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ENDOGENOUS INHIBITORS OF DOPAMINE-β-HYDROXYLASE IN RAT ORGANS*

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Abstract—Endogenous inhibitors of dopamine-β-hydroxylase (DBH) are present in organ homogenates from several mammalian species. An assay for these inhibitors which utilizes DBH partially purified from the bovine adrenal medulla has been developed. A rat spleen homogenate, diluted 1:8000 in Tris buffer, inhibited bovine DBH activity by more than 50 per cent. Similar amounts of inhibitory activity were present in other organs. The inhibitory activity in homogenates of rat heart was reduced by treatment at 95° while most of that in the spleen was resistant to heat denaturation. Mixing experiments showed that there were constituents in heart homogenates which could cause the inhibitory activity in spleen to become heat labile. Divalent cations and the heme containing protein cytochrome *c* also conferred heat lability on the inhibitory activity in rat spleen homogenates. *N*-Ethylmaleimide had no effect on the inhibitory activity in rat heart, spleen or adrenal gland but Cu^{2+} and *p*-chloromercuribenzoate completely reversed the effects of the endogenous inhibitors in these organs. Sulfhydryl reagents can inhibit DBH but there was no correlation between inhibitory activity and either total tissue sulfhydryl concentration or TCA soluble sulfhydryl concentration.

Endogenous inhibitors of dopamine-β-hydroxylase (EC 1.14.2.1; DBH) exist in many tissues [1, 2]. Their existence was first surmised from the observation that, although little or no DBH activity was detectable in homogenates of adrenal glands, activity was readily demonstrable when the homogenate was fractionated [3, 4, 5]. This observation led to the suggestion that endogenous inhibitors may limit the rate of dopamine hydroxylation in intact tissues [2, 4, 5].

During the β-hydroxylation reaction, Cu^{2+}, which is at the active site of the enzyme [6, 7] undergoes cyclic oxidation and reduction. The action of the endogenous inhibitors and probably of inhibitory compounds, such as cysteine and glutathione, appears to be exerted by interaction with the Cu^{2+} at the active site of the enzyme [1].

Endogenous inhibitors of DBH (DBHI) have been purified from heat denatured homogenates of bovine heart [8] and bovine adrenal gland [1, 9, 10]. The activity of the DBHI which was purified from bovine heart was not affected by *N*-ethylmaleimide (NEM), but it could be neutralized by the addition of Cu^{2+}. The purified inhibitory substance was reported to be heat stable, to have a molecular weight between 750 and 1200, and to contain organic phosphate and carbohydrate [8].

The inhibitor isolated from homogenates of bovine adrenal glands had an amino acid composition similar to that of glutathione but it differed from glutathione in the kinetics of the inhibition of DBH. Storage caused the inhibitor to become inactive but inhibitory activity was regenerated on exposure to H_2S gas [10]. The adrenal activity was reversed both by NEM [1] and by Cu^{2+} [9]. The difference in the effect of NEM on the heart and adrenal inhibitors suggests that the endogenous inhibitors differ in different organs.

In order to assess the potential physiological role of DBHI's in regulating DBH activity *in vivo* and thus of regulating norepinephrine biosynthesis, studies of the endogenous inhibitors of DBH in rat organs have been initiated. In this report an assay for DBHI's is described as are the basic characteristics of the endogenous DBHI's in several rat organs.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats (Simonsen) weighing 150–250 g were used in all experiments. They were killed by cervical fracture.

Chemicals. *S*-Adenosylmethionine-[^{14}C] (53 mCi/m-mole) was purchased from International Chemical and Nuclear Corporation, Irvine, California. The following compounds were obtained from Sigma Chemical Co.: octopamine HCl, tyramine HCl, bovine serum albumin, cytochrome *c*, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), l-arterenol HCl (l-norepinephrine), adenosine, adenosine 5′-monophosphate (AMP) and adenosine 5′-triphosphate (ATP); from Schwarz/Mann: *p*-chloromercuribenzoate (PCMB); from Abbott Laboratories: pargyline HCl (Eutonyl); from Boehringer Chemical Company: catalase (EC 1.11.1.6); from Calbiochem: Cleland's Reagent (dithiothreitol); from Eastman Kodak Company: diethylmaleate; from Aldrich Chemical Company: *N*-ethylmaleimide (NEM); from Pharmacia Fine Chemicals, Inc: DEAE Sephadex A-50 and Sephadex G-200, and from Regis Chemical Company: 6-hydroxydopamine.

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DBH purification. DBH was purified by a modification of the method of Foldes *et al.* [11]. Bovine adrenal medullae, dissected free of cortical tissue, were weighed and homogenized in 3 vol of cold 0.3 M sucrose. The supernatant obtained after centrifugation at 800 *g* for 15 min was centrifuged at 10,000 *g* for 20 min. The pellet thus obtained was resuspended in 0.3 M sucrose, layered on cold 1.6 M sucrose, and centrifuged at 100,000 *g* for 60 min in a Beckman SW 25.2 rotor. The pellet, which was highly enriched in chromaffin granules, was lysed by suspension in potassium phosphate buffer, 10 mM, pH 7.2. The membranes were sedimented by centrifugation (100,000 *g* for 1 hr) and the lysate was applied to a 40 × 2.5 cm column of DEAE Sephadex (A-50). DBH was eluted with a gradient running from 0 to 0.8 M NaCl in 10 mM phosphate buffer, pH 7.2. The fractions with the highest activities were combined and stored in polypropylene tubes in the presence of 0.1% bovine serum albumin at −10°. Enzyme stored in this manner retained full activity for at least 26 months. The DBH used in these experiments had a specific activity of 47 μmoles octopamine/hr/mg protein.

