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Regulation of Phenylethanolamine *N*-Methyltransferase Synthesis and Degradation

I. Regulation by Rat Adrenal Glucocorticoids

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SUMMARY

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The steady-state levels of rat adrenal phenylethanolamine *N*-methyltransferase are under the control of adrenal glucocorticoids. Following hypophysectomy, enzyme levels fall dramatically; they can be restored to normal by the administration of adrenocorticotrophin or dexamethasone. The decline in enzyme levels after hypophysectomy is accompanied by a decrease in the number of immunotitratable enzyme molecules; dexamethasone treatment restores the immunochemically reactive transferase. The decrease in number of enzyme molecules after hypophysectomy is due to accelerated proteolysis; this is reversed by glucocorticoid administration. Phenylethanolamine *N*-methyltransferase from hypophysectomized rats is more vulnerable to proteolysis *in vivo* and to denaturation at 50° *in vitro*, suggesting that hypophysectomy causes fundamental changes in the overall stability of the enzyme.

INTRODUCTION

Phenylethanolamine *N*-methyltransferase is the terminal enzyme in epinephrine biosynthesis (1). The adrenal gland levels of this enzyme are subject to dual regulation by splanchnic nerve impulses (2–4) and by adrenal cortical glucocorticoids (4, 5). Following hypophysectomy, phenylethanolamine *N*-methyltransferase levels fall markedly; enzyme activity can be restored nearly to control values by adrenocorticotrophin or dexamethasone administration, but neither drug elevates the enzyme above control levels in intact rats.

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In contrast, splanchnic nerve stimulation by reserpine or phenoxybenzamine effectively elevates phenylethanolamine *N*-methyltransferase levels in both intact and hypophysectomized rats (4). These drugs reflexively increase splanchnic nerve firing and cause doubling or tripling of the enzyme activity. The effectiveness of neural stimulation in elevating levels of this enzyme in normal rats is in marked contrast to the ineffectiveness of ACTH or dexamethasone, and suggests that the hormonal and neural control systems regulate phenylethanolamine *N*-methyltransferase levels by different biochemical mechanisms.

Previous studies have shown striking similarities between the regulation of phenylethanolamine *N*-methyltransferase and the regulation of dopamine β-hydroxylase,

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another catecholamine-synthesizing enzyme (4). Dopamine β-hydroxylase is also regulated by neural impulses (2, 4) and by adrenal glucocorticoids (4, 6). If normal rats are given reserpine, dopamine β-hydroxylase levels are doubled (4). After hypophysectomy, dopamine β-hydroxylase levels fall markedly (6); dexamethasone or ACTH administration will restore enzyme levels to nearly normal in operated animals, but neither drug alters enzymes levels in normal animals. The responses of phenylethanolamine *N*-methyltransferase and dopamine β-hydroxylase to hormonal and neuronal stimulation, therefore, are exactly analogous.

Our subsequent studies showed that the neurally mediated increase in dopamine β-hydroxylase levels seen after reserpine administration was due to induction of enzyme synthesis without an alteration in the rate of enzyme degradation (4). The decline in dopamine β-hydroxylase levels caused by hypophysectomy, on the other hand, is due to a marked acceleration in the rate of enzyme degradation, which is partially reversed by ACTH. Hypophysectomy has no effect on the rate of enzyme synthesis (4).

This led us to the proposition that the neuronal and hormonal regulation of dopamine γ-hydroxylase levels occurs by different biochemical mechanisms. Steady-state levels of this enzyme are controlled by adrenal glucocorticoids, which act by inhibiting its degradation. Under basal conditions this inhibition of degradation is maximal; further administration of ACTH or glucocorticoids therefore cannot elevate enzyme levels above control values. Elevation of dopamine β-hydroxylase levels above control values, on the other hand, is brought about by increased neural firing, occurs in both normal and hypophysectomized animals, and is due to an accelerated rate of enzyme synthesis. In a subsequent report we demonstrated that both hormonal and neuronal systems depend on cholinergic innervation, and speculate that the operation of the dual hormonal-neuronal control system might be important in the response of the animal to stress (7).

Enzyme levels reflect a balance between the rates of synthesis and degradation. Substantial evidence has accumulated in recent years that degradation is an important mode of regulating enzyme levels *in vivo*, and that powerful mechanisms exist within mammalian cells to control it (8). In view of our previous findings on dopamine β-hydroxylase, it became of interest to investigate whether the hormonal and neuronal controls operant on phenylethanolamine *N*-methyltransferase activity would affect the rates of synthesis and degradation of this enzyme as well.

