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Absence of a Relationship Between Sympathetic Neuronal Activity and Turnover of Serum Dopamine-β-Hydroxylase

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Summary. The effects of pharmacological alteration of adrenergic transmission on the rate of entrance of dopamine-β-hydroxylase (DBH) into the circulation were assessed in rats by an immunological method in which the kinetics of recovery of serum DBH activity were measured after depletion of the enzyme by treatment with anti-rat DBH antiserum. Neither α-receptor blockade with phenoxybenzamine nor ganglionic blockade with clorisondamine altered the rate by which DBH enters the bloodstream although both treatments markedly altered serum catecholamine levels. Prolonged treatment of newborn rats with guanethidine produced a severe peripheral sympathectomy but only a moderate decrease (30%) in serum DBH levels. In the sympathectomized rats, the rate of entrance of DBH into the circulation was significantly reduced whereas the half-life and rate of degradation of the enzyme was unchanged. These results indicate that the major portion of serum DBH does not enter the circulation by means of exocytotic release of the soluble enzyme.

Key words: Sympathetic neuronal activity – Noradrenaline – Dopamine-β-hydroxylase – Guanethidine – Exocytosis.

Introduction

Dopamine-β-hydroxylase (DBH) catalyzes the final step in the biosynthetic pathway for noradrenaline (NA) (Kaufman and Friedman, 1965). The enzyme is localized in noradrenergic neurons and in chromaffin cells where it exists in a soluble and membrane-bound form (Potter and Axelrod, 1963; Kirshner, 1957; Ross et al., 1972). DBH also occurs in the serum of several species including man (Weinshilboum and Axelrod, 1971; Goldstein et al., 1971). Experiments in vitro have provided compelling evidence that catecholamines and DBH are released from adrenergic tissue by an exocytotic process in which the soluble content of the storage vesicles is expelled into the extracellular space (Viveros et al., 1968; Gewirtz and Kopin, 1970; Weinshilboum et al., 1971). This observation has led to the hypothesis that serum DBH arises from the soluble enzyme in the storage vesicles and that therefore the steady-state levels of the enzyme in serum reflect the activity of sympathoadrenal medullary system (for reviews, see Geffen, 1974; Schanberg and Kirshner, 1976).

The possible relationship between serum DBH levels and sympathetic neuronal activity has been examined both clinically and experimentally. In a number of pathological states in which sympathetic neuronal activity may be altered – e.g., essential hypertension (Schanberg and Kirshner, 1976; Lamprecht et al., 1973), Huntington's chorea and Parkinson's Disease (Lieberman et al., 1972), Down's syndrome (Wetterberg et al., 1972), familial dysautonomia (Weinshilboum and Axelrod, 1971) and torsion dystonia (Wooten et al., 1973) – serum DBH activity has been found to deviate significantly from the norm. Although there have been reports that acute alterations in sympathetic neuronal activity in experimental animals cause a parallel change in the levels of serum DBH (Wooten et al., 1973; Weinshilboum et al., 1971), other have not been able to confirm these observations (Roffman et al., 1973; Ogihara and Nugent, 1974; Horwitz et al., 1973; Stone et al., 1974; Wetterberg et al., 1972). Recent studies have shown that other factors besides sympathetic neuronal activity play an important role in modulating the levels of the enzyme in serum. Weinshilboum et al. (1975) have demonstrated that genetic factors contribute to the 100-fold variation in serum DBH levels in humans. In examining the effects of cold pressor stress on serum DBH activity in

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human subjects, Stone et al. (1974) concluded that plasma volume changes are primarily responsible for acute alterations in serum DBH activity. Thus, the possible relationship between serum DBH activity and sympathetic neuronal activity remains unclear.

Like any serum protein, the steady-state level of DBH in serum reflects a balance between the rate of entrance of the enzyme into serum and its rate of degradation. To determine the contribution of exocytotic release from adrenergic tissues to serum DBH levels, one must demonstrate a correlation between sympathetic activity and the rate of entrance of DBH into the blood stream. We have devised an immunological method whereby the rate of entrance and of degradation of DBH in serum can be determined in vivo (Grzanna and Coyle, 1977). This method is based upon the observation that intravenous injection of anti-rat DBH antiserum into rats causes a rapid and profound reduction in the level of serum DBH without affecting the enzyme occluded within sympathetic neurons. This reduction in enzyme activity results from the formation of immune complexes of serum DBH molecules with the injected antibody, which are then rapidly cleared from the circulation. From the kinetics of the return of serum DBH activity after immune clearance of the circulating pool of enzyme, the rate of entrance of new DBH molecules and the rate of their degradation can be calculated. In this report, we have examined the effect of pharmacological alterations in sympathetic neuronal activity and of sympathectomy on the rate of entrance and turnover of DBH in the serum of rats.

