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Chemical, Enzymatic and Metabolic Studies on the Mechanism of Oxidation of Dopamine[1]

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N-Carbobenzyloxy-3,4-dihydroxyphenethylamine-β,β-H^3 suffers no loss of tritium activity when subjected to oxidation to the quinone, to 1,4-addition reactions involving catalysis by boron trifluoride etherate or to the conditions of decarbobenzyloxylation in 48% hydrobromic acid. N-Carbobenzyloxy-6-methoxydopamine quinone-β,β-H^3 (XI) shows only slight tritium lability at room temperature which increases at higher *p*H. The exchange of tritium in a comparable intermediary quinone of dopamine (III, IV) would be too slow to be detectable in the enzymatic synthesis of norepinephrine which has been studied with a number of tissues using dopamine labeled with C^{14} and H^3. The responsible enzyme (*"dopamine β-oxidase"*) effects conversion to norepinephrine with a H^3/C^{14} ratio approximating half of that of the starting material. The retention of the original H^3/C^{14} ratio in other experiments led to the discovery of the isomeric 2,4,5-trihydroxyphenethylamine (II), *isographic* with norepinephrine in more than ten different solvent systems. It is formed from dopamine by oxidation, autoxidation or metabolic processes. Whether nuclear hydroxylation proceeds enzymatically and whether it and the biogenesis of norepinephrine pass through quinoid intermediates III ⇄ IV must be left open, until direct studies with O^{18} become practicable.

The biosynthesis of the endogenous sympathetic hormone norepinephrine (V), which starts from tyrosine,[3] is generally believed to involve hydroxylation of the side chain of dopamine (I)[4] in the sequence of reactions (Chart I).[5,6] This conversion

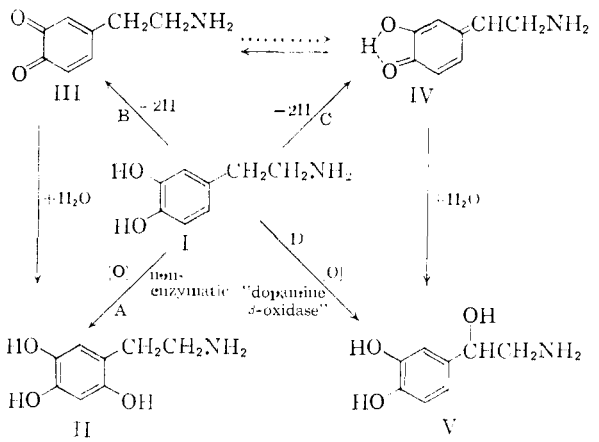
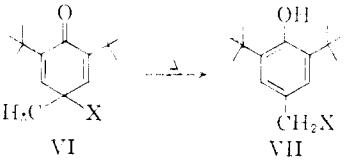


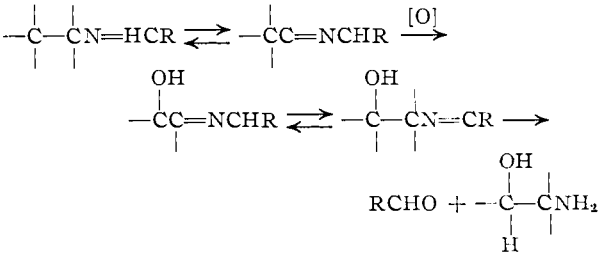
Chart I.—Enzymatic and non-enzymatic conversions of dopamine.

has been demonstrated in isolated tissues many times. However, it has not been possible so far to obtain enzyme preparations potent enough to permit studies on the mechanism of the reaction, *e.g.*, with O^{18}. The latter could enter directly into the benzyl position of the side chain (pathway D)[7] or, conceivably, could first dehydrogenate I to the quinone III (pathway B) which itself, or as the tautomeric quinone methine IV, is capable of 1,4- or 1,6-addition of water[1] to yield either 2,4,5-trihydroxyphenethylamine (II) or norepinephrine (V). The former (II) could also arise by pathway A, analogous to D, *i.e.*, direct hydroxylation, of I. The *p*-quinone methine IV, at least enzymatically, need not be formed *via* the *o*-quinone III (pathway B), but could arise directly by 1,6-abstraction of hydrogen (pathway C).

