as decrease in hydrophilicity or a competing inhibitory action were not excluded.

We also investigated the effects of several other agents that have been reported to induce platelet aggregation. Triethyl tin (18) is analogous to methyl mercury in that it is an alkyl metal cation (Et_3Sn^+). It induced aggregation after a prolonged delay, and this aggregation was either inhibited or very greatly prolonged by 5 μM indomethacin. Ethyl mercury was similar to methyl mercury as an aggregating agent but was 2-fold less active. Thimerosal (19) (a complex of ethyl mercury and thiosalicylic acid) induced aggregation after a longer delay than ethyl mercury, and the aggregation was inhibited by indomethacin. Mercuric chloride ($HgCl_2$) did not induce platelet aggregation, but at high concentration (>300 μM) it inhibited aggregation induced by ADP.

Methyl mercury had several other effects on platelets, as might be expected since it reacts with thiol groups. Some of these effects result from the activation of platelet via the cyclooxygenase and some are by a direct action. The accumulation of cyclic AMP by platelets exposed to PGE_1 was inhibited by methyl mercury, apparently by a direct action. This cannot explain the aggregating action of methyl mercury since the effect is not blocked by indomethacin (Fig. 7). The inhibition by methyl mercury took about 1 min to develop, contrasting sharply with the inhibition of adenylate cyclase by ADP or adrenaline, which occurs within 1 sec of exposure. The ADP receptor that mediates this action can be inactivated by exposure to *p*-mercuribenzene sulfonate or *N*-ethyl maleimide [>0.4 mM, (10)], but at concentrations up to 150 μM we did not observe any such blockade.

The uptake of radioactive 5-hydroxytryptamine was

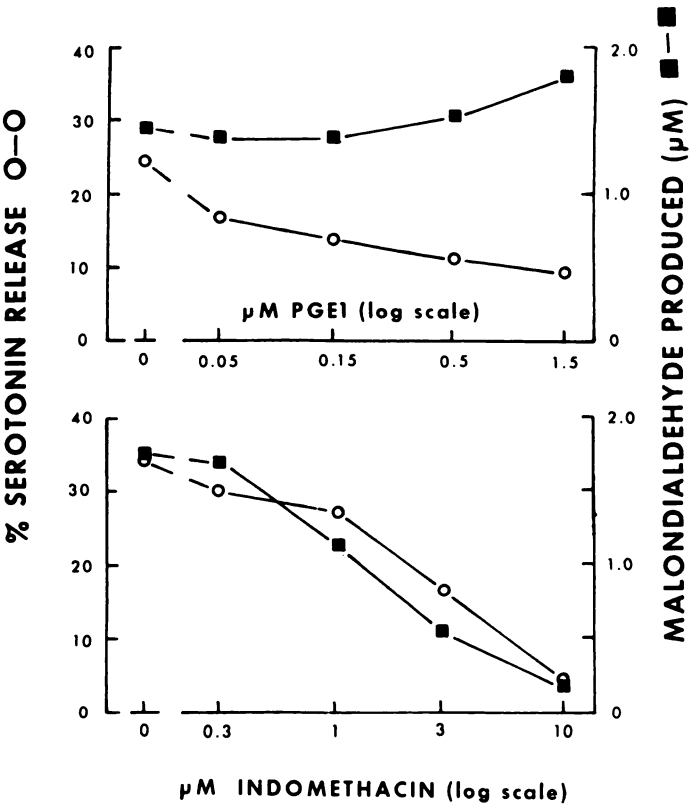

FIG. 5. *Effect of PGE_1 and indomethacin on serotonin release and malondialdehyde generation*

Platelets were labeled with [^{14}C]serotonin and exposed to PGE_1 or indomethacin for 3 min as indicated. EDTA, 10 mM, 0.4 mM *p*-mercuribenzene sulfonate, and 40 μM methyl mercury were then added. Three minutes later, release of radioactivity and generation of malondialdehyde were measured. Each point is the mean of duplicate determinations.