Materials and Methods

Treatment of Animals. Male Sprague-Dawley rats weighing 175–225 g were housed four to a cage with food and water available ad libitum. Drugs, dissolved in 0.9% NaCl, were administered by intraperitoneal injection. To alter sympathetic neuronal activity, rats received chlorisondamine (2 mg/kg; 3 times per day for 11 days) or phenoxybenzamine (10 mg/kg; 3 times per day for 11 days). Drug treatments were initiated one day prior to the injection of antiserum. DBH antiserum was administered by i.v. injection; 2 µl of the anti DBH antiserum was diluted in 0.2 ml of 0.9 % NaCl. This dose caused an acute 75% reduction in the steady-state levels of DBH and represents a subsaturating amount of antiserum which does not result in antibody excess. For pharmacological sympathectomy, the method of Johnson et al. (1976) was utilized; rats received guanethidine, 50 mg/kg, 6 days per weak between 7 and 28 days of age; litter mate controls received injections of vehicle alone.

Collection of Blood Samples. To obtain blood for assays of serum DBH, animals lightly anesthetized with chloral hydrate were bled by cardiac puncture; no more than 0.4 ml was withdrawn at each bleeding. After centrifugation at 2,000 × g × 15 min at 4° C, the serum was removed and stored at −30° C for up to 5 weeks until assayed. For assays of plasma catecholamines, animals were decapitated and the first 1.5 ml of arterial blood was collected in heparinized tubes; blood was obtained 4 h after the last injection of drug. After centrifugation at 2,000 × g × 15 min at 4° C, the plasma was removed and deproteinated with perchloric acid (60%, vol/vol; 20 µl per 1 ml of plasma). The deproteinated plasma was stored at −30° C until assayed for catecholamines.

DBH Assay. DBH activity was measured by a modification of the method of Molinoff et al. (1971) with 1 mM tyramine as substrate at pH 5.2. Serum samples were diluted 1:4 with 5 mM Tris-HCl, pH 7.4. Tissues samples were homogenized in 5 mM Tris-HCl, pH 7.4 containing 0.2% (vol/vol) Triton X-100; adrenal glands were homogenized in 400 volumes whereas all other tissues were homogenized in 40 volumes of buffer. The optimal concentration of Cu^{2+}, which inactivates endogenous inhibitors of the enzyme, was determined in separate assays for each tissue. One unit of DBH activity equals 1 nanomole of octopamine formed per h.

Assay of β-Hydroxylated Catecholamines. For determination of tissue catecholamines, organs were homogenized in 0.1 N perchloric acid; except for adrenal glands which were homogenized in 1:1,000 dilution, all other tissues were homogenized in 50 vol. Homogenates were centrifuged at 2,000 × g × 10 min to remove protein. β-Hydroxylated catecholamines (in the following text referred to as catecholamines) in plasma and tissue extracts were assayed by the method of Coyle and Henry (1973) as modified by Weise and Kopin (1976).

Antiserum to Rat DBH. Dopamine-β-hydroxylase was purified to homogeneity from rat adrenal medulla as previously described (Grzanna and Coyle, 1976). Antiserum was raised in guinea pigs; and immunoglobin fraction was separated from whole guinea pig serum by ammonium sulfate precipitation and redissolved in 0.025 M Na_2HPO_4 − 0.15 M NaCl, pH 7.3 to yield a final protein concentration of 33 mg/ml. Protein was measured by the method of Lowry et al. (1951).

Statistical Evaluation. Significance of the difference between two means was measured using a two-tailed *t*-test. Kinetic analysis of the recovery of serum DBH activity after immune inactivation was performed by the method of Swick et al. (1968) as previously described.

Drugs. Guanethidine sulfate was purchased from Regis Chemical Co.; chlorisondamine was the gift of Ciba Pharmaceutical Co. (Summit, NJ) and phenoxybenzamine was a gift of Smith, Kline and French Labs. (Phila., PA).

Results

Effect of Ganglionic Blockade on Plasma Catecholamines and Recovery of Serum DBH After Treatment with DBH Antiserum.