An initial *p*-quinol undergoing an allylic shift, such as observed in the easy rearrangement of VI → VII (X = Br),[8] is possible in a highly hindered *p*-substituted phenol but unlikely in a catechol.



A chemically possible mechanism, supplying additional activation for oxidative attack at the benzyl position, is the β-hydroxylation of a tautomeric Schiff base of dopamine by the pathway



where the β-hydroxyazomethine would be tautomeric with an oxazolidine.[9]

The preclusion of O^{18} [10] prompted the following studies of the fate of tritium in the non-enzymatic and enzymatic conversions depicted in Chart I.

Lability of Tritium in the Non-enzymatic Conversions of N-Carbobenzyloxy-2-methoxy-4,5-dihydroxyphenethylamine-β,β-H^3 Quinone (XI).—The catalytic reduction of arterenone (VIII)[11] in the

(1) Oxidation Mechanisms. XXIV. Preceding paper *cf.* THIS JOURNAL, **81**, 6231 (1959).

(2) Visiting Scientist of the USPHS on leave of absence from the Institute of Food Chemistry and Osaka City University, Japan.

(3) S. Udenfriend and J. B. Wyngaarden, *Biochim. Biophys. Acta*, **20**, 48 (1956).

(4) M. Goodall and N. Kirshner, *J. Biol. Chem.*, **226**, 213, 821 (1957).

(5) G. Rosenfeld, L. C. Leeper and S. Udenfriend, *Arch. Biochem. Biophys.*, **74**, 252 (1958).

(6) H. Blaschko, *Brit. Med. Bull.*, **13**, 162 (1957).

(7) *Cf.* formation of 3,5-diiodophenylglycolic acid on incubation of the parent acetic acid at *p*H 7.5 and 37°: T. Matsuura and H. J. Cahnmann, *Biochim. Biophys. Acta*, **29**, 216 (1958).

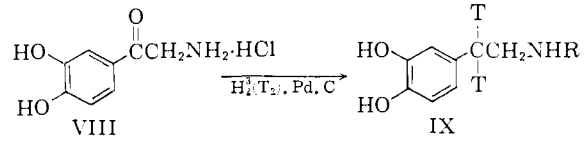
(8) C. D. Cook, N. G. Nash and H. R. Flanagan, THIS JOURNAL, **77**, 1783 (1955).

(9) Z. Földi, *Acta Chim. Acad. Hungaric.*, **10**, 1 (1955).

(10) O^{18} Tracer studies on a micro scale may be possible in the future if simplifications are found for the activation analysis according to I. Fogelström-Fineman, O. Holm-Hansen, B. M. Tolbert and M. Calvin, *Intern. J. Appl. Radiation and Isotopes*, **2**, 280 (1957).

(11) We are greatly indebted to Dr. Sydney Archer, Sterling-Winthrop Research Institute, for a liberal sample of this material.

presence of tritium in acidic medium proceeds with hydrogenolysis of the oxygen function and leads to



IX (R = H), a dopamine carrying tritium in the two crucial benzyl positions. The N-carbobenzyloxy derivative X of IX was taken through the steps previously carried out with cold material,[1] *i.e.*, silver oxide dehydrogenation to the quinone, 1,4-addition of methanol with boron trifluoride catalysis, sodium borohydride reduction of the methoxyquinone XI to the methoxycatechol XII, catalytic debenzylation to the methoxyamine XIII, and finally demethylation with 48% hydrobromic acid to 2,4,5-trihydroxyphenethylamine (XIV). None of the five steps led to significant loss of tritium (Table I). Even strong acid catalysis failed to labilize tritium in the benzyl position *para* to a phenolic hydroxyl.

