BBA 66298

CATECHOL *O*-METHYLTRANSFERASE

THE *para*- AND *meta*-O-METHYLATIONS OF NORADRENALINE

J. M. FRÈRE AND W. G. VERLY
Department of Biochemistry, University of Montreal, Montreal (Canada)

(Received October 13th, 1970)

SUMMARY

Rat liver catechol *O*-methyltransferase (*S*-adenosylmethionine:catechol *O*-methyltransferase, EC 2.1.1.6) was purified 350-fold without separating the *m*-O-methylating from the *p*-O-methylating activities which suggests that both activities belong to the same protein. No dimethylation of noradrenaline could be detected. The purified enzyme is unstable.

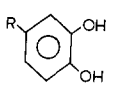
Two isoenzymes were separated by gel electrophoresis; they O-methylated the noradrenaline in the *meta* and *para* positions in the same ratio (*m*/*p* ratio).

The concentrations of noradrenaline or *S*-adenosylmethionine did not affect the *m*/*p* ratio. The identity of the K_m's for the two O-methylating activities is also in favor of the hypothesis that the same enzyme catalyzes the two reactions.

pH and ionic strength influenced the *m*/*p* ratio; the pH acts mostly through its effect on the ionization of the catechol moiety of noradrenaline. The norparanephrine was found to be chemically unstable.

Various O-methylated phenol and catechol derivatives had little or no inhibitory activity and did not affect the *m*/*p* ratio.

INTRODUCTION

In 1959, SENOH *et al.*[1] showed that crude preparations of catechol *O*-methyltransferase (*S*-adenosylmethionine:catechol *O*-methyltransferase, EC 2.1.1.6) can methylate catechol derivatives of the general formula 

in either the *para* or *meta* positions. This early work was later confirmed by a number of different investigators[2-8].

As yet, it is uncertain whether a single enzyme is capable of methylating both positions or if two distinct proteins are involved. ANDERSON[5], achieving a 6-fold purification of the enzyme by molecular sieve chromatography, did not observe any variation of the ratio of *m*-O-methylation to *p*-O-methylation (*m*/*p* ratio) for the

substrates 3,4-dihydroxycinnamic acid and 3,4-dihydroxyphenylethylamine. ANDERSON AND D'IORIO[9] reported a 200-fold purification of the enzyme but no *m/p* ratio determination was made at this high level of purification.

We have purified the enzyme 350-fold and determined the *m/p* ratio for the O-methylation of noradrenaline at each step of purification. We were able to separate two proteins exhibiting catechol *O*-methyltransferase activity and found no difference in the *m/p* ratio in the products of their O-methylating activity when noradrenaline was the substrate. Finally, factors influencing this *m/p* ratio were also investigated.

MATERIAL AND METHODS

(I) Chemical sources and assay methods

The enzyme was obtained from adult rat liver (Wistar strain, 150–200 g). Protein concentrations were determined spectrophotometrically (concn. (mg/ml) = 1.54 $A_{280\ nm}$ − 0.76 $A_{260\ nm}$). The incubation conditions are indicated in the tables and figures.

When the *m/p* ratio was not required, the activity of the enzyme was routinely determined using pyrocatecholphthalein as a substrate[10]. When the *m/p* ratio was to be measured, the substrate was [7-^{14}C]noradrenaline; the O-methylated amines were oxidized with periodate to aldehydes which were extracted with benzene and separated by gas chromatography[11].

[^{14}C]Normetanephrine and [^{14}C]norparanephrine were prepared enzymatically from [7-^{14}C]noradrenaline[11]; while the [^{14}C]normetanephrine preparation was radiochemically pure, the [^{14}C]norparanephrine preparation contained about 10% [^{14}C]-normetanephrine and 40% unidentified labelled impurities[11]. [7-^{14}C]Noradrenaline (48 mC/mmole) was obtained from Schwartz Bioresearch, pyrocatecholphthalein from Eastman Organic Chemicals, (−)-*S*-adenosyl-L-methionine from PL Biochemical, 3,4-dimethoxy-β-phenylethylamine and 4-methoxy-β-phenylethylamine from Calbiochem, vanillin from Fisher Scientific and isovanillin from K and K laboratories. Veratraldehyde[12], *N*-methyl-3,4-dimethoxy-β-phenylethanolamine and 3,4-dimethoxy-β-phenylethanolamine were prepared in our laboratory.

(II) Enzyme purification

All enzyme preparations were kept at 0–4° during the purification steps. Sodium phosphate buffers were used unless otherwise stated.

(a) Soluble fraction from rat liver. The method of AXELROD AND TOMCHICK[13] was used, but homogeneization of the livers was carried out using 0.25 M sucrose instead of isotonic KCl.

(b) $(NH_4)_2SO_4$ precipitation. The precipitate obtained between 40 and 60% saturation was dissolved in 25–35 ml 0.005 M sodium phosphate buffer, pH 7.0 (containing 32 mg cysteine · HCl per l) and dialyzed against the same buffer for 12 h. A precipitate which formed during dialysis contained no enzymatic activity and was eliminated by centrifugation. The supernatant was lyophilized (LI powder) or applied directly to a continuous paper electrophoresis curtain.

