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Methylation of inorganic mercury by methylcobalamin in aquatic systems

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The methylation of inorganic Hg(II) by methylcobalamin in aquatic systems was studied using high-performance liquid chromatography coupled with UV-digestion cold vapor atomic fluorescence spectrometry (HPLC-UV-CV AFS). Monomethylmercury (MMC) could be positively identified as the reaction product in the aqueous solution. The salinity and pH of the aquatic system have great effects on the formation of MMC, because they could change the species of the reactants in the solution. From an electrophile reaction point of view, salinity and pH alter the electron density of the methyl donor and the electrophilicity of metal ion in the reaction system. This methylation of inorganic Hg(II) is shown to be possible even in highly saline solutions, which indicates its importance in aquatic environments. Kinetic experiments showed that the methylation reaction was fast and first-order for Hg(II). The first-order reaction rate was determined to be 0.00612 and 0.000287 min^{-1} for pH 5.0 and 1.5, respectively. It is suggested that this methylation could occur in the absence of enzymes, in which Hg(II) acts as an electrophile to attack methylcobalamin with a subsequent transfer of carbanion methyl group to the higher oxidized state of Hg(II). **Copyright © 2007 John Wiley & Sons, Ltd.**

KEYWORDS: methylation; inorganic mercury; methylmercury; methylcobalamin; pH; salinity; electrophile reaction

INTRODUCTION

Mercury compounds have long been of great public concern because of their adverse effect on wildlife and humans. It is well known that the toxicity of mercury compounds depends on their species, which include inorganic, methyl, ethyl and phenylmercury. Amongst these compounds, methylmercury is the most toxic form in the environment. One famous case of severe methylmercury poisoning occurred in Minamata, Japan, which was caused by the consumption of seafood contaminated with methylmercury compounds discharged from a chemical plant in the 1950s and 1960s.

Methylation of elements such as Hg, As and Sn is an important transformation and transportation pathway of elements in the environment, and has been widely studied.[1] Natural conversion from inorganic mercury to methylmercury was first demonstrated by Jensen and Jernelov.[2] There exist two methylation pathways of inorganic mercury: the biotic process and the abiotic process in the aquatic environment.[3] Although many scientists have provided evidence for abiotic methylation of mercury in the environment,[4,5] it is widely accepted that biotic methylation accounts for most or all methylation in the environment, especially by sulfate-reducing bacteria (SRB).[6,7] However, the relative importance of abiotic methylation of inorganic Hg(II) should not be neglected.[3]

Chemical methylation of inorganic Hg(II) can occur only if suitable methyl donors exist in the environment. Nagase demonstrated that inorganic Hg(II) can be methylated by fulvic and humic acids, which are large organic components of dissolved organic matter.[5] It is also possible that inorganic mercury can be methylated by acetic acid, propionic acid and ethanol in aquatic environments under the irradiation of sunlight or ultraviolet light.[8,9] Transmethyl reaction between inorganic Hg(II) and other methylmetals, such as the methyl transfer from methyltin

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to inorganic Hg(II), have also been shown to be possible in aquatic environments.[10] Moreover, Wood demonstrated the formation of mono- and dimethylmercury from Hg(II) in cell-free extracts of a pure culture of a methanogenic bacterium using substrate concentrations of methylcobalamin under strict anaerobic conditions. Methyl transfer from methylcobalamin to inorganic Hg(II) was suggested as the most probable mechanism.[11] However, it is debated whether this methyl transfer reaction is an enzymatic or non-enzymatic processes.[12,13] Its importance in the aquatic environment, particularly in the presence of Cl^-, has not been established.[7] Elucidation of the methyl transfer mechanism from methylcobalamin to inorganic Hg(II) was further complicated by conflicting conclusions from Bertilsson and Imura, which respectively stated that monomethylmercury or dimethylmercury is the initial product of this methylation reaction.[13,14] Moreover, experimental results from Craig and Morton suggested that monomethylmercury (MMC) was the initial product, and the thermodynamic parameters of the methylation reaction could thereafter be calculated.[15]

In our paper, the methylation of inorganic Hg(II) by methylcobalamin in aquatic systems was investigated using a sensitive HPLC-UV-CV AFS system. The methylation reaction was carried out under different pH and salinity conditions. The effects of these two factors on the methylation reaction were emphasized and further explained in detail. A probable mechanism for the methylation of inorganic Hg(II) by methylcobalamin was proposed.

