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Immunochemical Studies on the Turnover of Rat Serum Dopamine β-Hydroxylase

R. GRZANNA[1] AND J. T. COYLE

Departments of Pharmacology and Experimental Therapeutics and of Psychiatry and the Behavioral Sciences, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

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SUMMARY

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Intravenous injection of homologous antiserum to rat dopamine β-hydroxylase causes a rapid, dose-dependent reduction in the activity of the enzyme. The enzyme-neutralizing effect of the antiserum is 1000 times greater *in vivo* than *in vitro*. The injected antiserum does not affect dopamine β-hydroxylase activity in peripheral adrenergic tissues. The kinetics of the rate of reappearance of dopamine β-hydroxylase activity in serum after treatment with the antiserum have been calculated. The rate constant for the entrance of enzyme into the circulation is 2.8 units/ml/day, the first-order rate constant for the degradation of the serum enzyme is 0.166 day^{-1}, and the half-life of serum dopamine β-hydroxylase in the rat is 4.2 days.

INTRODUCTION

Dopamine β-hydroxylase, the enzyme that catalyzes the conversion of dopamine to norepinephrine, is present in the serum of several species (1, 2). Studies *in vitro* have demonstrated that the enzyme is released along with catecholamines from adrenergic tissues by a stimulus-secretion coupled process of exocytosis (3-5). Accordingly, it has been postulated that *in vivo* dopamine β-hydroxylase ultimately enters the serum as a result of exocytotic release from sympathetic neurons (1, 6). Experiments showing that severe stress increases (7, 8) and acute chemical sympathectomy with 6-hydroxydopamine reduces (9) dopamine β-hydroxylase activity in rat serum lend support to this hypothesis. Consequently it has been suggested that serum dopamine β-hydroxylase might provide a convenient index of sympathetic neuronal activity (1, 10-12). The levels of serum enzyme have been examined in a number of disease states, such as hypertension (12, 13), Huntington's chorea and Parkinson's disease (14), Down's syndrome (15), familial dysautonomia (16), torsion dystonia (17), and schizophrenia and affective disorders (18).

In several recent studies, however, the influence of sympathetic neuronal activity on serum dopamine β-hydroxylase levels has been called into question (19-23). For example, Stone *et al.* (21) studied the effects of a cold pressor test on plasma dopamine β-hydroxylase in humans and found increases in some test subjects and declines in others. In humans, serum dopamine β-hydroxylase activity varies over a 1000-fold range, and Weinshilboum *et al.* (24) have recently demonstrated that genetic factors play a major role in determin-

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ing the levels of serum enzyme activity in humans. Reid and Kopin (25) have shown that marked alterations in sympathetic neuronal activity produced by pharmacological agents have little effect on the steady-state levels of serum dopamine β-hydroxylase in rats.

Although studies *in vitro* have demonstrated that dopamine β-hydroxylase is released from adrenergic tissue by an exocytotic process, other factors *in vivo* may play a much more important role in determining steady-state levels of the serum enzyme. Among these factors, the rates of entrance and degradation of the enzyme in serum are particularly important. Alterations in serum dopamine β-hydroxylase levels after experimental manipulations or in disease states will be interpretable only if it is known whether the change in steady-state levels is due to a change in the rate of entrance and/or the rate of degradation of the enzyme in the circulation. Such information can only be obtained by determining the turnover of the enzyme in the serum. In this report we demonstrate that intravenous injection of homologous antiserum to dopamine β-hydroxylase provides a method for specifically characterizing the turnover of the enzyme in rat serum. Using this immunochemical approach, the rates of entrance and degradation of serum dopamine β-hydroxylase have been calculated.

METHODS

Tissues. Sprague-Dawley rats (200 g) were obtained from a commercial supplier and were housed in our animal room for at least 1 week prior to experiments to preclude acute effects of the stress of transportation. To obtain blood, the animals were placed in a tubular Lucite rat holder, and 0.4 ml of blood was withdrawn from tail vein with a heparinized syringe. Antiserum was administered by injection in the tail vein, whereas drugs were administered by an intraperitoneal route.