To investigate the effects of prolonged sympathetic blockade on the recovery of serum DBH activity after administration of anti-DBH antiserum, rats were treated with chlorisondamine, a ganglionic blocking agent. The rats were divided into three groups: those receiving antiserum alone, those receiving chlorisondamine alone and those receiving antiserum and chlorisondamine. Blood samples for plasma catecholamines and DBH activity were obtained on days 1, 5, and 11 after initiation of treatment. As shown in Fig. 1, treatment with chlorisondamine resulted in a significant 3-fold reduction in the levels of plasma catecholamines throughout the 11 day period of the

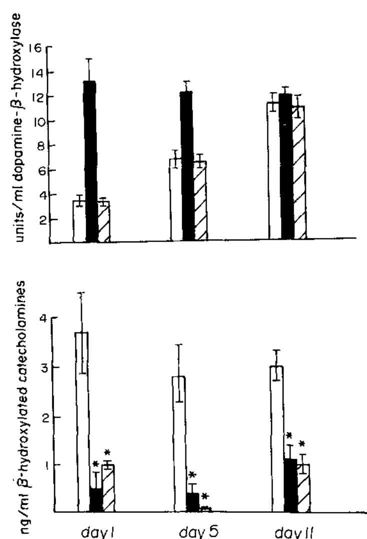

Fig. 1. Effect of prolonged treatment with chlorisondamine on the rate of recovery of serum DBH after antiserum injection and on plasma catecholamines. One group of rats received 2 µl of antiserum (open bars), a second group received chlorisondamine (black bars) and a third group received 2 µl of antiserum plus chlorisondamine (striped bars); S.E.M. is indicated by brackets with $N = 4$ for each treatment group. * $P < 0.01$ vs. drug-free

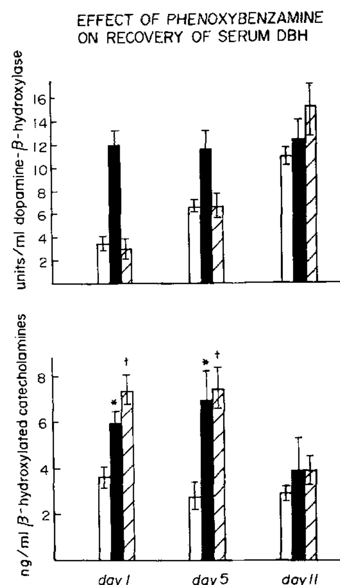

Fig. 2. Effect of prolonged treatment with phenoxybenzamine on the rate of recovery of serum DBH after antiserum injection and on plasma catecholamines. One group of rats received 2 µl of antiserum (open bars), a second group received phenoxybenzamine (black bars), and a third group received 2 µl of antiserum plus phenoxybenzamine (striped bars); S.E.M. indicated by brackets with $N = 4$ in each treatment group. * $P < 0.05$ vs. drug-free; $^{+}P < 0.01$ vs. drug-free

experiment, indicating that ganglionic blockade effectively reduced sympathoadrenal medullary release of catecholamines. Nevertheless, the recovery of serum DBH activity measured at three time points after depletion by antiserum treatment was essentially identical in the chlorisondamine and drug-free rats. Thus, in spite of a severe reduction in sympathetic tone, the turnover of serum DBH was not perceptibly altered.

Effect of Receptor Blockade on Plasma Catecholamines and Recovery of Serum DBH After Treatment with DBH Antiserum. Treatment with phenoxybenzamine, an α-adrenergic receptor blocker, causes a marked increase in sympathetic neuronal activity. To determine the effects of increased sympathetic activity on the rate of recovery of serum DBH after treatment with DBH antiserum, rats were chronically administered phenoxybenzamine. Animals were divided into three groups: those receiving 2 µl of antiserum alone, those treated with phenoxybenzamine alone and those receiving 2 µl of antiserum plus phenoxybenzamine. Blood was withdrawn on days 1, 5 and 11 after injection of antiserum. As shown in Fig. 2, the plasma levels of catecholamines were increased approximately 2-fold on days 1 and 5; they were not, however, significantly increased on day 11 in spite of the fact that the rats were severely symptomatic from treatment. This may reflect an exhaustion of catecholamine stores with chronic administration of the drug. In spite of the increased sympathetic tone, the recovery of serum DBH activity after antiserum injection did not differ significantly at any time point between the phenoxybenzamine-treated and drug-free rats.

