

## A Chemical Model of Catechol-*O*-methyltransferase. Methylation of 3,4-Dihydroxybenzaldehyde in Methanol Solution<sup>1)</sup>

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The reaction of 3,4-dihydroxybenzaldehyde (LH<sub>2</sub>) and dimethyl sulfate (DMS) in forming *m*- and *p*-*O*-methylated products (vanillin and isovanillin, respectively) in a methanol buffer solution was studied kinetically as a chemical model of catechol-*O*-methyltransferase (COMT). The *O*-methylations, especially *m*-*O*-methylations, were catalyzed by divalent metal ions such as Cu(II), Mg(II) and Zn(II). A clear Mg(II) catalysis was observed for the first time in this medium. As Mg(II) is an important metal in the COMT catalyzed reaction *in vivo*, this observation is very interesting. Kinetic analyses of the present data and recalculation of a part of the previous data offered the following evidence. In Cu(II) catalysis, a 1:2 complex (CuL<sub>2</sub>) was more active than the 1:1 complex (CuL). On the other hand, in Mg(II) catalysis and Zn(II) catalysis, ML was more active than ML<sub>2</sub>. These facts show that ML<sub>2</sub> is not always more active than ML, contrary to previous reports. Methanolysis of DMS, a significant side reaction of this model reaction, and dissociation of LH<sub>2</sub> were studied thoroughly as bases for these kinetic analyses.

**Keywords** catechol-*O*-methyltransferase; enzyme model; 3,4-dihydroxybenzaldehyde; dimethyl sulfate; metal chelate; *O*-methylation

Catechol-*O*-methyltransferase (COMT, EC 2.1.1.6), one of the important enzymes in catecholamine metabolism, occurs widely in mammalian tissues and catalyzes the transfer of the methyl group of *S*-adenosyl-*L*-methionine (SAM) to a hydroxyl group of catechol or substituted catechols.<sup>2)</sup> The quantity of this enzyme is elevated in human breast cancers and catabolizes catecholestrogens, which exert opposite effects on hormone-sensitive breast cancer cell growth and differentiation, into methoxy metabolites.<sup>3)</sup> Mg<sup>2+</sup> is required for the full activity of this enzyme, and can be substituted by other divalent metal ions such as Mn<sup>2+</sup>, Co<sup>2+</sup> and Zn<sup>2+</sup>.<sup>2,4,5)</sup> The *O*-methylation *in vivo* occurs almost exclusively on the *m*-hydroxyl group of the catechols,<sup>6,7)</sup> but *in vitro* it occurs at one of the *m*- or *p*-hydroxyl groups.<sup>5,7,8)</sup> The role of Mg<sup>2+</sup> was proposed as bringing substrates and COMT together in the bridge complex<sup>5)</sup> and so on,<sup>9)</sup> but is not fully understood at present.

A chemical model of COMT, nonenzymatic *O*-methylation, was studied by Senoh *et al.*<sup>5)</sup> They accounted for the catalytic effects of metal ions in terms of the formation of a 2:1 catechol-metal complex (this complex is expressed as ML<sub>2</sub> in this paper). However, because of the lack of an easy analytical method, sufficient kinetic studies have not been done.

We studied the reaction of 3,4-dihydroxybenzaldehyde (LH<sub>2</sub>) and dimethyl sulfate (DMS) in forming the *m*- and *p*-*O*-methylated products (vanillin and isovanillin, respectively) in an aqueous buffer solution, which is one of the model reaction of Senoh *et al.*,<sup>5)</sup> kinetically.<sup>1)</sup> Kinetic studies showed that CuL<sub>2</sub> was an active species. This was the same result that Senoh *et al.* showed.<sup>5)</sup> However, Zn-catalysis couldn't be analyzed thoroughly because of a lack of reliable stability constants of ZnL and ZnL<sub>2</sub>. This medium had two defects. One was the presence of a significant side reaction, that is, the rapid hydrolysis of DMS.<sup>1,10)</sup> Therefore, the amounts of *O*-methylated products were very small. The other was a low stability of the Mg(II) complex of LH<sub>2</sub>, with Mg(II) showing only a small catalytic effect. To overcome these defects, the same

reactions in a methanol solution were studied.

