Serum Dopamine-Beta-Hydroxylase Activity

By Richard Weinshilboum and Julius Axelrod

ABSTRACT

Dopamine-beta-hydroxylase, the enzyme which converts dopamine to norepinephrine, is released into the perfusate upon stimulation of the isolated perfused adrenal gland and after stimulation of the nerves to the isolated perfused spleen. This study was undertaken to determine whether dopamine-beta-hydroxylase activity could be detected circulating in blood. By using a sensitive new enzymatic assay, a dopamine-beta-hydroxylase activity was found in the blood of both man and the rat. It is located in the serum and is not associated with the formed elements of blood. The serum activity is similar to that of purified bovine adrenal dopamine-beta-hydroxylase in that it requires the presence of ascorbic acid, catalase, fumarate, and oxygen for full activity. Furthermore, as is also the case with the adrenal enzyme, serum activity is increased in the presence of cupric ions. The K_m values for substrate in human and rat sera are similar, and both are close to values determined in rat adrenal glands and stellate ganglia. The mean activity ± SE in the serum of six rats was 2.27 ± 0.04 nmoles/ml serum/20 min, and that of four normal humans ranged from 96.2 to 284 nmoles/ml/20 min.

KEY WORDS

adrenal medulla catecholamine release sympathetic nervous system

perfused spleen norepinephrine perfused adrenal gland

The neurotransmitter norepinephrine is formed by the beta-hydroxylation of 3, 4-dihydroxyphenylethylamine (dopamine) (1). This reaction is catalyzed by dopamine-beta-hydroxylase (DBH), an enzyme which is also capable of converting other phenylethylamines to their beta-hydroxylated derivatives (2). DBH activity is localized to the chromaffin granules in the adrenal medulla (3) and to the catecholamine-containing storage vesicles in sympathetic nerve terminals (4). About half of the enzyme activity can be released from rabbit chromaffin granules by lysis of the particles with distilled water (5). One explanation which has been offered for this finding is that a portion of the DBH activity is present in soluble form within the vesicle, and a portion is bound to the vesicular membrane (5). It has also been demonstrated that in the isolated perfused bovine adrenal gland, both DBH activity and catecholamines are released into the perfusate after stimulation with acetylcholine (6). Furthermore, several investigators have shown that stimulation of the nerves to the isolated perfused spleen releases DBH into the perfusate (7-9). These data raise the possibility that DBH is discharged into the circulation in vivo during periods of catecholamine release by either sympathetic nerves or the adrenal medulla. We have used a sensitive new enzymatic assay for DBH activity (10) and have been able to identify dopamine-beta-hydroxylase activity in the serum of both man and the rat.

Methods

Animals and Subjects.—All rats used in this study were 180- to 200-g male Sprague-Dawley rats obtained from Hormone Assay Laboratories, Chicago, Illinois. Human subjects were normal adult males and female volunteers.

Protein Assay.—Proteins were measured by the method of Lowry et al. (11).

Phenylethanolamine-N-Methyltransferase Assay.—Phenylethanolamine-N-methyltransferase activity was determined by the method of Axelrod (12) modified to use phenylethanolamine as a methyl acceptor rather than normetanephrine. All incubations were carried out for 5 minutes.

DBH Assay.—Dopamine-beta-hydroxylase acti-
ity was assayed by a method which depends upon the β-hydroxylation of tyramine to form octopamine. The octopamine formed by the DBH is enzymatically N-methylated by phenylethanolamine-N-methyltransferase (PNMT), an enzyme specific for β-hydroxylated amines (12), with a 14C-labeled methyl group donated by S-adenosyl-L-methionine-14C (Fig. 1).

Blood samples were obtained from experimental animals by decapitating and exsanguinating them into a test tube kept on ice. Human blood samples were obtained by venopuncture and were immediately placed on ice. Blood was centrifuged at 10,000 × g for 10 minutes at 4°C, and the serum was removed. The serum was then diluted appropriately with ice-cold distilled water, and 200-μl aliquots of the diluted sample were incubated at 37°C for 20 minutes in a reaction mixture containing 1.2 μmoles sodium fumarate, 1.2 μmoles ascorbic acid, 0.3 μmoles tyramine, 10 μmoles Tris-HCl buffer, pH 6, 0.16 μmoles pargyline, and 200 μg of catalase. Between 10⁻² to 10⁻¹ μmoles of CuSO₄ were also added to obtain maximal activation. The final reaction volume was 310 μl. Both the tyramine and ascorbic acid solutions were adjusted to pH 6 with NaOH. All Tris buffers were produced by titrating Tris (hydroxymethyl) aminomethane with HCl. Duplicate samples of serum heated to 95°C for 5 minutes were used as a blank.