Table 1. Effect of chronic guanethidine treatment on tissue DBH

	Control	Treated	% Control
Hearts (units/g)	184 ± 23	9.4 ± 4.4	5*
Pancreas (units/g)	79 ± 17	18 ± 5.0	23*
Spleen (units/g)	200 ± 30	6.1 ± 4.2	3*
Salivary (unit/g)	206 ± 25	83 ± 3.1	40*
Adrenal (unit/gland)	53 ± 5.1	58 ± 5.0	109

Results represent the means ± S.E.M. of 5 controls and 5 treated animals; * $P < 0.01$

Effects of Guanethidine-Induced Sympathectomy on Tissue DBH Activity and Catecholamine Levels and the Kinetics of Serum DBH Turnover. In adult rats which received chronic guanethidine treatment during the postnatal period, DBH activity was reduced by 80–95% in the heart, pancreas and spleen and by 60% in the salivary gland as compared to litter mate controls (Table 1). Similarly, catecholamine levels were depressed in these tissues by 75% or more (Table 2). In

Table 2. Effect of chronic guanethidine treatment on levels of β-hydroxylated catecholamines in tissues

	Control	Treated	% Control
Heart (ng/g)	410 ± 110	50 ± 40	12*
Pancreas (ng/g)	950 ± 110	270 ± 70	28*
Spleen (ng/g)	1140 ± 220	110 ± 40	10*
Salivary (ng/g)	1020 ± 130	240 ± 70	24*
Adrenal (µg/g)	579 ± 77	710 ± 34	123

Results represent the mean ± S.E.M. of 4 control and 4 treated animals; * $P < 0.01$

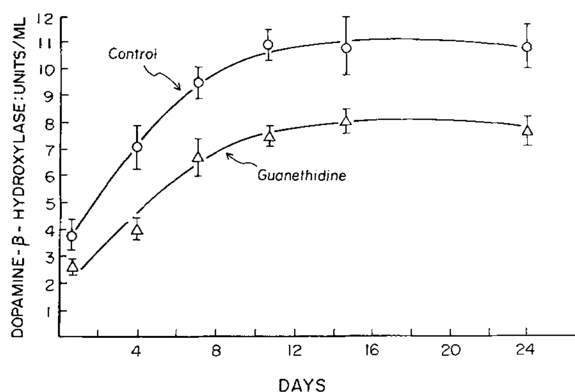

Fig. 3. Time course for recovery of serum DBH activity after antiserum injection in control and guanethidine sympathectomized rats 8 weeks after birth. 0 = control rats; each time point represents the mean of 6 animals. Δ = guanethidine treated rats; each time point represents the mean of 12 animals. S.E.M. are indicated by brackets

contrast, DBH activity and catecholamine levels were not significantly altered in the adrenal gland. Thus, the guanethidine treatment produced a severe sympathectomy that spared the adrenal medulla. Nevertheless, serum DBH activity was only slightly but significantly ($P < 0.05$) reduced by 30% in the guanethidine treated animals; however, total serum proteins were also reduced by 16% in the guanethidine treated rats (control 70 ± 3 mg/ml; treated 60 ± 2; $n = 20$; $P < 0.01$).

The effect of guanethidine-induced sympathectomy on the kinetics of serum DBH turnover was examined. Blood was obtained for measurement of serum DBH activity at various time intervals after injection of 2 µl of DBH antiserum. The rates of recovery of serum DBH activity as shown in Fig. 3 are quite similar for both the guanethidine and litter mate controls. The kinetics of serum DBH turnover for both groups were calculated by the method of Swick et al. (1968) (Table 3). The half-life did not differ between the two groups and the rate of degradation was only slightly reduced in the sympathectomized rats. However, the rate of entrance of DBH into serum in the sympathectomized animals was

Table 3. Turnover of serum DBH in guanethidine-treated and littermate controls

	Control	Treated	% Control
C_N	13.5 ± 0.9	9.5 ± 0.6	70*
k_D	0.167 ± 0.019	0.141 ± 0.012	84
k_S	2.19 ± 0.11	1.32 ± 0.12	60**
$t_{1/2}$	4.2 ± 0.5	4.9 ± 0.4	117

Data was calculated from a semilogarithmic plot of the equation $C_t = C_N(1 - e^{-K_D t})$ according to the method of Swick et al. C_t is the serum DBH activity at any time t (in days) after injection of 2 µl of anti-DBH antiserum; C_N is the enzyme activity after recovery. k_D is the first-order rate constant for the degradation of serum DBH expressed per day; k_S is the rate constant for the entrance of DBH into the circulation expressed in units/ml/day; $t_{1/2}$ is the half-life of serum DBH in days. N = 6 for controls, 12 for treated; * $P < 0.05$; ** $P < 0.01$

significantly decreased by 40%. Thus, the reduction in steady-state levels of serum DBH in the guanethidine sympathectomized rats reflected primarily a reduced rate of entry of DBH into serum.