Phenylethanolamine-N-methyl transferase purification. Phenylethanolamine-*N*-methyl transferase (EC 2.1.1.1; PNMT) was purified by a modification of previously described methods [12, 13]. Bovine adrenal medullae were homogenized in 3 vol of 1.15% KCl and centrifuged at 28,000 *g* for 30 min. The supernatant was adjusted to pH 5.3 with 1 N acetic acid and centrifuged at 28,000 *g* for 30 min. The resulting supernatant was readjusted to pH 7.0 with 2 M Tris buffer, pH 8.6, and solid ammonium sulfate was added to 30 per cent of saturation (176 g/l). After centrifugation the supernatant was brought to 50 per cent of saturation (127 g/l) with solid ammonium sulfate. The pellet obtained after centrifugation at 28,000 *g* for 30 min was resuspended in a minimal volume of 5 mM Tris buffer, pH 7.4, and dialysed against 100 vol of buffer. The dialysate was then layered on a Sephadex G-200 column (100 × 4 cm). The active fractions were collected and stored frozen at −10° in polypropylene test tubes. The enzyme was stable for at least 20 months when prepared and stored in this manner.

DBH assay. A modification of the assay of Molinoff *et al.*, [13] was used. The assay is based on the conversion of tyramine to octopamine by DBH and the subsequent conversion of the octopamine thus formed to synephrine[^{14}C] by PNMT using *S*-adenosylmethionine[^{14}C] as the methyl donor. Sciatic nerve sections (0.3 cm) were homogenized in 1 ml of 5 mM Tris buffer, pH 7.4. Other organs were weighed and homogenized in 25 vol of buffer. Homogenizations were with a Polytron homogenizer running at the highest speed for 20 sec. The homogenates were centrifuged at 12,000 *g* for 10 min, after which the supernatants were diluted as necessary with Tris buffer. When the effect of boiling was studied, the supernatants were subjected to a temperature of 95° for 5 min. Precipitated proteins were removed by centrifugation, after which the supernatants were diluted with Tris buffer. The reaction mixture contained 3.5 mM ascorbic acid, pH 5.5; 37 mM sodium fumarate, pH 5.5; 0.46 mM pargyline; 5 units of catalase per μl of reaction vol; 0.88 mM tyramine HCl, pH 5.5; 30 mM sodium acetate, pH 5.5; and either 100 μl or 200 μl of sample. The final reaction volume was either 160 or 310 μl. Stock solutions of octopamine, sodium fumarate and tyramine HCl were adjusted to pH 5.5 and stored at −10°. Sodium ascorbate, pH 5.5, and paragyline were prepared fresh daily. Catalase, stored at 4°, was diluted with 5 mM Tris, pH 7.4, on the day of each experiment. The first step of the assay was carried out for 20 min at 37° at pH 5.5, while the *N*-methylation step was carried out for 30 min at 37° at pH 8.6. Sodium borate, pH 10, was added to a final concentration of 0.2 M to stop the PNMT reaction. The synephrine[^{14}C] was extracted into either 4 or 6 ml (with 160 or 310 μl reaction vol, respectively) of a mixture of toluene and isoamyl alcohol (3:2, v/v, 310 μl reaction vol) or 5.5 ml of ethyl acetate (160 μl reaction vol) with a Vortex Genie mixer. After centrifugation at low speed, either 3 or 4 ml of the 3:2 toluene: isoamyl alcohol organic layer was dried at 80° as previously described [14] and 5 ml of a phosphor containing 150 ml of methanol and 4.29 g of 2,5-diphenyloxazole/l of toluene were added to each scintillation vial. When ethyl acetate was used for the extraction the radioactivity in 4 ml of the organic phase was determined with 6 ml of the toluene counting solution described above. Blanks and internal standards were included in each experiment. The internal standards (125 pmoles of octopamine) were used to calculate absolute amounts of DBH activity. They also correct for any variation in the activity of the PNMT.

DBHI assay. The reaction mixtures for DBHI assays were the same as those used in the DBH assay described above except that an aliquot of partially purified bovine adrenal medullary DBH, sufficient to convert 10–20 pmoles of tyramine to octopamine/min, was added to each assay tube. Blanks and octopamine internal standards were carried out as described above. DBHI's did not affect the second step of the reaction. Since the tissue homogenates were usually very dilute, only minimal amounts of endogenous DBH activity were seen. The activity of the partially purified DBH was determined in the absence of inhibitor in each experiment and the experimental results were compared to this activity with results being expressed as percent inhibition. The reproducibility of duplicate samples was unsatisfactory unless special precautions were observed to insure that glassware was scrupulously clean. Soaking glassware in cleaning solution (NoChromix [Godax Labs., N.Y.]) usually resulted in acceptable reproducibility. The best results were obtained, however, when new disposable glassware and pipettes (Van-Lab, VWR Scientific) were used throughout the experiment.

