Methylation of Mercury Compounds by Methylcobalamin*

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ABSTRACT: The methylation of mercuric chloride and certain organic mercury compounds by methylcobalamin in water solutions was studied by spectrophotometric and gas chromatographic methods. Methylcobalamin was found to react rapidly with mercuric chloride and at a slower rate with organic mercury compounds in water solutions. The ultimate products of the reaction with mercuric chloride were hydroxycobalamin and methylmercury cation as judged by spectrophotometric and gas chromatographic evidence. Under certain conditions of experiment this reaction is rather rapid in unbuffered aqueous solution going to 50% completion in 4 min.

Tris and phosphate buffers, thiol compounds, and cell proteins inhibit the reaction, presumably by binding Hg^{2+}.

Several cases of severe mercury poisoning have been observed during recent years, affecting either humans (Japan) or wild birds and fishes (Sweden); for review, see Expert Group Report (1971). In fishes, which were more thoroughly investigated than other materials, the poisonous metal was found to occur mainly as methylmercury (Westöö, 1966). This organic mercury compound was until 1966 extensively used in Sweden as a fungicide. Although its use was forbidden after 1966, the occurrence of this fungicide in fishes continued also after that year amounting to almost 100% of total mercury.

Jensen and Jernelöv (1969) were able to demonstrate a microbial methylation of mercuric chloride to both mono- and dimethylmercury using mixed cultures from aquaria sediments and from decaying fish. Kivimäe *et al.* (1969) found methylmercury in organs and eggs of hens fed wheat which contained mercuric nitrate, methoxyethyl-, or phenylmercury hydroxides.

Biochemical methylations have been studied thoroughly, especially methionine biosynthesis (*cf.* review by Shapiro and Schlenk, 1965). The methyl donors are tertiary sulfonium or quaternary ammonium compounds, which donate their methyl groups as CH_3^+ to nucleophils, *e.g.*, thiols.

Theoretically, one could envisage the following methylating agents for methylation of mercury

$$\begin{aligned} CH_3^+ + Hg^0 &\longrightarrow CH_3Hg^+ \\ CH_3\cdot + Hg^+ &\longrightarrow CH_3Hg^+ \\ CH_3{:}^- + Hg^{2+} &\longrightarrow CH_3Hg^+ \\ CH_3{:}^- + RHg^+ &\longrightarrow CH_3HgR \end{aligned}$$

where RHg^+ is an organic mercury cation. Accordingly, both Hg^{2+} and RHg^+ need a methylcarbanion donor to become methylated.[1] The only one with this property, hitherto found in Nature, is methylcobalamin, a vitamin B_{12} derivative (Ingraham, 1964).

Wood *et al.* (1968) demonstrated the formation of mono- and dimethylmercury from Hg^{2+} in cell-free extracts of a pure culture of a methanogenic bacterium using substrate concentrations of methylcobalamin in strictly anaerobic conditions. These authors suggest that the methyl transfer from methylcobalamin to mercury may be the result of both enzymatic and nonenzymatic processes.

The present paper describes the nonenzymatic methyl transfer from methylcobalamin to mercury compounds of toxicological interest. The reaction has been measured by a spectrophotometric method, based on the absorbance changes at 351 and 380 nm, which occur during the conversion of methylcobalamin into hydroxycobalamin. Methylmercury formed from Hg^{2+} has been analyzed by gas chromatography. Certain experiments were carried out in the presence of thiol compounds. Certain others in the presence of cell extracts from *Escherichia coli* 113-3.

Materials and Methods

Materials. Methylcobalamin was generously provided by Dr. L. Mervyn, Glaxo Laboratories Ltd., England, and by Dr. W. Friedrich, University of Hamburg, Germany. Methoxyethylmercury bromide, methylmercury dicyandiamide, and hydroxide as well as phenylmercury hydroxide were obtained from AB Casco, Stockholm, Sweden. Other materials were commercially available chemicals of reagent grade. *E. coli* 113-3 was obtained from American Type Culture Collection.

The concentration and purity of methylcobalamin solutions was estimated from the spectral data of Müller and Müller (1962) and by paper chromatography on Whatman No. 1 paper with 1-butanol–2-propanol–acetic acid–water (100:70:1:100, v/v) as solvent (Neujahr and Fries, 1966). The purity of the mercury compounds was checked by the methods developed by Östlund (1969). By those methods no impurities could be detected.