TABLE I

Structure	Type of the reaction: c.p.m./μmole X → XI → XII → XIII → XI XII XIII XIV
X	8140
XI	7830 7830
XII	8010 8010
XIII	7920 7920
XIV	7720
Ratio of the residual tritium	1:1 1:1 1:1 1:1

The quinone of N-carbobenzyloxydopamine was too unstable for characterization or tritium studies. Labilization of tritium was, therefore, followed in the comparable quinone of N-carbobenzyloxy-2-methoxy-4,5-dihydroxyphenethylamine (XI) in aqueous methanolic buffer solutions at 25° in the range *p*H 1–11. Figure 1 summarizes the results (see also Experimental part, Tables IV and V).

There was no significant loss of tritium below *p*H 2. Whether the values between *p*H 2–4 are statistically significant and the 10% loss of tritium is real cannot be decided without more data. The same reservation holds *vis-à-vis* the apparent plateau between *p*H 3.9–7.5. From then on a slow but significant loss of tritium was observed showing, as expected, the easy abstraction of a tritium from the activated methylene group and the formation of the

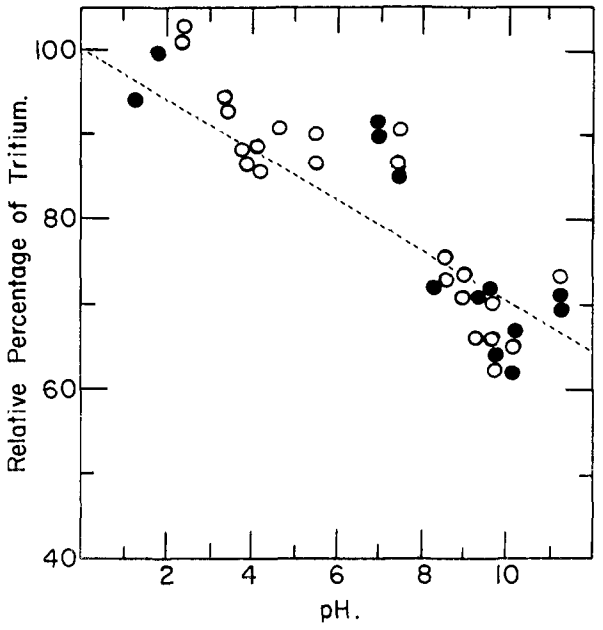
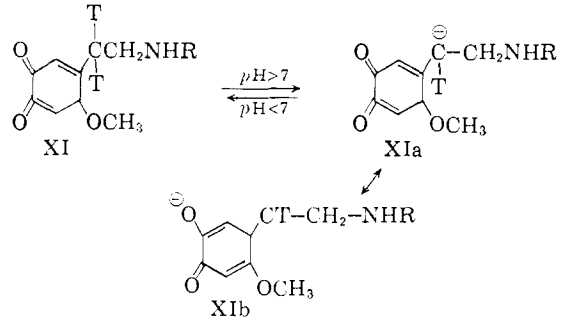


Fig. 1.—Ratio of the relative loss of tritium in the benzyl position of the quinone of N-carbobenzyloxy-2-methoxy-4,5-dihydroxyphenylethylamine in buffer solutions of varying *p*H on standing at 25°: ○, 0.5 *N* HCOOH-$(CH_3)_3N$ buffer in 50% aqueous methanol; ●, 0.5 *N* HCl-$(CH_3)_3N$ buffer in 50% aqueous methanol.

resonance-stabilized anion XI ↔ XIa, whose tautomer is the quinone methine.[12] The time and *p*H required for a significant loss of tritium to become noticeable make quite unlikely the discovery of a quinone intermediate in the enzymatic conversion of dopamine to norepinephrine by this tritium technique.



Enzymatic Studies with Dopamine Marked with C^{14} and H^3.—To determine the fate of the tritium atoms on the benzyl carbon during enzymatic conversion of dopamine to norepinephrine, mixtures of dopamine-β,β-H^3 and dopamine-α-C^{14} were incubated with tissue homogenates. Boiled tissues were used as controls.

As shown in Table II appreciable amounts of norepinephrine were formed in these studies. The last column in Table II presents the ratio

$$\frac{H^3/C^{14} \text{ isolated norepinephrine}}{H^3/C^{14} \text{ starting dopamine}}$$

(12) In this connection it may be mentioned that kinetic studies on the *p*H-dependent exchange of substituted phenols with deuterium [W. G. Brown, K. Wilzbach and W. H. Urry, *Can. J. Research*, **27b**, 398 (1949)] show a strong isotope effect as a result of steric demands in the formation of the quinoid tetrahedral transition state.