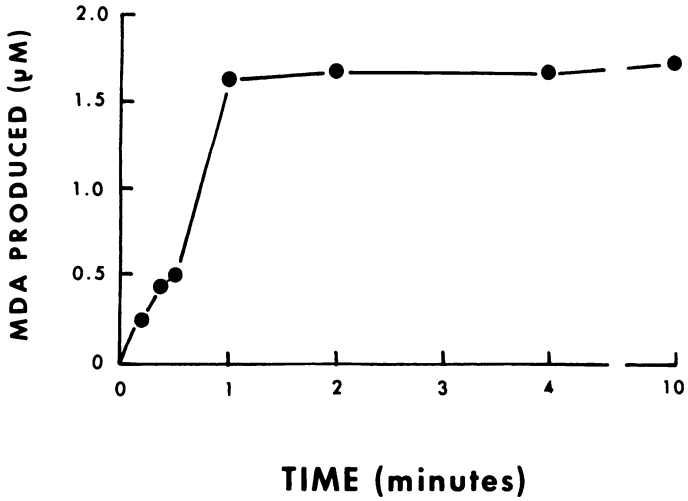

FIG. 4. *Generation of malondialdehyde (MDA) by platelets exposed to methyl mercury*

To platelet-rich plasma was added EDTA (10 mM), *p*-mercuribenzene sulfonate (0.4 mM), and at time zero 50 μM methyl mercury, and the suspension was briefly mixed. At the times indicated, samples were taken and assayed for malondialdehyde, one of two such experiments giving closely similar results. In another two experiments, using 100 μM methyl mercury without *p*-mercuribenzene sulfonate, platelets generated 0.6 μM and 0.4 μM, respectively, malondialdehyde within 4 min.

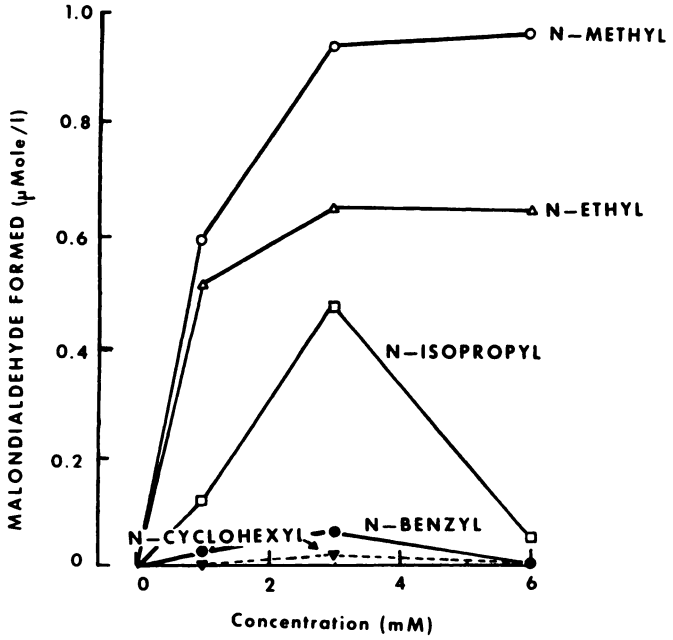

FIG. 6. *Generation of malondialdehyde by platelets exposed to N-substituted maleimides at the concentration shown for 4 min*

markedly inhibited by methyl mercury and this inhibition was blocked by indomethacin (Fig. 8). This apparent inhibition could be due to release of endogenous 5-hydroxytryptamine, resulting in dilution of the specific activity of the label. Adenosine uptake was progressively

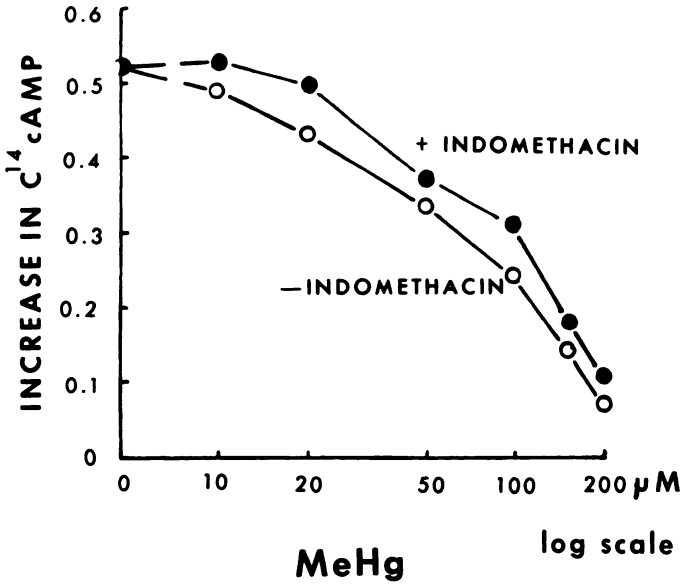

FIG. 7. *Effect of methyl mercury on adenylate cyclase*

Platelets were labeled with [^{14}C]adenine, and with either ethanol or ethanol and indomethacin (10 μM) for 30 min. PGE_1 (2 μM) plus methyl mercury at the concentration shown was then added and briefly mixed. Four minutes later the suspension was extracted and ^{14}C-labeled cyclic AMP was estimated. Each point is the mean of duplicates. ○, Ethanol controls; ●, indomethacin-treated. In this experiment, the rate of aggregation induced by methyl mercury (200 μM) was 93% inhibited by PGE_1, 2 μM, implying that the inhibition of adenylate cyclase was unlikely to be due to release of stored ADP. The inhibitory effect of methyl mercury was confirmed in other similar experiments.

