The Role of Anion in the Demethylation Reaction of Methylcobalamin with Mercuric Salts

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The demethylation of methylcobalamin with mercuric salts containing ClO_4^-, AcO^-, Cl^-, Br^-, SCN^-, or CN^- was kinetically investigated by a conventional spectroscopic technique. The rates of reaction for mercuric chloride were proportional to $[CH_3\text{-Co}]$ and $[HgCl_2]$ while those for acetate depended little on $[Hg(AcO)_2]$, but increased in proportion with $[AcO^-]$. The rates of reaction varied depending on the concentration and species of anion, and the following sequence was obtained: $AcO^- \gg Cl^- \geq SCN^- \geq Br^- \gg CN^-$. The sequence was reversely correlated with the stability constant of the equilibrium reaction, $HgL_{n-1}+L \rightleftarrows HgL_n$ ($n=1$ or 2), so that it was considered that HgL^+ is an attacking electrophile in the cleavage of cobalt-carbon bond by the mercuric salts. The activity of $HgCl_4^{2-}$ for the reaction was somewhat lower than that of $HgCl_2$ while that of HgClCN was lower to a greater extent.

Mercuric salts are known to be active for the cleavage of cobalt-carbon bond. Halpern and Maher[1] reported, as the first demonstration of this kind of reaction, the formation of methylchloromercury from the reaction of mercuric chloride with methylpentacyanocobaltate(III). Since then, the demethylation reaction of alkylcobalamins and their model compounds with mercuric salts has been investigated by a number of groups,[2-10] especially in connection with organo mercury poisoning.

So far, however, the authors have been primarily concerned with the identification of reaction products, the relationship between the reaction rates and the size of alkyl groups, and/or the influence of the sixth ligand of the alkyl cobalt complexes with respect to the demethylation reaction. Only a little work has been carried out on the effect of mercuric salt species on the reaction by Bertilsson and Neujahr,[9] who described the efficiency of some organo mercuric salts ($HgCl_2$, CH_3HgCl, C_6H_5HgCl, $CH_3COCH_2CH_2HgBr$, C_6H_5-HgOH, CH_3Hg dicyandiamide) for the reaction.

We have therefore studied the effect of anion in mercuric salts on the demethylation reaction. In this paper we describe the reaction features on using ClO_4^-, AcO^-, Cl^-, Br^-, SCN^-, or CN^- as ligands for the mercuric ion, the relationship between the reaction rates and the stability constants of mercuric complexes, and a possible reaction mechanism.

Experimental

Materials. Cyanocobalamin (*Daiichi Pure Chemical*) was used to synthesize methylcobalamin according to the accepted procedure.[11] The methylcobalamin sample was further purified on Amberlite XAD-2 resin. Its purity was checked by paper chromatography on Toyo Filter Paper No. 51 with 1-butanol–2-propanol–acetic acid–water (100-70-1-100, *v/v*) as a solvent.[12]

The concentration of methylcobalamin thus prepared was estimated from spectral data of the Müllers.[13] The sample of methylcalbalamin solution was transferred little by little into a micro tube and preserved in a frozen state. The sample tube was defrosted just before use.

Mercuric chloride and acetate (Koso Chemical GR) were used without further purification. Mercuric perchlorate was prepared from mercuric chloride and silver perchlorate (Kojima Chemical, GR). All the other chemicals used were commercial (GR).

Procedures. Fresh solutions of methylcobalamin and of mercuric salts were made up for a few experiments at a time. The reaction was started by adding a suitable amount of mercuric salt solution to 3 ml of methylcobalamin solution with a micro syringe (Jintan Thermo) and by stirring with a Teflon bar. The spectrum change in the range 340—600 nm was then traced with a Hitachi Recording Spectrophotometer EPS-3T. Finally, the spectrum of completely demethylated sample was obtained by 3 min irradiation of the reaction system with a 100 W projecter lamp.

Demethylation reactions were performed usually with excess mercuric salts over methylcobalamin in the cell of spectrophotometer around room temperature. The concentration range was $40—50\times10^{-6}$ M for methylcobalamin and $400—1600\times10^{-6}$ M for mercuric salts.

Results and Discussion

Mercuric Chloride. Since mercuric chloride reveals no spectral absorption in the region 340—600 nm, it was possible to observe the rates of demethylation of methylcobalamin by measuring the optical absorbance, A, of methylcobalamin at 350 nm. The spectral changes due to the demethylation reaction, $CH_3\text{-Co} \rightarrow H_2O\text{-Co}$, appeared to progress with isosbestic points.