### Experimental

The water content of methanol used was about 0.035% (w/v) (about 20 mM) by means of Karl Fischer's method. The methanol solution of hydrogen chloride was prepared as follows. Hydrogen chloride gas produced from conc. HCl-conc. H<sub>2</sub>SO<sub>4</sub> was desiccated through conc. H<sub>2</sub>SO<sub>4</sub> and dissolved in methanol. Other chemicals were the same as described in the previous paper.<sup>1)</sup>

Kinetic runs were carried out in 0.2 M bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane (Bis-Tris)-0.02 M HCl methanol buffer solution unless otherwise stated. The kinetic procedures were described in the previous paper.<sup>1)</sup> After removal of the solvent and the addition of water, the aqueous solution was treated in a similar manner to that described in the previous paper.<sup>1)</sup>

Conditions of the HPLC measurement were essentially the same as described in the previous paper.<sup>1)</sup>

### Results and Discussion

**Basic Study** The effect of the concentration of HCl in the buffer was studied in the absence of metal ions. The concentration of *O*-methylated products at the completion of the reaction (*S*<sub>∞</sub>) decreased with an increasing concentration of HCl, as shown in Fig. 1. The *m*-/*p*- ratio increased slightly. With an increasing concentration of HCl, the slope

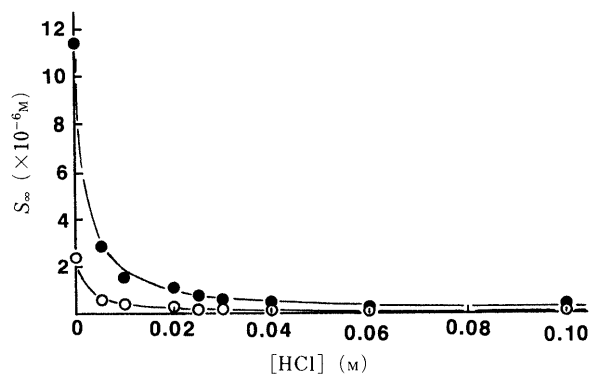


Fig. 1. *O*-Methylation of LH<sub>2</sub> as a Function of the Concentration of Added HCl at 37 °C in 0.2 M Bis-Tris Methanol Solution

Open circles, vanillin; closed circles, isovanillin. Lines are best fit curves of  $y = a/(x + b) + c$ . [LH<sub>2</sub>]<sub>0</sub> = [DMS]<sub>0</sub> = 2.5 mM.

TABLE I.  $S_\infty$  and First-Order Rate Constants ( $k_d$ ) of Methanolysis of DMS Calculated from the Time Dependence of *O*-Methylation

$T$ (°C)	$[\text{LH}_2]_0$ (mM)	$[\text{DMS}]_0$ (mM)	$[\text{M(II)}]_T^a$ (mM)	$n$	$S_\infty$ ( $\mu\text{M}$ )		$k_d^b$ ( $\times 10^{-5} \text{ s}^{-1}$ )
					Van	IVan	
30	5	2.5	0	2	$0.60 \pm 0.03$	$2.16 \pm 0.25$	$3.73 \pm 0.48$
37	2.5	2.5	0	2	$0.30 \pm 0.02$	$1.10 \pm 0.01$	$7.74 \pm 0.86$
	2.5	5	0	2	$0.67 \pm 0.01$	$2.39 \pm 0.05$	$8.08 \pm 0.46$
	5	2.5	0	2	$0.51 \pm 0.06$	$1.97 \pm 0.17$	$7.98 \pm 1.02$
	2.5	2.5	5 (Cu)	1	2.45	1.93	$7.94 \pm 0.01$
	2.5	2.5	10 (Cu)	1	2.71	1.96	$7.21 \pm 0.95$
	2.5	2.5	15 (Cu)	1	2.98	2.23	$7.85 \pm 0.22$
	2.5	2.5	5 (Mg)	1	1.87	1.82	$8.02 \pm 0.31$
	2.5	2.5	10 (Mg)	1	2.78	1.74	$7.63 \pm 0.66$
	2.5	2.5	15 (Mg)	1	3.05	2.14	$8.56 \pm 0.91$
	2.5	2.5	5 (Zn)	1	0.93	1.60	$7.23 \pm 0.07$
	2.5	2.5	10 (Zn)	1	2.18	1.76	$6.85 \pm 0.26$
	2.5	2.5	15 (Zn)	1	3.71	2.63	$7.21 \pm 0.35$
	2.5	2.5	20 (Zn)	1	5.33	2.88	$7.25 \pm 0.75$
	44	5	2.5	0	1 <sup>d)</sup>	0.43	1.47
5		2.5	0	1 <sup>e)</sup>	0.48	1.77	$8.37 \pm 0.38$
5		2.5	0	1 <sup>f)</sup>	0.55	1.69	$8.18 \pm 0.83$
5		2.5	0	3	$0.55 \pm 0.03$	$2.22 \pm 0.13$	$15.06 \pm 2.26$