(c) Continuous paper electrophoresis. A Beckman model CP continuous flow paper electrophoresis cell was used at a potential difference of 800 V. The buffer

contained, in 15 l, 4.0 g Na_2HPO_4, 0.25 g NaH_2PO_4, 0.5 g cysteine·HCl and 3.0 g of dithiothreitol and the pH was adjusted to 8.0 with NaOH. The dialyzed $(NH_4)_2SO_4$ fraction was applied at a rate of 2–3 ml/h, whereas the flow rate of the buffer was maintained at 27 ml/h.

(d) Molecular sieve chromatography. The method of ANDERSON AND D'IORIO[9] was used with the following modifications: Sephadex G-75 was used instead of Biogel P-60, with an elution speed of 40 ml/h and the buffer contained 0.15 g dithiothreitol per l.

(e) DEAE-cellulose chromatography. The DEAE-cellulose chromatography was realized according to ANDERSON AND D'IORIO[9].

(f) Preparative disc electrophoresis. We used a Canalco prepdisc apparatus: gels were cylindrical crowns, with a 13-mm outside radius and a 5-mm inside radius. Stacking and separating gels were standard, 2.5 and 7% gels, respectively, 5–7 mm and 3–7 mm thick. The thickness of the separating gel was very difficult to control accurately. Anode and cathode buffers were Tris–glycine buffers, pH 8.3 (3 g Tris and 14.4 g glycine per l), and the elution performed with an imidazole buffer (0.35 g imidazole, 0.07 ml *N,N,N',N'*-tetramethylethylenediamine and 0.15 g dithiothreitol per l, brought to pH 6.9 with HCl) flowing at a rate of 3 ml/min. Current was 6 mA.

Proteins were dissolved in a 0.15 M cysteine solution containing 0.0013% bromophenol blue and 10% sucrose. The total protein concentration in the sample was not over 10 mg/ml if the LI powder was used or 20 mg/ml with more purified preparations, otherwise a precipitate formed as soon as the protein solution came in contact with the Tris–glycine buffer.

(III) Analytical disc electrophoresis—detection of enzymatic activity in the gel

A Canalco Type 12 apparatus with standard gel system and buffers was used. The columns were 0.5 cm × 7 cm quartz tubes (General Electric). Current was 3 mA per column. Protein samples were prepared according to the method described above for preparative disc electrophoresis. After electrophoresis, proteins were detected in the gel by scanning the column at 280 nm using a transformed Hitachi–Perkin–Elmer spectrophotometer or at 510 nm after staining the gel with aniline black. Enzyme estimation was carried out as follows: after scanning at 280 nm, the separating gel was cut into 1.6-mm-thick slices (lateral gel slicer, Canalco); each slice was homogenized with 3.5 ml cold 0.06 M sodium phosphate buffer, pH 7.8, containing 1 mg cysteine·HCl per ml; the suspensions were centrifuged and the supernatants incubated together with [7-^{14}C]noradrenaline and the cofactors. The amount of O-methylation was then determined using the periodate oxidation method; complete oxidation of the normetanephrine and norparanephrine was obtained with 5 ml 5% periodate.

RESULTS AND DISCUSSION

(I) Purification and stability of the rat liver catechol O-methyltransferase

The soluble fraction of rat liver, prepared by a modification of the method of AXELROD AND TOMCHICK[13], was submitted to $(NH_4)_2SO_4$ fractionation; the precipitate obtained between 40 and 60% saturation contained 70–80% of the enzymatic activity and 12–14% of the total proteins. This 40–60% precipitate was redissolved, the solution dialyzed and the supernatant lyophylized giving the LI powder; 1 mg of LI powder catalyzed the methylation of p.p5–0.07 μmole of noradrenaline per minute

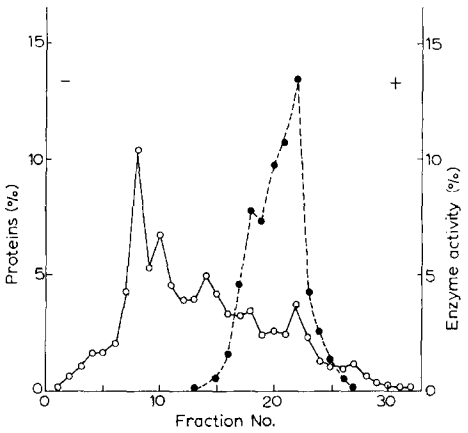


Fig. 1. Continuous paper electrophoresis purification of catechol *O*-methyltransferase. 1 g of proteins (dialysis supernatant) in 50 ml of buffer were applied at a rate of 2–3 ml/h on the paper curtain of a Beckman model CP continuous flow electrophoresis apparatus. The potential difference was maintained at 800 V and the buffer flow rate at 27 ml/h. The buffer contained, in 15 l, 4.0 g Na_2HPO_4, 0.25 g NaH_2PO_4, 0.5 g cysteine·HCl, 3 g dithiothreitol and the pH was adjusted to 8 with NaOH. ○—○, protein content measured spectrophotometrically; ●— — —●, enzyme activity determined with pyrocatecholphthalein[10]. Fractions 18–22 contained 49% of the enzymatic activity and 14% of the proteins, which accounted for a 3.5-fold purification.