After 20 minutes of incubation, the pH of the mixture was changed to 8.6 by adding to each tube 100 μl of a mixture of 80 μl 1.0x Tris-HCl buffer, pH 8.6, 10 μl containing 0.05 μg S-adenosyl-L-methionine-14C and 10 μl bovine adrenal PNMT, purified according to the method of Axelrod (12) through the ammonium sulfate precipitation and then passed over a Sephadex G-200 column. The final specific activity of the PNMT was 180 nmoles producting protein/hour. Approximately 40 μg of this protein were added to each assay tube. After 30 minutes of further incubation, the reaction was stopped by the addition of 0.5 ml 0.5M borate buffer, pH 10, and the radioactive 14C-N-methyl octopamine (synephrine) formed was extracted into 6 ml of toluene-isoamyl alcohol (3:2 v/v). After centrifugation at 750 × g for 10 minutes, 4 ml of the organic phase was transferred to a counting vial containing an additional 2 ml of toluene-isoamyl alcohol and dried in a chromatography oven at 80°C. As in the previously described assay (13) for octopamine using 14C-S-adenosyl-methionine and PNMT, this drying step was necessary to remove a volatile contaminant, probably 14C-S-adenosyl-methionine, which was carried through the organic extraction. After the vials were allowed to cool, 1 ml of ethanol and 10 ml of liquid toluene phosphor were added to each vial, and the radioactivity of the samples was determined in a Beckman liquid scintillation counter with a 14C counting efficiency of 80%.

In addition to a blank, a standard value was obtained with each assay by adding 20 or 40 ng of octopamine HC1 to a complete assay mixture including 200 μl of heated serum, and this sample was carried through the procedure. Results were expressed either as nanograms or nanomoles of octopamine formed per milliliter of serum per 20 minutes.

Standard methods of statistical analysis were used in these studies (14). Km values were determined by the method of Wilkinson (15) using an IBM 1620 digital computer and a FORTRAN program written by Cleland (16).

Thin Layer Chromatography.—Thin layer chromatography was carried out to identify the radioactive products of the enzyme assay. Eastman Chromagram sheets of silica gel, 100 μ thick, were used. n-Butanol saturated with 2N HCl and...
a mixture of toluene, acetic acid, ethylacetate and water (80:40:20:5) were the two solvent systems used. Sheets were prerun overnight in the system containing n-butanol saturated with 1N HCl. All sheets were activated by drying for 15 minutes at 95°C prior to application of the sample.

To prepare a sample for chromatography, it was dissolved in 1 ml of ethanol after being dried. It was then dried again under a stream of nitrogen and the residue was dissolved in 30 µl of ethanol for application to the chromatograms.

When the toluene, acetic acid, ethylacetate and water system was used, the plate was developed, dried and then developed again a total of four times to obtain the desired separation. After development, the sheets were sprayed with diazotized p-nitroaniline (17) followed by ninhydrin. They were then marked and cut into strips 1 cm wide, which were placed in counting vials containing 0.5 ml NCS (Amersham-Searle), a surface-active organic base. After 1 hour, 1 ml of ethanol and 10 ml of phosphor were added to each vial and the radioactivity was determined.

**TABLE 1**

<table>
<thead>
<tr>
<th></th>
<th>Plasma (nmoles/ml/20 min)</th>
<th>Cellular elements (nmoles/ml/20 min)</th>
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<tbody>
<tr>
<td>Rat</td>
<td>1.9 ± 0.8</td>
<td>&lt;0.3*</td>
</tr>
<tr>
<td>Man</td>
<td>116 ± 1.8</td>
<td>&lt;0.3*</td>
</tr>
</tbody>
</table>

*Below sensitivity of assay.

