TABLE V

Expt.	*p*H of the soln.	Residual tritium count after 74 hr. at room temp. C.p.m./μmole	Ratio of residual tritium against contrast (11, 17), %
1	1.35	5020	92.9
2	1.78	5390	99.8
3	7.02	5125	94.8
12	7.0	4770	90.6
4	7.45	4675	86.5
13	7.4	4480	85.0
5	8.42	3925	72.6
6	9.18	3820	70.7
14	9.1	3720	70.7
7	9.50	4010	74.1
8	9.80	3520	65.2
9	10.15	3630	67.2
15	10.1	3275	61.8
10	11.25[a]	3790	70.2
16	11.25	3790	71.8
11	1.0 mg. of the substance	5410	..
17	dissolved in 3 ml. of	5270	..
(contrast)	50% aq. methanol		

[a] The substance (1.0 mg.) was dissolved in 3 ml. of 0.5 *N* trimethylamine in 50% aqueous methanol.

The efficiency for tritium at the second setting was 5%. Internal standards were added and the observed count corrected (at both settings) for the over-all quenching due to norepinephrine, water and ethanol which varied from 15 to 25% of the observed count. All samples were counted for a sufficient period of time to give counting errors of less than 2%.

In Vivo Formation of 2,4,5-Trihydroxyphenethylamine (II).—Two 250-g. male rats were given 50 mg. of marsilid phosphate intraperitoneally and after 24 hours they were placed in all glass metabolism cages which had been previously washed in 1 *N* HCl. Each rat received 400 μg. of dopamine-C^{14} (sp. act. 4100 c.p.m./μg.) intraperitoneally and the urine was collected for 24 hours in a flask containing 1 ml. of 0.1 *N* HCl. The collection tubes were cooled in an ice-bath during the collection.

A preliminary separation of the catecholamine fraction was done on a Dowex-50 column (200-400 mesh) which was buffered at *p*H 5.7 with 1 *M* acetate buffer and washed with distilled water until the effluent was neutral. The *p*H of the urine was adjusted to 4.0 before being put on the column. The column then was washed with 50 ml. of distilled water and with 20 ml. of 1 *N* HCl. The catecholamines were finally eluted with 20 ml. of 2 *N* HCl and the eluate was concentrated to a small volume *in vacuo* at room temperature. It now was taken up in 0.5 ml. of methanol, streaked on Whatman No. 1 paper, and the chromatogram developed in the phenol system described above with external and internal standards of norepinephrine and 6-hydroxydopamine. After washing the chromatogram twice with anhydrous ether the area corresponding to the R_f of 6-hydroxydopamine was eluted with a solution of MeOH–HCl (3:1).

The eluate was divided into four portions. One was subjected to treatment with MeOH–HCl (as described in the preceding paper) to convert any norepinephrine to β-*O*-methylnorepinephrine; another portion was left untreated. Carrier amounts of norepinephrine and hydroxydopamine were added to the other two portions. One of the aliquots with carrier was subjected to treatment with anhydrous MeOH–HCl to form β-*O*-methylnorepinephrine. The four aliquots then were chromatographed on Whatman No. 1 paper in 2-butanol–formic acid–water (75:15:10) under nitrogen. In this system the R_f of hydroxydopamine is 0.25 and that of β-methylnorepinephrine 0.52.

BETHESDA 14, MD.

[CONTRIBUTION FROM THE NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES AND THE NATIONAL INSTITUTE OF MENTAL HEALTH, NATIONAL INSTITUTES OF HEALTH, PUBLIC HEALTH SERVICE]

Enzymatic *p*-O-Methylation by Catechol O-Methyl Transferase

BY SIRO SENOH,[1] JOHN DALY, JULIUS AXELROD AND BERNHARD WITKOP

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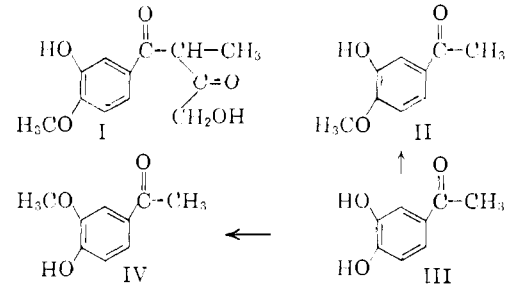
The enzymatic methylation by catechol O-methyl transferase of 3,4-dihydroxyacetophenone (III), 3,4-dihydroxyphenylmethylcarbinol (V) and 3,4-dihydroxyphenethylamine (dopamine, VI) has been studied as a function of *p*H. Spectrophotometric, colorimetric and chromatographic methods have been developed for the assay and resolution of *o*- and *p*-O-methylation mixtures. Depending on the nature of the side chain, *p*-O-methylation has been observed to occur to the extent of 10–56%.

The major metabolic pathway of catechol amines has been shown to involve *m*-O-methylation[2] by an enzyme, catechol O-methyl transferase.[3] In this paper evidence is presented that O-methylation occurs also at the phenolic hydroxyl group *para* to electron deficient as well as non-deficient side chains.

The isolation of "Substanz Y"[4] (presumably I) from beef adrenal glands is an example of the natural occurrence of a *p*-O-methylated catechol derivative. As a comparable model for enzymatic methylation studies we chose 3,4-dihydroxyacetophenone (III) which on the basis of previous studies[2] would be expected to be a substrate for catechol O-methyl transferase. The product of normal *m*-O-methylation would be acetovanillone (IV). Acetoisovanillone (II) would be observed if the novel *p*-O-methylation occurs. It was reasonable to expect a mixture of the two.