Enzyme assay. The activity of dopamine β-hydroxylase was measured by a modification of the method of Molinoff *et al*. (26) with 1 mM tyramine as substrate at pH 5.2. Plasma samples were diluted 1:4 with 5 mM Tris-HCl buffer, pH 7.4, and tissue samples were homogenized in 40 volumes (w/v) of 5 mM Tris-HCl containing 0.2% Triton X-100 (v/v). For each experimental condition, samples were assayed at different concentrations of cupric ion, which inactivates endogenous inhibitors, to achieve optimal activity. One unit of dopamine β-hydroxylase activity equals 1 nmole of octopamine formed per hour.

Antiserum to rat dopamine β-hydroxylase. Rat dopamine β-hydroxylase was purified to homogeneity from adrenal medulla as previously described (27). Adult male guinea pigs received an initial injection of 60–80 μg of dopamine β-hydroxylase protein in complete Freund's adjuvant and thereafter were boosted once a month with 30 μg of the enzyme protein in incomplete Freund's adjuvant. The immunoglobulin fraction was separated from whole guinea pig serum by ammonium sulfate fractionation and was dissolved in 0.025 M Na_2HPO_4–0.15 M NaCl, pH 7.3, to yield a final protein concentration of 33 mg/ml. Ouchterlony microdiffusion studies with serially diluted antiserum to rat dopamine β-hydroxylase revealed a single precipitant band with 5 μl of antiserum diluted up to 1:32 when run against a concentrated extract of rat adrenal medulla. Complement fixation assays with guinea pig anti-rat dopamine β-hydroxylase serum run against 1.25 μg of purified rat enzyme indicated complete fixation of complement at dilutions of antiserum up to 1:200,000. Immunoelectrophoresis of the antiserum run against rat serum revealed a single precipitant band.

Enzyme neutralization assay. To 1 ml of rat serum, various amounts of dopamine β-hydroxylase antiserum were added. The mixtures were incubated for 30 min at 37° and then for 18 hr at 4°. After centrifugation at 2000 × *g* for 30 min at 5°, 50-μl portions were assayed for dopamine β-hydroxylase activity at several different concentrations of cupric ion.

Serum catecholamines. Total β-hydroxylated catecholamines in serum were assayed by the method of Coyle and Henry (28) as modified by Weise and Kopin (29).

Protein. Protein was determined by the

method of Lowry *et al.* (30), with bovine serum albumin as standard.

Materials. Goat antiserum to guinea globulin fraction was obtained from Cappel Laboratories. Micro-Ouchterlony plates were purchased from Hyland Laboratories.

RESULTS

Effects of anti-rat dopamine β-hydroxylase serum in vitro and in vivo. When rat serum was incubated in the presence of various amounts of antiserum against rat dopamine β-hydroxylase, dose-dependent inhibition of enzyme activity was observed (Fig. 1). *In vitro*, 5 μl of antiserum produced 50% inhibition of enzyme activity in 1 ml of serum. Corresponding amounts of preimmune guinea pig serum did not significantly inhibit rat serum dopamine β-hydroxylase. For experiments *in vivo*, 0.1–200 μl of antiserum were injected intravenously into rats; blood samples were drawn immediately before and 2 hr after the injection. The antiserum exhibited potent inhibitory effects on serum enzyme activity, with as little as 0.1 μl causing a 44% reduction in activity. The injection of as much as 1 ml of preimmune guinea pig serum had no effect on serum enzyme activity. Assuming a plasma volume of 20 ml with a mean activity of 17 units/ml, the total activity of dopamine β-hydroxylase in the serum of a 200 g rat would be 340 units. Hence 0.1 μl of antiserum injected *in vivo* inactivated 170 units whereas 5 μl of antiserum incubated *in vitro* inactivated 8.5 units of enzyme activity. Thus the enzyme-neutralizing effect of the antiserum was 1000 times higher *in vivo* than *in vitro*.

