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The Role of Cations in Non-enzymatic and Enzymatic O-Methylations of Catechol Derivatives[1a]

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The non-enzymatic and enzymatic mono-O-methylations of certain catechol derivatives produce mixtures of isomeric mono-O-methyl ethers in a ratio dependent on *p*H and on the type of added bivalent metal cation. In enzymatic O-methylations with O-methyltransferase the total yield of O-methylation products as a function of metal ions *decreases* in the order: $Mg^{++} > Zn^{++} > Mn^{++} > Co^{++} > Ni^{++} > Fe^{++} > Cu^{++}$. The effectiveness of bivalent cations in promoting non-enzymatic *m*-O-methylations with dimethyl sulfate *increases* in the order: $Mg^{++} < Zn^{++} < Mn^{++} < Co^{++} < Ni^{++} < Fe^{++} < Cu^{++}$. These observations are discussed in terms of the 2:1 catechol–metal complex IV and the enzyme-metal bridge complex V operative in the enzymatic and non-enzymatic O-methylation reactions.

Enzymatic O-methylation by catechol-O-methyltransferase has been shown to play a significant role in the metabolism of catecholamines such as dopamine, norepinephrine and epinephrine.[2]

Catechol-O-methyltransferase, located[3] in the soluble fraction of cells of various mammalian organs, catalyzes the transfer of the active methyl group of S-adenosylmethionine[4] to the *m*-hydroxyl group of catecholamines, in the presence of a divalent cation such as Mg^{++}. So far only *m*-O-methylated ether derivatives have been found as the metabolites of catecholamines. However, *in vitro* enzymatic O-methylation has been observed not only in the *m*-position, but also in the *p*-position of catecholamines in a ratio approximating 10 to 1.[5] At the present time the possibility of "paranephrine" metabolites of catecholamines cannot be discarded.

Substrates with *m*-directing groups in the side chain such as 3,4-dihydroxyacetophenone, arterenone and adrenalone, underwent enzymatic *p*-O-methylation not only *in vitro* (35–45%) but also *in vivo* to the extent of 10–25%. Even apparent methyl migration from *para* to *meta* and *vice versa* has been observed *in vivo*.[5]

The mechanism of this enzymatic methylation has been pictured as a displacement reaction on S-adenosylmethionine by the more nucleophilic hydroxyl group of a given catechol. The present study shows the influence of cations of various bivalent metals on the O-methylation reaction under non-enzymatic as well as enzymatic conditions.

Materials and Methods. A. Non-enzymatic Studies.—Commercial preparations such as 3,4-dihydroxybenzaldehyde were purified by repeated recrystallization. Other substrates such as 3,4-dihydroxyacetophenone[6] and the two isomeric mono-O-methyl ethers[7] were synthesized by standard methods.

For non-enzymatic methylation studies 300 μmoles of the substrate in 10 ml. of 0.5 *M* phosphate buffer was allowed to react with an equivalent amount of dimethyl sulfate in 1 ml. of ethanol–chloroform (1:9 v./v.) solution under nitrogen and vigorous agitation of the heterogeneous system for 5 min. at 60°.

The reaction mixture was acidified (*p*H <2) with hydrochloric acid and the methoxy compounds formed were extracted with a total of 10 ml. of water-saturated chloroform on the shaking machine for 10 min. The phenolic components were re-extracted from the chloroform solution into 10 ml. of 0.1 *N* aqueous sodium hydroxide by the same procedure. The aqueous alkaline solution was adjusted to *p*H <2 by the addition of hydrochloric acid. The aqueous solution was extracted once more with 10 ml. of chloroform for 10 min., the chloroform layer was washed with distilled water. This repeated extraction process led to the quantitative separation of the methoxy ethers which stay in the chloroform phase. Because of its favorable partition coefficient, chloroform in this system was found to be superior to all other solvents (*e.g.*, benzene[5]) tested. Finally the residue of the evaporated chloroform extract was extracted into 10 ml. of 0.1 *N* NaOH; this solution was directly used for the ultraviolet spectrophotometric assay of the ratio of isomers and for the determination of the total yield of O-methylated products. This extraction method was also used for the assay of the enzymatic O-methylation mixture using only 5-ml. portions of chloroform and 0.1 *N* NaOH.

Method for the Calculation of the Yields of O-Methylated Products.—The 0.1 *N* NaOH solution containing the O-methylated products was made up to an arbitrary total volume and, in the case of acetovanillone–acetoisovanillone, the extinctions at λ_{max} 247–248 mμ and at λ_{max} 343–349 mμ were determined. The total yield of methylation products was calculated as follows:

V: total vol. (in ml.) of the 0.1 *N* NaOH soln. of O-methylated prod.

E_1: extinction at λ_{max} 247–248 mμ of the 0.1 *N* NaOH soln.

E_2: extinction at λ_{max} 343–349 mμ of the 0.1 *N* NaOH soln.

The ratio of *m*- and *p*-isomers in the total mixture of O-methylated compounds was determined with the aid of the calibration curve[5]: *meta:* *a*%, *para:* (100 − *a*)% (Fig. 1).