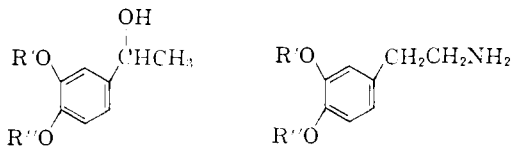
(1) Visiting Scientist of the U. S. Public Health Service on leave of absence from the Institute of Food Chemistry and Osaka City University, Japan.

(2) J. Axelrod, S. Senoh and B. Witkop, *J. Biol. Chem.*, **233**, 697 (1958).

(3) J. Axelrod and R. Tomchick, *ibid.*, **233**, 702 (1958).

(4) J. v. Euw, C. Meystre, R. Neher, T. Reichstein and A. Wettstein, *Helv. Chim. Acta*, **41**, 1510 (1958).

Enzymatic methylation studies also were carried out with 3,4-dihydroxyphenylmethylcarbinol (Va) and with 3,4-dihydroxyphenethylamine (dopamine) (VIa). In the case of V, enzymatic methylation would be expected to yield either 3-hydroxy-4-methoxyphenylmethylcarbinol (Vb) or 4-hydroxy-3-methoxyphenylmethylcarbinol (Vc) or a mixture of the two. Dopamine (VIa) could in the same way be methylated to either 3-hydroxy-4-methoxyphenethylamine (VIb) or 4-hydroxy-3-methoxyphenethylamine (VIc) or a mixture of the two.



Va, R′ = R″ = H
b, R′ = H, R″ = CH_3
c, R′ = CH_3, R″ = H

VIa, R′ = R″ = H
b, R′ = H, R″ = CH_3
c, R′ = CH_3, R″ = H

Materials.—3,4-Dihydroxyacetophenone (III) was prepared by the reduction[5] of 4-chloroacetylcatechol. After two recrystallizations from a mixture of chloroform–petroleum ether the colorless crystals had m.p. 119–120° (reported 114°[5]); $\lambda_{max}^{H_2O}$ (mμ): 227 (ε 13,800); 275 (ε 9,900); 306 (ε 7,300). $\lambda_{max}^{0.1\ N\ NaOH}$ (mμ): 262 (ε 9,900); 314 (ε 8,900); 383 (ε 10,600).

3-Methoxy-4-hydroxyacetophenone (Acetovanillone) (IV).[6]—The compound had m.p. 115–116°; $\lambda_{max}^{H_2O}$ (mμ): 227 (ε 14,000); 276 (ε 9,400); 306 (ε 7,500). $\lambda_{max}^{0.1\ N\ NaOH}$ (mμ): 248 (ε 8,900); 343 (ε 21,400).

Acetoisovanillone (II)[6] showed m.p. 67–68°; $\lambda_{max}^{H_2O}$ (mμ): 227 (ε 14,600); 275 (ε 10,000); 306 (ε 7,000). $\lambda_{max}^{0.1\ N\ NaOH}$ (mμ): 247 (ε 21,900); 287 (ε 6,500); 349 (ε 5,500).

3,4-Dihydroxyphenylmethylcarbinol (Va).—The dibenzyl ether of protocatechualdehyde was prepared in the usual manner and was recrystallized from petroleum ether (b.p. 90–100°), m.p. 91–92°.

Anal. Calcd. for $C_{21}H_{18}O_3$: C, 79.22; H, 5.70. Found: C, 79.23; H, 5.77.

A suspension of 2 g. of the 3,4-dibenzoxybenzaldehyde was added in 20 ml. of ether to a Grignard solution formed from 80 mg. of magnesium and 1.1 g. of methyl iodide in 20 ml. of ether. The reaction mixture was refluxed for 4 hours. Water then was added and the product was extracted with ether. The ether solution was dried and the ether removed *in vacuo* to afford 1.7 g. of oil.

This oil was dissolved in 75 ml. of ethanol containing 300 mg. of 10% palladium-on-charcoal. At atmospheric pressure 245 ml. of hydrogen (theoretical 250 ml.) was consumed. The catalyst was filtered off and the solution was taken to dryness *in vacuo*. The residual oil was extracted with 100 ml. of benzene. After cooling the benzene extract overnight, 250 mg., over-all yield 26%, m.p. 122–125°, of product was obtained. After repeated recrystallizations from benzene–petroleum ether an analytical sample was obtained, m.p. 130–131°; λ_{max}^{EtOH} (mμ): 223 (ε 4,560), 282 (ε 2,050).

Anal. Calcd. for $C_8H_{10}O_3$: C, 62.32; H, 6.54. Found: C, 62.36; H, 6.68.

3-Hydroxy-4-methoxyphenylmethylcarbinol (Vb).—To a solution of 830 mg. of 3-hydroxy-4-methoxyacetophenone (II) in a mixture of 10 ml. of *N* sodium hydroxide and 10 ml. of methanol was added 200 mg. of sodium borohydride. The solution was refluxed for 2 hours, diluted with 3 volumes of water and acidified with *N* hydrochloric acid. Extraction was carried out with ethyl acetate. The extract was washed with water, dried, and the ethyl acetate was removed *in vacuo*. The product was recrystallized 3 times from ethanol–water to yield 220 mg. (26%) of colorless needles of 3-hydroxy-4-methoxyphenylmethylcarbinol, m.p. 91–92°; λ_{max}^{EtOH} (mμ): 228 (ε 5,400); 280 (ε 2,300).

Anal. Calcd. for $C_9H_{12}O_3$: C, 64.41; H, 7.15. Found: C, 64.56; H, 7.38.

(5) H. Stephen and C. Weizmann, *J. Chem. Soc.*, **105**, 1046 (1914).
(6) T. Reichstein, *Helv. Chim. Acta*, **10**, 392 (1927).