This has been corrected by subtracting the radioactive contribution of the "norepinephrine-like" material in the boiled control experiments. It is apparent that the corrected ratios approximate the value 0.5, indicating that in the transformation of dopamine to norepinephrine not more than one of the two benzylic tritium atoms is lost. Obviously this finding alone is not sufficient to determine the mechanism of *dopamine β-oxidase*. However, as additional knowledge is gained concerning this enzyme, the present information will become more valuable.

TABLE II

RATIOS OF H^3/C^{14} IN THE ENZYMATIC CONVERSION OF C^{14}, H^3-LABELED DOPAMINE TO NOREPINEPHRINE AND 2,4,5-TRIHYDROXYPHENETHYLAMINE

Tissue[a]	Norepinephrine[b] formed, μg.	Counts/min.[c] C^{14}	Counts/min.[c] H^3	H^3/C^{14}	R/R^0[d]
Experiment 1: R^0 = 3.13					
Hypothalamus	4.8	13,884	24,513	1.75	0.56
Boiled hypothalamus	0.6	1,739	5,472	3.13	1.02
Experiment 2: R^0 = 3.13					
Caudate nucleus	0.88	2,281	5,038	2.19	0.69
Caudate nucleus	.73	1,826	3,514	1.92	0.61
Boiled caudate nucleus	.09	289	836	2.91	0.95
Cerebellum	.08	248	896	3.60	1.15
Cerebellum	.07	204	805	4.15	1.30
Experiment 3: R^0 = 3.72					
Hypothalamus	0.76	1,735	3,750	2.15	0.58
Hypothalamus + 50 μmoles dopamine	1.20	2,680	5,409	2.02	0.54
Cerebellum	0.04	96	345	3.60	0.97

[a] In each experiment tissues were incubated with a known mixture of dopamine-α-C^{14} and dopamine-β,β-H^3 (R^0). A total of 200 μg. of dopamine was incubated with 500 mg. of homogenized tissue in 3.5 ml. of solution at *p*H 7.4 (0.1 *M* phosphate buffer). This was fortified with a mixture containing 0.5 μmole each of DPN, TPN, ATP, pyridoxalphosphate, FAD, CoA, 0.3 mg. of citrate, 1 mg. of glucose and incubated for 3 hours at 37° in air. [b] The material formed in boiled controls and in cerebellum is found to cochromatograph with norepinephrine and coprecipitates with it during crystallization. Its properties are consistent with 2,4,5-trihydroxyphenethylamine. [c] In experiments 1 and 2 the measured counts/min. are corrected for boiled controls. Experiment 3 has not been corrected in this manner. [d] R^0 = H^3/C^{14} of starting dopamine.

A most interesting finding was that even in tissues devoid of *dopamine β-oxidase* and in boiled control experiments a small amount of material was formed, which, by chromatography and carrier recrystallization, did not separate readily from norepinephrine. 2,4,5-Trihydroxyphenethylamine (II) which is formed by (aut)oxidation of dopamine is as difficult to separate from norepinephrine. That the material formed under these conditions was 2,4,5-trihydroxyphenethylamine (II) is further indicated by its H^3/C^{14} ratio, which was found to be the same as that of the starting dopamine. The *in vitro* findings prompted *in vivo* experiments. When dopamine-α-C^{14} was administered to rats, an appreciable fraction was found to be excreted as the trihydroxy compound II in the urine. When a urine extract, subjected to conditions of O-methylation as described in the Experimental section, was chromatographed, a sharp radioactive peak, several times background, was found which corresponded exactly with the R_f of hydroxydopamine, indicating that the C^{14}-metabolite could *not* be norepinephrine. The hydroxydopamine was eluted and counted. It was found to represent at least 0.5% of the administered dopamine. However, this must represent a minimal conversion. Under similar conditions only 1% of administered hydroxydopamine was recovered in the urine. Thus, its formation can occur to a significant extent in the intact animal. It remains to be seen whether hydroxydopamine is formed endogeneously, whether it is formed *via* dopamine quinone and how much is normally excreted in the urine. Pharmacological studies using the anesthetized dog are in progress.[13] Few pharmacological data are available for other isomers of this kind, 2,3,4- and 3,4,5-trihydroxyphenethylamine.[14] It will be of interest to determine whether this compound can serve as a substrate of monoamine oxidase and of catechol O-methyl transferase. If so, then the corresponding metabolites, trihydroxyphenylacetic acid and 5-methoxy-2,4-dihydroxyphenylethylamine, should be sought. A further metabolite which is suggested by these studies is 6-hydroxy(nor)epinephrine.