When the natural logarithms of the absorbance change $\ln\{(A-A_\infty)/(A_0-A_\infty)\}$ were plotted against time, a straight line was obtained, A_0 and A being the absorbance at time zero and infinity (or irradiated), respectively. The slope of the straight line, denoted as k_1, will be used as a rate parameter. The dependence of k_1 on the concentration of mercuric chloride was then investigated. It was found that k_1 increases in proportion to the concentration of mercuric chloride. The results are shown in Figs. 1 and 2. Thus, the demethylation rate, r, for mercuric chloride satisfies the following rate law:

$$r = k[CH_3\text{-Co}][HgCl_2] \quad (1)$$

Methylcobalamin is known to conform to different molecular forms depending on the pH.[14] It exists as a "base-on" form in which benzimidazole is axially coordinated while a "base-off" exists at low pH and hence protonated. The corresponding pK_a was re-

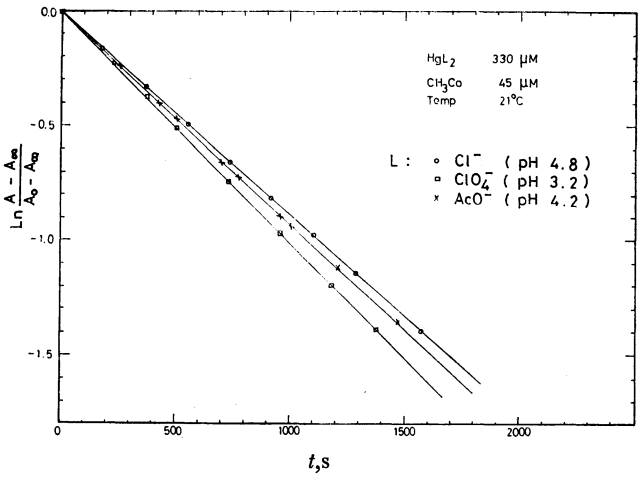


Fig. 1. Representative first-order plots for the demethylation of methylcobalamin with HgL_2.
□: L=ClO_4^-, pH=3.2, ×: L=AcO^-, pH=4.2.
○: L=Cl^-, pH=4.8
[HgL_2]=330 μM, [CH_3–Co]=45 μM, Reaction temperature 21 °C. A: measured at 350 nm

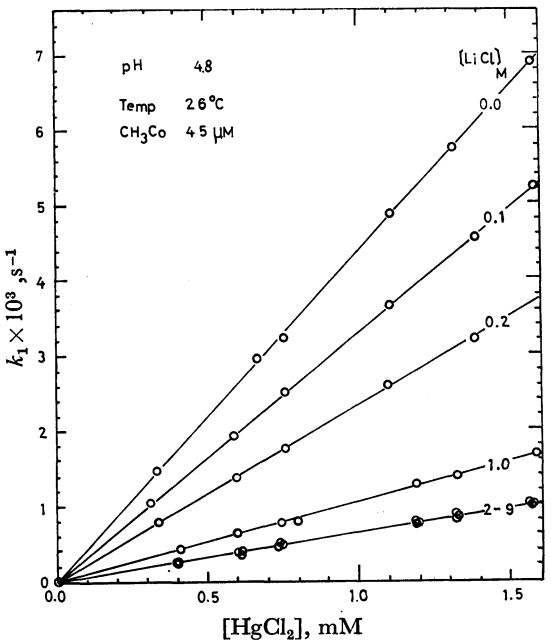


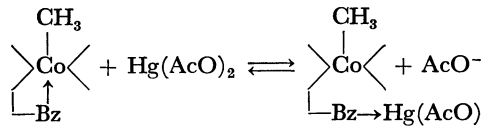
Fig. 2. Dependence of k_1 on the concentration of mercuric chloride and lithium chloride.
[CH_3–Co]=45 μM, pH=4.8. Reaction temperature 26 °C.

ported to be 2.72[14] so that the predominant species should be "base-on" forms above pH 3.5. Thus, the pH dependence of k_1 was investigated in the pH range 1—5 by adding perchloric acid, phosphoric acid or hydrochloric acid to the reaction system.

The spectra of methylcobalamin at the initial stage were explained by the reported pK_a value if pure "base-on" (λ_{max}=520 nm) and "base-off" forms (λ_{max}=460 nm) were respectively present at pH 5.0 and 1.0. Addition of hydrochloric acid to the reaction system usually caused a monotonous decrease of k_1; k_1 was about 25 times larger at pH 4 than at pH 1. A similar tendency was also observed with perchloric acid and phosphoric acid although the decreasing behavior was somewhat peculiar, being associated with the inexplicable action of oxo-acid anions.

