

Alkylation of ionic mercury to methylmercury and dimethylmercury by methylcobalamin: simultaneous determination by purge-and-trap GC in line with FTIR

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A new approach was used to determine the reaction products of methylcobalamin and ionic mercury: purge-and-trap gas chromatography in line with Fourier transform infrared spectroscopy (PT GC/FTIR). This technique simultaneously and specifically determines the spectrum of dimethylmercury (DMeHg) and methylmercury produced by the reaction. No interference from other known organic mercury species could be detected. The method is different from others because it does not require solvent extraction of the organomercurials from aqueous solution, but relies on immediate volatilization from the reaction vessel by addition of 100 μ l of 10 mM NaBH₄. The sample was purged with nitrogen for 10 min. The volatile species of mercury were trapped in a column at -120 °C, injected into the gas chromatograph and detected by FTIR. The efficiency of DMeHg and MeHg formation depended on different parameters: pH, temperature, reaction time, and the methylcobalamin/ionic mercury ratio. The initial reaction product was MeHg which was further transformed to DMeHg. The first methylation rate was two times faster than the second. MeHg formed first, reaching a maximum at higher temperatures (28 °C and 37 °C) and later decreasing as DMeHg formed. At lower temperatures (20 °C) the rate of MeHg formation was slower, being similar to the formation rate of DMeHg. Different species of inorganic mercury such as HgSO₄, Hg(NO₃)₂, Hg(SCN)₂, HgCl₂ and HgI₂ were used to study differences in methylation by methylcobalamin under standard conditions of acidity, temperature and cofactor Hg(II) ratio.

Keywords: Mercury, methylation, methylcobalamin, Vitamin B₁₂, Fourier transform IR spectroscopy

INTRODUCTION

Vitamin B₁₂ and similar compounds are widely produced by aerobic and anaerobic microbes: *Pseudomonas denitrificans*,^{1,2} *Rhodospirillum rubrum*,³ *Salmonella typhimurium*,⁴ *Escherichia coli*,⁵ *Propionibacterium shermanii*,⁶ *Eubacterium limosum*,⁷ methanogens,⁸⁻¹¹ acetogenic bacteria,¹² clostridia^{13,14} and sulphate-reducing bacteria.¹⁵ It is still commonly accepted that the methylated form of vitamin B₁₂ (methylcobalamin) is one of the few compounds responsible for mercury methylation in the environment.

Jensen and Jernelöv¹⁶ first detected methylmercury (MeHg) and dimethylmercury (DMeHg) in aquarium sludge and rotten fish spiked with HgCl₂, specifically determining them by GC/MS analysis. After four and seven weeks of incubation under anaerobic conditions both organomercurials were found, suggesting that mercury alkylation may be bacterial in origin and that anaerobic conditions could affect this reaction.

Wood *et al.*¹⁷ found that MeHg and DMeHg, determined by thin-layer chromatography (TLC), were produced *in vitro* by cell-free extracts of a methanogenic organism strain MOH isolated from symbiotic mixed cultures collected in canal mud at Delft, Holland. In the test for MeHg synthesis, methylcobalamin [CH₃-Co(III)-5,6-dimethylbenzimidazolyl cobamide] was added to the cell-free extract as a good substrate for methane production. Mercury(II) inhibited methane formation, but reduced vitamin B₁₂ to B_{12r} and produced two alkylated forms from Hg(II): MeHg and DMeHg. The rapid conversion suggested that the methyl transfer from Co(III) to Hg(II) was non-enzymic.