EXPERIMENTAL

Reagent and standards

All reagents were obtained commercially and used without further purification unless otherwise stated. De-ionized water (EASY Pure LF, Barnstead Co., USA) was used throughout. Methylcobalamin (CH_3B_{12}; 99.5%) was purchased from Phentex Corp. (USA). Methylmercury chloride and mercury chloride were obtained from Merck-Schuchardt (Germany). Stock solutions of methylmercury chloride (1 mg ml^{-1} as Hg) were prepared by dissolving appropriate amounts of methylmercury chloride in methanol and stored at 4 °C in darkness. Working solutions diluted with de-ionized water for analysis were prepared daily prior to use.

Analytical method

An HPLC-UV-CV AFS system was used to detect mercury species in aqueous solutions. A quaternary pump (P680 HPLC Pump, Dionex, USA) equipped with a Rheodyne Model 7715i injector valve (Rheodyne, Cotati, CA, USA) and a 20 µl sample loop was used for sample introduction. An Agilent Zorbax ODS column (150 × 4.6 mm i.d., 5 µm) was used to separate the mercury species. The effluent from the HPLC system was delivered to an 8 m PTFE digestion coil (i.d. 0.8 mm) wrapped around an 8 W UV lamp, where decomposition of methylmercury to inorganic mercury took place, with 0.5% $K_2S_2O_8$ in 10% HCl converged by a peristaltic pump as the oxidant. Following the introduction of a 0.5% KBH_4 in 0.2% KOH solution, cold vapors of mercury were produced and separated in the gas–liquid separator. Thereafter, it was carried to the detector by an argon stream, and detected with an AFS-610A non-dispersive atomic fluorescence spectrometer (Beijing Rayleigh Analytical Instrument Co., China). The spectrometer was equipped with a high-intensity hollow cathode mercury lamp at 253.7 nm line source (Beijing Tiangong Analytical Instrument Factory, China), running at 280 V of PMT voltage and 40 mA of lamp current. A personal computer with an AFS-610A software was used as a work station. The column, T-cross valve and the gas–liquid separator were connected by a PTFE tube. Detailed experiment conditions for HPLC-UV-CV-AFS are listed in Table 1.

Experimental design

In general, the methylation reaction of inorganic mercury with methylcobalamin took place in darkness at about 30 °C using 10 ml aqueous solution in 20 ml glass tubes. Stock solution of 100 mg L^{-1} $HgCl_2$ in 1% HNO_3 was used as the inorganic mercury source. The pH value of the reaction system was adjusted by 0.1 M NaOH and 0.1 M H_2SO_4, and determined using a pH meter (Hanna Instruments pH211C with HI 1200B glass body combination pH electrode). The salinity of the solution was adjusted with 5 M NaCl. The glass tubes were covered with aluminum foil to keep it dark and placed in a thermostatic bath to maintain a constant temperature.

Table 1. Experimental conditions of HPLC-UV-CV-AFS

HPLC	
Column	Agilent Zorbax ODS column, 4.6 × 150 mm, 5 µm
Mobile phase	10% (v/v) HCN; 60 mM CH_3COONH_4; 0.01% (v/v) 2-mercaptoethanol
Flow rate of Mobile phase	1.0 ml min^{-1}
Sample injection volume	20 µl
Hydride generation	
Oxidant solution	0.5% (m/v) $K_2S_2O_8$ in 10% (v/v) HCl, 1.8 ml min^{-1}
Reducing solution	0.5% (m/v) KBH_4, 3.6 ml min^{-1}
AFS	
Lamp	Hollow cathode mercury lamp, 253.7 nm
PMT voltage	280 V
Primary current	40 mA
Carrier gas	Argon, 500 ml min^{-1}

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Concentrations of the product in the reaction system were monitored during the reaction procedure for kinetic analysis.