Blood from a man and a rat were collected in heparinized tubes, and the plasma was separated by centrifugation. The formed elements were washed twice with an equal volume of normal saline and resuspected. Cells were lysed by freezing and thawing, and both plasma and lysed cells were assayed for DBH activity. No copper was added to the assay of the cellular elements because copper concentrations from 12 to 80 µM were found only to decrease the total cpm incorporated in the assay. Results are expressed as the mean of five determinations ± SD.

When the solvent system was toluene, acetic acid, ethylacetate and water (80:40:20:5), N-methyl tyramine was separated from synephrine. With this system the radioactivity in the blank was found to have the Rf of N-methyl tyramine, and most of the activity in the experimental sample was associated with the expected reaction product, synephrine (Fig. 3). The results of chromatography were the same for samples obtained from man and from rat.

**Effect of Time and Increasing Amounts of Serum on Activity.**—The activity present after assay of both rat and human blood proved to be directly proportional to the amount of
Chromatography of human serum extract. An extract of human serum after assay was applied to a silica gel, thin layer chromatograms and was developed in n-butanol saturated with 1N HCl. The abscissa represents distance from the origin in centimeters. The solid line represents radioactivity incorporated into the sample, and the broken line that incorporated into a "blank" specimen. The dark bars represent the Rf values of standards synephrine (SYN), metanephrine (META), N,N-dimethyloctopamine (Di-M-OCT), and epinephrine (EPINEPH).

Chromatography of human serum. A duplicate sample to that shown in Figure 2 was developed on a silica gel, thin layer plate using toluene, acetic acid, ethylacetate and water (90:40:30:5) as a solvent. Additional standards were applied to this chromatogram: N-methyltyramine (N-METH-TYR) and methoxytyramine (METHOXY TYR).
serum added to the reaction mixture when human samples were diluted 1 to 64 (Fig. 4, left), and samples from the rat were diluted 1 to 4. When the time course of the DBH portion of the assay was examined, activity was found to increase in a linear fashion for 50 minutes with rat as well as with human serum (Fig. 4, right).

Effect of Substrate Concentration on DBH Activity.—Increasing concentrations of substrate had different effects on the two steps of this enzyme assay. As would be expected,
increasing amounts of tyramine in the reaction mixture resulted in increased formation of octopamine until a maximum was reached at approximately $8 \times 10^{-3}$M (Fig. 5). However, before maximal concentrations of substrate for the DBH portion of the assay had been attained, increasing substrate concentrations had begun to inhibit the PNMT portion of the reaction, resulting in a decrease in the formation of radioactive N-methyl-octopamine (synephrine) (Fig. 5). Inhibition of PNMT by various amines, among them tyramine, has been reported previously (18). Because of the rapid decline in the formation of the radioactive product at high substrate concentrations, all reactions were carried out in the presence of tyramine at a concentration of $10^{-3}$M. Values for reaction rates reported here (nmoles/ml serum/30 min) are not maximal velocities and can only be compared with values obtained using identical substrate concentrations.

**Linearity of PNMT Reactions.**—To determine the range over which the PNMT portion of the assay was linear, varying amounts of octopamine were added to the reaction mixture containing 200 µl of human serum heated for 5 minutes to 95°C (Fig. 6). The reaction was linear up to 50 ng of added octopamine HCl.

**Requirement for Cofactors, Oxygen and Copper.**—Several cofactors such as ascorbic acid, catalase, and fumarate are needed for maximal adrenal DBH activity (1). To examine the requirements of the serum enzyme, DBH activity was assayed omitting the various components of the reaction mixture to determine whether they were necessary to attain maximal activity (Table 2). There was no activity in the absence of ascorbic acid, and the removal of catalase and fumarate reduced activity to 9.3% and 24% of control levels, respectively.

Bovine adrenal DBH, a mixed function oxidase, requires the presence of oxygen (19). To determine whether the DBH activity in the serum also had this requirement, a sample of human serum was assayed both in the presence of room air, and under a nitrogen atmosphere (Table 3). For the first minute of...
TABLE 2  
Effect of DBH Cofactors on Human Serum Activity

<table>
<thead>
<tr>
<th>Cofactor</th>
<th>Activity (nmoles/ml serum/20 min)</th>
<th>% Control</th>
</tr>
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<tbody>
<tr>
<td>None (control)</td>
<td>96.3 ± 2.0</td>
<td>100</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>8.9 ± 2.2</td>
<td>9.3</td>
</tr>
<tr>
<td>Catalase</td>
<td>22.7 ± 2.5</td>
<td>24</td>
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</table>

A sample of normal human serum was assayed for DBH activity in the usual way and also without several of the ingredients of the reaction mixture. Results are expressed as the mean of four determinations ± se.