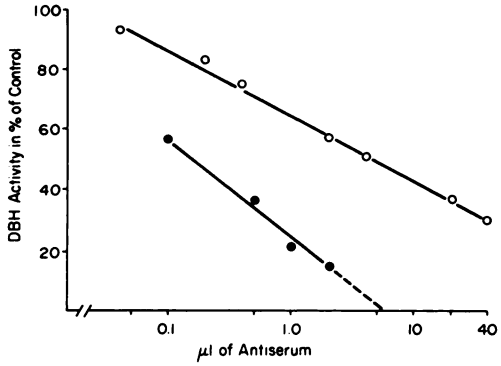

FIG. 1. *Inhibitory effects of dopamine β-hydroxylase antiserum in vitro and in vivo*

For the experiments *in vitro* (○), 1 ml of rat serum was incubated with various amounts of antiserum as described in METHODS, and the dopamine β-hydroxylase (DBH) activity remaining in the serum after centrifugation was measured in 50-μl aliquots. For the experiments *in vivo* (●), various amounts (0.1–2 μl) of antiserum were injected intravenously; 2 hr after treatment, the activity of serum dopamine β-hydroxylase was measured. Results are expressed as a percentage of the pretreatment specific activity of the serum enzyme, which was 17 units/ml. Each point is the mean of four preparations assayed in duplicate.

Time course of effect of antiserum in vivo. In experiments to determine how rapidly serum dopamine β-hydroxylase is inactivated *in vivo*, blood was withdrawn at various times after the injection of 2 μl of antiserum. Serum enzyme activity was reduced by 65% 1 min after injection and reached its nadir 9 min later (Fig. 2). Following the rapid fall after injection of antiserum, the enzyme activity gradually recovered to preinjection levels over several days. However, after injection of large amounts of antiserum (20 or 200 μl), the levels of serum dopamine β-hydroxylase activity remained depressed at about 10% of control for several days and then exhibited a recovery which paralleled that occurring with lower doses of antiserum (Fig. 3).

Role of circulating antibodies during recovery phase. It is possible that the sustained reduction in serum dopamine β-hydroxylase activity after injection of the antiserum was due to a direct inhibitory action of the antiserum remaining in the circulation. To evaluate this possibility, we examined the serum of rats treated with antiserum for inhibitory effects (Table 1). Twenty-four hours after the injection of the antibody, the serum enzyme activity was reduced to 0.8 unit/ml. When the serum from the treated rats was mixed with that of control rats, the dopamine β-hydroxylase activity of the combined sera was midway between the activity of the control and treated sera. Thus the gradual recovery of serum enzyme activity after a 2-μl injection of antiserum was not related

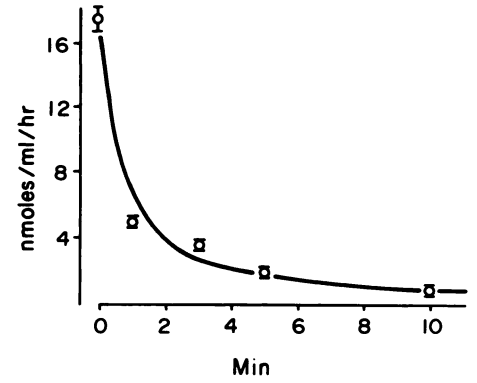

FIG. 2. *Time course for reduction in serum dopamine β-hydroxylase activity after injection of antiserum*

Rats received an intravenous injection of 2 μl of antiserum, and blood was drawn at several intervals afterward. Each point is the mean ± standard error for three rats.