(the conditions of incubation are described in Table I). The other purification procedures were studied most often using either the dialyzed solution or the LI powder as starting material. Applying directly the dialyzed solution, a continuous paper electrophoresis gave a 3–4-fold purification with a yield of 50% (Fig. 1). Starting with 50–100 mg of LI powder, a 6-fold enrichment and a 60–70% yield were obtained with the Sephadex G-75 chromatography. Starting with 20–30 mg of LI powder, the DEAE-cellulose chromatography also gave a 6-fold enrichment with a 60–70% yield, a result similar to that of ANDERSON AND D'IORIO[9]. With the preparative disc electrophoresis, using 30 mg of a sample 12-fold purified by $(NH_4)_2SO_4$ precipitation and continuous electrophoresis, two peaks of enzyme activity were found (Fig. 2); the amount of separation varied in different runs with the same sample, probably because it depended on the thickness of the separating gel which was difficult to control.

These methods were then used in succession and the purification level reached was always computed as the ratio of the specific activity (enzymatic activity per mg of protein) of the lyophilized powder from the last purification step, to the specific activity of the soluble fraction from rat liver (Table I). Powders obtained by lyophilization were stable for weeks when kept at or below 0°, but a difficulty was the great instability of the purified enzyme in solution already noted by earlier workers[13]. If the enzyme stability in crude preparations was increased in the presence of dithiothreitol or cysteine in agreement with earlier observations[13], these reagents were however unable to preserve the activity of highly purified enzyme solutions. Because it gave slightly better results, doubly distilled water was used throughout the purification steps. Addition of $1 \cdot 10^{-3}$ M EDTA or $1 \cdot 10^{-3}$ M $MgCl_2$ to the buffers did not stabilize the enzyme.

As the aim was to reach the highest possible purification level and because of the noted instability of the enzyme, the order of application of the purification techni-

TABLE I

m/p RATIO AS A FUNCTION OF THE PURIFICATION LEVEL OF THE RAT LIVER CATECHOL *O*-METHYLTRANSFERASE

The given quantities of proteins were incubated for 30 min at 37° together with 2.0 ml 0.06 M phosphate buffer, pH 7.8, 0.11 μmole cysteine·HCl, 0.57 μmole adenosylmethionine·HCl, 50 μmoles $MgCl_2$, 0.0083 μmole [^{14}C]noradrenaline (0.4 μC); 2.6 ml final volume, pH 7.5. After precipitation of the proteins with trichloroacetic acid, the supernatant was made alkaline with conc. NH_3, oxidized for 30 min at 25° with periodate, cooled down to 0°, brought to pH 6.5 and extracted with benzene; after addition of vanillin and isovanillin as carriers, the benzene solution was concentrated and 50 μl injected into a gas chromatograph coupled with a Nuclear Chicago proportional counter and a recorder[11]. The *m/p* ratio is the ratio of the areas under the vanilin and isovanillin radioactivity peaks; the error is computed as described in ref. 11.

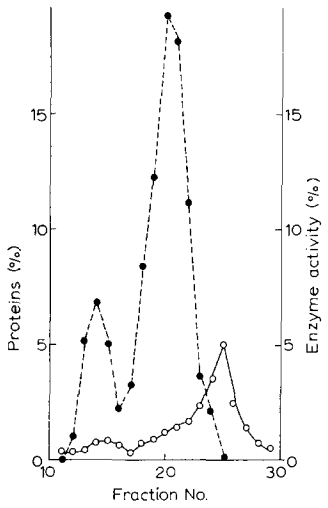
Sample No.	*Purification procedure*	*Purification level*	*Quantity of proteins incubated (μg)*	*m/p*
1	Liver homogenate	—	1 g liver	32 ± 6
2	SA	6	3800	14.4 ± 1.7
3	SA + S	26	280	15.4 ± 1.9
4	SA + S	36	130	12.9 ± 1.7
5	SA + C + D (powder A)	35	150	15.6 ± 3.5
6	SA + C + D (powder B)	42	70	13.0 ± 3.2
7	SA + C + S	56	170	15.6 ± 1.9
8	SA + D + S	77	100	13.0 ± 1.6
9	SA + C + S	81	40	10.1 ± 2.5
10	SA + C + S	112	240	14.0 ± 1.7
11	SA + S + D	121	60	14.6 ± 1.8
12	SA + S + D	122	250	16.3 ± 2.0
13	SA + C + D + S	168	20	14.7 ± 2.0
14	SA + D + S	182	50	13.4 ± 1.6
15	SA + Ce + S	250	20	13.0 ± 1.6
16	SA + S + D	266	20	14.2 ± 1.8
17	SA + C + S + D	345	4	13.3 ± 4.0
18	SA + C + D + S	350	5	13.6 ± 2.6

Abbreviations: SA, ammonium sulfate fractionation; C, continuous paper electrophoresis; D, preparative disc-electrophoresis; S, Sephadex chromatography; Ce, DEAE-cellulose chromatography.

ques was varied (Table I). The purest preparations (Prepns. 17 and 18) showed a 350-fold purification; neither of them was a pure enzyme as analytical disc electrophoresis revealed three protein bands with Prepn. 17 and one very wide band with Prepn. 18. Further attemps to purify these samples were unsuccessful because of a concurrent denaturation leading to a loss of most of the enzymatic activity. While this work was submitted for publication, ASSICOT AND BOHUON[14] reported a 450-fold purification of the catechol *O*-methyltransferase.