The molecular extinction coefficient of acetovanillone in 0.1 *N* NaOH at λ_{max} 248 mμ is ε 8,900, at λ_{max} 343 mμ, ε 21,400, and that of acetoisovanillone at λ_{max} 247 mμ is ε = 21,900 and at λ_{max} 349 mμ, ε 5,500.

The *apparent* molecular extinction coefficients of a mixture of acetovanillone and acetoisovanillone (*meta* *a*%, *para* (100-*a*)%) at λ_{max} 247–248 mμ and λ_{max} 343–349 mμ, respectively, is obtained from the equation

$$\epsilon'_{247-248\ m\mu} = 8{,}900 \times \frac{a}{100} + 21{,}900\left(1 - \frac{a}{100}\right) = 21{,}900 - 130a$$

(1) (a) Presented in part at the 14*th* Annual Meeting of the Chemical Society of Japan (Tokyo) *cf.* Siro Senoh, Yaeko Inoue (Tokuyama), Abstract of Papers, 334 (1961): and at the 13th Symposia on Enzyme Chemistry (Tokyo); Proceedings of Papers, 22 (1961). (b) Visiting Professor, Kyoto University, April-May, 1961.

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(7) T. Reichstein, *Helv. Chim. Acta*, **10**, 392 (1927).

$$\epsilon'_{343-349\ m\mu} = 21{,}400 \times \frac{a}{100} + 5{,}500\left(1 - \frac{a}{100}\right) = 5{,}500 + 159a$$

The total yield of O-methylated products is

$$\text{yield} = \frac{E_1 \times V}{\epsilon'_{247-248\ m\mu} \times 10^3}\ \text{mole} = \frac{E_1 \times V \times 10^3}{\epsilon'_{247-248\ m\mu}}\ \mu\text{mole}$$

or

$$= \frac{E_2 \times V}{\epsilon'_{343-349\ m\mu} \times 10^3}\ \text{mole} = \frac{E_2 \times V \times 10^3}{\epsilon'_{343-349\ m\mu}}\ \mu\text{mole}$$

The yield of a mixture (total volume V ml. in 0.1 N NaOH) of vanillin (b%) and isovanillin ($100-b$%) was calculated accordingly

E_3: extinction at λ_{max} 248 mμ of the O-methylation mixt. in 0.1 N NaOH.

E_4: extinction at λ_{max} 347–356 mμ of the O-methylation mixt. in 0.1 N NaOH.

The molecular extinction coefficient of vanillin in 0.1 N NaOH at λ_{max} 248 mμ is ϵ 9,320, and at λ_{max} 347 mμ is ϵ 26,200, and that of isovanillin at λ_{max} 248 mμ is ϵ 20,800, and at λ_{max} 356 mμ, ϵ 6,160.

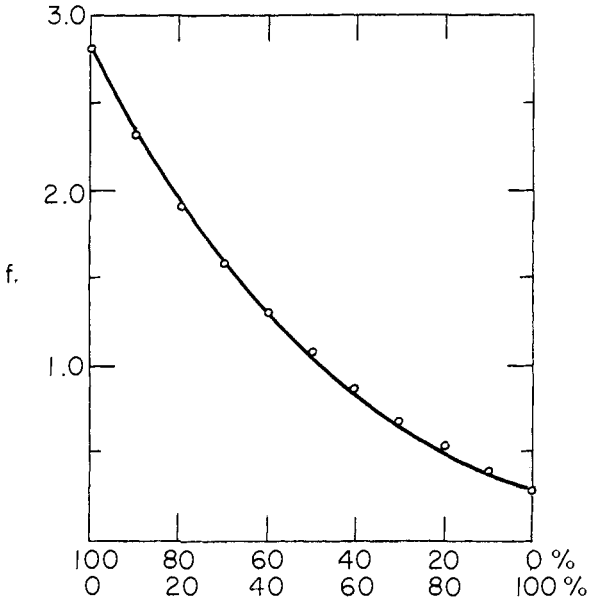


Fig. 1.—Calibration curve for the determination of the ratios of m- and p-O-methylation products obtained from spectrophotometric data of known mixtures of vanillin (top abscissa) and isovanillin (lower abscissa) measured at a concentration of 10γ in 1 ml. of 0.1 N NaOH. Factor f signifies the ratio $E_{347-356}m\mu/E_{247-8}m\mu$.

The apparent molecular extinction coefficients of a mixture of vanillin and isovanillin (*meta* b%, *para* $(100-b)$%) at λ_{max} 248 mμ and λ_{max} 347–356 mμ, respectively, is obtained from the equation

$$\epsilon'_{248\ m\mu} = 20{,}800 - 115b$$

$$\epsilon'_{347-356\ m\mu} = 6{,}160 + 200b$$

The equation of yield of a mixture was

$$\text{yield} = \frac{E_3 \times V}{\epsilon'_{248\ m\mu} \times 10^3}\ \text{mole}$$

or

$$= \frac{E_4 \times V}{\epsilon'_{347-356\ m\mu} \times 10^3}\ \text{mole}$$

B. Enzymatic Studies.—S-Adenosylmethionine was obtained from yeast.[8] The yield of active methionine from L-methionine was improved to 17.5–21% by the use of "Oriental" yeast and the application of vigorous aeration. The enzymatic O-methylation by partially purified catechol-O-methyltransferase was carried out in phosphate buffer with 3,4-dihydroxyacetophenone and 3,4-dihydroxybenzaldehyde as substrates. The O-methyl ethers in the reaction mixture were analyzed by the spectrophotometric method described above.