Spectrophotometric Methods. A special type of reaction vessel, consisting of a Thunberg tube with a standard 1-cm quartz cuvet sealed on to the lower end, was employed (LKB Instrument, Bromma, Sweden; Hellma cat. no. 100, QS). Methylcobalamin was pipetted into the side arm and the other reaction components into the cuvet. Total reaction volume was 2.0 ml. All operations were carried out in the dark, at 20°. Unless otherwise stated, the reactions were run in normal atmosphere. Each reaction was started by tipping the methylcobalamin solution into the cuvet while shaking by hand. The reaction course was followed by measuring the absorbance changes at 351 and 380 nm (A_{351} and A_{380}) with a Zeiss PMQ II spectrophotometer. When methylcobalamin reacts to form hydroxycobalamin, there is an increase in A_{351}

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[1] These theoretical considerations have also been put forward by L. B. in Expert Group Report (1971).

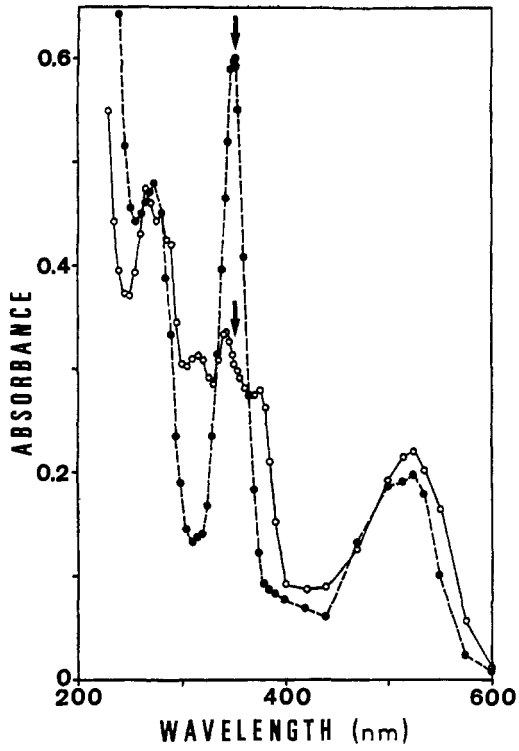

FIGURE 1: Absorption spectra of methylcobalamin (28 μM) in distilled H_2O before (○) and after (●) incubation with $HgCl_2$ (200 μM), 2 hr in the dark at room temperature. Arrows indicate 351 nm.

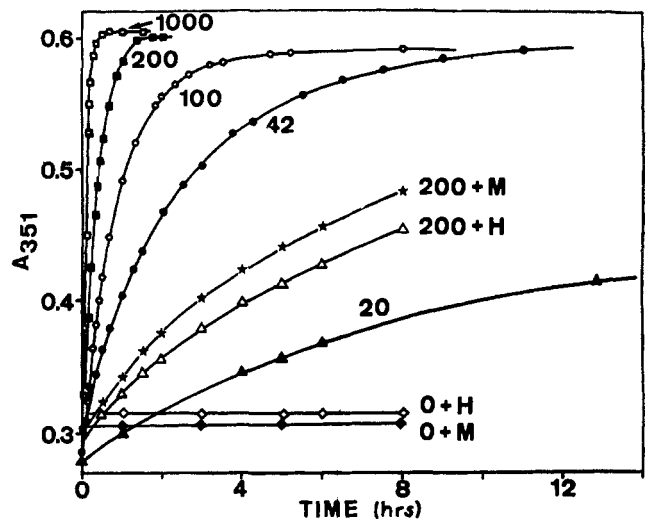

FIGURE 2: The time course of the reaction between methylcobalamin (28 μM) and varying initial concentrations of $HgCl_2$ (μM as indicated). Some of the experiments were carried out in the presence of 200 μM mercaptoethanol (M) or DL-homocysteine (H). The reaction was measured in standard 1-cm cuvets by the increase in absorbance at 351 nm (formation of hydroxycobalamin). All solutions in distilled water, total volume 2.0 ml; no arrangements for anaerobiosis. The reactions with 100 and 200 μM $HgCl_2$ were also carried out under nitrogen, which gave practically identical results.

and a concomitant decrease in A_{380} (Figure 1). Control cuvets without the mercury compound were included into each experiment to ascertain that the measured absorbance changes were due to the reaction between methylcobalamin and the mercury compound. After the absorbance at 351 and 380 nm had ceased to undergo changes, the ultraviolet and visible spectra of the reaction mixture (235–600 nm) were recorded.