A few years later it was demonstrated¹⁸ that Hg(II) could be methylated chemically by methylcobalamin in a few hours at 37 °C, pH 7, in the

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dark, under mild reducing conditions and in the absence of cell extract. A silica-gel thin-layer eluted with three solvent systems revealed two reaction products: DMeHg and MeHg. The initial product of this reaction was DMeHg, especially when equimolar amounts of HgCl_2 were used. DMeHg, thus synthesized, seemed to be converted into MeHg by further action of HgCl_2 . Because of the high volatility of DMeHg and owing to possible decomposition within the column when GC ECD (gas chromatography with an electron capture detector) was used, the reaction mixture of two organomercurials was treated with concentrated hydrochloric acid to quantitatively convert DMeHg to MeHg. The reaction proceeded at a high rate and Hg(II) was methylated almost quantitatively. Bertilsson and Neujahr¹⁹ carried out the same experiment, obtaining the same results for mercury methylation, but DMeHg could not be detected by the Westöö method.²⁰ Several reports in the 1970s²¹⁻²⁴ dealt with MeHg synthesis in pure cultures, and it seemed that both prokaryotes and eukaryotes could methylate Hg(II) . This synthesis was increased by addition of vitamin B_{12} to the media. In its methylated form, vitamin B_{12} was thus considered to be the main substrate for MeHg synthesis.

Corinoids are produced by many microorganisms, but today the sulphate-reducing bacteria are regarded as the best candidates for methylating inorganic mercury in the environment.^{25,26} It has been demonstrated that strains of *Desulfovibrio desulfuricans* are able to convert ionic mercury to MeHg in pure cultures by the methylcobalamin pathway.^{15,27}

The aim of this study was to determine by a new method the organic mercury species formed in the reaction of ionic mercury with methylcobalamin. The chemical forms were detected simultaneously as volatile species, which were purged from the aqueous solutions.

MATERIALS AND METHODS

Apparatus

The PTGC/FTIR system has been described previously.²⁸ A wide-bore fused silica column CP Sil 8 50 m \times 0.53 mm i.d., with 2 μm film thickness was used in the GC isothermally at 100 °C with a nitrogen flow rate of 10 $\text{cm}^3 \text{min}^{-1}$. A purge-and-trap apparatus (Chromopack) was used in line

with the GC and programmed as follows: precooling time 1 min, purge time 10 min at 80 °C with a flow rate (nitrogen) of 22 $\text{cm}^3 \text{min}^{-1}$, injection time 1 min at 250 °C.

Reagents

Solutions of 10 mM sodium borohydride (Carlo Erba) were prepared in double-distilled water (DDW). Methylcobalamin (Sigma) solution was prepared by dissolving 13.4 mg in 10 cm^3 DDW. The solution was kept in the dark and refrigerated until use. Aqueous solutions of other mercury compounds were prepared at the following concentrations as Hg : HgI_2 (8.81 $\mu\text{g cm}^{-3}$), $\text{Hg(NO}_3)_2$ (115.7 $\mu\text{g cm}^{-3}$), HgSO_4 (16.4 $\mu\text{g cm}^{-3}$) and Hg(SCN)_2 (37.9 $\mu\text{g cm}^{-3}$).

Standards

The stock solution of HgCl_2 (Carlo Erba) was prepared by dissolving 0.1354 g in 100 cm^3 of DDW. The stock solution of methylmercury chloride (BDH) was prepared by dissolving 0.1251 g in 100 cm^3 of ethanol and was stored at -10 °C. The stock standard solution of dimethylmercury (10 μl ; Aldrich) was mixed with 100 cm^3 of DDW to obtain a concentration of 276 $\mu\text{g cm}^{-3}$ as Hg .

Procedure

Portions (5 cm^3) of solution containing different concentrations of methylcobalamin were adjusted to different pH values. The solution was kept in the dark as much as possible in sealed tinted 7.5 cm^3 vials, to which 20 μg as Hg , were added to the methylcobalamin solution. The organomercurials formed (MeHg and DMeHg) were poured into the reaction vessel and 100 μl of 10 mM NaBH_4 solution was spiked; the sample was immediately purged with nitrogen. Under these conditions, MeHg was converted quantitatively to methylmercury hydride, as previously reported.²⁹ So DMeHg and methylmercury hydride were both volatilized by heating the sample to 80 °C and trapped in a column at -120 °C. After 10 min of nitrogen purging, the organomercurials were automatically injected into the gas chromatograph and detected by FTIR. The coefficients of variation of five replicates were 2.3% for MeHg and 3.8% for DMeHg. DMeHg could be detected if NaBH_4 was not used.