RESULTS AND DISCUSSION

The chromatograms of standard substances were compared with those of the samples from the reaction system (Fig. 1). This demonstrated that water-soluble mercury species could be successfully separated and detected by the HPLC-UV-CV AFS. As seen in Fig. 1, it could be judged that MMC is a water-soluble reaction product in the aqueous solution. This experimental result is consistent with that observed by Bertilsson and Craig.[13,15]

The effect of pH on the methylation reaction is depicted in Fig. 2. It could be clearly observed that the pH of the solution has a major effect on the methylation of inorganic Hg(II). Under strongly acidic conditions, a moderate amount of inorganic Hg(II) was methylated by methylcobalamin. With the increase of pH, the concentration of MMC in the reaction system peaked at pH 3. Henceforth, the methylation yield of inorganic Hg(II) dropped with the increase in pH. There are two probable explanations for the effect of pH on the methylation reaction. On one hand, it is due to inorganic Hg(II) complexing with OH^- in the reaction solution. According to the complexing stability constant of OH^- with Hg^{2+}, the ratio between Hg^{2+} and $[Hg(II)(OH)_2]^0$ in the solution is obtained from equation (1) and is shown in Fig. 3.

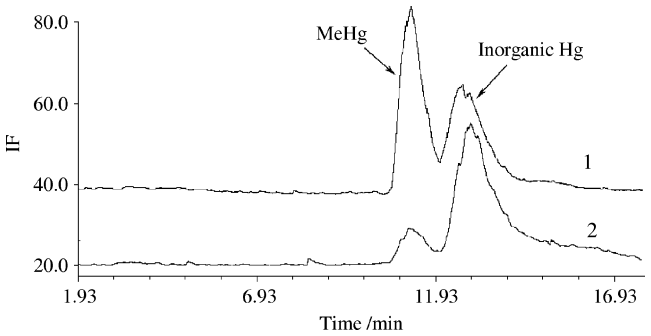


Figure 1. HPLC-UV-CV-AFS chromatograms of mercury species in water. 1, Standard substances; 2, sample from reaction system.

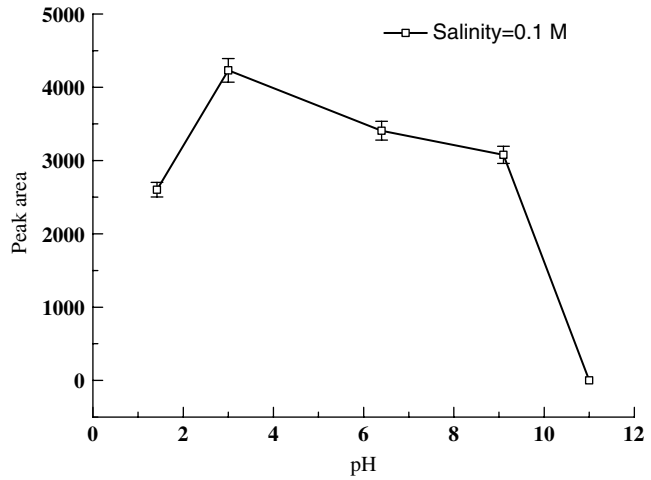


Figure 2. The effect of the pH on the methylation reaction. (Peak area refers to MMC detected by HPLC-UV-CV-AFS).