(II) m/p ratio and the number of enzymes; dimethylation

After incubation of the enzyme preparation with [7-^{14}C]noradrenaline, the ratio of labelled normetanephrine to norparanephrine in the reaction products (*m/p* ratio) was determined by gas chromatography after periodate oxidation to vanillin and isovanillin. The results obtained with the liver homogenate, the soluble fraction and the various enzyme preparations are given in Table I. With liver homogenate, a ratio of 32 was observed, while with the 17 purified preparations of the enzyme, the



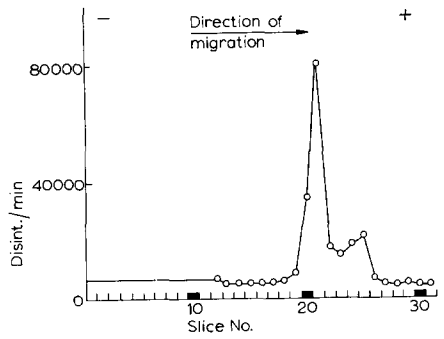


Fig. 2. Preparative disc electrophoresis purification of catechol *O*-methyl-transferase. 30 mg of a 12-fold purified enzyme preparation ($(NH_4)_2SO_4$ precipitation, continuous electrophoresis) were dissolved in 3.0 ml of the cysteine–sucrose mixture. The details are given in the text. ○—○, protein content measured spectrophotometrically; ●— — —●, enzyme activity determined with pyrocatecholphthalein[10].

Fig. 3. Separation by analytical disc electrophoresis of two catechol *O*-methyltransferases. 6 mg of a 20-fold purified enzyme preparation ($(NH_4)_2SO_4$ precipitation, continuous electrophoresis) were used. The details of the electrophoresis are given in the text. The enzymatic activity in the gel slices was determined as follows: the supernatant of the slice homogenate was incubated for 30 min together with 50 μmoles $MgCl_2$, 0.11 μmole cysteine·HCl, 0.2 μC [^{14}C]noradrenaline, in a total volume of 4.0 ml (pH 8.0–8.1). The rest of the procedure is described in Table I; the radioactivity (disint./min) is that of the benzene extract. Slices 1–12 were pooled before homogeneization. 58% of the enzyme activity put on the column was recovered.

mean value was 13.9 ± 1.5 with no individual result differing significantly from this mean.

Whole liver homogenates contain oxidative demethylases in the microsomes[2,15]; experiments have shown that normetanephrine was indeed demethylated to noradrenaline[16]. A differential action of these and other degrading enzymes on normetanephrine and norparanephrine may be responsible for the different *m*/*p* ratio observed with liver homogenate as compared with purified catechol *O*-methyltransferase preparations. A similar situation in rat whole blood (where there is no detectable norparanephrine formed after addition of [7-^{14}C]noradrenaline) has been investigated[16].

On the other hand, all the preparations with purification levels varying from 6- to 350-fold catalyzed the O-methylation of noradrenaline in the same *m*/*p* ratio. If the *m*-O- and *p*-O-methylations are catalyzed by two different enzymes, they must be very similar in charge and molecular weight since it was not possible to separate them with methods that did separate proteins on the basis of both charge and molecular weight.

As described in the preceding chapter on purification, two enzymatic components can however be separated by preparative disc electrophoresis (Fig. 2); the same result is obtained by analytical disc electrophoresis (Fig. 3). After the preparative disc electrophoresis (Fig. 2), Fractions 12–15 were pooled, 100 mg sucrose were added

to the solution which was then lyophilized (Powder A); Fractions 17–23 were likewise treated (Powder B). 40 mg of Powder A (about 60 μg proteins) and 10 mg of Powder B (about 100 μg proteins) were separately submitted to analytical electrophoresis; analysis of the gel slices showed that each preparation exhibited only one peak of enzymatic activity (Figs. 4 a and 4b). When a single solution was prepared with 35 mg of Powder A and 10 mg of Powder B, two peaks were observed (Fig. 4c) confirming that the two enzyme proteins are different. The two powders A and B catalyzed the O-methylation of noradrenaline in the same *m*/*p* ratio (Table I, Samples 5 and 6); it thus appeared that two isoenzymes had been separated and not the *p*-O-methylating from the *m*-O-methylating enzymes. This complicates the situation if we still insist that the *p*-O-methylating and the *m*-O-methylating activities must be separate; indeed, we must then postulate the existence of four enzymes grouped two by two so that each group separated by disc electrophoresis catalyzes the O-methylation of noradrenaline in the same *m*/*p* ratio. If not impossible, this is very unlikely.