4-Benzoxy-3-methoxyacetophenone.—To a solution of 11.5 g. of 4-hydroxy-3-methoxyacetophenone (IV) and 2.7 g. of sodium hydroxide in 15 ml. of an ethanol–water mixture (1:1) was added 8.4 g. of benzyl chloride. The solution was refluxed overnight and then diluted with a large volume of water. The precipitate was recrystallized from ethanol to yield 14.9 g. (84%) of 4-benzoxy-3-methoxyacetophenone, m.p. 87–88°.

Anal. Calcd. for $C_{16}H_{16}O_3$: C, 74.98; H, 6.29. Found: C, 75.20; H, 6.35.

4-Hydroxy-3-methoxyphenylmethylcarbinol (Vc).—Two grams of 4-benzoxy-3-methoxyacetophenone was dissolved in a mixture of 40 ml. of methanol and 10 ml. of 2 *N* sodium hydroxide. After addition of 300 mg. of sodium borohydride, the solution was refluxed for 30 minutes and then diluted with water. Extraction was carried out with ethyl acetate. The extract was washed with water, dried, and concentrated *in vacuo*. The residual oil was dissolved in 50 ml. of 95% ethanol containing 300 mg. of 10% palladium-on-charcoal. On reduction at atmospheric pressure, 180 ml. (theoretical 190 ml.) of hydrogen was consumed. The catalyst was removed by filtration, and the solvent was removed *in vacuo*. The crude material was sublimed at 50–60° and 0.005 mm. to yield 0.8 g. (61%) of 4-hydroxy-3-methoxyphenylmethylcarbinol, m.p. 100–101° (lit.[7] m.p. 101°); λ_{max}^{EtOH} (mμ): 228 (ε 6,200); 278 (ε 2,760).

Anal. Calcd. for $C_9H_{12}O_3$: C, 64.41; H, 7.15. Found: C, 64.75; H, 7.11.

3-Hydroxy-4-methoxyphenethylamine Hydrochloride (VIb).—The sample of this compound[8] had m.p. 206–208°; $\lambda_{max}^{H_2O}$ (mμ): 228 (ε 5,730); 278 (ε 2,570).

4-Hydroxy-3-methoxyphenethylamine Hydrochloride (VIc).[2]—The compound had m.p. 208–212°; $\lambda_{max}^{H_2O}$ (mμ): 228 (ε 6,050); 279 (ε 2,740).

Methods. Preparation of O-Methyl Transferase.[3]—Adult male rats were stunned, exsanguinated, and the livers removed immediately and chilled. All further operations were carried out at 0–5°. The livers (24 g.) were homogenized in 34 ml. of isotonic potassium chloride solution (1.15%) and centrifuged at a speed of 78,000 × g. for 45 minutes. The clear supernatant fraction was used for the enzymatic studies.

Results. Enzymatic O-Methylation of 3,4-Dihydroxyacetophenone (III).—3,4-Dihydroxyacetophenone (III) (3 mg., 18 μmoles), was incubated at 37° with 4 ml. of enzyme, 2 ml. of 0.5 *M* phosphate buffer, *p*H 7.8, 4 μmoles of S-adenosylmethionine (AMe) and 0.1 ml. of 2.0 *M* magnesium chloride in a total volume of 6.6 ml. (*p*H 7.4). After incubation for 1.5 hours the resulting mixture of methylation products was extracted twice with 2 volumes of benzene on the shaking machine for 30 minutes. Before the extraction the *p*H of the mixture was adjusted to less than 2. The phenolic components in the combined benzene extracts were re-extracted into 0.1 *N* aqueous sodium hydroxide. The aqueous layer was adjusted to *p*H <2 by the addition of hydrochloric acid. The aqueous solution was re-extracted with benzene, the benzene layer was washed twice with distilled water and evaporated to dryness under reduced pressure. An aliquot of the residue was dissolved in 0.1 *N* sodium hydroxide and its ultraviolet absorption spectrum measured in a self-recording spectrophotometer. The ratio of extinction at λ_{max} 343–349 mμ and λ_{max} 246–248 mμ yielded the empirical factor (*E*) which, by comparison with the calibration curve (Fig. 1), indicated the presence of 53.5% of acetovanillone (IV) and 46.5% of acetoisovanillone (II). In this determination the starting material III would interfere. However, benzene at *p*H 2 extracts only negligible amounts from aqueous solution. Its absence was established spectrophotometrically by the lack of the characteristic absorption at 383 mμ as well as by paper chromatography.

Control Experiment without S-Adenosylmethionine.—The enzyme solution (3 ml.) was incubated at 37° with 2 mg. of 3,4-dihydroxyacetophenone, 2 ml. of 0.5 *M* phosphate buffer, *p*H 7.8, and 0.1 ml. of 2.0 *M* magnesium chloride in a total volume of 5.5. ml. (*p*H 7.4). After incubation for 1.5 hours the reaction mixture contained no acetovanillone or acetoisovanillone.

(7) H. Finnemore, *J. Chem. Soc.*, **93**, 1520 (1908).
(8) This material was synthesized at the Sterling-Winthrop Research Institute and made available to us through the courtesy of Dr. Sydney Archer.

