pH-DEPENDENT DEUTERIUM SOLVENT ISOTOPE EFFECTS ON BOVINE ADRENAL MEDULLARY DOPAMINE-β-HYDROXYLASE

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Summary. - The effect of p^2H upon the dopamine-β-hydroxylase was studied. ^2H_2O inhibited the enzyme (50 % inhibition in 99.7 % ^2H_2O. A deuterium solvent isotope effect is observed which seems to correspond to an upward shift of 0.5 unit in both pK_a's of an active site group. Our present data confirm the assumption that this group is a histidyl residue. The results are consistent with the interpretation that a proton transfer step is mediated by this group. The proton was demonstrated to come from an ascorbate molecule. Attempts to demonstrate the presence of serine in the active centre were unsuccessful.

We recently reported some aspects of the pH dependence of the hydroxylation reaction of tyramine catalyzed by dopamine-β-hydroxylase (3,4-dihydroxyphenylethylamine, ascorbate : O_2 oxidoreductase (hydroxylating) EC 1.14.17.2) (1). This led to the conclusion that the basic form of an ionizable group at the active centre of the enzyme participates in the hydroxylation reaction process. The pK of this group was 6.2 in the free enzyme and 6.6 in the enzyme-substrate complex. These values were consistent with the identification of this group as the imidazole side-chain of a histidyl residue and in agreement with the inhibitory effect of diethylpyrocarbonate, a reagent specific for this histidine (2).

The object of the present work is to examine the effects of ^2H_2O and p^2H upon the kinetics of the hydroxylation reaction in order to provide supplementary evidence for the presence of a histidyl residue at the active site.

MATERIALS AND METHODS

The dopamine-β-hydroxylase was obtained from bovine adrenal medulla and purified as previously described by affinity chromatography (2). Protein was assayed by the method of Lowry *et al.* (3).

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The buffers used were 0.1 M potassium phosphate in the pH (p^2H) range 5.5 - 8.0 and 0.5 M sodium acetate in the pH (p^2H) range 4.7 - 6.0. Deuterium oxide of 99.75 % isotopic purity was obtained from Merck (Darmstadt, Germany). All substrates and buffers were freshly prepared in ^2H_2O. The enzyme in 0.5 M potassium phosphate buffer was diluted in ^2H_2O immediately before use. Values of p^2H were obtained from measured values of pH and calculated from the relationship p^2H = pH +0.40 (4,5). For each experiment the actual pH of the reaction mixture was controlled. The reaction mixture for the determination of dopamine-β-hydroxylase activity consisted of 100 µmoles of phosphate buffer or acetate buffer ; 100 µg of catalase (Boehringer, Mannheim) ; variable amounts of tyramine and ascorbate and 20 µmoles of fumarate in a final volume of 0.91 ml. The tubes were preincubated 10 min at 37°C and the reaction started by the addition of 1 to 2.5 µg of dopamine-β-hydroxylase in 100 µl. The reaction was carried out following the procedure described by Pisano *et al.* (6) as modified by us (7). The Km and Vm values were determined from Lineweaver-Burk plots (8), as described elsewhere (2).

RESULTS

Inhibition of dopamine-β-hydroxylase by ^2H_2O. Substitution of ^2H_2O for water resulted in an inhibition of dopamine-β-hydroxylase. The inhibition by ^2H_2O increased in a linear fashion with concentration and reached a value of 50 % at a concentration of 99.7 % at pH 5.5 (Fig. 1).

The inhibitory effect of ^2H_2O on the dopamine-β-hydroxylase was completely reversible (Table 1). The enzyme was allowed to stand in the presence of 79.75 % ^2H_2O and the activity was tested after diluting this enzyme with H_2O. Using enzyme in water as control, the activity after the ^2H_2O treatment was fully restored.

Effect of p^2H upon Km. The Km values for tyramine [solid line in ^2H_2O ; dashed line in H_2O (1)] are a function of pH, but those for ascorbate are not

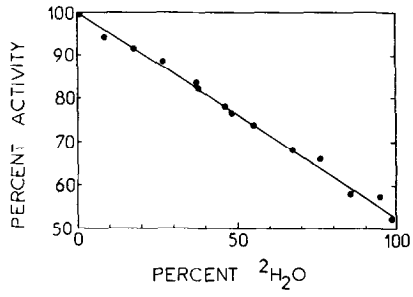

Fig. 1. - Effect of ^2H_2O on dopamine-β-hydroxylase. The percentage activities were calculated from control figures in the absence of ^2H_2O. Medium contained: 100 µmole of sodium acetate buffer (pH 5.5), 10 µmole of tyramine, 10 µmole of ascorbate, 20 µmole of fumarate and H_2O or ^2H_2O as indicated to a final volume of 0.91 ml. Tubes were preincubated 10 min at 37°C and the reaction was started by the addition of 100 µl of enzyme (2 µg) and incubated for 30 min.