This report describes studies on the regulation of phenylethanolamine *N*-methyltransferase by glucocorticoids. The fall in enzyme activity after hypophysectomy is caused by a decrease in the number of enzyme molecules, as measured by immunotitration of the enzyme with specific antiserum. Dexamethasone partially restores enzymatic activity; this is associated with an increase in the number of immunotitratable phenylethanolamine *N*-methyltransferase molecules. By combined radiolabeling and immunochemical methods it is shown that the decrease in enzyme levels following hypophysectomy is due to an accelerated rate of enzyme degradation, which is reversed by dexamethasone. Dexamethasone has no effect on the rate of synthesis of the enzyme but does inhibit its degradation. These results indicate that steady-state levels of phenylethanolamine *N*-methyltransferase as well as those of dopamine β-hydroxylase are controlled by adrenal glucocorticoids via mechanisms inhibiting degradation of those enzymes.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats weighing 160–200 g were obtained form Zivic-Miller Laboratories, Allison Park, Pa. Hypophysectomy was performed by the supplier. Animals were maintained on Purina laboratory chow in a facility with automatic temperature and lighting regulation (light from 6 a.m. to 6 p.m., dark from 6 p.m. to 6 a.m.). Hypophysectomized animals were given a commercial orange juice–water mixture.

Enzyme assay. Animals were killed by cervical dislocation. The adrenals were removed, cleaned of fat, and homogenized in 5 ml/pair of 5 mM Tris–0.2% bovine serum albumin–0.2% Triton X-100–150 mM NaCl

buffer, pH 7.3. The homogenates were centrifuged at 37,000 × *g* for 30 min at 1–4°. Phenylethanolamine *N*-methyltransferase activity was assayed in 100-μl portions of the supernatant by a previously described method, using phenylethanolamine as substrate (4).

Isotopes. *S*-Adenosyl-L-[*methyl*-^{14}C]methionine (52–59 mCi/mmole), L-[^{14}C]glutamic acid (265 mCi/mmole), and L-[^{3}H]-glutamic acid (30 Ci/mmole) were purchased from Amersham/Searle.

Immunoprecipitation procedures. Phenylethanolamine *N*-methyltransferase was purified to electrophoretic homogeneity from bovine adrenal medulla as previously described (9). The purified enzyme was injected intramuscularly into rabbits in complete (initial) and incomplete (all subsequent injections) Freund's adjuvant. Antiserum was obtained by ear venipuncture. The techniques employed and the specificity of the antiserum have been verified in previous reports (9).

Immunotitration reactions were carried out by incubating 0.5-ml portions of rat adrenal supernatants with various amounts of rabbit antiserum. Samples were incubated at 37° for 1 hr, then overnight in ice. They were then centrifuged at 37,000 × *g* for 30 min. The amount of enzyme activity remaining in the supernatant following removal of the immune complexes was then determined, and a titration curve such as that shown in Fig. 1 was constructed.

Double-label amino acid incorporation studies were carried out to determine the rates of phenylethanolamine *N*-methyltransferase synthesis and degradation relative to soluble adrenal protein. In these studies, adrenals from rats previously injected with radioactive amino acids were homogenized in 1.5–2 ml/pair of Tris–Triton–albumin–NaCl buffer and centrifuged at 37,000 × *g* for 30 min. Isotope incorporation into cell membrane protein was measured by washing the initial pellet twice in ice-cold buffer and digesting the pellet overnight in 1 ml of "NCS" solubilizer (Amersham/Searle). Incorporation of labeled amino acids into soluble adrenal protein was determined by adding 1 ml of 25% trichloroacetic acid to 0.5-ml portions of the adrenal supernatants. The precipitates were collected by centrifugation at 6500 × *g* for 30 min; pellets were washed once with 25% trichloroacetic acid and once with 150 mM NaCl before being digested overnight in 1 ml of "NCS."