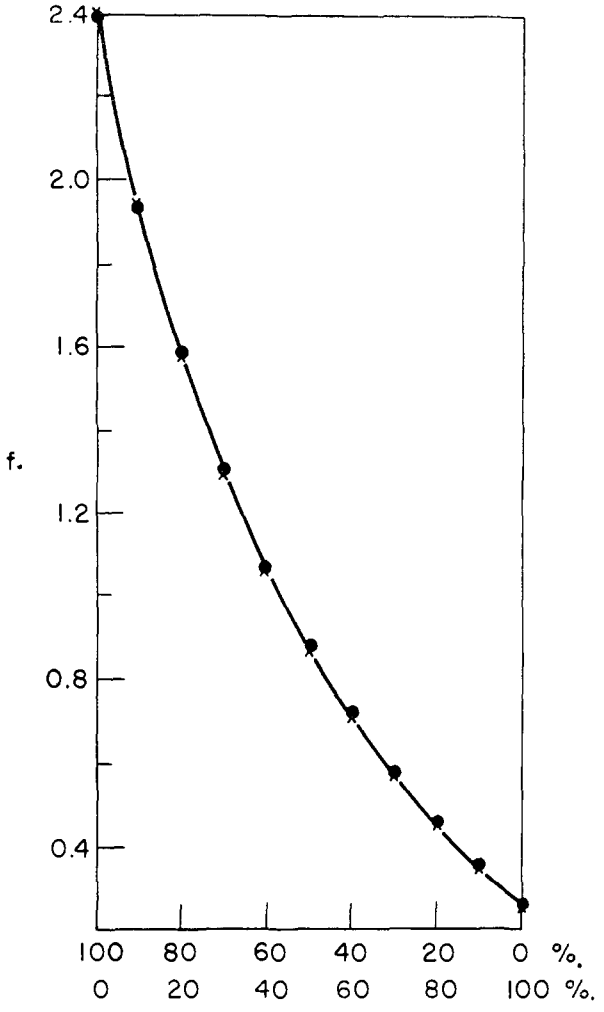


Fig. 1.—Calibration curve for the determination of the ratios of *m*- and *p*-methylation products obtained from spectrophotometric data of known mixtures of acetovanillone (top abscissa) and acetoisovanillone (lower abscissa) (10γ/ml. 0.1 *N* NaOH). The observed (X–X–X) and calculated (●–●–●) ratios coincide. Factor $f = E_{343-349\ m\mu}/E_{247-248\ m\mu}$.

Chromatographic Resolution of Acetovanillone (IV) and Acetoisovanillone (II) from the Enzymatic Methylation Mixture.—Table I and Fig. 2 show the conditions for, and results of, the chromatographic resolution of the enzymatic methylation products. After development in 1-butanol–15% aqueous ammonia (4:1), the two fractions corresponding to authentic samples of II and IV were cut off and eluted with benzene–ethanol (1:1). Each extract was evaporated to dryness under reduced pressure and examined spectroscopically in neutral and alkaline solution (Fig. 2, D and E). All of these methods confirmed the identities of the products.

Variations in the Enzymatic O-Methylation of 3,4-Dihydroxyacetophenone (III) as a Function of *p*H.—Table II summarizes the results of experiments in which the *p*H of the enzymatic incubation mixture was varied from 6.5–8. Erratic results were obtained below and above this *p*H range as a result of the inactivation of the enzyme. *meta*-Methylation decreased, *para*-methylation increased, with higher *p*H.

Enzymatic O-Methylation of 3,4-Dihydroxyphenylmethylcarbinol (V).—A sample of 3,4-dihydroxyphenylmethylcarbinol (1.5 mg., 9 μmoles) was incubated for 1.5 hours at 37° with 5 ml. of enzyme preparation, 2 ml. of 0.5 *M* phosphate buffer, *p*H 7.9, 4 μmoles of S-adenosylmethionine and 0.1 ml. of 2.0 *M* magnesium chloride in a total volume of 10 ml. (*p*H 7.5). After incubation the *p*H was adjusted to 4 and the suspension was extracted 3 times with 10-ml. portions of heptane. Each extraction was carried out for 20 minutes on the shaking machine. Under these conditions virtually none of the starting material was extractable. Aliquots from the heptane extract were used for subsequent isolation and determination of the products.

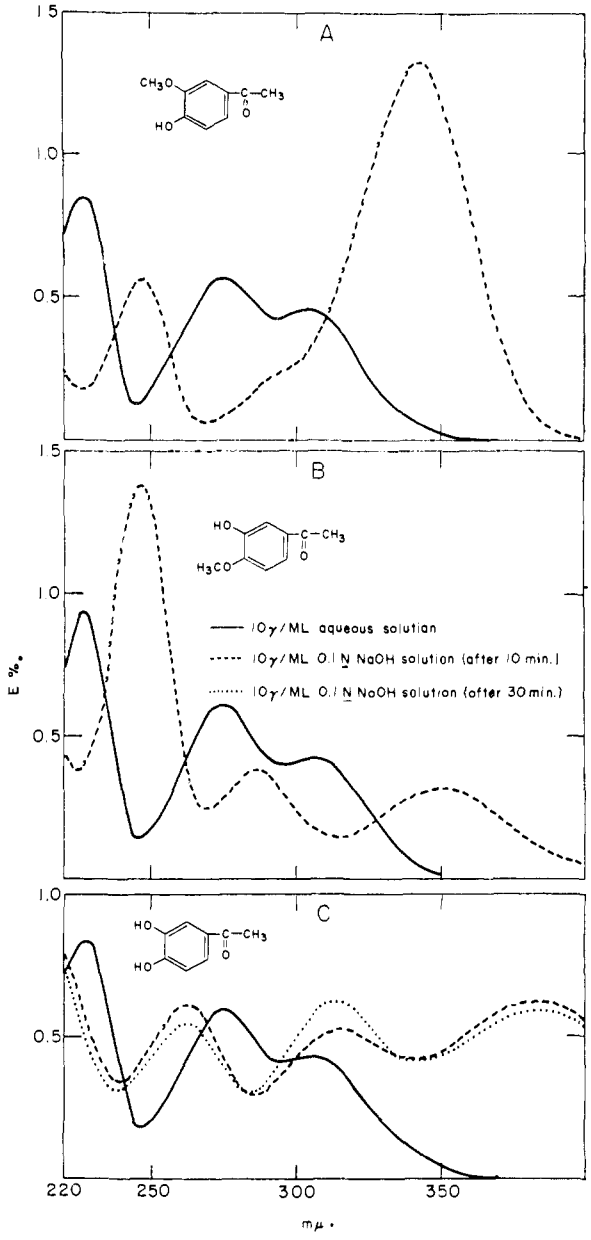


Fig. 2.—Spectroscopic differences of (A) acetovanillone (IV), (B) acetoisovanillone (II) and (C) 3,4-dihydroxyacetophenone in neutral and basic solutions.