Apparently dopamine is converted readily to 6-hydroxydopamine under simple conditions of autoxidation. The ascorbic acid–iron EDTA system[15] which can duplicate many aromatic hydroxylations carried out by intact animals was found to yield appreciable amounts of the hydroxy product when incubated with dopamine (Table III).

In this case use of a mixture of H^3- and C^{14}-dopamine permitted further identification by the H^3/C^{14} ratio of the isolated product.

The ease of formation of hydroxydopamine and the inability to separate it from norepinephrine by chromatography or recrystallization except after treatment with methanolic hydrogen chloride are important factors to consider in evaluating any studies concerning the biosynthesis of norepinephrine. The rapid excretion of labeled "norepinephrine" following administration of dopamine-C^{14} to patients with pheochromocytoma[16] may actually represent hydroxydopamine. This is now being investigated. Obviously the "norepinephrine" isolated in many enzymatic studies reported previously may often have contained or represented 2,4,5-hydroxyphenethylamine (II). Unless proper controls were used such findings must now be re-examined.

Experimental

N-Carbobenzyloxy-β-(3,4-dihydroxyphenyl)-ethylamine-β,β-H^3 (X).—To the mixture of 1.9 g. of 3,4-dihydroxyphenethylamine-β,β-H^3 (dopamine-β,β-H^3) hydrochloride specific activity 44,500 c.p.m./μmole, and 1.9 g. of sodium bicarbonate in 25 ml. of water and 10 ml. of ether there was added dropwise the solution of 1.8 g. of carbobenzyloxy chloride in

(13) Private communication from Dr. Leon Goldberg, National Heart Institute, National Institutes of Health.

(14) G. Barger and A. J. Ewins, *J. Chem. Soc.*, **97**, 2260 (1910); G. Hahn and F. Rumpf, *Ber.*, **71**, 2141 (1938); *cf.* O. Hinsberg, *ibid.*, **56**, 852 (1923); U. S. Patent 1,432,291 of Oct. 17, 1922.

(15) S. Udenfriend, C. T. Clark, J. Axelrod and B. B. Brodie, *J. Biol. Chem.*, **208**, 731 (1954).

(16) A. Sjoerdsma, L. C. Leeper, L. L. Terry and S. Udenfriend, *J. Clin. Investigation*, January (1959).

TABLE III

CONVERSION OF DOPAMINE TO 6-HYDROXYDOPAMINE BY THE ASCORBIC ACID SYSTEM[c]

Substance	R/R^0	R_{fA}[a]	R_{fB}[b]	Color with Fe(CN)$_6$ reagent Visible	Uv.	Color with Gibbs reagent
Dopamine	1.0	0.43	0.61	Pink	Blue fluor.	Brown
6-Hydroxydopamine (exp.)	0.99	.29	.25	Red	No fluor.	Reddish-orange
6-Hydroxydopamine (auth.)	..	.29	.25	Red	No fluor.	Reddish
β-O-Me-norepinephrine	..	.58	.51	Pink	(Fast) violet fluor.	Purple

[a] A, PhOH–0.02 *N* HCl–KCN (80 g.:20 ml.:trace) (satn. of SO_2 gas). [b] B, *sec*-BuOH–HCOOH–H_2O (75:15:10) (N_2 atmosphere). [c] Incubation mixture: 0.1 *M* phosphate buffer, *p*H 5.5; $FeSO_4$ 15 μmoles; ascorbic acid, 142 μmoles; EDTA 30 mg.; dopamine-C^{14} and $-H^3$ mixture 200 μg.; final volume 5.0 ml.; R^0, 3.72.