Sulfhydryl assay. Sulfhydryl concentrations in rat organ homogenates were determined by the method of Sedlak and Lindsay [15]. Total sulfhydryl concentration was determined by adding 0.5-ml samples of a 1:25 (w/v) organ homogenate in 20 mM EDTA, pH 4.7, to 1.5 ml of 0.2 M Tris buffer, pH 8.2, containing 0.1 ml of 10 mM DTNB. The volume of the mixture was adjusted to 10 ml with methanol and color was developed for 30 min, after which the samples were centrifuged at low speed. Absorbance was read at 412 nm in a Beckman DU spectrophotometer. Sulf-

hydryl concentration was calculated using an extinction coefficient of 13,600 M^{-1} cm^{-1}. Reagent and tissue blanks were run with each sample. Non-protein sulfhydryl, which is acid soluble and includes reduced glutathione as a major constituent [16], was determined by adding 5 ml of tissue homogenate in EDTA to 4 ml of distilled water. Trichloroacetic acid (1 ml of 50% w/v) was added and after 10 min of intermittent mixing the solution was centrifuged at low speed in an IEC centrifuge. An aliquot (2 ml) of the supernatant was added to 4 ml of 0.4 M Tris buffer, pH 8.9, containing 0.1 ml DTNB (10 mM). The absorbance at 412 nm was determined and the concentration of TCA soluble sulfhydryl was calculated as described above.

RESULTS

Characteristics of the DBHI assay. The assay for DBH and for DBHI's was linear for at least 30 min. Preincubation of the enzyme for up to 60 min with a preparation containing endogenous inhibitor from rat spleen did not affect the degree of inhibition.

In order to use bovine adrenal DBH to study the endogenous inhibitors found in rat organs, it was important to establish that the inhibitors had the same effect on exogenous bovine DBH as they had on the endogenous DBH present in homogenates of rat organs. Experiments were carried out in which DBH activity was determined in homogenates of heart, spleen, adrenal glands and brain as a function of the concentration of Cu^{2+}. It has been shown previously [2, 9, 13], that a very precise concentration of Cu^{2+}

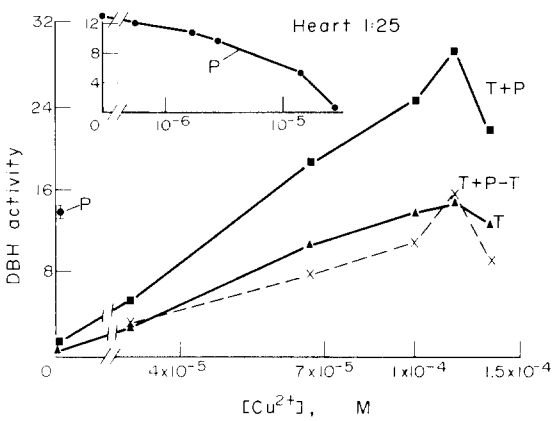

Fig. 1. The effect of Cu^{2+} on the DBH inhibitor from heart. The curves drawn with solid lines show the effect of Cu^{2+} on endogenous tissue DBH in a 1:25 homogenate of rat heart (T, ▲) and on partially purified adrenal DBH added to the heart homogenate (T + P, -■-). The dashed line is derived by mathematically subtracting the activities obtained with tissue from those obtained with partially purified bovine adrenal DBH in the presence of tissue (T + P − T, -- x --). The activities of tissue DBH (T) and tissue + partially purified DBH (T + P) in the absence of Cu^{2+} are shown by the intercepts on the ordinate. The activity of partially purified DBH in the absence of Cu^{2+} is shown on the ordinate (●) and is the mean of 5 determinations ± S.E.M. The data is expressed as the average of duplicate determinations. The inset shows the effect of increasing concentrations of Cu^{2+} on the activity of partially purified bovine adrenal DBH in the absence of tissue.

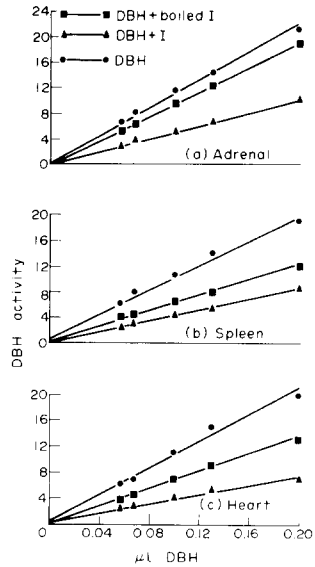

Fig. 2. Reversibility for DBH inhibitor from adrenal, spleen, and heart. Assays for DBH activity were carried out for 20 min as described in Materials and Methods. The tissues added to partially purified DBH (-●-) were as follows: (a) a 1:7000 dilution of rat adrenal supernatant (-▲-) or a 1:5000 dilution of a boiled rat adrenal supernatant (-■-); (b) a 1:10,000 dilution of rat spleen supernatant (▲), or a 1:500 dilution of boiled rat spleen supernatant (■); (c) a 1:6000 dilution of rat heart supernatant (-▲-) or a 1:300 dilution of boiled rat heart supernatant (-■-). The data represents the average of duplicate determinations.