Experiments at different pH values

Methylcobalamin and HgCl_2 solutions were adjusted to different pH values by adding sulphuric acid for acid pH and sodium hydroxide for alkaline pH. The values were monitored before and after the reaction with a pH-meter. In order to avoid interference by presumed complexes with mercury compounds in the aqueous solution, buffered solutions were not used.

Experiments at different temperatures

The tinted glass vials containing 20 μg as total Hg were incubated at 37°C, 28°C and 20°C in the dark. At the moment of organomercurial determination, the whole 5 cm^3 of sample was poured into the reaction vessel (20 cm^3) of the purge and trap apparatus and analysed after NaBH_4 addition.

RESULTS AND DISCUSSION

This study of inorganic mercury alkylation by methylcobalamin was similar to previous experiments,^{18,19} but the method for determining the organic mercury species was different. The organomercurials were not extracted by acid-solvent solutions but were determined simultaneously from the aqueous solution by purging with nitrogen.

The new technique enables us to determine both MeHg and DMeHg while interfering as little as possible with the reaction between methylcobalamin and ionic mercury. The determination was specific for each mercury species as far as retention times and FTIR spectra are concerned (Fig. 1). Methylmercury hydride has two close absorbance peaks at 1969 and 1943 cm^{-1} . DMeHg has two characteristic peaks, at 2914 cm^{-1} and 787 cm^{-1} (Fig. 1).

The variations of MeHg and DMeHg concentrations were investigated by changing the pH after a constant period (3 h) of incubation at 37°C (Fig. 2). Total organomercurials (MeHg + DMeHg) showed rapid formation with an optimum peak between pH 3 and 5 and average value of $11.78 \pm 0.62 \mu\text{g}$ ($n = 6$) in a 5- cm^3 sample, corresponding to 59% yield of total Hg(II) alkylation (MeHg + DMeHg). MeHg showed an amount ranging from 4.5 to 7.0 μg between pH 3 and 5; at the latter value the maximum peak was reached. DMeHg also showed optimum formation at acid pH values, with a peak at pH 3 with

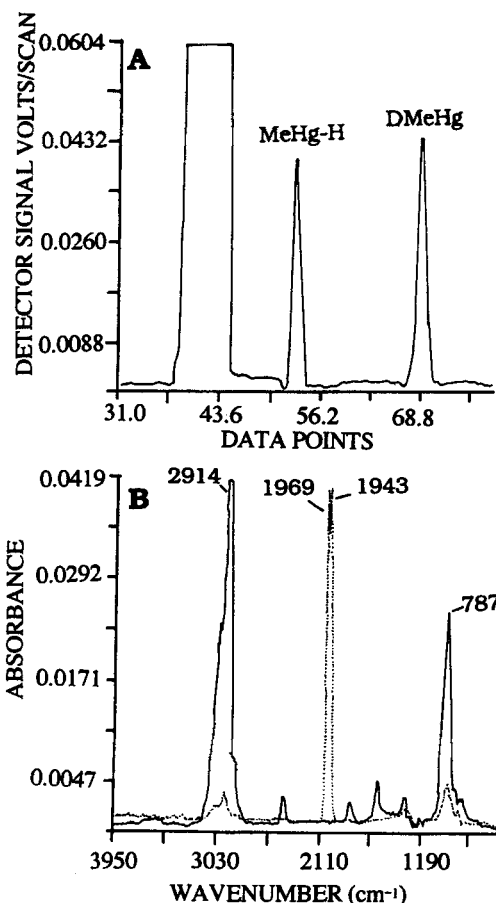


Figure 1 (A) Retention times (data points) from FTIR chromatogram of methylmercury hydride and dimethylmercury. (B) FTIR spectra:, 2.62 μg methylmercury hydride; —, 11.95 μg dimethylmercury.

the maximum total organomercurial concentration (13.75 μg). The methyl groups were transferred slowly from methylcobalamin to Hg(II) at pH values above 6. Both organomercurials were slowly formed; total organic Hg dropped to yields of 11.75% at pH 6.0 and to 4.35% at pH 8.5.