TABLE 3  
Effect of Oxygen on Human Serum DBH Activity

<table>
<thead>
<tr>
<th>Atmosphere</th>
<th>Activity (nmoles/ml/20 min)</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Room air</td>
<td>107 ± 5.6</td>
<td>100</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>18.9 ± 0.7</td>
<td>17.7</td>
</tr>
</tbody>
</table>

A sample of normal human serum was assayed for DBH activity in the presence of room air and in a nitrogen atmosphere. Results are expressed as the mean of four determinations ± se.

...the reaction, both sets of tubes were exposed to air as the serum was added, but, even so, the activity was reduced 82% in the absence of room air.

...The activity of tissue DBH is increased by the addition of cupric ions to the reaction mixture (20). It is thought that the copper inhibits the effect of tissue inhibitors of the enzyme (20). The activity of both rat and human serum DBH activity increased with the addition of CuSO4. Maximal activity for human serum diluted 1 to 64 was attained with a final copper concentration of 3 μM. Rat serum diluted 1 to 4 required a copper concentration of 32 μM for maximal activity (Fig. 7).

...Kinetics.—Homogenates of rat adrenal gland and stellate ganglia were assayed for DBH activity in the presence of eight different concentrations of substrate from 3 × 10^-4 to 10^-4 M under conditions which give maximal activity in this assay. DBH activity in rat serum was also assayed, and values for the Km of DBH activity using tyramine as a substrate at pH 6.5 in the presence of room air were determined. The Km values for adrenal gland, stellate ganglia and serum in the rat were 7.8 ± 0.7 × 10^-4 M, 6.8 ± 1.1 × 10^-4 M, and 7.0 ± 0.7 × 10^-4 M, respectively. The Km for human serum determined in a similar manner was 8.4 ± 0.7 × 10^-4 M.

Representative Values.—Serum DBH activity was determined on blood samples from six normal male Sprague-Dawley rats. Values ranged only from 2.16 to 2.38 nmoles/ml serum/20 min with a mean value ±SE of 2.27 ± 0.04. Values for fasting morning blood samples obtained from four normal human volunteers ranged from 98.2 to 284 nmoles/ml/20 min with a mean of 164.3. The serum activity of one normal subject was determined four times over a 6-week period. Values were consistent, with a range of 89.9 to 116 nmoles/ml/20 min and a mean of 99.2 ± 5.8. To determine the reproducibility of a given assay, one human serum sample was assayed six times. The value obtained was 88.4 ± 1.6 nmoles/ml serum/20 min. Thus, the standard error after six determinations on a single sample was 1.9% of the mean.
Discussion

A dopamine-ß-hydroxylase activity is present in the blood of both man and the rat. It is associated with serum and not with the formed elements of the blood. Serum DBH activity is similar to that which has been studied in purified form from the bovine adrenal gland in that it requires ascorbic acid, fumarate, and catalase and is inhibited in the absence of oxygen (1, 19). The Km for tyramine of serum DBH in the rat is similar to that of tissue DBH activity. Further studies will, of course, be necessary to establish the source and possible significance of the serum dopamine-ß-hydroxylase. Preliminary experiments in our laboratory have demonstrated a decrease of this activity in rats treated with 6-hydroxydopamine, a drug which destroys sympathetic nerve terminals (21), and a rapid increase of the levels in rats subjected to immobilization stress. If the serum activity proves to be released from sympathetic nerves, the adrenal medulla, or both, it might prove to be a valuable tool with which to study the role of catecholamines and their release in both normal and pathologic physiology. Presently such studies, particularly in human subjects, are hampered by the fact that even the best fluorometric assays for serum catecholamines have been extended to the limits of their sensitivity (22), and the more sensitive enzymatic assays are complex and laborious (23). There are many disease states, among others idiopathic orthostatic hypotension (24), familial dysautonomia (25), hypertension (26), and infantile hypoglycemia (27), in which catecholamines or their release may play important roles. In these situations it would be important to have available a convenient measure of sympathetic nervous system and adrenal medullary activity, and we hope to investigate serum DBH activity in these and other disease states.

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References