Experiments were done in 0.2 M Bis-Tris-0.02 M HCl methanol buffer solution, unless otherwise stated. Values are mean  $\pm$  S.D. a) The metal salts used were hexahydrates,  $\text{Cu}(\text{ClO}_4)_2 \cdot 6\text{H}_2\text{O}$ ,  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  and  $\text{Zn}(\text{ClO}_4)_2 \cdot 6\text{H}_2\text{O}$ . b) As values of  $k_d$  were obtained from both Van and IVan formation, the number of data is two-fold  $n$ . c) The average value of  $k_d$  ( $n=16$ ) at 37°C is used for calculations in this paper. d) Concentration of HCl in the buffer solution is 0.03 M. e) Concentration of added  $\text{H}_2\text{O}$  is 60 mM. f) Concentration of added  $\text{H}_2\text{O}$  is 120 mM.

of the  $S_\infty$  curve decreased. This fact assured the constancy of the reactivity of  $\text{LH}_2$  at a high HCl concentration. On the other hand, at a high HCl concentration, the catalytic effects of divalent metal ions decreased (data not presented). From these contrary facts, 0.02 M HCl was used as the appropriate buffer concentration.

The time dependence of the formation of *O*-methylated products was essentially the same as described in the previous paper,<sup>1)</sup> both in the presence and in the absence of divalent metal ions. That is, the formation of vanillin (Van) and isovanillin (IVan) followed first-order kinetics. The observed first-order rate constants ( $k_{\text{obs}}$ ) calculated from Van formations were almost the same as those of the IVan formation. The total yields of Van and IVan at the completion of the reaction were less than 1% of the amount expected from the yields of  $\text{LH}_2$  and DMS. Although the value of  $k_{\text{obs}}$  is more than 10 times smaller than in aqueous solution, these phenomena were essentially identical with those in aqueous solution and were explained in terms of the rapid solvolysis of DMS. Therefore,  $k_{\text{obs}}$  can be presented by  $k_d$ , which is the first-order rate constant of the solvolysis of DMS.<sup>1)</sup> The values of  $k_d$  and  $S_\infty$  are shown in Table I. Although the values of  $S_\infty$  and  $m/p$ -ratios were dependent on the conditions, the values of  $k_d$  were almost completely consistent if the temperature remained constant. Because only a small effect was caused by the addition of a small amount of water, the main solvolysis was methanolysis. Temperature affected the values of  $k_d$  clearly, but affected the values of  $S_\infty$  ambiguously. The Arrhenius plot of the values of  $k_d$  was almost linear. The activation parameters of the methanolysis of DMS are shown in Table II.