Radiolabeled phenylethanolamine *N*-methyltransferase was isolated by incubation of the adrenal supernatants with specific antiserum. A volume of antiserum previously determined by titration to remove 95–99% of the total adrenal phenylethanolamine *N*-methyltransferase was added to the supernatant preparations. Samples were incubated at 37° for 1 hr, then on ice for as long as 6 days. The immunoprecipitates were removed by prolonged centrifugation (up to 6 hr) at 700 × *g*, washed extensively in 150 mM NaCl, and digested overnight in 1 ml of "NCS." The longer incubation and centrifugation times, as contrasted with those used in the immunotitration procedures, were employed to minimize the nonspecific precipitation of radiolabeled adrenal protein.

All samples were counted in "PCS" counting medium (Amersham/Searle) in a liquid scintillation counting system in which the efficiency for tritium was 33–36% and for ^{14}C, 41–46%. The carbon spillover into the tritium channel was 15–18%.

Preparation of antienzyme immunoadsorbent. Anti-phenylethanolamine *N*-methyltransferase IgG was purified from crude rabbit antiserum. To each 100 ml of serum, 60 ml of saturated ammonium sulfate solution were added dropwise with stirring. All procedures were carried out at 0–4°. After stirring and equilibration, the solution was centrifuged at 30,000 × *g* for 30 min. The pellet was resuspended in a minimal volume of 20 mM Tris–150 mM NaCl buffer, pH 7.4. This solution was adjusted to 33% ammonium sulfate saturation by the dropwise addition of 60 ml of ammonium sulfate solution per 100 ml. After 30 min of equilibration, the solution was centrifuged at 30,000 × *g* for 30 min. The pellet was then resuspended in the original volume of 20 mM Tris–150 mM NaCl buffer, pH 7.4. The equilibration, centrifugation, and resuspension process was repeated three additional times. After the last centrifugation, the pellet containing purified IgG was resuspended in an original volume

of 0.25 M potassium phosphate buffer, pH 6.5.

The purified antienzyme IgG was covalently linked to Sepharose 4B (Pharmacia) by the method of Cuatrecasas and Anfinsen (10). First, 10 ml of packed Sepharose 4B were diluted with 20 ml of cold distilled water. The pH of this mixture was brought to 11 by the dropwise addition of 5 N NaOH. To the alkaline gel, 300 mg of cyanogen bromide per milliliter of gel were added. The pH was maintained between 11 and 11.5 by the addition of NaOH until no further base uptake occurred. The gel was quickly filtered and then washed on a Buchner funnel under suction with 1 liter of ice-cold distilled water, followed by 1 liter of iced 0.25 M phosphate buffer, pH 6. The purified IgG solution was added to the activated Sepharose gel and stirred gently overnight at 4°. The IgG gel was then filtered, extensively washed in buffer, and stored at 4° until use.

Use of IgG immunoadsorbent in dual-label enzyme turnover studies. The IgG-coupled immunoadsorbent gel was used in dual-label turnover studies as follows: 1-ml columns of IgG gel were prepared by pouring the gel into glass wool-stoppered Pasteur pipettes. The columns were washed with 25–50 ml of 0.25 M phosphate buffer, pH 6, followed by additional washing with 25–50 ml of 5 mM potassium phosphate–150 mM NaCl buffer, pH 7.4. The adrenal supernatants from rats receiving injections of radiolabeled amino acids were prepared as described above. The supernatants were applied to the column, and the flow was stopped for 2 hr. Then the column flow was adjusted to 10 ml/hr and the effluent was collected. The column was then washed with 25 ml of 5 mM phosphate–150 mM NaCl buffer, pH 7.4, and eluted with 3 ml of 6 M urea. One milliliter of the column effluent was dissolved in 3 ml of "NCS" and counted for isotope incorporation into protein. The urea eluate from the column was treated in the same way. Dual-label amino acid incorporation into phenylethanolamine *N*-methyltransferase was then determined by liquid scintillation counting. Counting efficiency of the urea eluates was 27% for tritium and 25% for ^{14}C, with 24% spillover of carbon into the tritium channel.

Statistics. Unless otherwise stated, data are presented as the mean and standard error of the mean. Levels of statistical significance were obtained by a two-tailed Student's *t*-test.