Discussion

In this study we have examined the rate of return of serum DBH activity after depletion by injection of an homologous antiserum against DBH. If serum DBH is primarily derived from adrenergic tissue by exocytotic release, its rate of recovery after antiserum treatment should be altered by pharmacological manipulations that chronically increase or depress sympathetic neuronal activity. Two drugs were utilized to alter sympathoadrenal medullary release of catecholamines: chlorisondamine, a nicotinic ganglionic receptor blocker, and phenoxybenzamine, an α-receptor blocker. The efficacy of these pharmacological treatments on sympathetic tone has been confirmed by the marked alterations in plasma catecholamine levels in arterial samples obtained from decapitated rats. In light of the recent report of Popper et al. (1977) demonstrating lower concentrations of catecholamines in samples obtained from an in-dwelling arterial catheter it must be emphasized that the control plasma catecholamine values in our study are higher than actual levels although relative differences among treatment groups should be valid.

Although plasma catecholamine were significantly elevated in the phenoxybenzamine treated rats and significantly depressed in the chlorisondamine-treated rats, the recovery of DBH activity after injection of antiserum was comparable to control in the drug-treated rats. More importantly, in spite of a ten-fold difference in plasma catecholamines levels with the two pharmacological treatments, there were no significant

differences between the two groups in serum DBH activity at any time point after clearance of the enzyme by antiserum injection. Thus, marked alterations in sympathoadrenal medullary activity did not significantly affect the rate of entrance of DBH into serum that is detectable with the modest variance of the present study. These results are compatible with the earlier findings of Reid and Kopin (1975) who could not detect any significant change in the steady-state levels of serum DBH after prolonged treatment of rats with chlorisondamine or phenoxybenzamine.

The absence of a correlation between sympathetic tone and recovery of serum DBH activity after antiserum depletion raises the question whether serum DBH is derived primarily from sympathetic tissues. To address this issue, we examined the effects of sympathectomy with guanethidine on the kinetics of serum DBH recovery. In accord with the results of Johnson et al. (1976), chronic treatment of rats with guanethidine between 7 and 28 days after birth caused a profound reduction in the activity of DBH and the levels of catecholamines in several organs receiving sympathetic innervation. In the guanethidine-treated rats, the adult levels of serum DBH activity were moderately but significantly decreased as previously reported by Grobecker et al. (1977). Kinetic analysis of serum DBH turnover indicated that the lower steady-state levels resulted from a 40% decrease in the rate of entrance of new DBH into the circulation; the rate of enzyme degradation, on the other hand, was not significantly affected. Thus, partial destruction of the sympathetic tissue results in a slower release of new DBH into serum, which is comparable with the concept that serum DBH is derived from sympathetic neurons. Nevertheless, the reduction in the steady-state levels and the rate of entrance of DBH into serum after guanethidine-induced sympathectomy was modest in comparison to the loss of peripheral sympathetic markers. It is conceivable that the extent of sympathectomy is over-estimated by taking spleen, heart and pancreas as the norm for the integrity of the sympathetic nervous system. In addition, DBH may also be released by cell types containing the enzyme but insensitive to guanethidine such as the adrenal medulla, small intensely fluorescent cells that proliferate with guanethidine treatment (Rybarczyk et al., 1976; Eranko and Eranko, 1971) and certain parasympathetic ganglia (Grzanna and Coyle, 1978).

The fact that exocytotic release of DBH has been demonstrated in vitro does not contradict our conclusion that exocytotic release is not a major contributor to serum DBH. In rat, only 2–20% of the total DBH activity in sympathetics and in the adrenal medulla is soluble (DePotter et al., 1970; Brimijoin, 1974); thus, the amount of soluble DBH available for exocytotic release constitutes a minor fraction of the total enzyme. Little is known about the intra-neuronal degradation of membrane-bound DBH; but, according to Brimijoin and Helland (1976), only 20% of the enzyme is transported in a retrograde fashion back to the sympathetic ganglia. Conceivably, the remaining membrane bound DBH may be a source of serum DBH. In recent studies of the fate of radiolabeled surface components of the lymphocyte, Marchalonis (1977) has shown that membrane proteins are released from the cell surface by a process which may be related to the turnover of membrane constituents. Hines and Garwood (1977) have observed the release of proteins from peripheral nerve axons; these neuronal proteins, when subjected to disc-gel electrophoresis, correspond in mobility to proteins normally present in plasma. It is noteworthy that in the experiments demonstrating the exocytotic release of DBH in vitro considerable amounts of the enzyme entered the perfusion bath in the absence of electrically induced neuronal activity (Weinshilboum et al., 1971). Accordingly, we speculate that the continuous "shedding" of membrane bound DBH, perhaps as a method of catabolism of vesicular components, is the primary source of DBH entering the serum. This hypothesis explains both the relationships between the integrity of the sympathetic nervous system and the rate of entrance of DBH into serum and the lack of a correlation between sympathetic neuronal activity and serum DBH turnover.

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