Kolesnikov *et al.* studied the methanolysis of DMS and reported that methanolysis produced monomethyl sulfate (MMS) and dimethyl ether, but further methanolysis of MMS didn't occur.<sup>11)</sup> The values of the first-order rate

TABLE II. Values of  $k_d$  and Activation Parameters of Methanolysis of DMS at 37°C

Source	$k_d$ ( $\text{s}^{-1}$ )	$E_a$ ( $\text{kJ mol}^{-1}$ )	$\Delta H^\ddagger$ ( $\text{kJ mol}^{-1}$ )	$\Delta S^\ddagger$ ( $\text{JK}^{-1} \text{ mol}^{-1}$ )
This work <sup>a)</sup>	$7.71 \times 10^{-5}$	79.7	77.1	-75.3
Ref. 11 <sup>b)</sup>	$6.07 \times 10^{-5}$	83.2	80.7	-65.9

a) Reproduced and calculated from the data in Table I. b) Calculated from the data at 35°C in ref. 11.

constant and the activation parameters at 37°C calculated from their data were almost the same as the values of this work, as shown in Table II. These facts support the validity of the interpretation of the time dependence of *O*-methylation, as mentioned above.

In the methanol solution, methanolysis of DMS, a significant side reaction, was observed. Therefore, kinetics were discussed in terms of the initial rate ( $V_0$ ) of *O*-methylation calculated as follows.<sup>1)</sup>

$$V_0 = S_\infty \cdot k_d \quad (1)$$

In the absence of metal ions,  $S_\infty$  was measured at various concentrations of  $\text{LH}_2$  and DMS as shown in Fig. 2 at 37°C. The values of  $k_d$  are almost constant, and  $S_\infty$  is almost proportional to the initial concentration of these substances,  $[\text{LH}_2]_0$  and  $[\text{DMS}]_0$ , except at excess  $[\text{DMS}]_0$ . Therefore,  $V_0$  can be expressed as

$$V_0 = k_1 [\text{LH}_2]_0 [\text{DMS}]_0 \quad (2)$$

where  $k_1$  indicates the second-order rate constant, and the values are listed in Table III. At excess  $[\text{DMS}]_0$ , the constancy of  $[\text{H}^+]$  may not be maintained during the reaction. The values of  $k_1$  at different temperatures were calculated from the data in Table I and Eqs. 1 and 2, and the results are shown in Table III. Although the Arrhenius

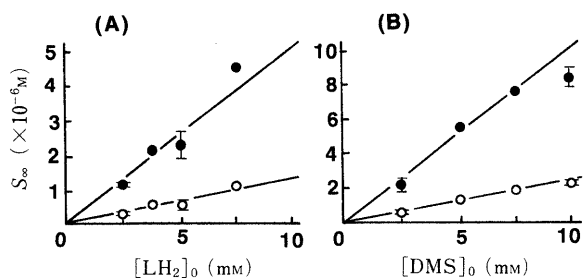


Fig. 2. *O*-Methylation of LH<sub>2</sub> as a Function of the Concentration of the Reactants, LH<sub>2</sub> (A) or DMS (B), at 37°C in Methanol Buffer Solution

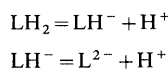
Open circles, vanillin; closed circles, isovanillin. Error bars represent S.D. of mean, and lines are best fit lines of  $y = ax$ . (A): [DMS]<sub>0</sub> = 2.5 mM. (B): [LH<sub>2</sub>]<sub>0</sub> = 5 mM. Lines were obtained without the data of [DMS]<sub>0</sub> = 10 mM.

TABLE III. Apparent Second-Order Rate Constants ( $k_1$ )

$T$ (°C)	$k_1$ ( $\times 10^{-5} \text{ M}^{-1} \text{ s}^{-1}$ )	
	Van	IVan
30	0.179	0.643
37	0.400	1.57
44	0.659	2.68

plots of the values were almost linear in both Van and IVan formation, activation parameters weren't calculated. Because, as  $k_1$  is an apparent constant, the calculation is meaningless.

In this reaction system, the dissociation equilibrium can be written as follows.