Activities of enzyme preparations were determined by measuring the amount of O-methyl ether derivatives formed from 3,4-dihydroxyacetophenone under the following condition: the mixture containing a known aliquot of enzyme solution, 1 μmole of substrate, 1 μmole of $MgCl_2$, 0.5 μmole of S-adenosylmethionine, 150 μmoles of 0.1 M phosphate buffer at pH 7.8 and water, made up to a final volume of 3 ml., was incubated for 30 min. at 37°.

Preparation of Catechol-O-methyltransferase from Rat Liver.[4]—Fresh livers (100 g.) from 10 adult male rats were homogenized with 400 ml. of isotonic KCl and centrifuged. The supernatant fraction (320 ml.) was adjusted to pH 5.0 with 1.0 N acetic acid, allowed to stand for 10 min., and centrifuged. To 300 ml. of the supernatant was added 52 g. of ammonium sulfate (~30% saturation). The precipitate was discarded and 34 g. of ammonium sulfate (~50% saturation) was added to the supernatant fraction. After centrifugation, the precipitate was dissolved in 50 ml. of water and dialyzed for 15 hr. against 0.001 M phosphate buffer (pH 7.0). To 100 ml. of this solution, 45 ml. of calcium phosphate gel (18 mg. of dried material/ml.) was added. The enzyme adsorbed on the gel was eluted with 0.02 M phosphate buffer at pH 6.9. After dialysis against 0.001 M phosphate buffer at pH 7.0 for 12 hr., this preparation was used for methylation experiments. It was stable for at least 6 months at $< -10°$. The enrichment is summarized in Table I.

Properties of the Partially Purified Enzyme.—In the absence of adenosylmethionine, O-methylation did not occur at all (Table II). The enzyme preparation seemed to contain a small amount of divalent cations, for even without addition of Mg^{++} O-methylation to the extent of 50% of the complete system was observed. Pretreatment with 0.1 μmole of ethylenediamine tetraacetic acid (EDTA) inhibited the reaction completely.

TABLE I

PURIFICATION OF CATECHOL-O-METHYLTRANSVERASE

Purification step[a]	Total activity,[b] units	Amount of protein, mg.	Spec. act., units/mg. of protein
1. Crude extract (supernatant at 60,000 g, 1 hr.)	103	11,000	0.0094
2. Supernatant of pH 5.0 ppt.	127	8,140	.015
3. Ammonium sulfate ppt. (30–50%)	118	1,120	.105
4. Calcium phosphate adsorption (pH 5.0) and elution (pH 6.9), and dialysis	80	193	.398

[a] All operations were carried out at 0–5°. [b] One unit equals 1 μmole of methoxy derivative formed in 30 min. under the conditions described.

TABLE II

ENZYMATIC O-METHYLATION OF 3,4-DIHYDROXYACETOPHENONE

System	Yield of O-methylated prod., μmole
Complete system[a]	0.20
Adenosylmethionine omitted	.0
Mg^{++} omitted	.10
Mg^{++} omitted + EDTA 0.001 μmole	.095
Mg^{++} omitted + EDTA 0.01 μmole	.062
Mg^{++} omitted + EDTA 0.1 μmole	.0

[a] The mixture containing 0.5 mg. (protein weight) of catechol-O-methyltransferase, 1 μmole of substrate, 1 μmole of $MgCl_2$, 0.5 μmole of adenosylmethionine, 150 μmoles of phosphate buffer (pH 7.8) and water to make a final volume of 3 ml. was incubated at 37°. At the end of 30 min. the amount of O-methylated product was measured.

(8) J. A. Stekol, E. I. Anderson and S. Weiss, *J. Biol. Chem.*, **233**, 425 (1958).

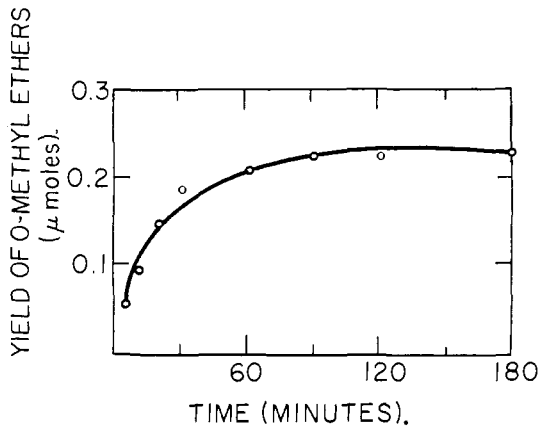


Fig. 2.—Rate of enzymatic O-methylation of 3,4-dihydroxyacetophenone in the complete system (Table II) with purified O-methyltransferase (Table I).

The rate of O-methylation is shown in Fig. 2. The methylation reaches completion after 1.5 hr.