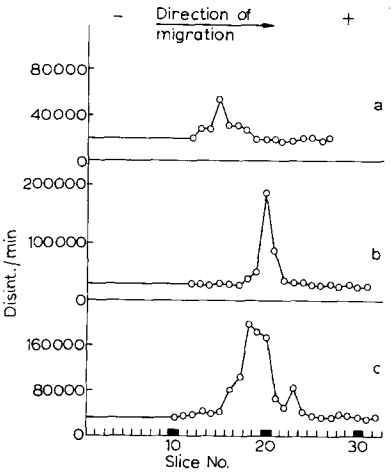


Fig. 4. Study by analytical disc electrophoresis of the catechol *O*-methyltransferases separated by preparative disc electrophoresis. The fractions corresponding to each of the two enzyme peaks of Fig. 2 were pooled and lyophilized after addition of sucrose. The enzyme activity of the gel slices was determined as described in Fig. 3, except that 0.5 μC [^{14}C]noradrenaline was used and the incubation time increased to 60 min. a. 40 mg of Powder A. The stacking step was very slow (30 min) due to high salt content of the sample; electrophoresis was terminated after 90 min; bromophenol blue had migrated 4.5 cm in the separating gel. b. 10 mg of Powder B. Electrophoresis was terminated after 90 min (stacking step: 5 min); bromophenol blue had migrated 7.0 cm. c. 35 mg of Powder A + 10 mg of Powder B. Electrophoresis was terminated after 120 min (stacking step: 30 min); bromophenol blue had migrated 6.8 cm. In Samples a and b, Slices 1–12 were pooled and processed as one sample. In c, Slices 1–10 were also pooled.

It thus seems that a single protein is able to carry out both the *m*-O- and *p*-O-methylations of catecholamines. The existence of several catechol *O*-methyltransferase isoenzymes has already been described by ANDERSON AND D'IORIO[10].

The formation of 3,4-dimethoxy phenylethanolamine by catechol *O*-methyltransferase was never observed: no radioactive veratraldehyde was ever detected after periodate oxidation; the detection limit was 1% of the more abundant radioactive aldehyde, vanillin. In a specially designed experiment 0.3 μC [^{14}C]noradre-

TABLE II

EFFECT OF NORADRENALINE AND *S*-ADENOSYLMETHIONINE CONCENTRATIONS ON THE *m/p* RATIO

2 ml 0.06 M phosphate buffer, 20 μg cysteine·HCl, 50 μmoles $MgCl_2$, 1.5 mg enzyme (LI powder) in a total volume of 4 ml, pH 7.5.

(^{14}C)Noradrenaline (M)*	*S-Adenosylmethionine (M)*	*m/p*
$0.975 \cdot 10^{-6}$	$5 \cdot 10^{-4}$	12.5 ± 1.6
$1.95 \cdot 10^{-6}$		12.8 ± 1.7
$3.90 \cdot 10^{-6}$		13.4 ± 1.8
$8.95 \cdot 10^{-6}$		11.8 ± 1.5
$1.79 \cdot 10^{-5}$		11.9 ± 1.5
$3.58 \cdot 10^{-5}$		11.1 ± 1.4
$8.95 \cdot 10^{-5}$		11.8 ± 1.5
$1.79 \cdot 10^{-4}$		12.0 ± 1.5
$1.4 \cdot 10^{-6}$	$0.4 \cdot 10^{-4}$	10.6 ± 1.4
	$0.8 \cdot 10^{-4}$	11.4 ± 1.4
	$1.6 \cdot 10^{-4}$	11.4 ± 1.4

* Specific radioactivity = 40 mC/mmole.

naline was enzymatically methylated to an extent of more than 80%; after periodate oxidation and benzene extraction, the whole sample was injected in the gas-chromatographic column: no radioactive veratraldehyde could be detected although this procedure lowered the detection limit for the dimethylation to 0.15%.

(III) Factors affecting the m/p ratio

Throughout this part of the investigation, the LI powder was used as a source of enzyme.

(a) Substrate concentration

No significant variation of the *m/p* ratio was obtained by varying the noradrenaline concentration from $0.95 \cdot 10^{-6}$ to $1.79 \cdot 10^{-4}$ M and the adenosylmethionine concentration from $0.4 \cdot 10^{-4}$ to $1.6 \cdot 10^{-4}$ M (Table II). The *p*-O-methylating and the *m*-O-methylating activities have thus, towards noradrenaline, the same $K_m = 1.27 \cdot 10^{-4}$ M (phosphate 0.27 M, *S*-adenosylmethionine $4.5 \cdot 10^{-4}$ M, $MgCl_2$ $1 \cdot 10^{-2}$ M, cysteine·HCl $3 \cdot 10^{-5}$ M; pH 7.5) similar to that observed by D'IORIO[17]; this observation is also in agreement with the hypothesis that the same enzyme(s) catalyze(s) the two reactions.

(b) pH

Fig. 5 shows the variations of enzymatic activity and of the *m/p* ratio as functions of pH. The *m/p* ratio is affected by pH; similar results were previously reported with other substrates[1,3].

To account for the variation of the *m/p* ratio with pH produced by non-enzymatic methylations of 3,4-dihydroxybenzaldehyde and 3,4-dihydroxyacetophenone, SENOH *et al.*[3] assumed that the methylating agents react more readily with the *para* oxygen when the first dissociation of the catechol moiety has been completed.