is required to obtain maximal DBH activity *in vitro*. If an insufficient amount of Cu^{2+} is used, the inhibitors will decrease the activity of the enzyme. If too high a concentration of Cu^{2+} is added, the Cu^{2+} will itself inhibit either the partially purified DBH (Fig. 1, inset) or the DBH which is present in the homogenate (Fig. 1). The partially purified DBH did not require Cu^{2+} for maximal activity since there was no inhibitor in this preparation (Fig. 1, inset). In these experiments, when the activity of exogenous bovine adrenal DBH was measured in the presence of an organ homogenate, as a function of the concentration of Cu^{2+}, the bovine adrenal enzyme was affected in the same way as was the DBH in the homogenate (Fig. 1). These results, which were obtained with heart (Fig. 1), brain [2], spleen and adrenal homogenates (not shown), suggest that it is valid to use partially purified bovine adrenal medullary DBH in an assay of the DBH inhibitors which are found in rat organs.

To determine whether the inhibitors of DBH act reversibly, reaction velocity was determined as a function of enzyme dilution in the presence and in the absence of inhibitor [17]. This analysis was performed for the unboiled and boiled rat spleen, heart and adrenal homogenates (Fig. 2).

When inhibitor was added to partially purified DBH the reaction velocity was decreased. The percent inhibition was constant, however, regardless of the amount of DBH activity in the assay. This suggests that the effect of the inhibitors is reversible.

Amount of DBHI activity in various rat organs. The amount of inhibitory activity was such that in order to obtain an I_{50}, dilution by several thousand-fold

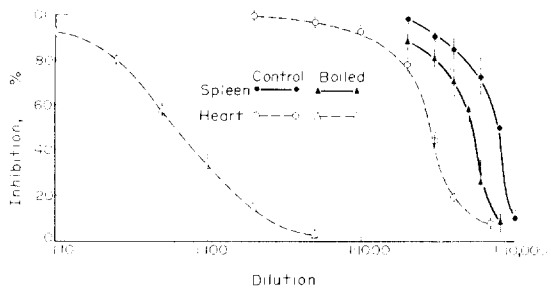

Fig. 3. Effect of dilution on DBHI activity in homogenates of rat heart and spleen. Rat spleen (●) and heart (--○--) homogenates were prepared. A portion of each homogenate served as the control while other aliquots of the spleen homogenate (▲) and heart homogenate (--△--) were studied after being at 95° for 5 min. Dilution curves were performed on the 2 preparations from each homogenate. The per cent inhibition of an aliquot of partially purified DBH is represented on the ordinate and dilution of the supernatant or boiled supernatant on the abscissa. Values are means of 5 organs ± S.E.M.

was required for all organs examined. The dilution curves for spleen and heart are shown in Fig. 3. In these experiments a mean dilution of 1:2950 for the heart and of 1:8000 for the spleen was required to inhibit the partially purified DBH by 50%. (Fig. 3). When these homogenates were heated at 95° for 5 min, an average of only 2 per cent of the inhibitory activity remained in the heart homogenate while 60 per cent of the inhibitory activity in the spleen appeared to be heat stable (Fig. 3). Heating for an additional 15 min had no further effect on the heat stability of the inhibitor present in either the spleen or heart homogenates. The I_{50} and percentage of the inhibitory activity which was heat stable were determined for homogenates of several rat organs (Table 1).

The differences in the heat lability of the DBHI in homogenates of heart from that observed in spleen homogenates (Table 1) could indicate that the DBHI's are organ specific [2] or it could be due to the presence of a constituent in the heart homogenate which rendered the inhibitor labile to heat. Homogenates of heart and spleen were mixed in equal proportions

Table 1. DBHI levels in rat organs

Organ	Dilution (I_{50})	Percentage heat stable (5' at 95°)
Heart	1:3470 ± 640 (11)	2.7 ± 0.6 (11)
Spleen	1:8510 ± 605 (19)	60.5 ± 7.6 (17)
Adrenal	1:7730 ± 1750 (3)	62.0 ± 13.3 (3)
Sciatic nerve	1:1300 ± 175 (6)	52.6 ± 3.7 (3)
Salivary gland	1:2185 ± 300 (8)	81.0 ± 3.5 (3)

Dilution curves of boiled and unboiled inhibitor were determined (see Fig. 3). I_{50}'s were calculated from the dilution curves. The percentage heat stable was calculated using the following formula:

$$\left(\frac{I_{50}\text{ boiled}}{I_{50}\text{ unboiled}}\right)100.$$

The numbers in parentheses are the numbers of separate determinations. Results are mean ± S.E.M.