Dissociation constants and total concentration of LH<sub>2</sub> are defined as follows.

$$K_{a1} = \frac{[\text{LH}^-][\text{H}^+]}{[\text{LH}_2]} \quad (3)$$

$$K_{a2} = \frac{[\text{L}^{2-}][\text{H}^+]}{[\text{LH}^-]} \quad (4)$$

$$[\text{LH}_2]_T = [\text{L}^{2-}] + [\text{LH}^-] + [\text{LH}_2] \quad (5)$$

$[\text{LH}_2]_T$  is equal to  $[\text{LH}_2]_0$  at  $t=0$ , and is kept almost constant during the reaction. Therefore, Eq. 2 is replaced by Eq. 6.

$$V_0 = k_1[\text{LH}_2]_T[\text{DMS}]_0 \quad (6)$$

On the other hand,  $V_0$  can be expressed using concentrations of three species of LH<sub>2</sub> as follows.

$$V_0 = (k_{a1}[\text{L}^{2-}] + k_{a2}[\text{LH}^-] + k_{a3}[\text{LH}_2])[\text{DMS}]_0 \quad (7)$$

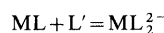
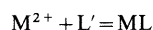
In this equation,  $k_{a1}$ ,  $k_{a2}$ , and  $k_{a3}$  are second-order rate constants of each species, respectively. Eq. 7 is replaced by Eq. 8 using Eqs. 3–5.

$$V_0 = \frac{(k_{a1}K_{a1}K_{a2} + k_{a2}K_{a1}[\text{H}^+] + k_{a3}[\text{H}^+]^2)}{(K_{a1}K_{a2} + K_{a1}[\text{H}^+] + [\text{H}^+]^2)}[\text{LH}_2]_T[\text{DMS}]_0 \quad (8)$$

From the comparison of Eqs. 6 and 8,  $k_1$  is equal to the part of  $\{ \}$  in Eq. 8. Therefore,  $k_1$  is an apparent second-order rate constant. Determination of the exact concentration of  $[\text{H}^+]$  in this methanol buffer solution is difficult technically. Therefore, determination of these constants in

Eq. 8 by the method of least squares is difficult. Because the difference of HCl concentration in the buffer solution caused almost no effect to the values of  $k_d$  as shown in Table I,  $V_0$  is inferred to be proportional to  $S_\infty$ . The simulation of Eq. 8 accounts for Fig. 1, approximately, and shows  $K_{a1} \gg K_{a2}$  and  $k_{a1} \gg k_{a2}, k_{a3}$ . This order of rate constants is accordant with the result of Senoh *et al.*<sup>5)</sup> This simulation is well expressed by a more simple but less rational equation,  $y = a/(x+b) + c$ , which is fitted to the data by the method of least squares for a rough estimate of  $S_\infty$  variation.

**Kinetics of the Metal Iron Catalysis**  $S_\infty$  was measured in the concentrations of  $[\text{LH}_2]_0 = [\text{DMS}]_0 = 2.5 \text{ mM}$  and various concentrations of metal ions, and  $V_0$  was calculated according to Eq. 1. The results are shown in Fig. 3. The *O*-methylations, especially *m*-*O*-methylations, were catalyzed by divalent metal ions. Variations of  $V_0$  in both Cu(II) and Zn(II) catalysis were almost the same as those in aqueous solution.<sup>1)</sup> On the other hand, the Mg(II) catalysis was clearly observed for the first time in this medium. As Mg(II) is an important metal in the COMT catalyzed reaction *in vivo*, this observation is very interesting. These effects were explained in terms of the formation of the complexes, ML and  $\text{ML}_2^{2-}$ , as follows.



In these equations, L' is free L not combined with any metal, and denotes a mixture of  $\text{L}^{2-}$ ,  $\text{LH}^-$  and  $\text{LH}_2$ . In the following description,  $\text{M}^{2+}$  and  $\text{ML}_2^{2-}$  are abbreviated as M and  $\text{ML}_2$ , respectively. In a given buffered methanol solution, the conditional stability constants are defined as follows.

$$K_1 = \frac{[\text{ML}]}{[\text{M}][\text{L}']} \quad (9)$$

$$K_2 = \frac{[\text{ML}_2]}{[\text{ML}][\text{L}']} \quad (10)$$

Total concentration of M and L are expressed as follows.

$$[\text{M}]_T = [\text{M}] + [\text{ML}] + [\text{ML}_2] \quad (11)$$

$$[\text{L}]_T = [\text{L}^{2-}] + [\text{LH}^-] + [\text{LH}_2] + [\text{ML}] + 2[\text{ML}_2] \\ = [\text{L}'] + [\text{ML}] + 2[\text{ML}_2] \quad (12)$$

$[\text{L}]_T$  is equal to  $[\text{LH}_2]_0$  at  $t=0$ , and is kept almost constant during the reaction. Therefore, in the metal ion-catalyzed reactions,  $V_0$  can be expressed as follows,

$$V_0 = (k_1[\text{L}'] + k_2[\text{ML}] + k_3[\text{ML}_2])[\text{DMS}]_0 \quad (13)$$

where  $k_2$  and  $k_3$  are second-order rate constants, respectively.