The formation of MeHg and DMeHg at acid pH is apparently in contradiction with the results from Beijer and Jernelöv.³⁰ In a 10-day experiment, they found that DMeHg was formed in sediments at neutral and alkaline pH values. The species was detected in the gaseous phase by trapping after aeration. Other authors³¹ have also determined DMeHg in the gaseous phase but not in solution. Recently Mason and Fitzgerald³² determined DMeHg in open ocean waters with a new analytical technique, based on volatilization of all mercury species with sodium tetraethylbor-

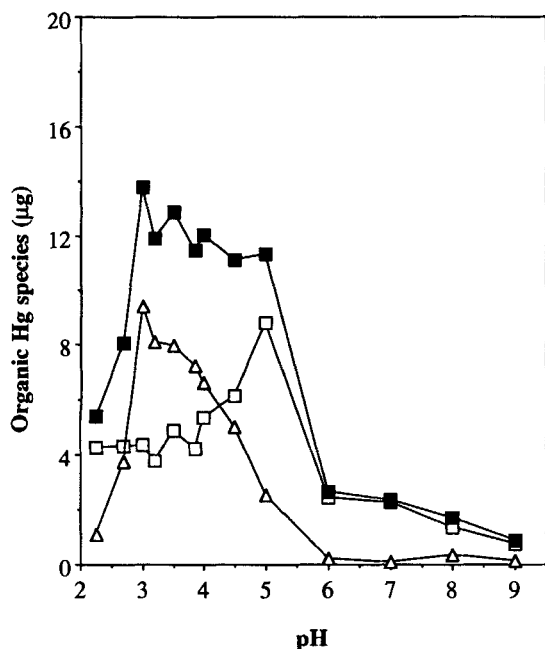


Figure 2 Concentration changes of (□) MeHg, (△) DMeHg and (■) total organic mercury species (MeHg + DMeHg) with pH, after 3 h of incubation in darkness with a vitamin/Hg(II) ratio of 10:1.

ate, followed by atomic fluorescence detection. In the present study DMeHg was measured in the liquid phase under closed conditions; the effect of pH on dimethylmercury volatilization was not investigated, but we checked the mercury losses. A partial recovery of 20 μg total Hg ($67.7 \pm 4.03\%$) was achieved with a headspace volume of 2.5 cm^3 after 24 h incubation at pH 4 and 37 $^\circ\text{C}$. A higher recovery ($89.8 \pm 0.36\%$) was obtained with the lowest headspace volume. A further experiment was performed under the same conditions to determine the recovery starting from a solution at pH 4 with 20 μM methylcobalamin, adding 20 μg DMeHg from the stock solution. After 3 h of incubation at 37 $^\circ\text{C}$ the recovery was $61.2 \pm 0.3\%$, and at 20 $^\circ\text{C}$ it was $80.9 \pm 0.22\%$. This finding demonstrates that a percentage of the volatile DMeHg was lost during the procedures of mercury speciation, mainly when pouring the 5- cm^3 sample from the tinted vials to the purge-and-trap reaction vessel. The underestimated concentration of total Hg(II) transformed was due to the chemical equilibrium reached between DMeHg concentrations in the aqueous and gaseous phases.

The formation rate of DMeHg also depended on the ratio between methylcobalamin and Hg(II)