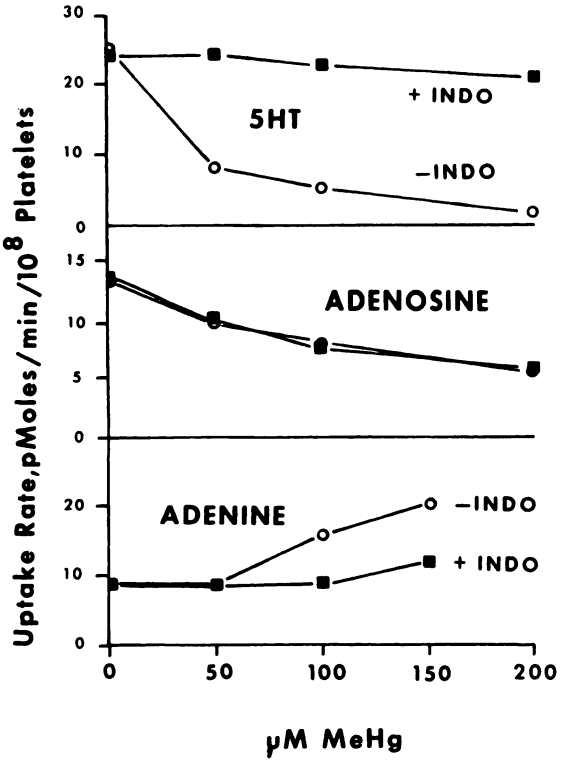

FIG. 8. *Effect of methyl mercury with or without indomethacin (INDO) on the uptake of serotonin (5HT), adenosine, or adenine*

○, Results obtained with ethanol control; ■, with 10 μM indomethacin. EDTA, 10 mM was added before the radioactive label, and uptake was assessed at several times therefter as described under Materials and Methods.

inhibited and this inhibition was unaffected by indomethacin, suggesting a direct action (Fig. 8B). Adenine uptake was accelerated in the absence of indomethacin.

After a 1-min exposure to methyl mercury (50–150 μM), a small decrease in the adenylate energy charge (from approximately 0.92 to 0.89) occurred, which substantially reversed after 10 min. This rapid drop, which is seen with other aggregating agents (3), was abolished by indomethacin. On the other hand, methyl mercury (25–200 μM) accelerated in a concentration-dependent manner the production of [^{14}C]hypoxanthine and/or inosine by platelets to a rate of up to 1% of [^{14}C]adenine nucleotide/min, and this effect was not blocked by indomethacin. Control platelets generated [^{14}C]hypoxanthine and inosine at a rate of about 0.1%/min.

In four experiments, platelets were washed by suspension (to a count of 3×10^8/ml) into calcium-free Tyrode's solution, pH 7.4, of pellets prepared by centrifuging platelet-rich plasma to which either EDTA (10 mM) or ethylene glycol bis(β-aminoethyl ether)-*N-N-N'-N'*-tetraacetic acid (5 mM) had been added. Methyl mercury (25–150 μM) induced malondialdehyde production in three of these suspensions (1–3 nmoles/10^9 platelets). The fourth donor's platelets generated no measurable malondialdehyde, and her platelets aggregated poorly in their own plasma to even 200 μM methyl mercury. Malondialdehyde production was virtually abolished by indomethacin (10 μM). In another experiment, release of radioactive serotonin was measured in platelets suspended in calcium-

free Tyrode's solution containing 10 mM citrate (pH 7.4). A concentration of 50 μM methyl mercury induced 29% release, which was reduced to 16% and 6% by 5 mM and 10 mM added $CaCl_2$, respectively. In contrast, release induced by thrombin (0.1 unit/ml, Parke Davis, topical) was increased from 1% to 80% by 5 mM added $CaCl_2$.