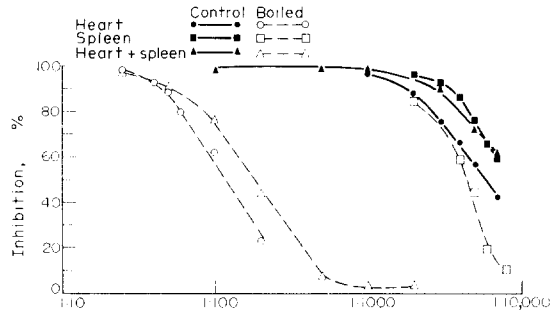

Fig. 4. Effect of rat heart supernatant on the heat lability of rat spleen DBHI. Dilution curves were performed for heart (●), boiled heart (--○--), spleen (■) and boiled spleen (--□--) as Fig. 3. Control curves and curves after heat denaturation (5 min at 95°) are shown. Dilution curves were also performed for boiled (--△--) and unboiled (▲) mixtures of equal volumes of the heart and spleen homogenates. The heart plus spleen boiled curve represents the dilution of the spleen homogenate in the mixture (e.g. a 1:25 heart + 1:25 spleen homogenate would produce a 1:50 spleen dilution). The data represents the average of duplicate determinations.

and dilution curves were determined for the spleen, the heart and for a 1:1 mixture of the two homogenates (Fig. 4). When a mixture of heart and spleen homogenates was placed in a boiling water bath for 5 min, there was a marked decrease in the inhibitory activity present in the spleen homogenate. This suggested that a constituent present in the heart homogenate was responsible for the heat lability observed with these mixed homogenates. Similar results were obtained when adrenal and heart homogenates were combined and subjected to a temperature of 95° for 5 min.

Effect of metals on the heat lability of DBHI. In view of the possibility that DBHI's may be sulfyhdryl compounds [1], and the ability of metals, particularly divalent cations, to inactivate sulfhydryl compounds [18, 19, 20], the effect of metals on the heat stability of spleen DBHI was investigated. When a concentration of Fe^{2+} greater than 10^{-4} M was added to

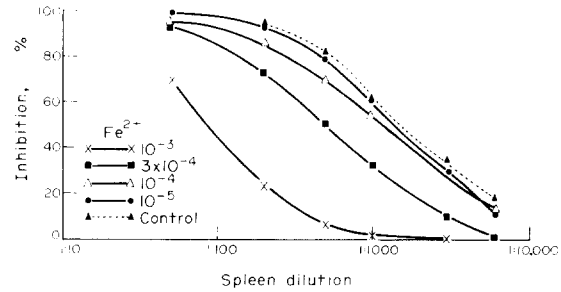

Fig. 5. Effect of Fe^{2+} on the heat lability of rat spleen inhibitor. $FeSO_4$ was added to a 1:25 rat spleen homogenate to produce concentrations between 10^{-3} and 10^{-5}M. The supernatants from these homogenates were boiled for 5 min at 95°. Precipitated proteins were removed by centrifugation and the supernatant was diluted with buffer to produce the desired final dilution. Fe^{2+} does not inhibit DBH at concentrations below 10^{-3} M. The control curve is the dilution curve for boiled spleen supernatant in the absence of exogenous Fe^{2+} (--▲--). The data represent the average of duplicate determinations.

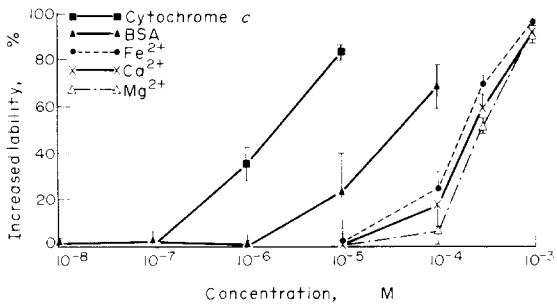

Fig. 6. Effect of Ca^{2+}, Mg^{2+}, Fe^{2+}, cytochrome *c* and bovine serum albumin on increasing the heat lability of rat spleen inhibitor. $CaCl_2$ (×), $FeSO_4$ (--●--), $MgCl_2$ (-△-), cytochrome *c* (Cc, ■--) or bovine serum albumin (BSA, -- ▲) were added to a 1:25 spleen homogenate to produce final concentrations between 10^{-8} and 10^{-3} M. These homogenates were centrifuged and an aliquot of the supernatant was heated at 95 for 5 min. Denatured proteins were removed by centrifugation and dilution curves were performed with each of the resulting supernatants. Ca^{2+}, Fe^{2+} or Mg^{2+} did not inhibit DBH at or below 10^{-3} M. At those concentrations of Cc or BSA which inhibited DBH directly ($\geq 10^{-5}$ or $\geq 10^{-4}$ M, respectively) 100% values were obtained by adding the appropriate concentration of protein to Tris buffer, 5 mM, pH 7.4, and assaying DBH in the presence of this solution. In order to eliminate exogenous non-protein metals the solutions of Cc and BSA were heated for 5 min at 95 , dialyzed against Tris buffer 5 mM, pH 7.4, EDTA (2.9 mM) and deferoxamine (0.3 mM) (Desferal, Ciba Pharmaceutical Co., Summit, N.J.) for 48 hr. The proteins were then dialysed against Tris buffer 5 mM, pH 7.4, for an additional 48 hr to remove the chelating agents. The per cento increased lability was calculated using the following formula:

$$\left(1 - \frac{I_{50} \text{ boiled in presence of metal or protein}}{I_{50} \text{ boiled in absence of metal or protein}}\right) 100$$