RESULTS

Titration of rat adrenal phenylethanolamine N-methyltransferase with specific antiserum. Rabbit anti-bovine phenylethanolamine *N*-methyltransferase antiserum cross-reacted completely with the enzyme from rat adrenals. Figure 1 shows a titration curve in which enzyme from hypophysectomized or sham-operated rats was precipitated from adrenal supernatants with this antiserum. Although the antiserum is effective in removing the rat adrenal enzyme from solution, the avidity of the antibody

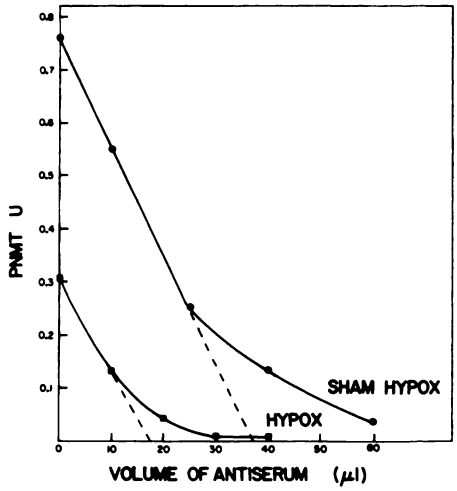

FIG. 1. *Immunoprecipitation curve of phenylethanolamine N-methyltransferase (PNMT) from hypophysectomized and control rats using antiserum to bovine enzyme*

Two hypophysectomized and two control rats were used. The animals were killed 10 days after surgery, and their adrenals were removed and homogenized as described under MATERIALS AND METHODS. Various volumes of antiserum were added to 0.5-ml portions of the adrenal supernatant. Following incubation the immunoprecipitates were removed by centrifugation, and enzymatic activity remaining in the supernatant was determined. Data are expressed as units (U) of activity, where 1 unit is the formation of 1 nmole of [*N*-*methyl*-^{14}C]phenylethanolamine per hour. Extrapolation of the linear portion of the titration curve to the abscissa gives the equivalence point, i.e., the volume of antiserum required to remove the enzyme from solution completely.

for the homologous (bovine) antigen is much greater than for the heterologous antigen. Nonetheless, since the antiserum completely removed the enzyme from rat adrenal preparations, it was deemed suitable for use in these studies. The volume of antiserum required to remove the enzyme from solution completely was estimated by regression analysis of the linear portion of the titration curve, and was termed the equivalence point.

Effect of hypophysectomy and dexamethasone administration on phenylethanolamine N-methyltransferase activity. Hypophysectomy caused a marked reduction in transferase activity (Table 1). This was associated with a proportionate decline in the number of enzyme molecules, as measured by immunotitration. Treatment of the animals with dexamethasone caused a partial restoration of enzyme activity (Table 1), which was associated with a proportional increase in the amount of immunotitratable transferase (Fig. 2). Table 2 shows the statistical analysis of these titration curves. Comparison of the ratios of enzyme activity with immunochemical equivalence points confirms that the differences in activity among the experimental groups are due solely to differences in the numbers of enzyme molecules, and not to the presence of activators or inhibitors of the enzyme.

TABLE 1

Effects of hypophysectomy and dexamethasone on phenylethanolamine N-methyltransferase

The number of animals in each group is shown in parentheses. Beginning 10 days after hypophysectomy, some rats received 5 mg/kg of dexamethasone (Decadron; Merck Sharp & Dohme) intraperitoneally daily for 3 days. One day after the last injection the animals were killed and their adrenal phenylethanolamine *N*-methyltransferase levels were determined as described in the text. Enzyme activity is expressed as units per adrenal pair, where 1 unit is the formation of 1 nmole of [*N-methyl*-^{14}C]phenylethanolamine per hour. The two-tailed Student's *t*-test was used to analyze the data.

Treatment	Enzyme activity
	units/adrenal pr
Sham hypophysectomy (4)	8.74 ± 0.70
Hypophysectomy (6)	2.42 ± 0.10[a]
Hypophysectomy + dexamethasone (6)	4.87 ± 0.10[b, c]

[a] Significantly different from sham-hypophysectomized rats; $p \ll 0.0001$.

[b] Significantly different from untreated hypophysectomized rats; $p \ll\ll 0.0001$.

[c] Significantly different from sham-hypophysectomized rats; $p < 0.001$.