Isolation and Determination of Enzymatically Formed 3-Hydroxy-4-methoxyphenylmethylcarbinol (Vb) and 4-Hydroxy-3-methoxyphenylmethylcarbinol (Vc).—Investigation of a variety of chromatographic conditions had shown that the isomeric hydroxymethoxyphenylmethylcarbinols are isographs[9] in the solvent systems used. Table III presents the results of descending paper chromatography with both synthetic and enzymatically formed hydroxymethoxyphenylmethylcarbinols. The spraying reagent was dichloroquinonechlorimide prepared as given in Table I. Both of the synthetic hydroxymethoxyphenylmethylcarbinols and the mixture, formed enzymatically, gave blue spots with this reagent.

(9) *Cf.* S. Senoh and B. Witkop, THIS JOURNAL, **81**, 6222 (1959).

TABLE I

R_f VALUES OF 3,4-DIHYDROXYACETOPHENONE (III) AND ITS METHOXY DERIVATIVES II, IV

Ascending technique and Whatman No. 1 filter paper was used. The spraying reagent was 0.1% of freshly prepared dichloroquinone chlorimide solution in ethanol followed by 0.5% of aqueous sodium carbonate. The catechol III gave a brown spot under visible and a blue fluorescent spot under ultraviolet light. Synthetic and enzymatically formed acetoisovanillone gave a blue spot (intensive bright violet fluorescence), and synthetic or enzymatically formed II gave an intensive sky-blue spot (intensive greenish-yellow fluorescence), respectively.

Solvent systems	3,4-Dihydroxyacetophenone (III)	Acetovanillone (IV) Synthetic	Acetovanillone (IV) Formed enzymatically	Acetoisovanillone (II) Synthetic	Acetoisovanillone (II) Formed enzymatically
1-Butanol–15% aq. NH_3, 4:1	0.22	0.36	0.37	0.58	0.59
2-Propanol–15% aq. NH_3–H_2O, 8:1:1	.40	.48	.48	.65	.64
1-Butanol–methanol–15% aq. NH_3–H_2O, 8:2:1:1	.33	.49	.49	.66	.66
Cyclohexane–2-propanol–H_2O, 2:2:1 (aq. layer)	.75	.84	..	.89	..
Butanone–propionic acid–H_2O 15:5:6	.79	.87	..	.87	..

TABLE II

pH of incubation mixture Init.	Final (after 1.5 hr.)	Relative yields of methylation products, % Acetovanillone (IV)	Acetoisovanillone (II)	Δ (IV − II)
6.5	6.5	62 (59)[a]	38 (41)	+24 (+18)
6.6	6.7	63 ...	37 ...	+26 ...
7.4	7.2	53.5 (52)	46.5 (48)	+7 (+4)
7.5	7.3	53 (54)	47 (46)	+6 (+8)
8.3	7.5	44 (45.5)	56 (54.5)	−12 (−9)
8.4	7.6	49 (50)	51 (50)	−2 (0)
Control expt. (without active methionine)				
7.4	7.2	0 (0)	0 (0)	.. ...

[a] The figures in parentheses represent relative yields of individual methylation products *after* separation of IV and II by paper chromatography (1-butanol–15% NH_3, 4:1). The other figures are assay values of the mixture of methylation products.

TABLE III

R_f VALUES OF 3-HYDROXY-4-METHOXYPHENYLMETHYLCARBINOL (Vb) AND 4-HYDROXY-3-METHOXYPHENYLMETHYLCARBINOL (Vc)

Solvent systems	3-Hydroxy-4-methoxyphenylmethylcarbinol (Vb)	4-Hydroxy-3-methoxyphenylmethylcarbinol (Vc)	Enzymatically formed mixture
Methanol–butanol–benzene–H_2O, 2:1:1:1	0.88	0.86	0.87
2-Propanol–15% aq. NH_3–H_2O, 8:1:1	.85	.85	.86
1-Propanol–formic acid–H_2O, 7:1:2	.93	.95	.92
Methyl ethyl ketone–propionic acid–H_2O, 15:5:6	.96	.96	.96

Chromatographic Separation of 3-Hydroxy-4-methoxyphenylmethylcarbinol (Vb) and 4-Hydroxy-3-methoxyphenylmethylcarbinol (Vc) after Coupling with Diazotized Sulfanilic Acid.—Since no separation of the isomers Vb and Vc could be obtained by paper chromatography, the chromatographic properties of the derivatives formed by coupling with diazotized sulfanilic acid were investigated.[10,11]