Results are expressed as mean ± S.E.M. of 3–5 experiments.

a spleen homogenate, a marked reduction in DBHI activity occurred on exposure to a temperature of 95 for 5 min (Fig. 5). Ca^{2+} and Mg^{2+} caused similar increases in the heat lability of DBHI in spleen homogenates (Fig. 6). In order to determine whether metalloproteins could also cause the inhibitor in the spleen to become heat labile, the heme protein cytochrome *c* (Fig. 6) was added to a spleen homogenate. Exposure of the mixture to a temperature of 95 for 5 min resulted in a marked increase in the lability of the inhibitor. Similar results were obtained with the heme containing proteins yeast lactic dehydrogenase and hemoglobin. Bovine serum albumin, a non-iron containing protein, was able to labilize DBHI, but a much higher concentration was required. (Fig. 6).

Relation of glutathione concentration to endogenous DBHI levels. Sulfhydryl compounds such as cysteine, glutathione and mercaptoethanol have been shown to inhibit DBH [1, 10]. Furthermore, the effects of at least some of the endogenous inhibitors of DBH are reversed by appropriate concentrations of sulfhydryl reactive agents such as Cu^{2+}, Hg^{2+}, Ag^{+}, PCMB, and NEM [9]. To examine the possibility that DBH inhibitory activity might be related to levels of reduced glutathione, rats were injected with the glutathione depleting agent diethylmaleate [16]. A dose response curve for diethylmaleate and the time course of trichloroacetic acid (TCA) soluble sulfhydryl depletion were determined for rat liver. A dose of 1.3 mg/kg was chosen and the animals were killed 30 min after the administration of the drug. At higher doses or longer times increased mortality was observed. This treatment resulted in a marked decrease in total sulfhydryl content in rat heart and spleen (Fig. 7) as well as in the liver. Under conditions in which there was a highly significant decrease in TCA soluble sulfhydryl concentration, no change was seen in the amount of inhibitory activity in either the rat spleen or heart (Fig. 7).

Reversibility of endogenous DBHI. The ability of homogenates of spleen, heart and adrenal to inhibit partially purified bovine adrenal DBH was fully reversed by appropriate concentrations of Cu^{2+} and PCMB (Fig. 8). NEM failed to reverse inhibition in any of these rat organ homogenates. In these experiments dithiothreitol was added during the *N*-methylation step in order to reverse the inhibitory effects of PCMB and NEM on PNMT.

Inhibitor activity stability. The stability of DBHI activity was determined for the rat heart, spleen and adrenal (Table 2). Dilution curves were performed at the indicated times and I_{50}'s were determined from these curves. The heart and boiled heart homogenates and the unboiled spleen homogenates retained nearly full inhibitory activity for at least 3 days when stored at −10°. On the other hand, the inhibitory activity in boiled spleen homogenates and in adrenal homogenates (either boiled or unboiled) was labile when stored at −10° for from 1–3 days. In other experiments exposure of inhibitor preparations to a pH

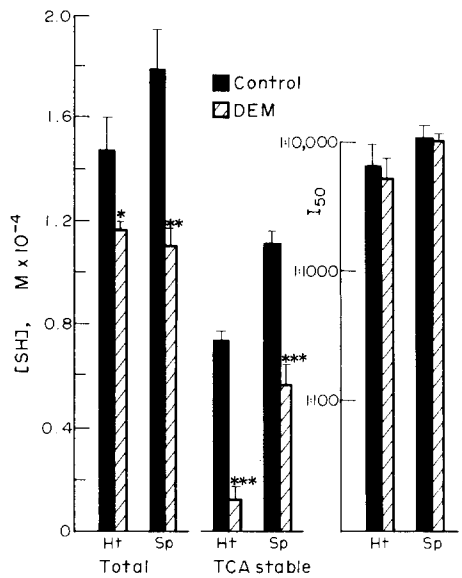

Fig. 7. Effect of sulfhydryl depletion on rat heart and spleen inhibitor. Diethylmaleate (DEM) (1.3 mg/kg) was injected i.p. in cottonseed oil. The controls were injected with cottonseed oil alone. After 30 min the animals were killed and their spleens and hearts were removed and assayed for total sulfhydryl, TCA soluble sulfhydryl, and ability to inhibit DBH (I_{50}). Sulfhydryl concentration is expressed as the molar concentration in a 1:25 organ homogenate. Ht = heart, Sp = spleen. Results are expressed as mean ± S.E.M. with n = 5 for experimental and control animals. *P < 0.5, **P < 0.005, ***P < 0.0005.