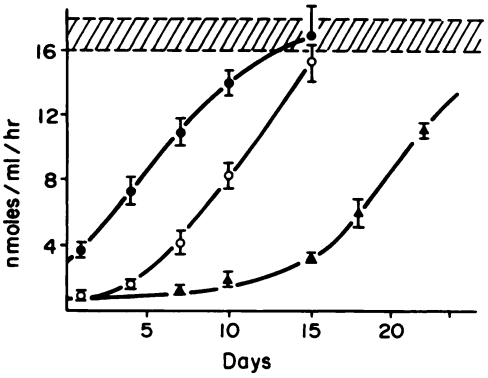

FIG. 3. *Time course for recovery of serum dopamine β-hydroxylase activity after intravenous injection of antiserum* (●, *2 μl*; ○, *20 μl*; ▲, *200 μl*)

Blood was withdrawn for serum dopamine β-hydroxylase assay at various times after injection. Each point represents the mean ± standard error for four rats. The hatched area represents one standard error above and below the mean for the activity of the serum enzyme prior to treatment.

to a residual inhibitory effect of dopamine β-hydroxylase antibodies in the circulation.

Since the dose-response curve *in vivo* indicated that 6 μl of antiserum should inactivate all the circulating dopamine β-hydroxylase, we hypothesized that the prolonged depression of enzyme activity occurring after injections of 20 and 200 μl reflects circulating antibodies that are free to complex with newly released enzyme as it enters the serum. To test this hypothesis, 500 μl of antiserum against guinea pig IgG (goat anti-guinea pig IgG) were injected 1 hr after the injection of 200 μl of the dopamine β-hydroxylase antiserum. This treatment abolished the prolonged plateau phase, and the recovery of serum dopamine β-hydroxylase activity exhibited the same slope as that observed after injection of 2 μl of antiserum (Fig. 4). Therefore the progressive recovery of serum dopamine β-hydroxylase activity after 2 μl of antiserum was not due to an excess of circulating dopamine β-hydroxylase antibodies.

Effect of antiserum treatment on tissue dopamine β-hydroxylase activity. Since it has been reported that heterologous antibodies to dopamine β-hydroxylase are taken up into sympathetic neurons (31), we evaluated the possibility that the gradual reappearance of serum enzyme activity after treatment with antiserum might be due to inactivation of the enzyme at intracellular sites. Accordingly, dopamine β-hydroxylase activity in extracts of several organs was examined before and 1, 5, and 13 days after the injection of 2 μl of antiserum. As shown in Table 2, the anti-

TABLE 1

Effect of serum from dopamine β-hydroxylase antiserum-treated rats on serum dopamine β-hydroxylase activity

Rats received an intravenous injection of 2 μl of dopamine β-hydroxylase antiserum (B); 24 hr after treatment, the rats were bled by the tail vein and their serum dopamine β-hydroxylase activity was determined. Serum from control rats was also assayed (A). Equal portions of serum from control (A) and treated (B) rats were mixed, incubated at 5° for 2 hr, and centrifuged at 12,000 × *g*, and the dopamine β-hydroxylase activity of the mixture was measured (A + B). The expected activity of the mixture equals the activity of both components divided by 2.

Rats	Activity
	units/ml
Control (A)	12.9 ± 0.1
Antiserum-treated (B)	0.8 ± 0.0
Combined (A + B)	6.7 ± 0.1
Expected [(A + B)/2]	6.8 ± 0.1

serum had no significant effects on enzyme activity in the various tissues examined. Also, the concentration of β-hydroxylated catecholamines in plasma 5 days after treatment with 2 μl of antiserum (4.5 ± 0.2 ng/ml) did not differ from preinjection levels (5.3 ± 0.6 ng/ml; $N = 6$); thus peripheral sympathetic function was not affected by the antiserum. Since these experiments demonstrate that the gradual reappearance of dopamine β-hydroxylase activity in serum is due neither to the continued presence of antibody in serum nor to inhibition of the enzyme in tissue, it can be concluded that the gradual return in enzyme activity reflects the entrance of new enzyme molecules into the circulation.