A solution of diazotized sulfanilic acid was prepared as described by Ames and Mitchell[12] and 0.6 ml. of this solution was added to 2–4 ml. of a strongly basic ethanol–water (1:1) solution containing 400 γ of hydroxymethoxyphenylmethylcarbinol. The enzymatically formed hydroxymethoxyphenylmethylcarbinol was first purified by preparative paper chromatography (descending) using methanol–butanol–benzene–water (2:1:1:1). After drying, the hydroxymethoxyphenylmethylcarbinol fraction was extracted into a small volume of ethanol. The solutions containing the orange sodium azobenzenesulfonate derivatives were concentrated to a small volume *in vacuo* and an aliquot of this solution was chromatographed (descending) with methanol–butanol–benzene–water (2:1:1:1) on Whatman No. 1 paper that had been saturated with 2.5% sodium carbonate and dried. The results are shown in Table IV. The relative intensities of the two orange spots derived from the enzymatically formed hydroxymethoxyphenylmethylcarbinol indicated that only a small amount of the *p*-O-methylated compound had been formed, the preponderant product being 4-hydroxy-3-methoxyphenylmethylcarbinol. A similar isolation and assay was carried out on the products from a control enzymatic experiment in which no S-adenosylmethionine was present. A small amount of material which reacted with the diazotized sulfanilic acid had been carried through the isolation and gave a yellow coupling product with an R_f of 0.69. Because of this, no attempt was made to assay quantitatively the exact proportions of *m*- and *p*-O-methylation of 3,4-dihydroxyphenylmethylcarbinol.

TABLE IV

R_f VALUES AND COLOR OF SODIUM AZOBENZENESULFONATE DERIVATIVES OF 3-HYDROXY-4-METHOXYPHENYLMETHYLCARBINOL (Vb) AND 4-HYDROXY-3-METHOXYPHENYLMETHYLCARBINOL (Vc).

Azo coupling derivatives of 3-Hydroxy-4-methoxyphenylmethylcarbinol (Vb) Synthetic	Azo coupling derivatives of 3-Hydroxy-4-methoxyphenylmethylcarbinol (Vb) Enzymatically formed	Azo coupling derivatives of 4-Hydroxy-3-methoxyphenylmethylcarbinol (Vc) Synthetic	Azo coupling derivatives of 4-Hydroxy-3-methoxyphenylmethylcarbinol (Vc) Enzymatically formed
0.65 (orange)	0.65 (orange)	0.76 (orange)	0.77 (orange)

Enzymatic O-Methylation of 3,4-Dihydroxyphenethylamine (Dopamine, VIa).—A sample of 3,4-dihydroxyphenethylamine hydrochloride (VIa) (4 mg., 20 μmoles) was incubated at 37° with 5 ml. of enzyme preparation, 2 ml. of 0.5 *M* phosphate buffer, *p*H 7.9, 4 μmoles of S-adenosylmethionine and 0.2 ml. of 2.0 *M* magnesium chloride in a total volume of 10 ml. (*p*H 7.3). After incubation for 1.5 hours the *p*H was adjusted to 10.0 and the solution was extracted twice with 25 ml. of an isoamyl alcohol–toluene (3:2) mixture. Each extraction was shaken for 20 minutes. The amines were reextracted into 5 ml. of 0.1 *N* hydrochloric acid. This solution was used for subsequent purification. Under the conditions described none of the starting material is extracted from the enzyme mixture.

Chromatographic Isolation of 3-Hydroxy-4-methoxyphenethylamine (VIb) and 4-Hydroxy-3-methoxyphenethylamine (VIe) from the Enzymatic Methylation Mixture.—Investigation of 3-hydroxy-4-methoxyphenethylamine hydrochloride (VIb) and 4-hydroxy-3-methoxyphenethylamine hydrochloride (VIc) under a variety of conditions of paper chromatography led to no separation of the two isomers. Table V presents the results of chromatographic studies with synthetic and enzymatically formed hydroxymethoxyphenethylamines. Synthetic 3-hydroxy-4-methoxyphenethylamine (VIb) gave an intense blue spot with the dichloroquinone-chlorimide reagent (see Table I), 4-hydroxy-3-methoxyphenethylamine (VIc) gave a brown spot and the enzymatically formed product gave a pale blue spot.

Preparative chromatography (descending) was carried out with the enzymatically formed hydroxymethoxyphenethylamines with all precautions to prevent oxidation (SO_2 atmosphere, drying with nitrogen).[9] After development of the

(10) R. Hossfeld, THIS JOURNAL, **73**, 852 (1951).

(11) W. Chang, R. Hossfeld and W. Sandstrom, *ibid.*, **74**, 5766 (1952).

(12) B. Ames and H. Mitchell, *ibid.*, **74**, 252 (1952).

TABLE V

R_f VALUES FOR 3-HYDROXY-4-METHOXYPHENETHYLAMINE HYDROCHLORIDE (VIb), 4-HYDROXY-3-METHOXYPHENETHYLAMINE HYDROCHLORIDE (VIc) AND ENZYMATICALLY FORMED HYDROXYMETHOXYPHENETHYLAMINE HYDROCHLORIDES

Solvent systems	3-Hydroxy-4-methoxy-phenethylamine·HCl (VIb)	4-Hydroxy-3-methoxy-phenethylamine·HCl (VIc)	Enzymatically formed mixture
Methanol–butanol–benzene–H_2O, 2:1:1:1	0 64	0 64	0.65
2-Propanol–15% aq. NH_3–H_2O, 8:1:1	.76	.78	.76
1-Propanol–formic acid–H_2O, 7:1:2	.63	.62	.63
Butanone–propionic acid–H_2O, 15:5:6	.78	.76	.77

chromatograms with methanol–butanol–benzene–water (2:1:1:1) on Whatman No. 1 paper, the fraction corresponding to the *m*- and *p*-methylated product was extracted into 0.1 *N* hydrochloric acid and the amount of hydroxymethoxyphenethylamine was determined from the absorption at 280 mμ. When the control enzymatic experiment, in which no S-adenosylmethionine was present, was assayed in the same manner no formation of material corresponding in R_f to compounds VIb and VIc occurred.