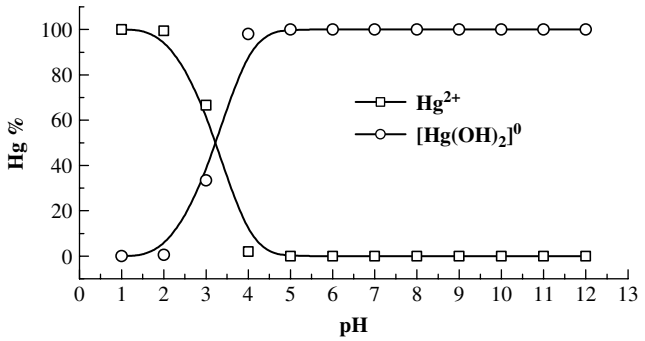
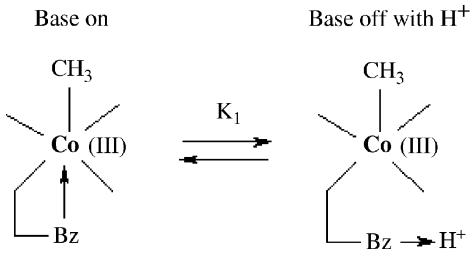


Figure 3. The relative amount of different inorganic mercury species vs pH [calculation from the complexing stability constant of OH^- with Hg^{2+} using equation (1)[16]].

$$\delta_{HgL_n} = \frac{\beta_n [L]^n}{1 + \sum_{i=1}^{n} \beta_i [L]^i} \quad (1)$$

where L is the complexing ligand, β is the complexing stability constant and n is complexing level.[16] On the other hand, methylcobalamin in aquatic solutions is subjected to an equilibrium between the 'base on' form (5,6-dimethylbenzimidazole coordinated to the cobalt atom), and the uncoordinated 'base off with H^+' form.[17] The equilibrium is shown in equation (2).



(2)

Different species of inorganic Hg(II) and methylcobalamin show different reactivities. In strongly acidic solutions, the main methylation pathway of inorganic Hg(II) would be the reaction between $[Hg(II)Cl_n]^{2-n}$ and the 'base off with H^+' form of methylcobalamin. However, a more complex system exists at pH 3, which involves $[Hg(II)Cl_n(OH)_m]^{2-n-m}$, 'base off with H^+', and 'base on'. Under higher pH conditions, the only methylation reaction is between $[Hg(II)(OH)_2]^0$ and the 'base on' form of methylcobalamin.

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Since the salinity of aqueous solution is of great environmental interest, its effect on the methylation of Hg(II) was also investigated at two pH levels (Fig. 4). In our experiments, the range of the salinity was 0–1.0 M, which covers the salinity of most aquatic environments (the salinity of seawater 0.5 M). The salinity exerts a different influence on the methylation reaction under different pH conditions. In strongly acidic solutions, the yield of MMC decreased with the increase of the salinity, because of the different reactivity of Hg^{2+}, $[HgCl]^{+}$, $[HgCl_2]^{0}$, $[HgCl_3]^{-}$ and $[HgCl_4]^{2-}$ with methylcobalamin. The order of reactivity is as follows: $Hg^{2+} > [HgCl]^{+} > [HgCl_2]^{0} > [HgCl_3]^{-} > [HgCl_4]^{2-}$. A large amount of Cl^{-} would increase the electron density of Hg(II) and decrease the electrophilicity of Hg(II) towards the carbanion methyl group of methylcobalamin. Figure 5 shows relative amount of different inorganic mercury species at different salinity, which can be computed by equation (1) using the complexing stability constant of Hg^{2+} with Cl^{-} listed in Table 2. At pH 5, the concentration of MMC rose and quickly became constant with the increase of the salinity. The reason for this phenomenon is that OH^{-} has stronger ability to complex with Hg^{2+} than Cl^{-}. Two explanations for stronger complexing ability of OH^{-} with Hg^{2+} than Cl^{-} were proposed: (1) OH^{-} has higher complexing stability constant with Hg^{2+} than Cl^{-} (Table 2); (2) the nucleophilicity of OH^{-} is higher than that of Cl^{-}.[18] Therefore, the increase in Cl^{-} influences only slightly the methylation of inorganic Hg(II) at pH 5.0. Celo *et al*. concluded that methylcobalamin was unlikely to methylate Hg(II) in moderately or highly saline environment.[3] However, our results are inconsistent with above conclusion. In the range of salinity from 0 to 1.0 M, all inorganic Hg(II) can be methylated by methylcobalamin. In particular, the salinity has a slight influence on the high yield of MMC at pH 5.0, which is more environmentally relevant.