Table 1. - Reversibility of ^2H_2O inhibition of dopamine-β-hydroxylase

Enzyme activity (µmole/30 min/mg protein)

Enzyme treatment	Duration of preincubation in ^2H_2O					
	0	5 min	10 min	15 min	20 min	30 min
Control	143	136	134.5	132	132	132
79.8 % ^2H_2O	136*	136	128	126	124	124

25 µg of enzyme (suspended in 0.1 ml of phosphate buffer, pH 6.5) was diluted with 0.9 ml of H_2O containing 0.1 ml of catalase (control) or with 0.9 ml of ^2H_2O containing 0.1 ml of catalase and incubated for various times at 37°C. At the end of the indicated preincubation times, an 0.1 ml aliquot (2.5 µg of enzyme) was removed and assayed for enzyme activity at pH 5.5 in acetate buffer.
*The calculated concentration of ^2H_2O in the assay system is 7.9 %.

pH-dependent (Fig. 2). It appears that the pK values of one ionizable group at the active centre are shifted upwards in ^2H_2O. Table 2 gives the pK values found in H_2O and in ^2H_2O. The experimental points for ^2H_2O fitted curve of the same shape as the data in H_2O. No changes are observed for the pK of the ionizable group at 5.4.

Effect of p^{2}H upon Vm. It appears that 99 % ^2H_2O decreased the Vm for tyramine by near 50 % in the pH range 4.8 to 6.2 and that the inhibition de-

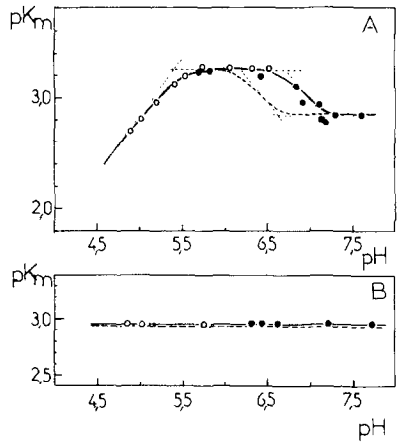

Fig. 2. - Effect of pH on Km : (A) of tyramine, (B) of ascorbate. Reaction was performed at 37°C in H_2O (dashed line, see ref. 1) and in 99.7 % ^2H_2O (solid line), in phosphate buffer (-O-O) and ascorbate buffer (-●-●) as described in Methods. Km^{-1} are expressed in M^{-1}.

Table 2. - pK values obtained from pKm and log Vm plots as a function of pH and p^2H

	pK of free enzyme		pK of enzyme substrate complex	
	H_2O*	^2H_2O	H_2O*	^2H_2O
pKm	5.4	5.4		
	6.2	6.7	6.6	7.15
log V	-	-	6.6	7.1

*pK values in H_2O are taken from our previous results (1).

creased inversely to the affinity for tyramine over the pH value of 6.2 (Fig. 3).

Incubation of dopamine-β-hydroxylase in ^3H_2O. Incubations of dopamine-β-hydroxylase in ^3H_2O were performed in order to determine the origin of the proton mediated by the histidine residue.

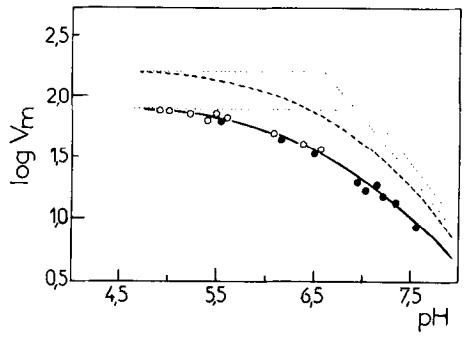

Fig. 3. - pH-dependence of $\log_{Vm}$ for the dopamine-β-hydroxylase catalyzed hydroxylation of tyramine in H_2O (dashed line, see ref. 1) and in ^2H_2O (–○–○–, phosphate buffer ; –●–●–, acetate buffer). Vm are expressed in µmole of octopamine formed per 30 min per mg of protein.