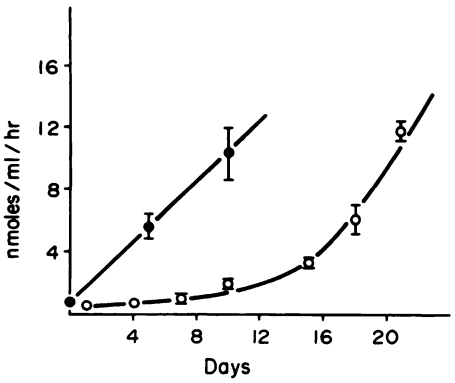

FIG. 4. *Reversal of prolonged inhibitory effect of 200 μl of antiserum by anti-guinea pig IgG*

Two hours after injection of 200 μl of dopamine β-hydroxylase antiserum, 500 μl of goat anti-guinea pig IgG were administered; this represents the equivalence point for optimal precipitation of guinea pig IgG by goat anti-guinea pig IgG. Blood was drawn at several intervals after treatment for determination of serum dopamine β-hydroxylase activity. ○, 200 μl of antiserum; ●, 200 μl of antiserum and 500 μl of goat anti-guinea pig IgG.

Kinetics of rate of reappearance of serum dopamine β-hydroxylase activity. Upon reaching a minimum level of activity 10 min after injection of 2 μl of antiserum, dopamine β-hydroxylase activity in the serum returned to pretreatment levels over a 15-day period. The rate of return, i.e., the entrance of new enzyme molecules into the circulation, was calculated by making the following assumptions: (a) the enzyme enters the circulation at a constant rate, and (b) a constant fraction of the active enzyme in the circulation is being degraded per unit of time. With these assumptions the reappearance of the enzyme activity can be described by the following general equation:

$$C_t = C_N (1 - e^{-k_D t})$$

in which C_t is the enzyme activity at any time t (in days) after the injection of 2 μl of antiserum, C_N is the enzyme activity in the serum before the injection of antiserum, and k_D is the first-order rate constant for the degradation of serum enzyme. This equation can be transformed into

$$\log(C_N - C_t) = -\frac{k_D}{2.303} \cdot t + \log C_N$$

By plotting the difference between $C_N - C_t$

TABLE 2

Effects of dopamine β-hydroxylase antiserum on tissue dopamine β-hydroxylase activity

Rats were treated with 2 μl of dopamine β-hydroxylase antiserum and killed 1, 5, and 13 days later for determination of dopamine β-hydroxylase activity in organs and serum. Results are the means ± standard errors of eight control and four or five treated rats.

Organ	Control activity	Experimental activity		
		Day 1	Day 5	Day 13
	units		*% control*	
Salivary gland	262 ± 6/g	92 ± 10	103 ± 8	100 ± 8
Heart	209 ± 9/g	111 ± 9	101 ± 5	104 ± 11
Pancreas	73 ± 5/g	120 ± 11	104 ± 11	101 ± 15
Spleen	220 ± 9/g	77 ± 10	97 ± 7	99 ± 8
Adrenal gland	45 ± 4/gland	126 ± 13	107 ± 12	109 ± 11
Serum	17 ± 1/ml	14 ± 1[a]	47 ± 7[a]	92 ± 9

[a] $P < 0.001$ compared with control.

on a semilogarithmic graph against t (31), a straight line was obtained (Fig. 5). The slope of this line equals $k_D/2.303$, or 0.166 day^{-1}. At normal dopamine β-hydroxylase levels, C_N, the amount of enzyme entering the serum per unit of time equals the amount being destroyed. Hence, with k_D known, k_s, the rate constant for the entrance of new enzyme molecules into the circulation, can be calculated from the equation

$$k_s = k_D \cdot C_N$$

When $k_D = 0.166$ day^{-1}, $k_s = 2.82$ units/ml/day. The biological half-life of serum dopamine β-hydroxylase either can be derived from Fig. 5 directly or can be calculated using the equation

$$t_{1/2} = \frac{\ln 2}{k_D}$$

By both methods, the half-life for serum dopamine β-hydroxylase is 4.2 days.