20 ml. of ether under vigorous agitation in an ice-bath. Stirring was continued for 5 hours at 0°. The isolation and purification of the reaction product was carried out as described in the preceding paper.[17] Two recrystallizations from methanol–ether–petroleum ether yielded 1.3 g., m.p. 132.5–133.5°, specific activity 42,000 c.p.m./μmole.

The mother liquors were evaporated to dryness. The residual sirup was mixed with 5 g. of cold N-carbobenzyloxydopamine and recrystallized twice from the same solvent; yield 3.1 g., m.p. 132–133°, specific activity 4,400 c.p.m./μmole.

For the following syntheses, N-carbobenzyloxydopamine-β,β-H^3 (X) was diluted with cold N-carbobenzyloxydopamine.

Quinone of N-Carbobenzyloxy-β-(2-methoxy-4,5-dihydroxyphenyl)-ethylamine-β,β-H^3 (XI).—To 2 g. of N-carbobenzyloxydopamine-β,β-H^3 (X), specific activity 8,140 c.p.m./μmole, dissolved in a mixture of 25 ml. of anhydrous methanol containing 0.7 ml. of 98% formic acid and 4.5 g. of anhydrous sodium sulfate, was added at 0° 4.5 g. of dried freshly prepared silver oxide. The flask was stoppered and shaken vigorously for 2 minutes. The reaction mixture was filtered through anhydrous sodium sulfate and washed twice with methanol. To the resulting red quinone solution was added 10 ml. of boron trifluoride etherate. After 10 minutes the mixture was boiled gently on the steam-bath for 2 minutes, poured into a mixture of ice and water, well mixed and left in the refrigerator overnight. The solid, after decantation, was washed with water and dried. Trituration with methanol produced crystals. Two recrystallizations from methanol gave 350 mg. of the yellow quinone XI, m.p. 133–134°,[17] specific activity 7,830 c.p.m./μmole (Table I).

β-(2-Methoxy-4,5-dihydroxyphenyl)-ethylamine-β,β-H^3 Hydrochloride (XIII).—The catalytic decarbobenzyloxylation of 150 mg. of the catechol (XII), specific activity 8,010 c.p.m./μmole, was carried out in 20 ml. of ethanol containing 0.2 ml. of concentrated hydrochloric acid with 500 mg. of 10% palladium-on-charcoal as described in the preceding paper.[17] One obtained 115 mg., m.p. 186–188°, specific activity 7,920 c.p.m./μmole (Table I).

N-Carbobenzyloxy-β-(2-methoxy-4,5-dihydroxyphenyl)-ethylamine-β,β-H^3 (XII).—To the suspension of 200 mg. of the quinone XI, specific activity 7,830 c.p.m./μmole, in 2 ml. of methanol was added 30 mg. of sodium borohydride with agitation. The reaction mixture was filtered and diluted with water and cooled in ice. The colorless crystals were collected, washed with water and dried, yielding 195 mg., m.p. 143–144°,[17] specific activity 8,010 c.p.m./μmole (Table I).

β-(2,4,5-Trihydroxyphenyl)-ethylamine-β,β-H^3 Hydrobromide (XIV).—The solution of 50 mg. of 6-methoxydopamine-β,β-H^3 (XIII) hydrochloride, specific activity 7,920 c.p.m./μmole, in a mixture of equal volumes of 48% aqueous hydrobromic and glacial acetic acid was refluxed gently under nitrogen for 8 hours. The solution was evaporated to a small volume and dried over sodium hydroxide in a vacuum desiccator. The crystalline solid was suspended in a small volume of glacial acetic acid, filtered and washed twice with the same solvent. The crystals (47 mg.) had m.p. 218.5–219.5° and specific activity 7,720 c.p.m./μmole (Table I).