Table 3. - Incubation of dopamine-β-hydroxylase in ^3H_2O

Tyramine (mM)	Octopamine (mM)	cpm	Octopamine formed (µmole)	expected cpm
1.25	-	70	0.6	540
2.5	-	74	0.79	711
5	-	50	0.9	810
10	-	82	1.0	900
-	0.5	40	-	-
-	1	80	-	-

Reaction mixtures contained 10 µl of catalase, 20 µmole of fumarate, 0.2 mmole of acetate buffer (pH 5.5), 10 µmole of ascorbate, 500 µl of ^3H_2O (^3H_2O with a specific activity of 165 µc/ml was obtained from CEA, France) and variable amounts of tyramine as indicated, in a final volume of 0.91 ml. The reaction was started with the addition of 10 µg of enzyme and the tubes were incubated at 37°C for 30 min. The incubation was stopped by the addition of 0.1 ml of trichloracetic acid and after centrifugation to remove proteins, the mixture was passed through a 0.5 x 1 cm column of Dowex 50 (H^+). After extensive washings with 20 ml of water, octopamine was eluted with 2 ml of 1 N ammonia. An aliquot (1 ml) was dissolved in a liquid scintillation solution. A reagent blank in which all additions were made except for the enzyme was used to correct the results. Controls, in which octopamine was added, were made in order to test endogenous exchange with ^3H_2O.

The octopamine formed was not radioactive when exchanges occurring in incubation media are taken into account (Table 3).

Effect of diisopropylfluorophosphate upon dopamine-β-hydroxylase. Dopa-

Table 4. - Effect of diisopropylfluorophosphate upon dopamine-β-hydroxylase activity

Diisopropylfluorophosphate† (μM)	0	25	50	80	125
Control*	21	21	21.5	16.5	8.6
Diisopropylfluorophosphate	21	22	19	14	8.6

Activities are expressed in μmole of octopamine formed per 30 min at 37°C and per mg of protein.
Dopamine-β-hydroxylase was diluted in 0.5 M potassium phosphate buffer (pH 6.5) at a concentration of 60 μg/ml. Diisopropylfluorophosphate was diluted in dry isopropanol (2.5 nmole per 10 μl). Aliquots of this solution from 10 to 100 μl were added to the enzyme solution. Mixtures were left at room temperature for 30 min. Aliquots of the mixtures were tested for the enzymatic activities as described in Methods.
*Aliquots of the enzyme solution were treated with equivalent amounts of isopropanol.
†Micromolar final concentration.

mine-β-hydroxylase was phosphorylated in order to test if serine residues participate at the active site.

Diisopropylfluorophosphate did not inhibit the enzyme even when the inhibitor/enzyme ratio was 1250/1 (Table 4).

DISCUSSION

As shown in Fig. 2, the pK values of an ionizable group at the enzyme active centre are shifted upwards by 0.5 pK units and 0.55 pK units in $^{2}H_{2}O$ respectively for the enzyme and the enzyme-substrate complex. Previous work from our laboratory led to the suggestion that a histidyl residue is involved in the catalytic interaction between the enzyme and tyramine (1,2). The observed pK shifts in $^{2}H_{2}O$ are consistent with this conclusion because it is known that the pK of imidazole groups change from 6.11 to 6.54 on going from $H_{2}O$ to $^{2}H_{2}O$ (9). In the range of $p^{2}H$ from 4.8 to 6.2 where no change of Km for tyramine was observed, the hydroxylation rate was decreased by 50 %. This inhibition does not seem to be due to an irreversible denaturation of the enzyme

protein by $^{2}H_2O$, or to changes in affinity of the enzyme for ascorbate. Thus, the experimental results are consistent with the interpretation that a proton transfer step is mediated by the basic imidazol group of the enzyme during the hydroxylation reaction. It appears that histidine serves as a proton source. Goldstein *et al.* (10) have shown that one of the two side-chain benzylic tritiums is lost during the enzymatic β-hydroxylation of [β,β-^{3}H] dopamine and is released as water according to the following reaction :

[β,β-^{3}H] dopamine + ascorbate + O_2 ⟶

[β-^{3}H] norepinephrine + dehydroascorbate + [^{3}H] OH

As one of the β proton is eliminated from dopamine (or tyramine) as water, the proton bound to the oxygen atom in the hydroxyl group of norepinephrine (or octopamine) could be derived from the imidazole side-chain of the histidyl residue. Furthermore, this proton could come either from the aqueous medium since low pH is necessary for the enzymatic activity, or from ascorbate. Our study of dopamine-β-hydroxylase in $^{3}H_2O$ has shown that the proton originates from the ascorbate molecule. The reduction of copper present in the active centre is caused by ascorbate (11), and one of the liberated protons could be captured by the imidazole side-chain of the histidyl residue.

Is is tempting to consider possible detailed mechanisms for the hydroxylation of dopamine (or tyramine) by dopamine-β-hydroxylase. The reaction occurs in two steps since a ping-pong mechanism has been demonstrated by Goldstein *et al.* (12) and by us (2). The first step is the reduction of copper by ascorbate and the second step is the appearance of ternary complex : enzyme-tyramine-oxygen. Our previous results and the present data favor the idea that one histidyl residue is involved in the reaction process, but the involvement of other residues must not be neglected. Serine residues do not seem to participate in the hydroxylation reaction on the basis of our results obtained with diisopropylfluorophosphate.

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