Quantitative Determination of Methylmercury. Methylmercury formed in the reaction between methylcobalamin and mercuric chloride was estimated by a simplified method according to Westöö (1966). The aqueous solution (2.0 ml) containing methylmercury was mixed with 2.0 ml of 6 M HCl (preextracted with benzene) and the mixture was extracted with 5.0 ml of redistilled benzene. By this procedure 89% of the methylmercury chloride was extracted into the organic phase. The benzene extracts were stored at 20° until analyzed, as it was found that frozen solutions gave lower methylmercury values. The amount of methylmercury chloride in the benzene solutions was determined on an Aerograph HY-FI Model 600-D gas chromatograph with an electron capture detector. The analyses were carried out with a 5 ft × 1/8 in. glass column with 10% Carbowax 20M on Chromosorb W at 140–150°. The temperatures of the injector and detector were 30° higher and the carrier gas flow rate was 60 cc of nitrogen/min. The benzene solution (3–8 μl) was injected and the peak height was compared to those from standard solutions of methylmercury chloride. These were prepared from methylmercury dicyandiamide run through the extraction procedure.

Cell extracts were prepared by disruption of *E. coli* 113-3 grown in a medium containing methionine and cyanocobalamin, essentially as described by Hatch *et al.* (1961). The extracts were included at protein levels up to 1-mg/ml reaction mixture.

Results

Figure 1 shows the spectra of 28 μM methylcobalamin before and after it has been incubated with mercuric chloride during 2 hr at room temperature in the dark. The absorption curve of the reaction product is lacking two of the characteristic peaks of methylcobalamin, *viz.*, those at 343 and 375 nm. The product has, instead, a peak at 351 nm. Its spectrum closely correlates with that of a 28 μM solution of hydroxycobalamin (Dolphin and Johnson, 1965). It can thus be concluded that in a solution of methylcobalamin and mercuric chloride the methyl group is removed giving as the final cobalamin product hydroxycobalamin. Therefore, the increase in absorbance at 351 nm was in the following taken as a convenient measure of the reaction between methylcobalamin and various mercury compounds. The concomitant decrease in absorbance at 380 nm was used as a control of the reaction course.

Figure 2 summarizes results of experiments, in which the time course of the reaction between methylcobalamin and mercuric chloride was studied using different initial concentrations of mercuric chloride. Some experiments were carried out in the presence of thiol compounds. It is seen that, *e.g.*, with 1000 and 200 μM $HgCl_2$, *i.e.*, a 35-fold and a 7-fold excess of Hg^{2+}, the reaction goes to 50% completion within 4 and 15 min, respectively. With decreasing concentrations of Hg^{2+} the reaction rate gradually slows down.

All the experiments shown in Figure 2 were carried out without any special arrangements to create and/or maintain anaerobiosis. The experiments with 100 and 200 μM $HgCl_2$ were repeated under nitrogen after the dissolved oxygen was removed. They gave practically identical results.

A curious feature of Figure 2 is that the plateau values of absorbances obtained in the experiments with concentrations lower than 200 μM $HgCl_2$ are slightly but significantly lower than those in experiments with 200 and 1000 μM $HgCl_2$. The absorbance values obtained in the two latter experiments are closer to that of 28 μM hydroxycobalamin than those in other

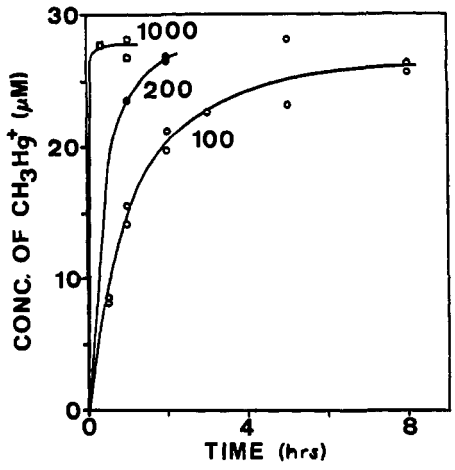

FIGURE 3: The time course of the reaction between methylcobalamin (28 μM) and different initial concentrations of $HgCl_2$ (μM as indicated). The reaction was measured by the formation of CH_3Hg^+. Three series of identical reaction mixtures, one for each level of $HgCl_2$, were prepared in 12-ml test tubes containing 2.0 ml of distilled water. The tubes were kept in normal atmosphere, in the dark at 20°. Samples were taken after varying lengths of time and analyzed by gas chromatography.