Results and Discussion.—Non-enzymatic mono-O-methylations of 3,4-dihydroxyacetophenone were carried out as described above with variations in the *p*H from 5.5 to 13. The results are shown in Figs. 3A, curve I, and Table III; the figures show the extent of formation of *m*-O-methyl ethers as a function of *p*H and type of bivalent cations which were added as sulfates or chlorides. The presence of divalent cations, such as Cu^{++}, Fe^{++} and Ni^{++}, led to markedly different ratios of products (Fig. 3A, curves II, III and IV). Likewise, the ratio of vanillin–isovanillin was significantly changed in the presence of nickelous, ferrous and cupric ions (Fig. 3B, Table III). According to effectiveness, the cations fall into three groups: (i) Cu^{++} and Fe^{++}; (ii) Ni^{++}, Co^{++} and Mn^{++}; and (iii) Zn^{++} and Mg^{++}. These ions affect the ratio of methylation products in different degree and manner by complexing with the substrates.

TABLE III

EFFECTS OF CATIONS ON THE NON-ENZYMATIC O-METHYLATION OF 3,4-DIHYDROXYBENZALDEHYDE AND -ACETOPHENONE

	3,4-Dihydroxybenzaldehyde			3,4-Dihydroxyacetophenone		
Cation	*p*H 7.2–7.4	*p*H 8.3–8.5	*p*H 9.3–9.5[a]	*p*H 7.3–7.4	*p*H 8.2–8.4	*p*H 9.2–9.3[a]
Na^+, K^+	15.0%	14.5%	16.0%	22.0%	22.0%	22.0%
Cu^{++}	70.5	73.0	72.0	70.0	72.5	72.5
Fe^{++}	57.5	65.0	76.0	64.0	60.0	61.0
Ni^{++}	41.5	60.5	67.0	28.5	44.0	53.0
Co^{++}	20.5	37.5	52.5	19.5	28.5	41.5
Mn^{++}	16.0	27.0	37.0	18.5	28.0	37.0
Zn^{++}	14.0	19.0	31.5	18.0	22.0	26.5
Mg^{++}	15.5	19.0	24.5	22.0	21.5	24.0

[a] The initial *p*H of the mixture of substrate and metal (M^{++}) salt in 0.2 *M* phosphate buffer.

In the process of chelation the ultraviolet absorption spectra are shifted to longer wave lengths and the molar extinction coefficients are affected significantly (Figs. 4A, 4B). Ni^{++}, Co^{++}, Mn^{++}, Zn^{++} and Mg^{++} ions belong to the second group. They cause small changes in λ_{max} of the ultraviolet absorption but large differences in the molar extinction coefficients of the catechol substrates. These cations have a small but noticeable effect on the

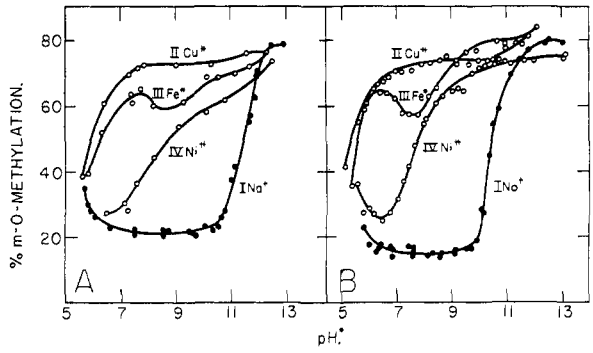


Fig. 3.—*Non-enzymatic m*-O-methylation of 3,4-dihydroxyacetophenone (A) and 3,4-dihydroxybenzaldehyde (B) as a function of *p*H and cations added: I, Na^+, K^+; II, Cu^{++}; III, Fe^{++}; IV, Ni^{++}. The mixture containing 300 μmoles of substrate, 5,000 μmoles (10 ml.) of 0.5 *M* phosphate buffer (or NaOH), 300 μmoles of metal (M^{++}) salt (aqueous solution) and 300 μmoles of dimethyl sulfate (1 ml. of ethanol–chloroform solution, 1:9 mixture) was vigorously stirred under nitrogen for 5 minutes at 60°.

* Indicates initial *p*H of the reaction.

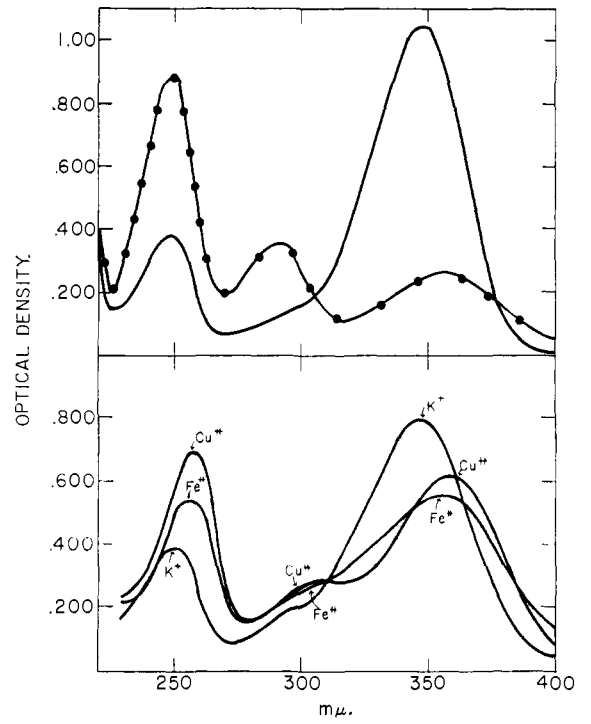


Fig. 4A.—Ultraviolet absorption spectra of vanillin (———) and isovanillin (–●–●–●–) (above) in 0.1 *N* NaOH (above) and of 3,4-dihydroxybenzaldehyde (below) 2:1 substrate–metal complexes with K^+, Cu^{++} and Fe^{++} ions in phosphate buffer *p*H 9.7.