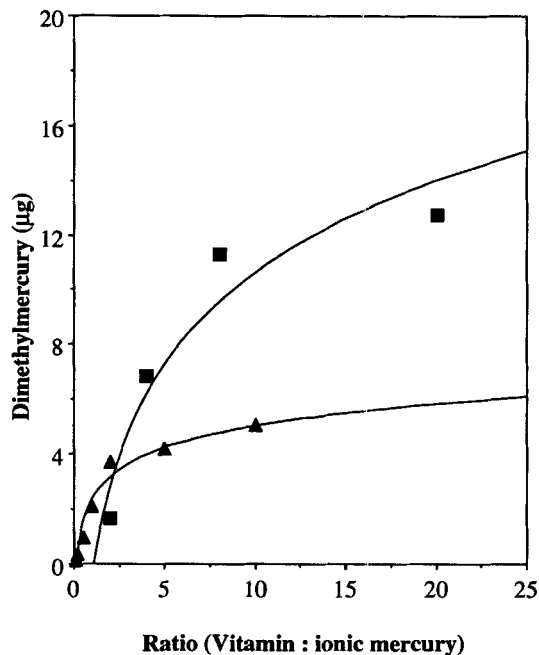


Figure 3 Formation of dimethylmercury at different vitamin/Hg(II) molar ratios at (■) 37 $^\circ\text{C}$ and (▲) 28 $^\circ\text{C}$ at pH 4, after 3 h of incubation in darkness.

(from 0.1 to 20). The experiment was carried out at two different temperatures (37 $^\circ\text{C}$ and 28 $^\circ\text{C}$) at pH 4 after 3 h of incubation (Fig. 3). At higher ratios with more methylcobalamin than ionic mercury, the rate of DMeHg formation was favoured, whereas at higher concentrations of ionic mercury with respect to the cofactor, MeHg formation was favoured: in other words the reaction rate was slower and hence mainly MeHg was produced. Vitamin B₁₂ and its methylated forms can reach concentrations from tens of nM to hundreds of μM inside the cells, depending on the species and strain of bacteria.^{3,7,33} Methylcobalamin is a constitutive cofactor, at least in methanogens¹⁰ and acetogenic bacteria¹² for the production of methane and acetate, respectively. Hence the vitamin/Hg ratio is a factor to be considered.

The formation of both organic mercury species depended strictly on temperature. The kinetics of organomercurial formation was studied at three different temperatures (20 $^\circ\text{C}$, 28 $^\circ\text{C}$ and 37 $^\circ\text{C}$) at pH 4 and at a vitamin/Hg ratio of 10:1, to speed up the mercury alkylation experiments. At 37 $^\circ\text{C}$ total organic mercury species reached a maximum after 1 h with a 77% recovery of mercury. At 28 $^\circ\text{C}$ the maximum was reached at 8 h with a lower recovery (65%) of total mercury. At 20 $^\circ\text{C}$, there was slow and continuous production of

organomercurials after 24 h of incubation (Fig. 4).

MeHg, as the intermediate compound between Hg(II) and DMeHg, was formed quickly (Fig. 5) and, after reaching a maximum at 37 °C and 28 °C, its concentration decreased because of partial transformation to DMeHg. At 20 °C the rate of MeHg formation was similar to that of DMeHg. DMeHg was produced at different rates in relation to temperature (Fig. 6) and after 10 h of incubation its production leveled off. This species of mercury was stable in solution at pH 4 and 37 °C in the sealed vials. No degradation of DMeHg was detected after seven days of experiment.

To understand better the relation between organomercurial production and temperature, the formation rates were calculated from the first linear part of the respective plots (Figs 5 and 6) and also from data obtained from DMeHg produced from MeHg (not plotted) in 3-h experiments at the respective temperatures (T) of 20 °C, 28 °C and 37 °C, pH 4, and at a vitamin/Hg ratio of 10:1. The natural logarithm of methylmercury (MeHg_r) and dimethylmercury (DMeHg_r) formation rates ($\mu\text{g h}^{-1}$) from Hg(II) were correlated with the reciprocal of each tem-