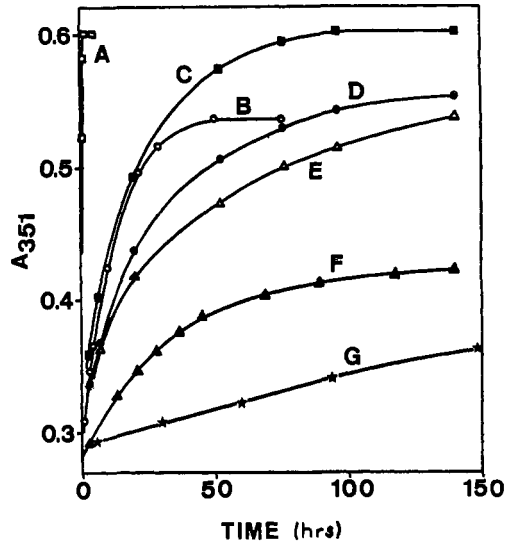

FIGURE 4: The reaction between methylcobalamin (28 μM) and various mercury compounds (200 μM). Conditions as in Figure 2: (A) $HgCl_2$, (B) CH_3HgCl,[2] (C) C_6H_5HgCl,[2] (D) $CH_3OCH_2CH_2HgBr$, (E) C_6H_5HgOH; (F) CH_3HgOH, and (G) CH_3Hg–dicyandiamide.

experiments in Figure 2. This indicates the occurrence of side reactions involving methylcobalamin or its conversion products. If such reactions occur, however, the products amount to only a few per cent of the main reaction products.

Both of the investigated thiols had a profound effect on the methyl transfer from methylcobalamin to Hg^{2+}, presumably by binding the latter and making it unreactive (Figure 2). At levels stoichiometrically equivalent to that of Hg^{2+} (200 μM) both mercaptoethanol and homocysteine decreased the rate of the reaction by more than 60%. Without added mercury, those thiols only very slightly affected the A_{351} of methylcobalamin.

The reaction rate was highest in unbuffered aqueous solutions or in physiological sodium chloride. Tris·Cl and potassium phosphate (0.05 M, pH 7.5), significantly decreased the rate of the reaction and the plateau values. The reaction was completely abolished by cell extracts from *E. coli* 113-3 present at a level of 1 mg of protein/ml.

We have shown in Figures 1 and 2 that one of the products in the reaction between methylcobalamin and mercuric ion is hydroxycobalamin. Figure 3 demonstrates that the other product is methylmercury cation as judged by gas chromatographic evidence. The progress curves with 1000, 200, and 100 μM $HgCl_2$ closely resemble those in Figure 2. The plateau values are very close to 28 μM CH_3Hg^+ thus corresponding to the initial concentration of methylcobalamin.

It can thus be concluded from Figures 2 and 3 that, in water solutions, there occurs a fairly rapid transfer of the methyl group from methylcobalamin to mercuric ion, the ultimate products of the reaction being methylmercury cation and hydroxycobalamin.

Several organic mercury compounds are in use or have been used as fungicides in agriculture or pulp industry. We considered it therefore of interest to investigate the reactivity of such compounds toward methylcobalamin. Results of experiments with organic mercury compounds are summarized in Figure 4. It is seen that all of the compounds investigated are by far less reactive than mercuric chloride. The reactivity of the organic mercury compounds is very much dependent on the accompanying anion. Thus, methylmercury chloride reacts faster than the hydroxide, which in turn has a higher reactivity than the dicyandiamide. The same correlation between reaction rate and plateau values of A_{351} is seen here as in Figure 2.

Discussion

According to Wood *et al.* (1968) mercuric chloride can be methylated by methylcobalamin using cell-free extracts of a methanogenic bacterium. As the reaction mixture employed by these authors was rather complex, containing large amounts of protein, ATP, phosphate buffer, etc., it was difficult to evaluate whether the methyl transfer was enzymatic or not. Further, the above authors could not exclude or heat the cell extract because the time course of their methylation reaction was measured by the decrease in enzymatic methane formation.