product ratio depending on the nature of the divalent cation (Table IV).

Methylation experiments carried out in the presence of monovalent cations (Na^+, K^+) led to three different ratios of products, depending on the *p*H of the medium (curves I, Figs. 3A and 3B). The methylation mixture obtained between *p*H 6 and 10 contained preferentially *p*-isomers (*ca.* 80%), while the proportion of *m*-isomers gradually

TABLE IV

THE EFFECT OF VARIOUS METAL CATIONS ON THE λ_{max} AND ϵ_{max} OF 3,4-DIHYDROXYACETOPHENONE AND 3,4-DIHYDROXYBENZALDEHYDE AT *p*H 9.7 (IN PHOSPHATE BUFFER)

	3,4-Dihydroxyacetophenone					3,4-Dihydroxybenzaldehyde				
Metal	ϵ_1	λ_{max}, mμ	ϵ_2	λ_{max}, mμ	ϵ_2/ϵ_1	ϵ_1	λ_{max}, mμ	ϵ_2	λ_{max}, mμ	ϵ_2/ϵ_1
K^+, Na^+[a]	9,750	248	16,000	342	1.64	9,750	248	19,800	347	2.03
Cu^{++}	20,200	257	13,300	352	0.609	17,100	258	15,500	359	0.908
Fe^{++}	15,000	256	12,000	352	0.795	13,500	257	14,000	357	1.04
Ni^{++}	10,400	251	14,100	344	1.35	9,750	253	16,500	351	1.69
Mn^{++}	7,500	250	10,600	332	1.41	7,000	252	11,000	348	1.57
Co^{++}	9,000	251	12,900	343	1.43	9,380	252	16,700	351	1.78
Zn^{++}	10,000	250	15,200	343	1.52	9,950	251	18,400	349	1.85
Mg^{++}	9,500	248	15,500	342	1.63	9,150	250	19,200	347	2.10

[a] The ratio of concentrations of catechol *versus* metal ion was 2:1.

increased at *p*H <6. As the *p*H increased over 10, *m*-O-methylation became prevalent and the proportion was finally reversed (*para ca.* 20%, *meta ca.* 80%) at *p*H >12.

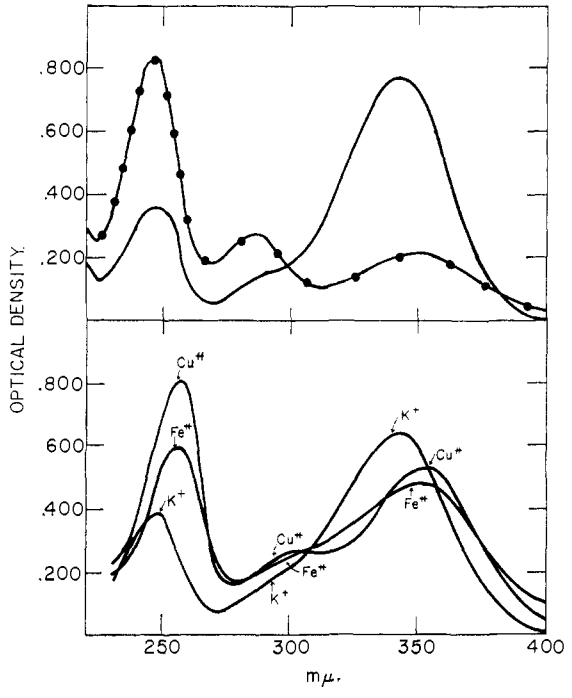
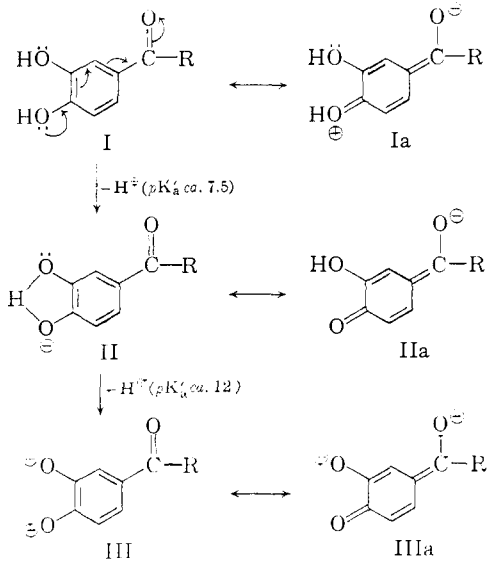


Fig. 4B.—Ultraviolet absorption spectra of acetovanillone (———) and acetoisovanillone (-●-●-●-) (above) in 0.1 *N* NaOH (above) and of 3,4-dihydroxyacetophenone (below) 2:1 substrate–metal complexes with K^+, Cu^{++} and Fe^{++} ions in phosphate buffer *p*H 9.7.