It is necessary to know the values of  $K_1$  and  $K_2$  for the analyses of these kinetics. Although ultraviolet absorbance at about the 350 nm region increased with the formation of complexes ML and  $\text{ML}_2$ ,<sup>12)</sup> reliable stability constants were not obtained. Therefore, these metal ion catalyses were analyzed by the method of nonlinear least squares from the data in Fig. 3 using Eqs. 9–13 directly. Previously reported Zn(II) catalysis in aqueous solution<sup>1)</sup> was inserted again in Fig. 4, in which reliable stability constants were not obtained, and was analyzed similarly. From Figs. 3A, 3B and 4, sets of values of  $K_1$ ,  $K_2$ ,  $k_2$  and  $k_3$  were obtained from Van formation, but not from IVan formation because

of the relatively small variation and large deviation of  $V_0$ . Therefore,  $k_2$  and  $k_3$  of IVan formation were calculated using  $K_1$  and  $K_2$  obtained from Van formation. Results are shown in Table IV. In these figures, the calculated curves fitted well in the data, and these facts support the proposed mechanism mentioned above. In Cu(II) catalysis, the order of magnitude of the constants in *m*-*O*-methylation is  $k_2 < k_3/2$ , and a prominently convex  $V_0$  curve is observed similar to that observed in aqueous solution.<sup>1)</sup> Since  $k_3$  is the constant of  $ML_2$ ,  $k_3/2$  was compared with  $k_2$  of the

constant of ML. In Mg(II) catalysis, the order is  $k_2 > k_3/2$ , and a slightly convex  $V_0$  curve is observed. In Zn(II) catalysis in aqueous solution, the order is  $k_2 \gg k_3/2$ , and a sigmoidal  $V_0$  curve is observed. These relationships between the order of rate constants and the shape of the  $V_0$  curve can generally be simulated using Eqs. 9–13. On the other hand, from Fig. 3C, no set of these values was obtained from either Van or IVan formation because of a relatively large deviation. In Zn(II) catalysis in methanol solution, a sigmoidal  $V_0$  curve was observed; the order is inferred to be  $k_2 \gg k_3/2$ .

In conclusion, the observation of clear Mg(II) catalysis in methanol solution is an important progress in the study of the COMT model, though the amount of *O*-methylated products were very small similarly to the aqueous solution.<sup>1)</sup> On the other hand, if metal complexes were formed, the difference of the solvents, water or methanol, was not considered to be large. In Cu(II) catalysis,  $ML_2$  is more active than ML in *m*-*O*-methylation. As  $ML_2$  is an anionic species, the nucleophilicity of the *m*-phenoxide ion<sup>5)</sup> may be increased. On the other hand, in Mg(II) catalysis and Zn(II) catalysis, ML is more active than  $ML_2$ . In these cases an alternative interpretation is needed, but distinct interpretation is not possible because of the lack of sufficient data. This difference of metal ions shows that  $ML_2$  is not always more active than ML, contrary to the conclusions of previous reports.<sup>1,5)</sup> Further, we are studying the effect of another bidentate ligand(R) to this chemical model.<sup>13)</sup> In the presence of R, the formation of the ternary complex MLR, which is a good model of a bridge complex<sup>5)</sup> and so