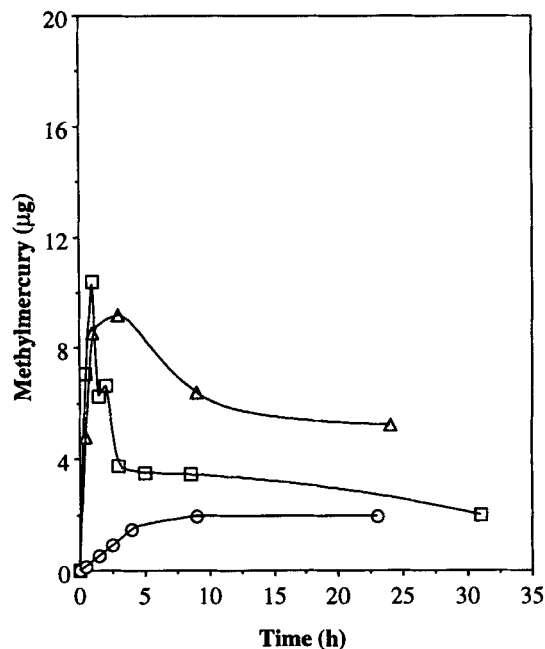


Figure 5 Formation of MeHg at pH 4 and 20 °C (○), 28 °C (Δ) and 37 °C (□) with a vitamin/Hg(II) ratio of 10:1 after different periods of incubation in darkness.

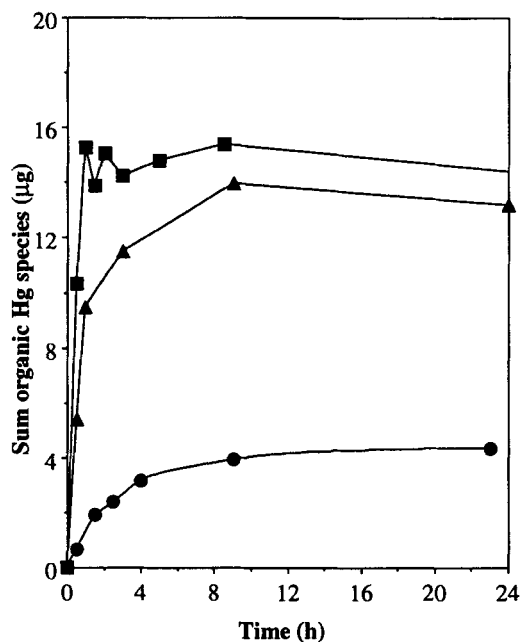


Figure 4 Formation of organic mercury species (MeHg + DMeHg) at pH 4 and (●) 20 °C, (▲) 28 °C and (■) 37 °C with a vitamin/Hg(II) ratio at 10:1 at different times of incubation in darkness.

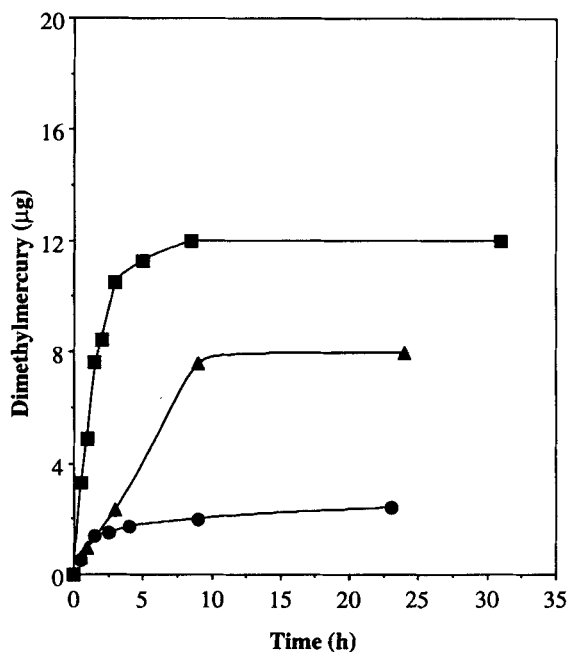


Figure 6 Formation of DMeHg at pH 4 and (●), 20 °C, (▲) 28 °C and (■) 37 °C with a vitamin/Hg(II) ratio of 10:1, after different periods of incubation in darkness.