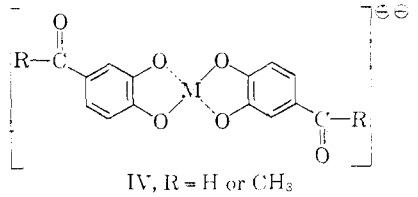
This phenomenon is understandable in terms of the *p*H-dependent equilibrium between uncharged catechol (I↔Ia), its mono- (II ↔ IIa) and dianion (III ↔ IIIa). Two proton-donating groups of *pK'*a 7.5 and *ca.* 12 were ascertained in 3,4-dihydroxyacetophenone by titration under nitrogen. This means that the first dissociation becomes noticeable at *p*H 6 and the second at *p*H 10. In support of this, the ultraviolet absorption spectra of 3,4-dihydroxyacetophenone and -benzaldehyde (Figs. 4A and 4B) at the first dissociation stage (II) were found to be very similar to that of acetovanillone and vanillin, respectively, in aqueous alkaline solution.



The dominant species at stage II is the *p*-phenoxide anion which acts as a nucleophile on the methylating agent leading to *p*-O-methylation between *p*H 6 and 10. This mechanism also explains the nearly constant ratio of *para* and *meta* isomers throughout this *p*H region. In more acidic medium (*p*H <6), where the substrate is mainly present as the uncharged catechol I, the resonance equilibrium I ↔ Ia favors *m*-O-methylation.

On the other hand, within the di-anion the *m*-phenoxide anion III ↔ IIIa is the stronger nucleophile favoring *m*-O-methylation in strongly alkaline medium.

The addition of divalent cations generally promotes the production of *m*-O-methyl ethers to a variable degree dependent on the *pH* and the nature of the cation (Fig. 3A and B, Table III). The effect of the added cation reaches a maximum at the ratio of one mole of cation per two moles of substrate, indicative of the formation of a complex IV (Table V). In the acidic region, the influence



IV, R = H or CH_3

of divalent cations decreases rapidly. If one assumes increasing covalency in the metal–*p*-phenoxide linkage in the order $Mg^{++}\ldots\rightarrow Cu^{++}$, it follows that the nucleophilicity of the (coördinately bound) *m*-phenoxide ion should increase in the same direction. In fact, the ratio (ϵ_2/ϵ_1) of molar extinction coefficients at λ_{max} 247–258 mμ and λ_{max} 342–359 mμ of the catechol–metal complex IV in comparison with that of the *p*-phenoxide anion II almost continuously decreases in the order Mg^{++} to Cu^{++} (Table IV). The characteristic ultraviolet spectra of the catechol–Cu^{++} complexes are comparable to those of acetoisovanillone and isovanillin in aqueous alkaline solution (see Figs. 4A, 4B). The position of the ferrous ion is anomalous. According to metal complex stability it should rank between Mn^{++} and Co^{++}.[9]

TABLE V

EFFECT OF THE CONCENTRATION OF CATIONS ON THE NON-ENZYMATIC O-METHYLATION OF 3,4-DIHYDROXYACETOPHENONE AND 3,4-DIHYDROXYBENZALDEHYDE AS A FUNCTION OF MOLAR CONCENTRATION OF ADDED DIVALENT CATIONS *versus* THAT OF SUBSTRATE AT *p*H 8.0–8.2

In each experiment the mixture containing 100 μmoles of substrate, 10 ml. of 1.5 *M* phosphate buffer (pH 8.0), 10 to 300 μmoles of metal (Me^{++}) salt in aqueous solution, 100 μmoles of dimethylsulfate in ethanol–chloroform (1:9) solution, made up to a final volume of 13 ml., was vigorously stirred under nitrogen for 5 min. at 60°.

	3,4-Dihydroxyacetophenone		3,4-Dihydroxybenzaldehyde	
Molar ratio of added cation *versus* substrate	Rate of *m*-O-methylation	Total yield of O-Me-products relative to control (100)	Ratio of *m*-O-methylation	Total yield of O-Me-products relative to control (100)
No metal	22.0	100	14.5	100
Cu^{++} 0.1	25.5	81.0	34.3	99.0
.3	47.5	64.0	52.0	73.5
.5[a]	69.5	46.6	72.0	61.0
1.0	70.5	44.3	74.0	57.5
3.0	70.2	40.5	72.5	52.2
Fe^{++} 0.1	39.3	125	37.5	211
.3	57.3	168	58.0	321
.5[a]	64.8	176	66.5	304
1.0	64.0	194	66.5	316
3.0	63.7	176	66.2	280
Ni^{++} 0.1	27.5	100	31.2	146
.3	41.7	124	50.5	149
.5[a]	45.3	120	57.5	168
1.0	45.0	123	56.2	160
3.0	44.5	115	53.0	151

[a] At this ratio the formation of the 1:2 metal–catechol complex is complete. A further increase in metal cation concentration does not significantly affect the ratio of O-methyl isomers nor the relative yields of total O-methylation products.