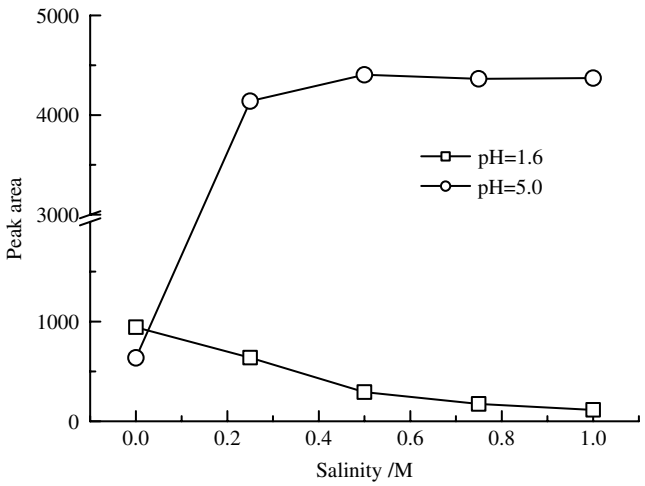

Figure 4. The effect of salinity on the methylation reaction.

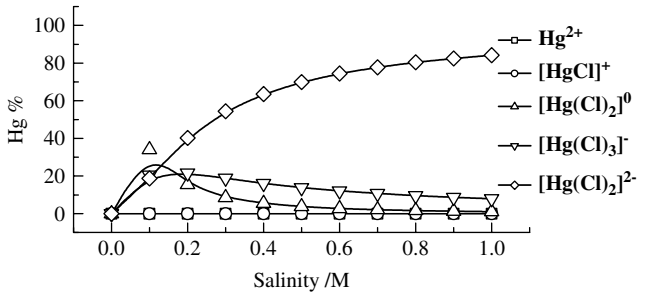

Figure 5. The relative amount of different inorganic mercury species vs salinity [calculation from the complexing stability constant of Cl^{-} with Hg^{2+} using equation (1)[16]].

Table 2. Stability constant of Hg^{2+} complexing with OH^{-} and Cl^{-}

Equilibrium reaction	β (complexing stability constant)[16]
Complexing with OH[a]	
$Hg^{2+} + 2OH^{-} = [Hg(OH)_2]^{0}$	21.7
Complexing with Cl[b]	
$Hg^{2+} + Cl^{-} = [HgCl]^{+}$	6.74
$Hg^{2+} + 2Cl^{-} = [HgCl_2]^{0}$	13.22
$Hg^{2+} + 3Cl^{-} = [HgCl_3]^{-}$	14.07
$Hg^{2+} + 4Cl^{-} = [HgCl_4]^{2-}$	15.07

[a] Ionic strength is 0.5.
[b] Ionic strength is 0.5.

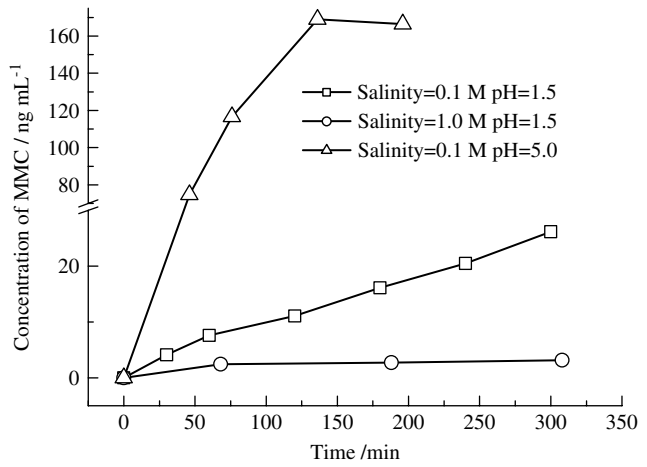

Figure 6. Formation of MMC vs. the time under different conditions.