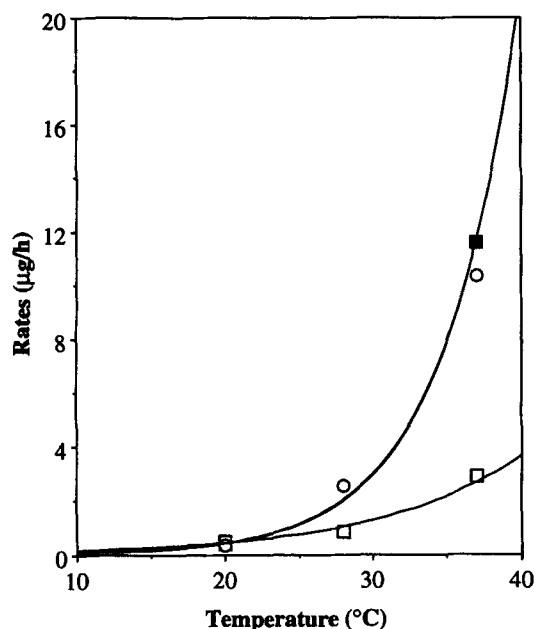


Figure 7 Exponential relationships between different organic mercury species formed and temperature (°C): □, DMHg formed from HgCl₂; ■ DMHg formed from MeHgCl; ○, MeHg formed from HgCl₂.

perature according to Arrhenius equations (Eqns [1] and [2] and Fig. 7

$$\ln(\text{MeHg}_t) = 6.161 - 143.5/T \quad [1]$$

$$R^2 = 0.997$$

and

$$\ln(\text{DMeHg}_t) = 2.758 - 71.34/T \quad [2]$$

$$R^2 = 0.944$$

The alkylation rate of DMHg formation from methylmercury chloride (MeHgCl) was also calculated at 20 °C and 37 °C (Fig. 7) and is expressed by Eqn [3].

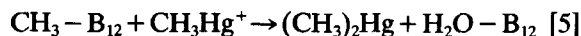
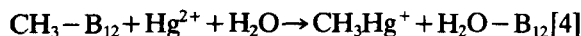
$$\ln(\text{DMeHg}_t) = 6.431 - 146.9/T \quad [3]$$

This rate was similar to that obtained from the first methylation step of Hg(II). The MeHg formation rate from Hg(II) was 2.01 times greater than for DMHg. This finding is in contrast with that of Wood *et al.*³⁴

The temperature parameter began to be important at temperatures above 25 °C. Below

this temperature similar amounts of MeHg and DMHg were formed.

However, the final product of the overall temperature- and pH-dependent reaction was DMHg (Eqns [4] and [5]).



The formation of organomercurials also depended on the chemical species of inorganic mercury interacting with methylcobalamin (Table 1). The production of DMHg was faster with inorganic mercury compounds such as HgSO₄, which had an ionic bond, whereas in the presence of a covalent bond the transalkylation reaction was slower.

This new method to detect MeHg and DMHg simultaneously by PT GC/FTIR in aqueous solutions at different pH values, temperatures and vitamin/Hg ratios makes a new contribution to the kinetics of the methylcobalamin reaction with Hg(II).

- (1) MeHg and DMHg were formed together in the aqueous solution even though MeHg was formed twice as fast (empirical ratio = 1.84:1) as DMHg;
- (2) the final product of the reaction was DMHg and this was stable in this closed system for up to seven days at pH 4;
- (3) an acid pH between 3 and 5 was important for the formation of both organomercurials;
- (4) temperature was important and an empirical relationship was found for MeHg and DMHg formation rates;
- (5) inorganic mercury species reacting with methylcobalamin gave different production rates of MeHg and DMHg; if the bond was more ionic, DMHg formation was faster than with a covalent bond.

Table 1 Alkylation of several inorganic species at a concentration of 20 µg with vitamin/Hg(II) molar ratio 10:1, after 3 h of incubation at 37 °C and pH 4 in the dark

Mercury species	MeHg (µg)	DMeHg (µg)	Total organic Hg (µg)	Ratio MeHg/DMeHg
HgSO ₄	5.80	9.50	15.30	0.6
Hg(NO ₃) ₂	9.61	6.17	15.78	1.55
Hg(SCN) ₂	11.47	6.06	17.53	1.89
HgCl ₂	11.36	3.71	15.16	3.06
HgI ₂	4.02	0.45	4.47	8.93