The easiness with which the methyl group is transferred from methylcobalamin to various mercury compounds in the entirely nonenzymatic system described in this paper is rather striking. It can be visualized that both, Hg^{2+} and compounds of the type R-Hg^+ are methylated by a nucleophilic attack from a methylcarbanion ($CH_3:^-$), *i.e.*, a Grignard-like reagent. The cobalt–carbon bond of alkylcobalamins has been postulated to function as such in some biological alkyl-transfer reactions (Ingraham, 1964). Evidence of a methylcarbanion transfer in our system is obtained from the results that Hg^{2+} is methylated considerably faster than R-Hg^+ (*cf.* Figure 4). This conforms to the established course of alkylation by Grignard reagents (Coates, 1960).

Guest *et al.* (1962) have reported a nonenzymatic methylation of homocysteine by methylcobalamin. The methyl transfer amounted to about 5% of that observed in the pres-

[2] Water solutions of methyl- and phenylmercury chlorides were prepared by mixing the corresponding hydroxides with equimolar amounts of HCl (200 μM). Controls with HCl (200 μM) instead of the mercury compound gave no change in the ultraviolet and visible spectra of methylcobalamin.

ence of a purified B_{12} containing transmethylase. The nonenzymatic methyl transfer to homocysteine observed in our experiments (Figure 2) is considerably slower than the transfer to equimolar concentrations of $HgCl_2$.

After our experiments have been completed, Hill *et al.* (1970) reported that Hg^{2+} reacts faster with methylcobalamin than with the corresponding ethyl and propyl derivatives, and that 5,6-dimethylbenzimidazole has a trans-labilizing effect as compared to H_2O.

Jensen and Jernelöv (1968) have put forward the hypothesis that dimethylmercury is the primary product of biological methylation of mercury, monomethylmercury being the decomposition product of it. Also Wood *et al.* (1968) supports this theory. If the methylation of mercury in Nature is a nonenzymatic process it is reasonable to visualize it as a two-step reaction. In such a case the postulate of dimethylmercury formation as the main methylation product is contradicted by the much slower reaction between methylcobalamin and monomethylmercury as compared to inorganic mercury (*cf.* Figure 4). As pointed out above, there is no conclusive evidence of enzymatic methylation of mercury, so far.

The concentration 28 μM methylcobalamin was selected with view to convenience in absorbance readings. This concentration is probably much higher than those usually occurring in, *e.g.*, bacteria. Although synthetic methylcobalamin serves as an excellent methyl donor in the formation of methane by cell-free extracts of certain methane bacteria, no cobalt methylcorrinoids could be detected in cells of these organisms (for review, *cf.* Stadtman, 1967). However, unspecified amounts of methylcobalamin have been isolated from an industrial extract of a streptomycete (Lindstrand, 1964) and minute amounts of certain other cobalt methylcorrinoids from a CO_2-reducing *Clostridium thermoaceticum* (Ljungdahl *et al.*, 1965). The biological significance of the nonenzymatic methyl transfer between methylcobalamin and mercury is very difficult to estimate, however. An obscuring circumstance is the effect of thiols (*cf.* Figure 2) and cell proteins. These substances inhibit the reaction, presumably by binding mercury and thereby making it unavailable to methylation. There are indications in the literature that mercury can be bound to proteins not only through sulfur groups, but also, in some other unspecified manner(s) (Hughes, 1949). Thus, when a molecule of a mercury compound penetrates into the cell of a microorganism it is likely that it will be taken care of by cell proteins before it becomes methylated by methylcobalamin. It is not excluded, however, that methylcobalamin, when associated with some proteins inside the cell, has quite different methylating potential with respect to mercury than that, which can be demonstrated *in vitro*.

Acknowledgments

We wish to express our sincerest thanks to Professor Torbjörn Westermark, Department of Nuclear Chemistry, this Institute, for having inspired this work and for stimulating discussions and valuable advices. The generous gifts of methylcobalamin from Dr. L. Mervyn, Glaxo Laboratories Ltd., England and from Professor Dr. Wilhelm Friedrich, University of Hamburg, Germany, are greatly acknowledged. We are indebted to the Isotoptekniska Laboratoriet for the permission to use their gas chromatographic equipment.

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