Colorimetric Determination of 3-Hydroxy-4-methoxyphenethylamine (VIb) in the Presence of 4-Hydroxy-3-methoxyphenethylamine (VIc).—The reaction of 4-hydroxy-3-methoxyphenethylamine (VIc) with dichloroquinonechlorimide in basic solution formed a light brown solution with a spectrum having no strong absorption in the visible region, whereas the spectrum of the reaction mixture, an intense blue solution containing 3-hydroxy-4-methoxyphenethylamine (VIb), had a strong maximum at 600 mμ. This difference allowed an assay to be developed for VIb in the presence of VIc as described below.

To 4 ml. of a solution containing 0.5 ml. of 5% sodium bicarbonate and a total of 100 γ of hydroxymethoxyphenethylamine, graded from 0 to 40% 3-hydroxy-4-methoxyphenethylamine, was added 0.2 ml. of an alcoholic solution containing 200 γ of dichloroquinonechlorimide. The enzymatic methylation mixture after careful purification by paper chromatography was assayed in the same manner. The tubes were allowed to stand for 10 minutes and the optical density at 600 mμ then was measured. From the standard curve, the percentage of 3-hydroxy-4-methoxyphenethylamine in the enzymatic methylation mixture was calculated to be 13%.

Chromatographic Separation of 3-Hydroxy-4-methoxyphenethylamine and 4-Hydroxy-3-methoxyphenethylamine after Coupling with Diazotized Sulfanilic Acid.—A solution of diazotized sulfanilic acid was prepared as described by Ames and Mitchell[12] and 0.2 ml. of this solution was added to 2.5 ml. of water containing 250 γ of hydroxymethoxyphenethylamine and 0.3 ml. of 10% sodium hydroxide. The solution was concentrated to dryness *in vacuo* and extracted into 1 ml. of methanol–water (1:1). An aliquot of this solution was chromatographed (descending) with methanol–butanol–benzene–water (2:1:1:1) on Whatman No. 1 paper saturated with 2.5% sodium carbonate and then dried. The results given in Table VI confirm the formation of both 3-hydroxy-4-methoxyphenethylamine (VIb) and 4-hydroxy-3-methoxyphenethylamine (VIc) on enzymatic O-methylation of 3,4-dihydroxyphenethylamine (dopamine VIa).

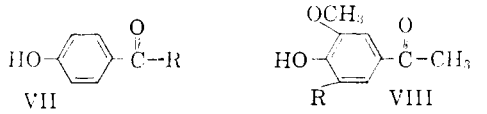
Variations in the Enzymatic O-Methylation of 3,4-Dihydroxyphenethylamine (Dopamine, VIa) as a Function of *p*H.—When enzymatic methylation was carried out at *p*H values of 6.5, 7.4, and 8.2 and the percentage of *p*-O-methylated isomer was determined as described above, values of 11, 15 and 10%, respectively, were obtained.

Discussion

The well-known displacement of the ultraviolet absorption bands of *p*-hydroxyphenones, *e.g.*, VII and VIII, to longer wave length on basification is influenced by the presence of methoxy groups.[13,14] These differences (Fig. 2) permitted the spectrophotometric assay of mixtures of acetovanillone and -isovanillone.

TABLE VI

Azo coupling derivatives of 3-Hydroxy-4-methoxy-phenethylamine (VIb)		Azo coupling derivatives of 4-Hydroxy-3-methoxyphenethylamine (VIc)	
Synthetic	Enzymatically formed	Synthetic	Enzymatically formed
0.45 (orange)	0.44 (orange)	0.31 (rose red)	0.32 (rose red)

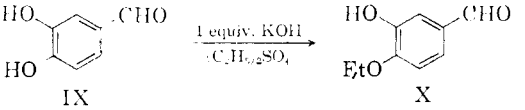


VII VIII

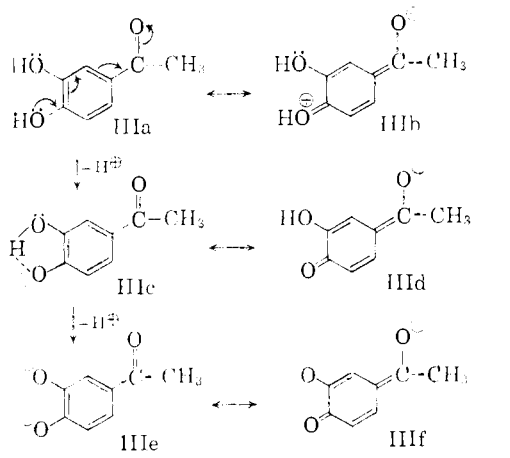
R = H: $\lambda_{max}^{0.1\ N\ NaOH}$ 330 mμ (ϵ 27,900)[13]
R = CH_3 324 (ϵ 23,100)[13]

R = H: $\lambda_{max}^{0.1\ N\ NaOH}$ 348 mμ[14]
R = OCH_3 362 mμ[14]

The influence of the side chain on the ratio of *o*- and *p*-O-methylation has some precedent. Protocatechualdehyde (IX) with one equivalent of alkali undergoes, under appropriate conditions, *p*-O-alkylation to X.[15] The explanation is that one



IX X

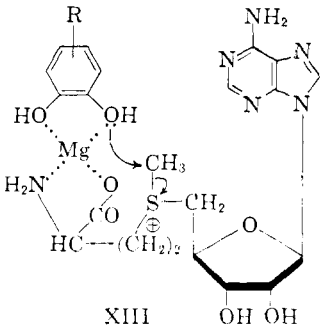


mole of base forms the *p*-phenoxide ion IIIc by abstraction of a proton from the more acidic phenolic hydroxide group in the *p*-position of IIIa. In the presence of excess alkali the di-anion IIIe is formed which undergoes preferential methylation at the more nucleophilic *m*-phenoxide. Catechol O-methyltransferase operates best in the neutral *p*H region. The observed preference for enzymatic *m*-O-methylation of IIIa under neutral conditions is in agreement with the stronger nucleophilic character of the *m*-hydroxyl group. Increasing yields of *p*-O-methylation product at higher *p*H show that the very effective nucleophilic-electrophilic monocate-

(13) L. Doub and J. M. Vandenbelt, THIS JOURNAL, **69**, 2714 (1947).