Kinetic experiments were also performed under different conditions of salinity and pH (Fig. 6). It was found that both pH and salinity have strong influences on the methylation rate. In the solution of pH 5.0, the rate of the methylation

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reaction was fast, and the yield of MMC was more than 50%. The methylation reaction of inorganic Hg(II) reached equilibrium after 150 min. However, under strongly acidic conditions, all methylation reactions of inorganic Hg(II) became slower and the yield of MMC was lower than that at pH 5.0.

A large excess of methylcobalamin over inorganic Hg(II) was designed in the kinetic experiments so that the concentration of methylcobalamin remained essentially constant in the methylation system. The methylation reaction can be considered as pseudo-first-order for Hg(II), i.e. $m = 1$ because of the linear relationship between $\ln[(C_0 - C)/C_0]$ and the reaction time, where C_0 is the initial concentration of inorganic Hg(II) and C is the concentration of CH_3Hg in the methylation system. The results of the first order kinetic fit are visualized in Fig. 7. The high correlation coefficient ($R = 0.99$) corroborates the linear relationship. Thus, it could be deduced that this methylation reaction was first-order for inorganic Hg(II). The first-order reaction rates were 0.00612 and 0.000287 min^{-1} in the solution at pH 5.0 and 1.5, respectively.

The reaction mechanism for inorganic Hg(II) and methylcobalamin can be considered as a transfer of carbanion methyl group to the higher oxidized state of inorganic Hg(II). The inorganic Hg(II) acts as an electrophile, and heterolytic cleavage of the Co–C bond in methylcobalamin occurs.[19] According to our experimental results, it is suggested that the methylation of inorganic Hg(II) by the methylcobalamin may occur in the absence of the enzyme. The schematic of this methylation reaction is presented in equation (3).

$$[Hg(II)Cl_n(OH)_m]^{2-n-m} + CH_3B_{12}(III) \longrightarrow [CH_3Hg(II)Cl_n(OH)_m]^{1-n-m} + B_{12} \quad (3)$$

In the reaction system, inorganic Hg(II) exists in the form $[Hg(II)Cl_n(OH)_m]^{2-n-m}$, in which Hg^{2+} forms a complex with inorganic ligands such as Cl^- and OH^-.

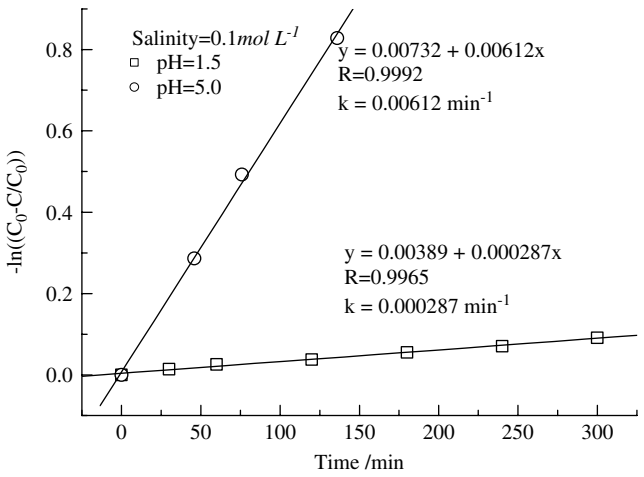
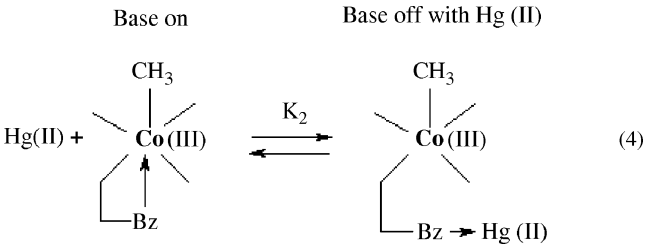


Figure 7. First-order rate plots at different pH.