The salt effect was investigated under a constant pH. No significant difference in k_1 was observed in 0.1—1 M acetate buffer (pH=4.2), while the effect of lithium chloride was remarkable; k_1 appeared to decrease gradually with increasing [LiCl], falling down to one-seventh of the initial k_1 (Fig. 2). The depression of k_1 at higher [LiCl] (2—9 M) approached a constant value. It might be associated with the formation of $(HgCl_4)^{2-}$.

Mercuric Acetate. The demethylation of methylcobalamin in the presence of mercuric acetate took place similarly as in the case of chloride; the spectral change due to the demethylation progressed with isosbestic points. It was found, however, that the "base-off" type of methylcobalamin is present during the reaction even at pH=4.2. From the results obtained by Desimore *et al.*[10] and by us, an equilibrium between base-off and base-on methylcobalamin upon addition of mercuric acetate can be written as follows:



(i)

The demethylation rates, as expressed by $\ln\{(A-A_\infty)/(A_0-A_\infty)\}$ *versus* time, gave a straight line similar to the case of mercuric chloride. However, unlike the chloride case, k_1 was little dependent on the concentration of mercuric acetate (0.2 power), increasing nearly in proportion to the concentration of acetate ion in the acetate buffer (Fig. 3). Thus, 1 mM mercuric acetate is far more active than 1 mM mercuric chloride for the cleavage of cobalt-carbon bond provided that acetate ions were present sufficiently in the solution. The rate of demethylation for mercuric acetate, r', is given by

$$r' \fallingdotseq k'[CH_3\text{–Co}][AcO^-] \quad (2)$$

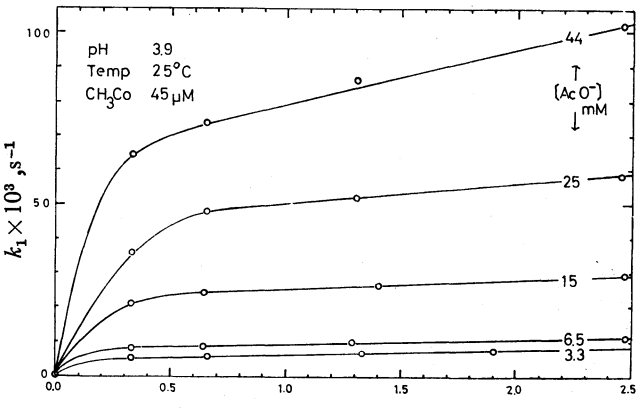
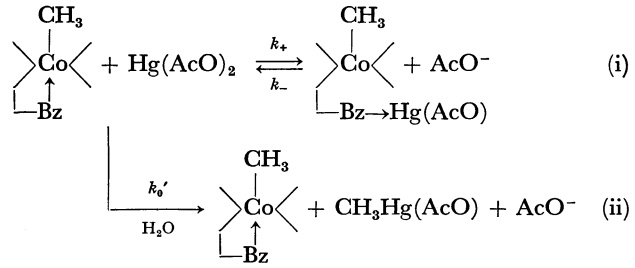


Fig. 3. Dependence of k_1 on the concentration of mercuric acetate and sodium acetate.
[CH_3–Co]=45 μM, pH=3.9, Reaction temperature 25 °C.

The effect of foreign anions was examined. When 3-fold excess of lithium chloride was present over 0.5 mM mercuric acetate in 44 mM acetate buffer, the reactivity of mercuric acetate was found to decrease remarkably, falling down nearly to that of mercuric chloride. Such a phenomenon can be interpreted if the anions of mercuric salt are replaced according to the stability constant of mercury complex (Table 1).

Desimore *et al.*[10] have so far investigated the mechanism of demethylation with mercuric acetate, based on the finding that methylcobiamide (a model compound of base-off methylcobalamin) is far less reactive than base-on methylcobalamin. In the light of their studies and our observations, the following reaction scheme is proposed.



If the equilibration (i) is fast as compared with (ii) and tends to the right-hand side, the rate of demethylation, r', can be approximately given by

$$r' = \frac{k_0'}{K}[CH_3\text{-}Co][AcO^-] \qquad (3)$$

where $K=k_+/k_-$ and $[CH_3\text{-}Co]$ is the total amount of methylcobalamin. The derived expression 3 is essentially the same as the observed one 2.