Effect of Divalent Cations and *p*H on Enzymatic O-Methylation.—The ratio of *m*- and *p*-isomers in the enzymatic methylation mixture was significantly influenced by divalent cations and by the variations in *p*H. Figures 5A and B show the results for 3,4-dihydroxyacetophenone and 3,4-dihydroxybenzaldehyde. Experiments with Fe^{++} and Cu^{++} which are not shown in the figures revealed only low activity of these ions on the course of O-methylation. The *m*,*p*-ratio of O-methylation as a function of *p*H and metal ions is much more complex than in the non-enzymatic series. In previous cases[10,11] this has been explained by the fact that metals are capable of either positive or negative catalysis by specific or non-specific, reversible or irreversible binding with the prosthetic group or the protein of the enzyme. The relative inhibitory or activating contributions of a given metal will be different in each case and may markedly depend on concentration and *p*H. However in the enzymatic O-methylation the ratio of products is *not* much affected by the concentration of Mg^{++}, Zn^{++} and Mn^{++} ions within the range of 0.2–100 μmole (Table VI).

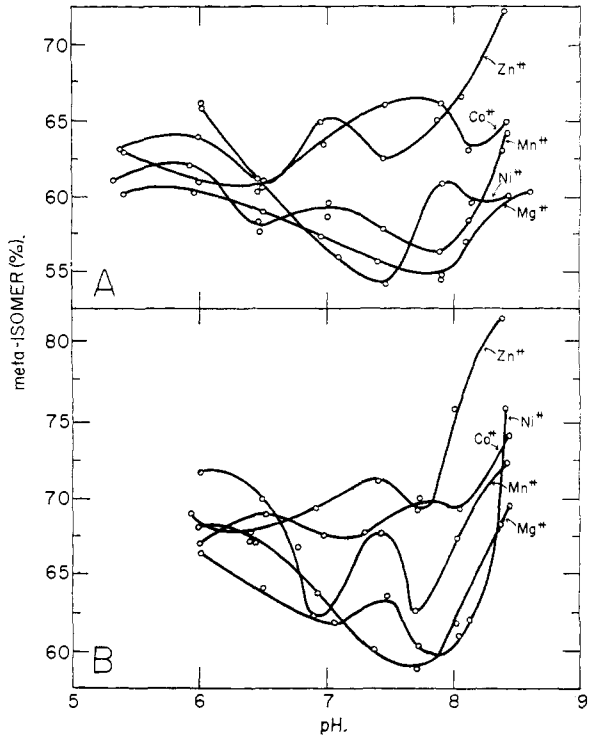


Fig. 5.—Effect of divalent cations on the *enzymatic* O-methylation of 3,4-dihydroxyacetophenone (A) and of 3,4-dihydroxybenzaldehyde (B). The mixture containing 0.5 mg. (protein weight) of catechol O-methyltransferase and 1 μmole of EDTA was pre-incubated for 10 minutes at 37°. After the addition of 0.5 μmole of adenosylmethionine and 1 μmole of metal salt (M^{++}) and water to make a final volume of 3 ml., the mixture was incubated further for 50 minutes at 37° and assayed for ratios of *m*- and *p*-isomers as well as for total yields (see Fig. 6).

The order with regard to relative activities of divalent cations based on total yield of O-methylated product (Fig. 6A and B) at optimal *p*H (7.8) places magnesium at the beginning and cupric ion at the end of the sequence: Mg^{++}, Zn^{++}, Mn^{++}, Co^{++}, Ni^{++}, Fe^{++}, Cu^{++}. Apparently the (hypothetical) magnesium complex V, operative in the enzymatic transfer of methyl, satisfies best the spatial, electronic and stability requirements for nucleophilic displacement within the complex which must dissociate as soon as the substrate has been methylated. The primary function of mag-

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(10) *Cf.* J. F. Speck, *J. Biol. Chem.*, **178**, 315 (1949).

(11) G. L. Eichhorn, *Fed. Proc.*, **20**, 40 (1961).

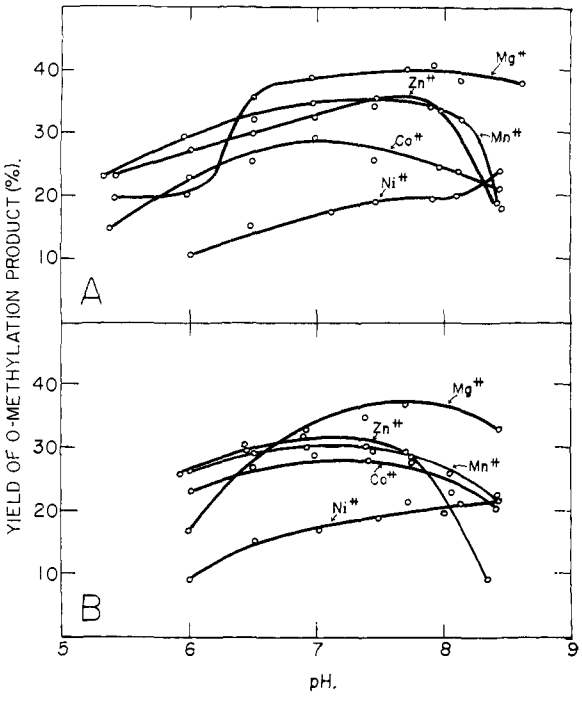
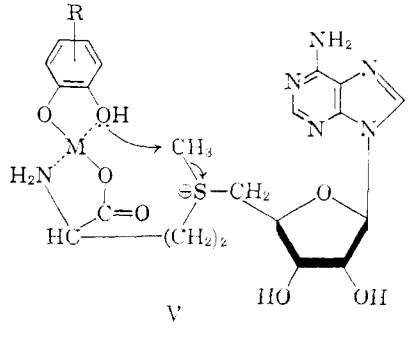