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REFERENCES

1. Lago, B D and Demain A L J. *Bacteriol.*, 1969, 99: 347
2. Kusel, J P, Fa, Y H and Demain, A L J. *Gen. Microbiol.*, 1988, 172: 459
3. Hirayama, O and Katsuta, Y *Agric. Biol. Chem.*, 1984, 159: 206
5. Guest, J R, Friedman, S and Woods, D D *Nature (London)* 1962, 195: 340
6. Brady, R O and Barker, H A *Biochem. Biophys. Res. Commun.*, 1961, 4: 464
7. Vogt, J R A and Renz, P *Eur. J. Biochem.*, 1988, 171: 655
8. Blaylock, B A and Stadtman, T C *Arch. Biochem. Biophys.*, 1966, 116: 138
9. Wolin, M J, Wolin, E A and Wolfe, S *Biochem. Biophys. Res. Commun.*, 1964, 15: 420
10. Blaylock, B A *Arch. Biochem. Biophys.*, 1968, 124: 314
11. Stupperich, E and Kräutler, B *Arch. Microbiol.*, 1988, 149: 268
12. Stupperich, E, Eisinger, H J and Kräutler, B *Eur. J. Biochem.*, 1988, 172: 459
13. Peterkofsky, A, Redfield, B and Weissbach, H *Biochem. Biophys. Res. Commun.*, 1961, 5: 213
14. Pan-Hu, H S K, Hosono, M and Imura, N *Appl. Environ. Microbiol.*, 1980, 40: 1007
15. Choi, S C and Bartha, R *Appl. Environ. Microbiol.*, 1993, 59: 290
16. Jensen, S and Jernelöv, Å *Nature (London)*, 1969, 223: 753
17. Wood, M J, Kennedy, F S and Rosen, C G *Nature (London)*, 1968, 220: 173
18. Imura, N, Sukegawa, E, Pan, S K, Nagao, K, Kim, J Y, Kwan, T and Ukita, T *Science*, 1971, 172: 1248
19. Bertilsson, L and Neujahr, H *Biochemistry*, 1971, 10: 2805
20. Westöö, G *Acta Chem. Scand.*, 1966, 20: 2131
21. Yamada, M and Tonomura, K *J. Ferment. Technol.*, 1972, 50: 159
22. Vonk, J W and Siipesteijn, A K J. *Ant. van Leeuwenhoek*, 1973, 39: 505
23. Spangler, W J, Spigarelli, J L, Rose, J M and Miller, H M *Science*, 1973, 180: 192
24. Hamdy, M K and Noyes, O R *Appl. Microbiol.*, 1975, 30: 424
25. Compeau, G C and Bartha, R *Appl. Environ. Microbiol.*, 1985, 50: 498
26. Compeau, G C and Bartha, R *Appl. Environ. Microbiol.*, 1987, 53: 261
27. Padberg, S, Iverfeldt, Å, Lee, Y H, Baldi, F, Filippelli, M, May, K and Stoeppler, M In: *Mercury as Global Pollutant: Toward Integration and Synthesis*, Lewis, 1993 (in press)
28. Baldi, F, Cozzani, E and Filippelli, M *Environ. Sci. Technol.* 1988, 22: 836
29. Filippelli, M, Baldi, F, Brinckman, E F and Olson, G J *Environ. Sci. Technol.*, 1992, 26: 1457
30. Beijer, K and Jernelöv, Å In: *The Biogeochemistry of Mercury in the Environment*, Nriagu, J O (ed) Elsevier/North-Holland Biomedical Press, Amsterdam, 1979, pp. 203–210
31. Bisogni, J J and Lawrence, A W J. *Water Pollut. Control Fed.*, 1975, 47: 135
32. Mason, R P and Fitzgerald, W F *Water Air Soil Pollut.*, 1991, 56: 779
33. Cameron, B, Briggs, K, Pridmore, S, Brefort, G and Crouzet, J J. *Bacteriol.*, 1989, 171: 547
34. Wood, J M, Ridley, W P, Cheh, A, Chudik, W and Thayer, J S In: *Proc. Int. Conf. Heavy Metals in the Environment*, Hutchinson, E C (ed), University of Toronto Press, Toronto, 1977, pp 49–68.