It is of interest to compare the intrinsic reactivity of mercuric acetate with that of mercuric chloride. For this the ratio k_0'/k was taken into consideration. It follows from Eqs. (1) and (3) that

$$\frac{k_0'}{k} = \frac{r'[CH_3\text{-}Co][HgCl_2]K}{r[CH_3\text{-}Co][AcO^-]} \qquad (4)$$

This can be transformed into an experimentally accessible one by introducing the rate parameter k_1 (k_1' is used for mercuric acetate):

$$\frac{k_0'}{k} = \frac{k_1'}{k_1}\cdot\frac{[HgCl_2]}{[Hg(AcO)_2]}\cdot\frac{K[Hg(AcO)_2]}{[AcO^-]} \qquad (5)$$

The right side of Eq. (5) consists of three factors. The first factor, k_1'/k_1, can be obtained from the observed data in Figs. 2 and 3, and the third one might correspond to the ratio of "base-off" to "base-on" methylcobalamin which can be estimated from the optical absorption.

When the second factor is unity or $[HgCl_2]=[Hg(AcO)_2]=1$ mM at $[AcO^-]=44$ mM, the first factor can be estimated to be *ca.* 20. The major part of methylcobalamin in the case of mercuric acetate was "base-off" so that equilibrium (i) shifted fairly to the right. From an inspection of the optical absorption we see that the third factor, $K[Hg(AcO_2]/[AcO^-]$ is *ca.* 10. Thus, we have

$$\frac{k_0'}{k} \geq 10^2$$

The result shows that mercuric acetate is about 100 times more reactive than mercuric chloride.

Mercuric Perchlorate. Addition of mercuric perchlorate to a methylcobalamin solution caused instantaneous formation of base-off form even at pH 4.5, followed by demethylation reactions. A good first-order plot was obtained in the lower concentration range (below 0.5 mM) as shown in Fig. 1 together with ones for mercuric chloride and acetate. However, at higher concentrations (above 0.5 mM) the plot was found to deviate from linearity, the rate decreasing. The reactivity of mercuric perchlorate was not studied further because of the complicated nature of the reaction.

Mercuric Cyanide, Bromide, and Thiocyanate. Since mercuric salts are less soluble in water, attempts were made to investigate their reactivities indirectly, *viz.* to investigate the effect of potassium cyanide, bromide and thiocyanate added to the reaction system of methylcobalamin and mercuric perchlorate. It was anticipated that mercuric salts in interest would be immediately formed.

30 μl of 0.1 M mercuric perchlorate was added to a 3 ml aqueous or acetate-buffered methylcobalamin solution containing 16 mM potassium salt, namely, CN^-, Br^-, or SCN^-. The role of Cl^- and AcO^- was also investigated in a similar manner. The rate parameter k_1 was obtained for each case (Table 1).

TABLE 1. KINETIC DATA FOR THE DEMETHYLATION OF METHYLCOBALAMIN WITH MERCURIC COMPLEXES AND THEIR STABILITY CONSTANTS

L	k_1 10^4 s^{-1} a)		Stability constants M^{-1} b)				
	in H_2O	in 0.2 M AcO^-	K_1	K_2	K_3	K_4	
AcO^-	260	$\geq 10^4$	10^5	10^4	—	—	15,16)
Cl^-	32	28	10^7	10^6	10	10	17)
Br^-	7.6	3.5	10^9	10^8	10^2	10^2	18)
SCN^-	8.9	5.4	10^9	10^7	10^3	10^2	15,19)
CN^-	<0.01	<0.01	10^{18}	10^{17}	10^4	10^3	15)

a) $CH_3\text{-}Co=40$ μM, $[L]=16$ mM, $[Hg(ClO_4)_2]=1$ mM, Reaction at 27 °C. b) $HgL_{n-1}+L \rightleftarrows HgL_n$, $K_n = \frac{[HgL_n]}{[HgL_{n-1}][L]}$

In the unbuffered reaction system, k_1 of acetate ion was in good agreement with that derived from mercuric acetate (Fig. 3) while k_1 of chloride ion was slightly less than that of mercuric chloride. It seems that the added anion rapidly combines with mercuric ion.

The k_1 value of Br^- and SCN^- did not differ much from that of mercuric chloride obtainable at high lithium chloride concentrations (≥ 2 M). Addition of CN^- (16 mM) inhibited the demethylation to a considerable extent.

In the 0.2 M acetate buffered solution without the addition of anions, demethylation took place too fast to be followed by a spectroscopic technique in accordance with the finding described. Rate parameter k_1 was also estimated like the unbuffered one for 16 mM Cl^-, Br^-, SCN^-, and CN^-. In these reactions methylcobalamin was of base-on form.