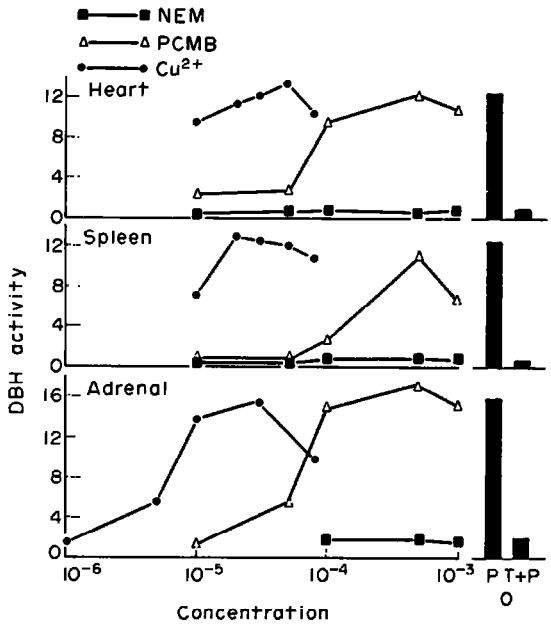

Fig. 8. Reversal of DBHI activity by Cu^{2+}. NEM and PCMB. Cu^{2+} (·●·), *N*-ethylmaleimide (NEM, ·■·) and *p*-chloromecuribenzoate (PCMB, Δ·) were added to a mixture of partially purified DBH plus an aliquot of a homogenate to produce final concentrations of between 10^{-6} and 10^{-3} M. Spleen (1:2000), heart (1:400) and adrenal (1:4000) homogenates were used. These dilutions were chosen so as to obtain preparations with only negligible amounts of DBH activity. Dithiothreitol was added to the *N*-methylation step to reduce the inhibition of PNMT by PCMB and NEM. The partially purified DBH (P) and tissue + partially purified enzyme (T + P) activities were obtained in the absence of PCMB, NEM or Cu^{2+}.

as low as 2.0 or as high as 10.0 did not affect the DBHI activity.

Effect of norepinephrine, ATP, AMP and adenosine on pure DBH. Since ATP and norepinephrine are found in high concentrations in adrenergic storage granules, the ability of norepinephrine, ATP, AMP and adenosine to inhibit DBH was examined. The concentration of norepinephrine in the spleens of rats used in these experiments was about 0.4 μg/gm. The concentration of norepinephrine in a 1:7000 dilution of rat spleen would therefore be about 3.4×10^{-10} M. Norepinephrine up to 10^{-5} did not inhibit partially purified DBH. The concentration of ATP is one-fourth that of norepinephrine in the adrenal medullary chromaffin granule [21]. The concentration of ATP and its degradation products should therefore be no greater than 8.5×10^{-11} M in a 1:7000 dilution of a spleen homogenate. Neither ATP, AMP nor adenosine at concentrations up to 10^{-4} M had any effect on DBH activity.

Table 2. Stability of rat organ DBHI's

Organ	Percentage original inhibitory activity remaining after 72 hr.
Heart	88.8 ± 4.3
Boiled heart	96.0 ± 2.6
Spleen	92.8 ± 2.4
Boiled spleen	9.4 ± 3.9
Adrenal	62.0 ± 1.8
Boiled adrenal	52.8 ± 8.6

Dilution curves for the indicated organs were performed on the same day that the animals were killed and again, 72 hr later, using homogenates which had been stored at −10°. The boiled samples were prepared and assayed on day 1 and were also stored at −10°. Results are expressed as per cent of original inhibitory activity remaining after 72 hr ± S.E.M. of 4 determinations. Per cent of original inhibitory activity is calculated by the following formula:

$$\left(\frac{I_{50} \text{ at 72 hr}}{I_{50} \text{ at day 0}}\right) 100.$$

DISCUSSION

It has been suggested that tyrosine hydroxylase (EC 1.14.3) is the rate-limiting step in the biosynthesis of catecholamines [22]. It is likely that tyrosine hydroxylase limits the maximum rate of catecholamine synthesis, but it is probably an oversimplification to suggest that tyrosine hydroxylase is the only regulated step in this biosynthetic pathway [2]. There are at least two potential regulatory mechanisms which may function *in vivo* to limit the expression of DBH activity. The first of these derives from the fact that DBH is contained within and bound to the membranes of adrenal chromaffin granules [23, 24] and adrenergic storage granules [25, 26]. Thus, access of dopamine to DBH may be restricted and may limit the expression of DBH activity. Endogenous inhibitors of DBH provide a second potential means of regulating DBH activity. These inhibitors have been described in several organs [1, 8] but little is known concerning their physiological role in terms of a possible contribution to the regulation of norepinephrine biosynthesis *in vivo*.

As assay for the endogenous inhibitors of DBH has been established. This assay is based on the ability of various dilutions of organ homogenates to inhibit the activity of a standard preparation of DBH. The DBH used in these experiments was obtained from bovine adrenal medullae and it had an activity of approximately 47 μmoles octopamine/hr/mg protein. This activity compares with an activity of 93–105 μmoles octopamine/hr/mg protein reported by Foldes *et al.* [11] for enzyme that was biochemically homogenous. It is important to point out that the assay depends on having a preparation of DBH which is free of endogenous inhibitors. It is not necessary, however, to have biochemically pure enzyme. The fact that Cu^{2+} does not lead to an increase in the activity of the DBH preparation used indicates that it is not contaminated with inhibitors (Fig. 1, inset).