The observed variation of the *m/p* ratio with pH in the enzymatic O-methylation of noradrenaline could similarly be accounted for by assuming a preferential

p-O-methylation of the ionized form. The equation expressing this theoretical model can be fitted to the experimental data if one gives a proportion of 0.059 for the *p*-O-methylation of the undissociated form and 0.218 for the dissociated form, and a pK_a value of 8.56 for the catechol moiety of noradrenaline (Fig. 5). This model, which gives a great influence on the *m*/*p* ratio to the ionization of the catechol moiety of the substrate when the O-methylation is catalyzed by the catechol *O*-methyltransferase, is supported by experimental results showing that substances with a carbonyl in position 7 have a lower pK_a and undergo a higher *p*-O-methylation than substances with a saturated carbon in the same position[2,3,5,7,8]. The excellent agreement between the pK_a value of 8.56 for the catechol moiety of noradrenaline derived from the model proposed above, with the reported value of 8.7 (ref. 18), seems to indicate that the influence of pH on the *m*/*p* ratio is mostly through its action on the catechol substrate; its action on the enzyme is likely of minor importance for this issue.

It should finally be pointed out that the *m*/*p* ratio of 10.0 ± 1.2 obtained at pH 7.9 in this study is somewhat higher than the value observed by DALY *et al.*[2] with a different technique.

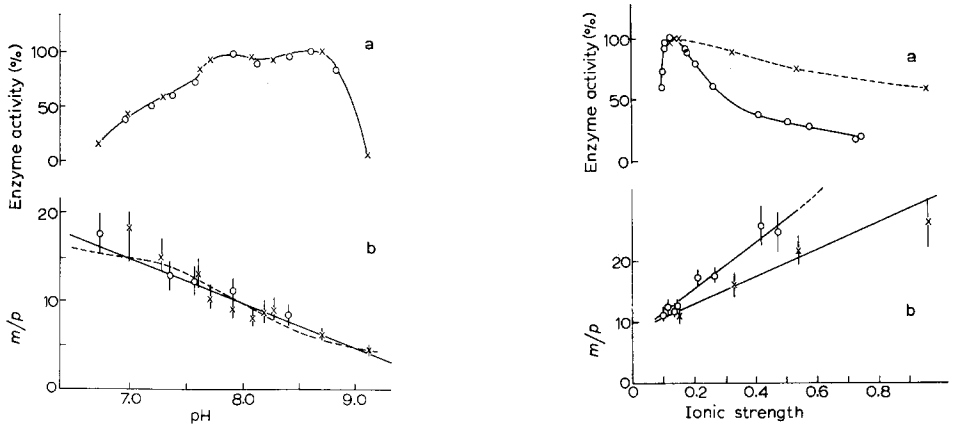


Fig. 5. Action of pH on enzyme activity and *m*/*p* ratio. 2 mg enzyme LI powder, 2 ml 0.06 M phosphate buffer of varying pH, 50 μmoles $MgCl_2$, 0.46 μmole adenosylmethionine, 0.06 μmole cysteine·HCl, 0.6 μC (×) or 0.06 μC (○) [^{14}C]noradrenaline; total volume: 2.6 ml. The incubation time was either 20 min (×) or 10 min (○). The enzyme activity was measured by the radioactivity of the benzene extract after periodate oxidation; details can be found in Table I. In each set, (×) or (○), the sample with the highest activity was assigned 100% relative activity. Gas-chromatographic analysis was used for the determination of the *m*/*p* ratio. b also contains a theoretical curve (— — —) representing the equation:

$$\frac{m}{p} = \frac{(1 - a)\,[H^+] + (1 - b)\,K}{a[H^+] + Kb}$$

where K = dissociation constant of the catechol moiety of noradrenaline; a = proportion of *p*-O-methylation of the undissociated form; b = proportion of *p*-O-methylation of the dissociated form; the three values calculated from points of the regression continuous line corresponding to pH 7, 8 and 9, are $K = 2.77 \cdot 10^{-9}$ g-ions/l ($pK = 8.56$), $a = 0.059$, $b = 0.218$.

Fig. 6. Action of ionic strength on enzyme activity and *m*/*p* ratio. 1 mg enzyme LI powder, 1.0 ml 0.12 M phosphate buffer, 0.46 μmole adenosylmethionine, 0.06 μmole cysteine·HCl, 0.2 μC [^{14}C]-noradrenaline; 4.5 ml total volume, pH 7.5. The incubation lasted 15 min. Enzyme activity and *m*/*p* ratio measurements as usual (Table I). The ionic strength due to phosphate was 0.088; increasing concentrations of $MgCl_2$ (×) or NaCl (○) with a uniform 0.011 M $MgCl_2$. When the ionic strength was higher than 0.6 with $MgCl_2$, the *p*-O-methylation became undetectable.

(c) $MgCl_2$ concentration and ionic strength

Ionic strength was first increased by addition of $MgCl_2$. In a second experiment, the concentration of $MgCl_2$ was maintained at 0.011 M (optimal concentration) and the ionic strength was increased by addition of NaCl. The activity of the enzyme and the *m/p* ratio were measured as functions of ionic strength (Fig. 6).