DISCUSSION

Antibodies have been produced against a number of enzymes; in some cases enzymatic activity is inhibited by the antibody while in other cases it is not (for review, see refs. 33–35). Some enzymes have been found to be active when combined with specific antibody even after precipitation

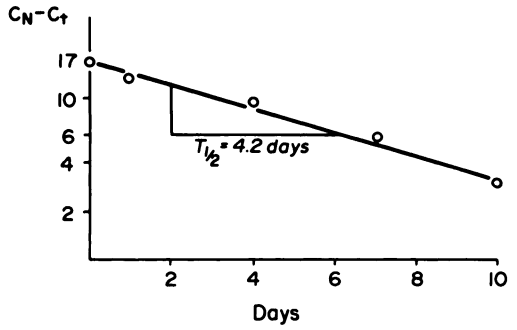

FIG. 5. *Half-life of serum dopamine β-hydroxylase*

Data from Fig. 3 for the recovery of serum enzyme activity after treatment with 2 μl of antiserum are plotted as C_N-C_t vs. time (see the text) on a semilogarithmic scale according to the method of Swick *et al.* (32). K_D is the first-order rate constant for serum enzyme degradation and equals 0.166 day^{-1}.

of the immune complex. The extent of diminution of enzyme activity by a specific antibody *in vitro* is dependent upon the ratio of antibody to enzyme and is maximal in a reaction mixture containing a large excess of antibody (36). This is consistent with the inhibitory effects of homologous dopamine β-hydroxylase antiserum when incubated with rat serum *in vitro*. In contrast, enzyme-antibody complexes which are formed *in vivo* are rapidly removed from the circulation by the reticuloendothelial system (37). This process corresponds to the "immune phase," when formation of specific antibodies begins 1 week after the injection of heterologous proteins in a host (see p. 548 of ref. 38). This removal of enzyme-antibody complex from the circulation accounts for the 1000-fold disparity between the enzyme-neutralizing effects of dopamine β-hydroxylase antiserum *in vivo* and *in vitro* (Fig. 1).

The rapidity with which the dopamine β-hydroxylase–antibody complex is removed from the circulation is striking. The half-time of this process is 45 sec, which is comparable to the rate of removal of injected particulate material such as colloidal chromic phosphate (39) by the reticuloendothelial system. Francis *et al.* (40) have shown that 90% of an injected protein is eliminated from the blood of rabbits immune to this protein by a "fast reaction" having a half-life of approximately 10 min. Thus we can conclude that intravenous administration of dopamine β-hydroxylase antiserum leads to the rapid removal of the enzyme from the circulation.

The dose-response curve for dopamine β-hydroxylase antiserum injected *in vivo* demonstrates an acute reduction of serum enzyme activity, proportional to the amount of antiserum injected up to 6 μl. Doses of antiserum in excess of this amount result in a prolonged reduction of serum enzyme levels, which can be abolished by treatment with an antiserum to the dopamine β-hydroxylase antibody, and the rate of recovery is identical with that occurring after injection of 2 μl of antiserum. These experiments suggest

that with injection of "submaximal" amounts of antiserum, the enzyme-antibody complexes are rapidly cleared and no specific antibody remains in the circulation. Mixing experiments confirmed the absence of a circulatory inhibitor in rat serum 1 day after the injection of 2 μl of dopamine β-hydroxylase antiserum. The gradual return of serum enzyme activity was also not due to a direct effect of the antibody on the tissues stores of the enzyme, since tissue dopamine β-hydroxylase and serum catecholamine levels remained unaltered from 1 to 13 days after injection of 2 μl of dopamine β-hydroxylase antiserum. Thus the gradual recovery of serum enzyme activity after injection of 2 μl of the antiserum must be due to the entrance of enzyme molecules into the circulation.