The activity of endogenous inhibitors in various rat organs is extremely high. There is, for example, enough inhibitor present in a 1:8000 homogenate of rat spleen to inhibit partially purified bovine adrenal medullary DBH by approximately 50 per cent. The endogenous inhibitors derived from several organs differed markedly in terms of their apparent heat stability. We thus suggested that there are organ specific differences in DBHI [2]. Mixing experiments demonstrated, however, that there are constituents in heart homogenates which can confer heat lability on both the heart and spleen inhibitors. It is likely that this

effect is due to the presence of divalent cations perhaps in the form of metalloproteins. Divalent cations such as Fe^{2+}, Ca^{2+}, and Mg^{2+} confer heat lability on DBHI derived from rat spleen. Similar results were obtained with Fe^{2+} containing proteins such as cytochrome *c* and hemoglobin. The concentration of cytochrome *c* is approximately ten times higher in the heart than in the spleen [27]. The heart may also contain large amounts of other metalloproteins which are involved in oxidative phosphorylation. It is likely that this may explain the difference in the heat lability of the DBHL in heart as opposed to spleen homogenates.

The greater heat lability of the inhibitory activity in heart than in spleen homogenates does not, therefore, imply the existence of organ specific DBHI's. On the other hand, the inhibitor isolated from the bovine heart did not react with NEM [8] while that isolated from the bovine adrenal gland could be neutralized by NEM [1, 9]. This suggests that there may be organ specificity in the DBHI's in bovine tissue. The inhibitory activity of rat heart, spleen, and adrenal was uniformly insensitive to NEM while inhibition could be completely reversed by both Cu^{2+} and PCMB. Even though pharmacologic approaches did not demonstrate the existence of multiple DBHI's, several lines of evidence do suggest that there is more than one endogenous inhibitor present in the homogenates being studied. While most of the inhibitory activity in rat heart was destroyed by exposure to 95 for 5 min, there is a portion of the inhibitory activity which was not destroyed by heating for even 20 min. Furthermore, when homogenates of various rat organs were chromatographed on Sephadex G-200 at least four discrete peaks of inhibitory activity were seen. Two of the peaks are of high stokes radius and are heat labile, while two of lower apparent molecular weight are heat stable (Orcutt and Molinoff, unpublished).

Additional evidence with regard to the possible multiplicity of endogenous inhibitors comes from consideration of the effects of glutathione. Glutathione is present in the homogenates being studied and it is able to inhibit DBH. Depletion of glutathione with diethylmaleate did not, however, affect the total level of inhibitory activity in either rat heart or spleen. This suggests that glutathione contributes only a small fraction of the total inhibitory activity which is present in these homogenates.

These results do not rule out the possibility that the inhibitors are sulfhydryl compounds since most of the depletion in total sulfhydryl can be accounted for by the decrease in glutathione (TCA soluble sulfhydryl). Experiments have been carried out to test the ability of glutathione to inhibit partially purified DBH. Concentrations below 0.2 mM had no effect while 50 per cent inhibition was seen at 1 mM. The concentrations of reduced glutathione in 1:25 homogenates of rat heart and spleen were determined to be 73 μM and 110 μM respectively. This means that glutathione cannot be contributing significantly to the inhibitory activity which is observed even in concentrated homogenates. The lack of potency of glutathione stands in marked contrast to the more active endogenous inhibitors which are able to inhibit DBH after a dilution of several thousand-fold.

For endogenous inhibitors of DBH to have a significant physiological role they must have access to DBH which is contained within and bound to the membranes of adrenergic storage vesicles and adrenal chromaffin granules [23, 24, 25, 26]. The enormous amount of inhibitory activity which is present in the homogenates examined suggests that most of it is located in an extragranular pool since if all or even most of the total inhibitory activity had access to DBH, it would result in almost complete inhibition of the enzyme. On the other hand, if even a small percentage of the DBHI has access to DBH, then the inhibitors may be playing a very significant physiological role in regulating DBH activity and thus the synthesis of norepinephrine.

Attempts to document the presence of DBHI's in vesicles have not led to clear-cut results. The experiments are complicated by the large amount of inhibitory activity which is present in the homogenates and is apparently derived from non-neuronal tissues. Surgical sympathetic denervation or treatment with 6-hydroxydopamine [28] had no effect on the I_{50} of iris or heart, respectively. On the other hand, several lines of evidence suggest that the endogenous inhibitors of DBH do have access to the enzyme which is sequestered in adrenergic storage granules. When DBH activity is measured in perfusates of the isolated cat spleen [29], the perfused cat adrenal (Mosimann and Molinoff, unpublished), or in vesicle-rich fractions obtained from density gradients of homogenates of rat heart [26], exogenous Cu^{2+} must be added in order to obtain full expression of DBH activity. It is not possible to use the concentration of Cu^{2+} which is required to obtain maximal DBH activity as a quantitative measure of the amount of inhibitor present in a preparation since most of the Cu^{2+} is apparently bound nonspecifically. However, the fact that some Cu^{2+} is required implies that there is at least some inhibitor present. Experiments are now under way which are designed to explore the possibility that a small percentage of the total amount of DBHI does have access to DBH.

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