DISCUSSION

Several groups of workers have investigated the effects of methyl mercury on defined biological systems. It was found to be a powerful inhibitor of the adenylate cyclase of liver plasma membrane adenylate cyclase, and is more potent in this regard than a number of other thiol agents (20). It also inhibits phosphodiesterases of a variety of tissues, but this effect is common to many thiol agents (21).

Disturbances of synaptosomal respiration, including loss of respiratory control, have been demonstrated after both administration of methyl mercury to rats and *in vitro* incubation of synaptosomes with the toxin (22). Methyl mercury depresses frog muscle contraction and increases spontaneous transmitter release from presynaptic nerve terminals after administration to guinea pigs, leading to the generation of sporadic action potentials. The effect was not due to inhibition of acetyl cholinesterase (23). It is difficult to assess the significance of these findings, since one would expect organic mercurials to influence any biological system dependent on the integrity of thiol groups.

We have shown here a variety of effects of methyl mercury on platelets, some of which were prevented by the cyclooxygenase inhibitor, indomethacin, and some were not. The inhibition of the PGE_1-stimulated platelet adenylate cyclase by methyl mercury occurred at a higher added concentration than similar inhibition of the liver enzyme (20), but it is not possible to establish the free concentration of methyl mercury in either system, since the majority in each case will be bound to other protein and nonprotein thiols. This inhibition was not blocked by indomethacin, suggesting a direct action. Adenosine uptake was also inhibited by a non-cyclooxygenase-dependent mechanism.

The ability of methyl mercury to induce the shape change, aggregation and release, and the associated decrease in adenylate energy charge was blocked by indomethacin, and we can present a persuasive case that these effects of methyl mercury are due to the activation of arachidonate cycloperoxidation. It is well established that free arachidonic acid is converted by platelets to the endoperoxides PGG_2 and PGH_2, and to thromboxane A_2. These unstable products are capable of inducing platelet shape change, aggregation, and the release reaction (13). Thus the activation of this pathway gives an adequate and plausible explanation for the ability of methyl mercury to induce aggregation and release.

Present evidence suggests that the activity of cyclooxygenase is regulated by the availability of its substrates. Platelet phospholipids are richly endowed with arachidonic acid totaling about 0.2 μmole/10^9 platelets esterified in position 2 [calculated from data of Cohen and Derksen (24), assuming that 10^9 platelets have 0.5 μmole of lipid phosphorus (25)]. This appears to be the source of the substrate, since very little free archidonate is found in platelets (26), and methyl mercury induced malondialdehyde production in washed platelets.

In our experiments we observed up to 6 nmoles of malondialdehyde generated per 10^9 platelets. Under normal circumstances, about 4 moles of arachidonate are required to generate 1 mole of malondialdehyde (13). If this is true in the presence of methyl mercury, about 24 nmoles of arachidonate must be liberated per 10^9 platelets, i.e., more than 10% of the total platelet arachidonate. There are at least three mechanisms by which methyl mercury could bring this about. First, it could stimulate the action of one or more of the phospholipases A_2 which have been demonstrated in platelet membranes (27) or in intact cells (26, 28). Second, it could catalyze (29) the hydrolysis of arachidonate-containing plasmalogen, thus releasing arachidonyl-lyso-phospholipid which could be further hydrolyzed to arachidonate. Third, methyl mercury could inhibit the reincorporation of spontaneously liberated arachidonate by blocking the resynthesis of arachidonyl-coenzyme A or the action of the acyl-coenzyme A transferase. Further investigation is required to determine whether any of these mechanisms can account for the effect of methyl mercury.

We have shown a rapid release of (presumably) arachidonic acid from platelets. If a similar mobilization of fatty acids occurs in other tissues, toxic effects may result, and one can predict that the tissues at most risk are those with a high concentration of lipid, a low turnover of cells and membrane-bound enzymes, and tissues which depend on the maintenance of the physical properties of their membranes for their function. Clearly the nervous system satisfies these predictions. Mobilization of fatty acids will result in the generation of lysophospholipids which "stabilize" membranes at low concentrations and lyse them at high concentrations, and the oxidation of polyunsaturated fats to generate free radicals, malondialdehyde, thromboxanes, prostaglandins, and hydroxy fatty acids, any or all of which may be toxic and account for functional and morphological changes.

This investigation clearly demonstrates the need for the study of the effect of methyl mercury on the lipid metabolism of other tissues as well as the use of a more direct approach to the elucidation of its effects on the phospholipids of platelets, and it also suggests the possibility that inhibitors of brain cyclooxygenase such as acetaminophen (30) might modify the course of methyl mercury poisoning.

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