Lability of Tritium of N-Carbobenzyloxy-2-methoxy-4,5-dihydroxy-phenethylamine-β,β-H^3-quinone (XI). (A) In Formic Acid–Trimethylamine Buffer.—The results are shown in Table IV (see Fig. 1). Each sample solution was prepared by the following procedure: The solution of 1.0 mg. of N-carbobenzyloxy-2-methoxy-4,5-dihydroxyphenethylamine-β,β-H^3-quinone (XI) in 3 ml. of a certain *p*H of formic acid–trimethylamine in 50% aqueous methanol buffer (a mixture of a certain proportion of 1 *N* aqueous formic acid and 1 *N* aqueous trimethylamine and addition of the same volume of methanol) was allowed to stand at room temperature with occasional agitation for 74 hours under nitrogen in a closed system. Then the solution was subjected to lyophilization. The residue was dissolved in methanol and the tritium count was measured by the usual method in the scintillation spectrophotometer.

TABLE IV

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 (14). %
1	2.40[a]	18,500	102.7
2	3.38	16,700	92.7
3	3.78	15,500	86.2
4	4.10	15,400	85.6
5	4.57	16,600	92.2
6	5.51	15,500	86.2
7	7.42	15,500	86.2
8	8.60	13,100	72.4
9	8.95	12,700	70.5
10	9.38	11,900	66.1
11	9.70	11,200	62.2
12	10.08	11,900	66.1
13	11.25[b]	13,300	73.8
14 (contrast)	1.0 mg. of substance dissolved in 3 ml. of 50% aq. methanol	18,000	...

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

(B) In Hydrochloric Acid–Trimethylamine Buffer.—The results are shown in Table V (see Fig. 1). Each sample solution was prepared as described under procedure A, using a certain *p*H of the mixture of 0.5 *N* hydrochloric acid and 0.5 *N* trimethylamine in 50% aqueous methanol.

Enzymatic Studies Using Dopamine Labeled with C^{14} and H^3. Materials and Methods.—The preparation of dopamine-C^{14} was described previously.[3] 3,4-Dihydroxyphenethylamine-β,β-H^3 (IX) was prepared by the catalytic reduction of arterenone in the presence of tritium as described above. It was purified by preparative chromatography on paper using the system phenol (80 g.) and 0.02 *N* HCl (20 ml.) in the presence of a trace of potassium cyanide in an atmosphere of sulfur dioxide, and stored as a solution in 0.1 *N* hydrochloric acid in the cold.

Tritium and carbon were counted in the Packard Tri-Carb model 314–DC liquid scintillation counter. Toluene containing 0.3% 2,5-diphenyloxazole and 0.01% 1,4-di-(5-phenyloxazolyl)-benzene was utilized as a counting medium. Since the norepinephrine was not soluble in toluene, 5 ml. of ethanol and 0.05 ml. of 0.1 *N* HCl were added to 10 ml. of the toluene solution. Carbon activity was obtained at a photomultiplier tube voltage of 900 using a 10–25 volt window, and carbon-plus-tritium activity was counted at a tube voltage of 1310 using a 10–100 volt window. The efficiency for carbon at the first setting was 35%; at the second it was 15%. No counts for tritium appeared at the first setting.

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

TABLE V

Expt.	*p*H of the soln.	C.p.m./ μmole	Residual tritium count after 74 hr. at room temp. 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.

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[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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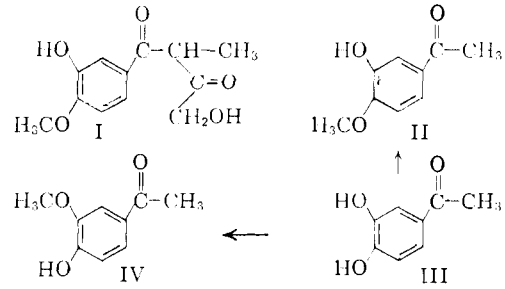
The enzymatic methylation by catechol O-methyl transferase of 3,4-dihydroxyacetophenone (III), 3,4-dihydroxyphenylmethylcarbinol (V) and 3,4-dihydroxyphenethylamine (dopamine, VI) has been studied as a function of *p*H. Spectrophotometric, colorimetric and chromatographic methods have been developed 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.



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