The enzyme is inhibited by high concentrations of $MgCl_2$ or NaCl. This inhibition was studied by varying the noradrenaline concentration: Lineweaver–Burk plots showed that NaCl inhibition had non-competitive characteristics, while $MgCl_2$ inhibition was of mixed type. The inhibition of the enzyme by high NaCl concentrations, which is non competitive with respect to noradrenaline, might be explained

TABLE III

NET YIELD OF O-METHYLATION (%) AND *m/p* RATIO AS FUNCTIONS OF INCUBATION TIME

150 μmoles $MgCl_2$, 1.8 μmoles adenosylmethionine, 0.6 μmole cysteine·HCl, 2 ml 0.12 M phosphate buffer, 10 mg enzyme powder and 0.6 μC [^{14}C]noradrenaline (Sample I) or 1.8 μC [^{14}C]-noradrenaline (Sample II), in a total volume of 8.0 ml, pH 7.5; 1.0-ml aliquots were removed at various times. After the penultimate removal, solutions were sterilized by ultrafiltration. The method for the determination of the *m/p* ratio has been described in Table I; for the computation of the error, see ref. 11. The benzene-extractable radioactivity after periodate oxidation gave the O-methylation yield.

Time (min)	*Sample I: pH 7.3*		*Sample II: pH 7.7*	
	%	*m/p*	%	*m/p*
5	29.5	15.7 ± 3.8	29.7	9.8 ± 1.3
10	51.0	13.6 ± 1.8	50.2	10.7 ± 1.4
20	76.5	15.6 ± 2.0	75	11.4 ± 1.5
40	92.5	16.6 ± 2.2	90	11.8 ± 1.5
80	94.5	14.3 ± 1.9	96	14.0 ± 1.8
160	94.0	17.9 ± 2.3	95	12.5 ± 1.6
245	93.5	20.3 ± 3.1	—	—
1440	—	*p* undetectable	88	*p* undetectable

by conformational changes in the enzyme. Inhibition of the catechol *O*-methyltransferase by high concentrations of $MgCl_2$ is in agreement with earlier results[3,19,20]; the non-competitive component might also be explained by conformational changes in the enzyme, while the competitive component could be due to complexation of the catechol substrate by Mg^{2+} outside the active site of the enzyme.

The ionic strength has also an influence on the *m/p* ratio (Fig. 6); this effect could be related to conformational changes in the enzyme.

(d) Incubation time

Experiments were carried out at pH 7.3 and 7.7 (Table III). After 80 min, the yield of the O-methylation reaction reached a maximum and then slowly decreased while the *m/p* ratio increased. The total yield decrease seemed thus to be due to the disappearance of the *p*-O-methylated isomer which was complete after 24 h.

When 0.1 μC [^{14}C]normetanephrine was, after sterilization by ultrafiltration, incubated in 2 ml 0.06 M sodium phosphate buffer, pH 7.8, containing 0.12 μmole cysteine·HCl and 1 mg enzyme (LI powder), 89 ± 5% of the initial normetanephrine could be recovered after 24 h; omission of enzyme powder did not affect the result.

Incubation of 0.035 μC [^{14}C]norparanephrine[11] in the same conditions induced a total disappearance of this labelled compound (detected as isovanillin after periodate oxidation) after 24 h, in the absence as well as in the presence of enzyme powder. A second experiment was made with norparanephrine and shorter incubation times: the presence of the enzyme preparation did not influence the rate of disappearance of norparanephrine. These experiments showed that the disappearance of norparanephrine was not due to an enzymatic process, but to chemical degradation. Moreover, a slow degradation of norparanephrine was also observed when this amine was kept at 0° in 0.4 M ammonium acetate buffer, pH 5.2: a freshly synthesized mixture of [^{14}C]normetanephrine and [^{14}C]norparanephrine gave a *m/p* ratio of 1.06 $\pm$ 0.14; after 4 weeks, the ratio increased to 1.50 $\pm$ 0.20 and, 2 weeks later, to 1.75 $\pm$ 0.23.

We also observed that the presence of catechol *O*-methyltransferase cofactors ($MgCl_2$ and adenosylmethionine) together with the enzyme during incubation did not decrease the disappearance of norparanephrine or induce the formation of new normetanephrine. This seemed to indicate that the chemical degradation of norparanephrine was not a demethylation to noradrenaline.

It is important to notice that, with short incubation times (1 h or less), no significant degradation of norparanephrine was recorded; the instability of the amine could not influence the results obtained with those incubation times.