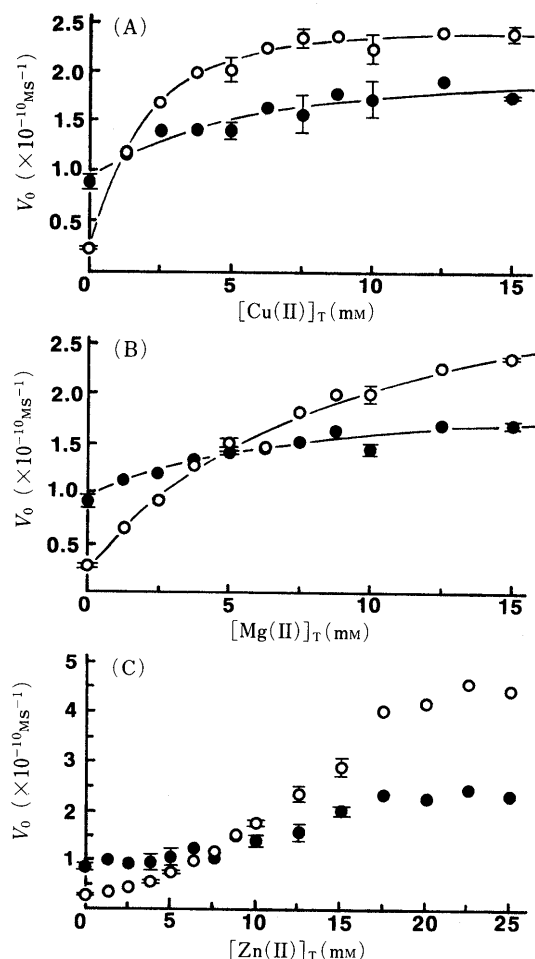


Fig. 3. M(II)-Catalyzed *O*-Methylation of  $LH_2$  at 37°C in Methanol Buffer Solution

Open circles, vanillin; closed circles, isovanillin. Error bars represent S.D. of mean, and lines are best fit curves calculated by Eqs. 9–13 with parameters in Tables III and IV.  $[LH_2]_0 = [DMS]_0 = 2.5$  mM (A): Cu(II)-catalyzed (B): Mg(II)-catalyzed (C): Zn(II)-catalyzed. Best fit curves were not obtained.

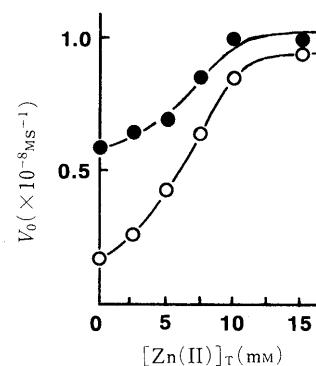


Fig. 4. Recalculation of Zn(II)-Catalyzed *O*-Methylation of  $LH_2$  at 37°C in Aqueous Buffer Solution<sup>1)</sup>

Open circles, vanillin; closed circles, isovanillin. Lines are best fit curves calculated by Eqs. 9–13 with parameters in Table IV and other parameters in the previous report.<sup>1)</sup>  $[LH_2]_0 = [DMS]_0 = 10$  mM.

TABLE IV. Conditional Stability Constants ( $K_1$ ,  $K_2$ ) and Second-Order Rate Constants ( $k_2$ ,  $k_3$ ) at 37°C

Metal	Medium	Source	Product	Stability const.		Rate const. ( $\times 10^{-5} M^{-1} s^{-1}$ )	
				$\log K_1$	$\log K_2$	$k_2$	$k_3$
Cu(II)	Methanol	Fig. 3A	Van	2.53	2.04	3.73	26.4
			IVan		N.D.	3.36 <sup>a)</sup>	3.23 <sup>a)</sup>
Mg(II)	Methanol	Fig. 3B	Van	2.37	3.21	8.87	0.405
			IVan		N.D.	4.33 <sup>a)</sup>	3.10 <sup>a)</sup>
Zn(II)	Aqueous	Fig. 4	Van	4.22	1.93	9.66	0.117
			IVan		N.D.	10.37 <sup>a)</sup>	8.53 <sup>a)</sup>

These values were obtained by the method of non-linear least squares. N.D., not determined. a) Calculated using the values of  $K_1$  and  $K_2$  obtained from the data of Van formation.

on,<sup>9)</sup> is expected. Details of the study will be reported shortly. In view of MLR, the R of ML is solvent. For a better chemical model of COMT, a combination of M(II) and R will be important.

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