(14) H. W. Lemon, *ibid.*, **69**, 2998 (1947).

(15) D. Beke and C. Szantay, *Acta Chim. Acad. Sci. Hung.*, **14**, 325 (1958).

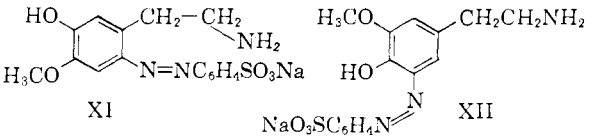


XIII

cholate anion[16] IIIc starts competing successfully with the uncharged species IIIa. To what extent binding by additional functional groups enters into the (magnesium-requiring) nucleophilic displacement on S-adenosylmethionine (hypothetical formulation XIII[17]) is not known. The steric requirements with respect to the sulfonium center are very selective and are reported to be identical for catechol O-methyl transferase, guanidinoacetate methylpherase and the S-adenosylmethionine cleaving system from yeast.[18]

The mechanism of the enzymatic *p*-O-methylation of 3,4-dihydroxyphenylmethylcarbinol (Va) or of dopamine (VIa) *in vitro* cannot readily be rationalized. The physiological implications are of great interest, should this process also occur *in vivo*.

The azo coupling products, *e.g.*, of the O-methylated dopamines VIb and VIc may be formulated as



XI XII

XI and XII. The different points of attachment of the azo part and possible (non-bonded) interactions with the side chain in XI or the phenolic hydroxyl in XII facilitate chromatographic separation and may cause the striking differences in color. "Paranephrine," *i.e.*, *p*-O-methylepinephrine,[19] with regard to pressor effects, is no more active than metanephrine.[20] Investigations now in progress[21] are aimed at establishing the ratio of formation of (nor)-paranephrine *in vitro* and *in vivo* as well as any possible indications for its being a better substrate than (nor)metanephrine for the demethylase that reconverts such ethers to (nor)epinephrine.[22] Previous data indicate that *p*-O-alkyl ethers are cleaved more rapidly than their *m*-O-methyl analogs.[23]

Acknowledgment.—We are greatly indebted to Dr. Sydney Archer, Sterling-Winthrop Research Institute, for his interest and donation and preparation of compounds.

(16) *Cf.* J. W. Churchill, M. Lapkin, F. Martinez and J. A. Zaslowsky, THIS JOURNAL, **80**, 1944 (1958).

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(19) *Cf.*, F. Külz and W. Hornung, German Patent 682,394 (1939); *C. A.*, **36**, 3011 (1942).

(20) We are greatly obliged for this information to Drs. S. Archer and A. M. Lands, Sterling-Winthrop Research Institute.

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BETHESDA 14, MD.

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Chemical and Enzymatic Studies of the Labile Metabolite 4(5H)-Imidazoline-5-acetic Acid[1]

BY HERMANN KNY[2] AND BERNHARD WITKOP

RECEIVED MAY 15, 1959

Carefully controlled intramolecular cyclization of α-ethyl formimino-L-aspartate (II) yielded 4(5H)-imidazolone-5-acetic acid (III) which had a half-life time of close to one hour at *p*H 8. Its *enzymatic degradation* by imidazoleacetic acid oxidase from *Pseudomonas* was followed *in situ* in a self-recording spectrophotometer by the disappearance of λ_{max} 259 mμ; it was found to be significantly faster than the *spontaneous hydrolysis* and produced more formiminoaspartic acid (VII) than the spontaneous decomposition which yielded more (formyl)isoasparagine, pointing to two different sites for cleavage on the imidazolone ring. Translactamization of III to 5,6-dihydro-4-pyrimidone-6-carboxylic acid (VIII) could not be ruled out. The highly unstable dihydropyrimidone VIII was the intermediate in the partial hydrogenation of the pyrimidone IX with a rhodium catalyst as evidenced by the appearance of formiminoaspartic acid (VII) besides asparagine which probably arose from the accompanying tetrahydropyrimidone XII *via* methyleneasparagine (XI). The internal cyclization of formylasparagine (XV) or -isoasparagine (IV) with acetic anhydride did not yield the dihydropyrimidone VIII but 3-formaminosuccinimide (XVII).

Imidazoleacetic acid is degraded by a strain of *Pseudomonas fluorescens*, adapted to imidazoleacetic acid, to formiminoaspartic (VII)[3] and formylaspartic acid (VI).[4] It is known that O^{18} is directly incorporated into imidazoleacetic acid[5] by a "mixed function oxidase."[6] The presumed intermediate

(1) Labile Metabolites. VIII. Preceding paper, *cf.* THIS JOURNAL, **81**, 1768 (1959).

(2) Visiting Scientist of the USPHS from the University of Basle, Switzerland.

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(6) *Cf.* H. S. Mason, "Advances in Enzymology," Interscience Publishers, Inc., New York, N. Y., Vol. 19, 1957, p. 179.