(IV) Action of some O-methylated substances on catechol O-methyltransferase

The influence of various dimethoxy- and methoxy-phenyl derivatives upon the activity of catechol *O*-methyltransferase and the *m/p* ratio of the noradrenaline O-methylation was studied (Table IV). Only vanillin and isovanillin exhibited some inhibitory action and none of the substances influenced significantly the *m/p* ratio. Furthermore, no inhibition was observed when the four first substances listed in Table IV were incubated at a higher concentration (500 μg/ml) with lower concen-

TABLE IV

ACTION OF DIFFERENT SUBSTANCES ON THE RAT LIVER CATECHOL-O-METHYLTRANSFERASE

In a total volume of 4.0 ml (pH 7.6) each sample contained: 2 ml 0.12 M phosphate buffer, 50 μmoles MgCl 0.12 μmole cysteine·HCl_2, 24 μmoles adenosylmethionine, 1.2 μmoles [^{14}C]noradrenaline (2 mC/mmole), 1.5 mg enzyme LI powder and 100 μg of the tested substance. After 15-min incubation, the reaction was stopped with trichloroacetic acid and the samples processed as indicated in Table I. The relative activities relayed on radioactivity measurements made on the benzene extract before concentration; gas chromatographic analysis gives the *m/p* ratio; the error is computed as in ref. 11.

Substance	*Relative activity (%)*	*m/p ratio*
None	100	11.3 $\pm$ 1.5
N-Methyl-3,4-dimethoxy-β-phenylethanolamine	97.5 $\pm$ 5	11.9 $\pm$ 1.5
3,4-Dimethoxy-β-phenylethanolamine	96.5 $\pm$ 5	12.7 $\pm$ 1.7
3,4-Dimethoxy-β-phenylethylamine	98 $\pm$ 5	12.3 $\pm$ 1.6
4-Methoxy-β-phenylethylamine	98.5 $\pm$ 5	13.9 $\pm$ 1.8
Vanillin	70 $\pm$ 5	9.8 $\pm$ 1.3
Isovanillin	76 $\pm$ 5	9.8 $\pm$ 1.3
Veratraldehyde	98 $\pm$ 5	13.6 $\pm$ 1.8
Normetanephrine	95 $\pm$ 5	11.4 $\pm$ 1.5

trations of substrates (noradrenaline, $7.5 \cdot 10^{-6}$ M; *S*-adenosylmethionine, $5.3 \cdot 10^{-5}$ M) and $MgCl_2$ ($2 \cdot 10^{-3}$ M). These negative results indicate that the pharmacological activity of the 3,4-dimethoxy- and of the 4-methoxy-phenylamines[21] cannot be explained by an action on liver catechol *O*-methyltransferase. The lack of inhibition by 3,4-dimethoxy-β-phenylethylamine is in agreement with earlier results[22].

ACKNOWLEDGEMENTS

This work was supported by a grant from the Canadian Medical Research Council. The authors wish to thank Mr. P. Methot for his excellent technical assistance.

One of the authors (J.M.F.) is an Aspirant du Fonds National Belge de la Recherche Scientifique.

REFERENCES

1 S. SENOH, J. DALY, J. AXELROD AND B. WITKOP, *J. Am. Chem. Soc.*, 81 (1959) 6240.
2 J. W. DALY, J. AXELROD AND B. WITKOP, *J. Biol. Chem.*, 235 (1960) 1155.
3 S. SENOH, Y. TOKUYAMA AND B. WITKOP, *J. Am. Chem. Soc.*, 84 (1962) 1719.
4 H. M. FALES, J. MANN AND S. H. MUDD, *J. Am. Chem. Soc.*, 85 (1963) 2025.
5 P. J. ANDERSON, Thèse de doctorat, University of Ottawa, 1967.
6 F. A. KUEHL, M. HICHENS, R. E. OSMOND, M. A. P. MEISINGER, P. H. GALE, V. J. CERILLO AND N. G. BRINK, *Nature*, 203 (1964) 154.
7 M. S. MASRI, A. N. BOOTH AND F. DE EDS, *Biochim. Biophys. Acta*, 65 (1962) 495.
8 M. S. MASRI, D. J. ROBBINS, O. H. EMERSON AND F. DE EDS, *Nature*, 202 (1964) 278.
9 P. J. ANDERSON AND A. D'IORIO, *Biochem. Pharmacol.*, 17 (1968) 1943.
10 P. J. ANDERSON AND A. D'IORIO, *Can. J. Biochem.*, 44 (1966) 347.
11 J. M. FRÈRE AND W. G. VERLY, *J. Chromatog.*, 49 (1970) 425.
12 W. H. PERKIN AND R. ROBINSON, *J. Chem. Soc.*, 91 (1907) 1079.
13 J. AXELROD AND R. TOMCHICK, *J. Biol. Chem.*, 233 (1958) 702.
14 M. ASSICOT AND C. BOHUON, *European J. Biochem.*, 12 (1970) 490.
15 J. AXELROD, *Biochem. J.*, 63 (1956) 634.
16 J. M. FRÈRE AND W. G. VERLY, *Biochim. Biophys. Acta*, 235 (1971) 85.
17 A. D'IORIO, *Biochem. Pharmacol.*, 12 (1963) 1307.
18 T. KAPPE AND M. D. ARMSTRONG, *J. Med. Chem.*, 8 (1965) 368.
19 B. BELLEAU AND J. BURBA, *J. Med. Chem.*, 6 (1963) 755.
20 H. OZAWA AND H. KAWASHIMA, *J. Pharm. Soc. Japan*, 87 (1967) 345.
21 R. MICHAUX AND W. G. VERLY, *Life Sci.*, 3 (1963) 175.
22 A. BARBEAU, P. SINGH AND M. JOUBERT, *Life Sci.*, 5 (1966) 757.