Fig. 6.—Yields of total enzymatic O-methylation products from 3,4-dihydroxyacetophenone (A) and from 3,4-dihydroxybenzaldehyde (B).

nesium consists in bringing substrate and enzyme together in the bridge complex V rather than in



V

TABLE VI

EFFECT OF THE CONCENTRATION OF CATIONS ON THE RATIO OF PRODUCTS IN THE ENZYMATIC O-METHYLATION OF 3,4-DIHYDROXYACETOPHENONE IN PHOSPHATE BUFFER AT *p*H 7.8

Metal,[a] μmoles		*m*-O-Methylation, %	Relative yield of O-methylated prod.,[c] %
Mg^{++}	0.2	55.0	66.2
	1.0[b]	55.3	100.0
	10.0	55.2	100.6
	100.0	55.4	68.7
Zn^{++}	0.2	63.3	82.1
	1.0	63.0	81.2
	10.0	62.0	81.3
	100.0	0	0
Mn^{++}	0.2	58.7	71.5
	1.0	58.0	76.2
	10.0	58.5	70.5
	100.0	54.5	21.8

[a] The figures express the molar ratio of added cations *versus* 1.0 μmole of substrate. [b] The yield obtained with 1.0 μmole of Mg^{++} ions was taken as standard (100%) for comparison. [c] The mixture containing 0.5 mg. (protein weight) of enzyme, 1 μmole of substrate, 150 μmoles of phosphate buffer (0.1 *M*, *p*H 7.8) and 0.1 μmole of EDTA was pre-incubated for 10 minutes at 37°. After the addition of 0.5 μmole of adenosylmethionine and 0.2 to 100 μmoles of metal salt and of sufficient water to a total volume of 3 ml., the mixture was incubated further for 50 min. and assayed for the ratio of *m*-isomer as well as for total yields of O-methylated product as described in the Experimental part.

distorting the electron densities of the phenolic hydroxyls. In fact the transition metal cations that do withdraw electrons from the catechol hydroxyls most strongly prevent nucleophilic displacement of the methyl of adenosylmethionine (Fig. 6).

A second less likely explanation is the formation of the 2:1 catechol complex IV as a process competitive with the formation of the enzyme–metal–substrate complex V. Such an assumption fails to rationalize the observed order of activity of the various cations since both types of complexes would be expected to have the same relative stabilities.

[CONTRIBUTION FROM THE MIDWEST RESEARCH INSTITUTE, KANSAS CITY 10, MO.]

Antibiotics. I. Synthesis of 1,6-Dimethyl-5,7-dioxo-1,5,6,7-tetrahydropyrimido[5,4-e] *as*-Triazine (Toxoflavin) and Related Compounds[1a]

BY G. DOYLE DAVES, JR., ROLAND K. ROBINS AND C. C. CHENG[1b]

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The synthesis of 1,6-dimethyl-5,7-dioxo-1,5,6,7-tetrahydropyrimido[5,4-e]-*as*-triazine (II) and related compounds is described. Compound II was found to be identical with the antibiotics toxoflavin and xanthothricin. The structure of a third related antibiotic, fervenulin, has been definitely established as 6,8-dimethyl-5,7-dioxo-5,6,7,8-tetrahydropyrimido[5,4-e]-*as*-triazine (XIX) which is isomeric with II.

The numerous mass fatal food poisonings in the province of Banjumas in central Java were found by van Veen and Mertens[2] to be due to a highly poisonous yellow crystalline substance called "toxoflavin," which periodically occurs in "bong-

(1) (a) This investigation was supported by research contract SA-43-ph-3025 from the Cancer Chemotherapy National Service Center, National Cancer Institute of the National Institutes of Health, Public Health Service. (b) To whom inquiries should be directed.

(2) (a) A. G. van Veen and W. K. Mertens, *Proc. Akad. Wetenschappen Amsterdam*, **36**, 666 (1933); (b) W. K. Mertens and A. G. van Veen, *Geneesk. Tijdschr. Ned. Indië*, **73**, 1223, 1309 (1933); (c) W. K. Mertens and A. G. van Veen, *Meded. Dienst Volksgezondheid Ned. Indië*, **22**, 209 (1933); (d) A. G. van Veen and W. K. Mertens, *Rec. trav. chim.*, **53**, 257, 398 (1934).