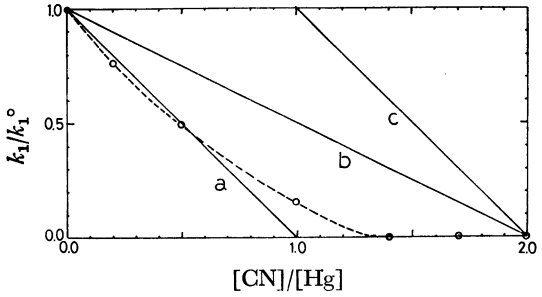


Fig. 4. Inhibition effect by cyanide ion.
···o···: observed values, a, b and c: see text.
k_1°: rate parameter for $HgCl_2$
k_1: rate parameter for $HgCl_2$–KCN
$[CH_3\text{–}Co]=45\ \mu M$, $[HgCl_2]=1$ mM, pH=5.0.
Reaction temperature 26 °C.

The inhibition of CN^- was further investigated in the concentration range 0—2 equivalent of KCN for the added $HgCl_2$ (1 mM). The result is shown by the open circles in Fig. 4, and compared with the solid lines a, b, and c which were drawn under the following assumptions (a), (b), and (c) and the anion replacement reactions iii, iv, and v.

$$HgCl_2 + CN^- \longrightarrow HgClCN + Cl^- \quad \text{(iii)}$$

$$HgClCN + CN^- \longrightarrow Hg(CN)_2 + Cl^- \quad \text{(iv)}$$

$$HgCl_2 + 2CN^- \longrightarrow Hg(CN)_2 + 2Cl^- \quad \text{(v)}$$

(a): Reaction iv occurs after the completion of Reaction iii. Reactivities of HgClCN and $Hg(CN)_2$ are negligibly small as compared with that of $HgCl_2$.

(b): Ligation of CN^- occurs according to Reaction v. $HgCl_2$ is much more reactive than $Hg(CN)_2$.

(c): Ligation of CN^- occurs according to (a). Both $HgCl_2$ and HgClCN have the same reactivity, $Hg(CN)_2$ being inactive.

As shown in Fig. 4, assumption (a) is closest to the observed one. It seems that HgClCN and $Hg(CN)_2$ are inactive for the cleavage of cobalt-carbon bond as compared with $HgCl_2$.

Reactivity and Stability Constant of Mercuric Complex. The stability constants of various mercury complexes in the literature[15–19] are shown in Table 1. From these values we can infer the main mercury species in the solution as well as the concentration of anions (shown in parentheses).

$Hg(ClO_4)_2$[15]	Hg^{2+}	ClO_4^- (<1M)
$Hg(AcO)_2$	$Hg(AcO)_2$	AcO^- (1—100 mM)
$HgCl_2$	$HgCl_2$	Cl^- (<100 mM)
$HgCl_2$	$(HgCl_4)^{2-}$	Cl^- (>1 M)
HgBr, $Hg(SCN)_2$, $Hg(CN)_2$	$(HgL_3)^-$ and/or $(HgL_4)^{2-}$	L^- (≥10 mM)

The transformation of $HgCl_2$ into $(HgCl_4)^{2-}$ was inferred when the concentration of Cl^- was increased from 100 mM to 2 M. This is in line with the fact that rate parameter k_1 for mercuric chloride decreased with increasing concentration of lithium chloride as shown in Fig. 2. Addition of mercuric perchlorate or mercuric acetate gave rise to base-off methylcobalamin even at pH 4 while the addition of mercuric chloride retained base-on methylcobalamin. It indicates that the axial benzimidazole can displace the anion in the mercuric complex to yield base-off form when the stability constant K_2 is less than $10^4\ M^{-1}$.

It has been pointed out that the reaction scheme and the type of mercuric complex really depend on the anion species present in the solution. In the light of these observations and in particular from the data shown in Table 1, the reactivities of HgL_2 for the cleavage of cobalt-carbon bond are estimated to be in the following sequence.

$$Hg(AcO)_2 \gg HgCl_2 \geq Hg(SCN)_2 \geq HgBr_2 \gg Hg(CN)_2$$

It is apparent that the above sequence is reverse to the stability constant K_2. It seems to indicate that the elimination of L from HgL_2 is required prior to the cleavage of cobalt-carbon bond. In this connection, it is noteworthy that an equivalent mixture of $HgCl_2$ and KCN is a less active reagent for the cleavage than $HgCl_2$. The cyanide in the mixed ligand complex, HgClCN, might strengthen the Hg–Cl bond.

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