Several techniques have been developed to study the turnover of enzymes *in vivo*. These include the rate of disappearance of radioactive enzyme molecules after a pulse label with radioactive precursors, the rate of disappearance of enzyme activity after induction, and the rate of reappearance of enzyme activity after administration of an irreversible inhibitor of the enzyme. With regard to the last of these techniques, Price *et al.* (41) treated rats with an irreversible inhibitor of catalase and calculated the rates of synthesis and degradation of the enzyme from the progressive return of catalase activity. Erwin and Deitrich (42) used a similar approach with an irreversible inhibitor to study the turnover of monoamine oxidase in rat liver. The effects of injection of a "submaximal" dose (2 μl) of dopamine β-hydroxylase antiserum on the serum enzyme activity can be considered analogous to the action of an irreversible inhibitor. Assuming that the marked reduction in serum dopamine β-hydroxylase levels does not influence the rate of entrance of enzyme into the circulation, the recovery of serum enzyme activity represents the rate of entrance of new enzyme into serum minus the rate of its degradation.

When the reappearance of dopamine β-hydroxylase activity in serum is plotted semilogarithmically according to the method of Swick *et al.* (32), a straight line is obtained. The linear decrement allows calculation of the first-order rate constant for degradation of serum dopamine β-hydroxylase which is 0.166 day^{-1}. Thus approximately 17% of the total serum enzyme is inactivated per day. The rate at which new enzyme enters the circulation each day is 2.8 units/ml, or approximately 60 units/rat. The half-life of the enzyme in serum is 4.2 days, which is similar to that of several other serum proteins.

The half-life for serum dopamine β-hydroxylase determined by our method is considerably longer than the values previously reported. Rush and Geffen (43) measured the turnover of serum dopamine β-hydroxylase by intravenous infusion into lambs of ^{125}I-labeled sheep enzyme and estimated a half-life for the enzyme of 2.7 hr, based upon the rate of disappearance of radioactivity from the serum. However, after infusion of labeled proteins into circulation, there are at least two distinct phases of elimination. During the first phase, equilibration between intra- and extravascular pools occurs; this phase lasts approximately 1 day and is followed by a period in which clearance of the protein occurs at a logarithmic rate. It is this latter rate which must be used for the calculation of half-life. Since it is questionable whether Rush and Geffen observed a steady-state level after perfusion for 90 min, it is likely that these authors measured the rate of redistribution of infused dopamine β-hydroxylase between the intra- and extravascular pools rather than the actual degradation of the enzyme. From the increase and subsequent decline to basal levels of serum dopamine β-hydroxylase activity resulting from stress, Roffman *et al.* (8) calculated a half-life for the enzyme of 3 hr in rats. Stone *et al.* (21) have shown, however, that severe stress causes increases not only in serum dopamine β-hydroxylase activity but also in other serum proteins and enzymes; thus stress-induced changes may simply reflect a transient redistribution of the serum enzyme. Using inhibition of protein synthesis, Molinoff *et al.* (44) determined a half-life of 15–16 hr for dopamine β-hydroxylase

in sympathetic ganglia. A net accumulation of dopamine β-hydroxylase in serum would not seem possible if the turnover of the serum enzyme is several times faster (e.g., the previously reported $t_{1/2}$ of 2.7 hr) than that of the enzyme in tissue, the source of the serum enzyme.

A particularly attractive aspect of the immunoinactivation of serum dopamine β-hydroxylase is that it provides a technique for selectively reducing the serum enzyme level without affecting tissue stores. With this technique, it will be possible to examine the effects of drugs or treatments that alter sympathetic neuronal activity on the rate of release and turnover of the serum enzyme. Accordingly, the contribution of sympathetic neuronal activity to the regulation of serum dopamine β-hydroxylase levels can now be assessed directly.

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