(4)

$[Hg(II)Cl_n(OH)_m]^{2-n-m}$ acts as an electrophile would be available to coordinate with the nitrogen atom in the 5,6 dimethylbenzimidazole moiety of methylcobalamin. Thus, there is another equilibrium of methylcobalamin, shown in equation (4), in the aqueous solution besides that in equation (2). The methylation reaction may also take place between the 'base off with Hg(II)' form and $[Hg(II)Cl_n(OH)_m]^{2-n-m}$. However, the equilibrium in equation (4) is not the dominant equilibrium in relation to that in equation (2) due to low concentration of inorganic Hg(II). The reaction of the 'base on' form with $[Hg(II)Cl_n(OH)_m]^{2-n-m}$ is, in this reaction system, the predominant pathway in producing MMC. It has been reported that the 'base on' form reacts with inorganic Hg(II) at least 1000 times faster than 'base off with Hg(II)'.[20] It is widely accepted that the 'base on' form has higher activity than the 'base off' form [including 'base off with H^+' and 'base off with Hg(II)']. The difference in their reactivity is presumably due to the difference in electron density at the Co–C bond where the 'base on' form has a greater electron density because of the coordination of the benzimidazole nitrogen in the sixth ligand site.

There are two kinds of anionic ligand in the reaction systems: Cl^- and OH^-, which complexes with Hg^{2+}. However, the nucleophilicity of OH^- is stronger than that of Cl^-,[18] which is also demonstrated by the magnitude of the complexing stability constant with Hg^{2+}. In $[Hg(II)Cl_n(OH)_m]^{2-n-m}$, more complexing ligand and replacement of Cl^- by OH^- will reduce the electrophilicity of inorganic Hg(II). As a result, the effects of pH and salinity on the methylation reaction are due to the changes of electron density of the methyl donor and the electrophilicity of inorganic Hg(II). With the increase in pH from 1.0 to 3.0, more OH^- complexing with Hg(II) would reduce the nucleophilicity of inorganic Hg(II) and the reaction rate. However, the reaction rate still rise because of the increase of the 'base on' form which has higher reactivity than the 'base off' form. Base on this fact, it is easy to judge that pH dependency of the methylcobalamin is a dominant factor for methylation rate of inorganic Hg(II).

The kinetic experiments at two pH levels also explain that there are two entirely different reactions in different pH solutions because of their great difference in first order reaction rate. In the strongly acidic solution (pH 1.5), the methylation occurs between $[Hg(II)Cl_n]^{2-n}$ and 'base off with H^+' form. However, $[Hg(II)(OH)_m]^{2-m}$ is methylated by the 'base on' form in the reaction system at pH 5. The methylation rate at pH 5.0 is over 20 times faster than that at pH 1.5. It

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validates the conclusion by DeSimone,[20] which is that the 'base on' form has higher activity than the 'base off' form.

CONCLUSION

A sensitive HPLC-UV-CV-AFS method was applied in studying the methylation of inorganic Hg(II) by methylcobalamin in aqueous systems. This methylation reaction has been shown to occur even in the absence of the enzyme. It is proposed that inorganic Hg(II) acts as an electrophile and attack on methylcobalamin, resulting in the transfer of carbanion methyl group from methylcobalamin to the higher oxidized state of Hg(II). The salinity and pH of the reaction system influence this methylation reaction because of their effects on the species of the reactants. The salinity and the pH change the electron density of the methyl donor and the electrophilicity of the metal ions in the reaction system. The methylation reaction of inorganic Hg(II) with methylcobalamin is fast and first-order for Hg(II). This methylation reaction has been shown to be of importance in aquatic environments, even in the presence of Cl^-, since it can still occur under high salinity conditions (salinity between 0.5 and 1.0 M).

Acknowledgments

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