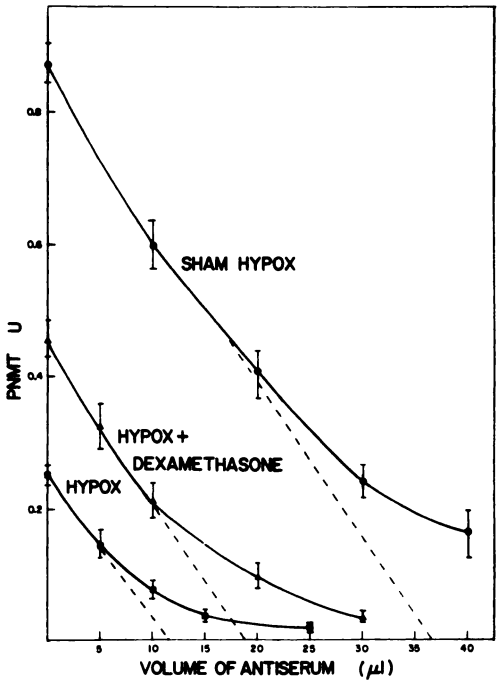

FIG. 2. *Equivalence points for phenylethanolamine N-methyltransferase (PNMT) activity in control, hypophysectomized, and dexamethasone-treated hypophysectomized rats*

Each group contained six rats. Ten days after hypophysectomy some animals received 1 mg of dexamethasone intraperitoneally, daily for 3 days. The rats were then killed and their adrenals were removed and prepared as described in the legend to Fig. 1. The titration curve shown above was then constructed.

Effect of hypophysectomy on phenylethanolamine N-methyltransferase synthesis and degradation. Steady-state enzyme levels reflect a balance between the rates of enzyme synthesis and degradation. The effect of hypophysectomy on phenylethanolamine *N*-methyltransferase levels, therefore, could be brought about by a decline in the rate of enzyme synthesis, an acceleration of enzyme degradation, or a combination of both processes. Accordingly, the double-label amino acid incorporation technique used in our earlier studies (4, 9) was employed to distinguish among these possibilities and to elucidate the role of

TABLE 2

Immunotitration analysis

Immunotitration analysis was performed on the animals described in Table 1. To 0.5-ml portions, representing 10% of the total adrenal phenylethanolamine *N*-methyltransferase, various volumes of antiserum was added. The titration curves shown in Fig. 2 were obtained, and regression analyses were performed on the linear portions of the curves. The *y* intercept of the curve represents the enzyme level in the absence of antibody, and when corrected for volume does not differ significantly from the levels obtained in Table 1. Comparison of the ratio of *y* intercepts (enzyme activity) with the ratio of equivalence points shows a close similarity, and the slopes of the curves are identical. This indicates that the differences in enzyme levels reflect differences in the number of enzyme molecules and in the volume of antiserum required to remove those molecules.

Treatment	Regression analysis of titration curves		
	y intercept	Slope	Equivalence point
	units/0.5 ml	*units/μl*	*μl*
Sham hypophysectomy (4)	0.843 ± 0.004	−0.021 ± 0.001	40 ± 1
Hypophysectomy (6)	0.250 ± 0.002	−0.021 ± 0.001	12 ± 1[a]
Hypophysectomy + dexamethasone (6)	0.441 ± 0.004	−0.022 ± 0.001	20 ± 2[a, b]
Sham/hypophysectomy	3.37	1.00	3.25
Hypophysectomy (dexamethasone)/hypophysectomy	1.76	1.01	1.67
Sham/hypophysectomy (dexamethasone)	1.91	0.99	1.94

[a] Significantly different from sham-hypophysectomized rats; $p \ll 0.0001$.

[b] Significantly different from untreated hypophysectomized rats; $p < 0.005$.

the pituitary-adrenal axis in controlling phenylethanolamine *N*-methyltransferase steady-state levels.

The technique involves sequential administration of the tritiated form of an amino acid, followed 24–96 hr later by administration of the ^{14}C-labeled form of the same amino acid. The animals are killed at the peak of ^{14}C incorporation into the enzyme (usually 1–2 hr after injection). Total protein and phenylethanolamine *N*-methyltransferase are then isolated, and the radioactivity in each is determined. The tritium activity remaining in each fraction is taken as a measure of the rate of enzyme degradation relative to that of total protein, while the carbon activity reflects the relative rate of enzyme synthesis. The ratio of carbon to tritium in the enzyme is compared with that in protein and expressed as a second ratio, termed the turnover index (11).

When this technique was used to study the effect of hypophysectomy on phenylethanolamine *N*-methyltransferase, the results shown in Table 3 were obtained. Hypophysectomy caused a decrease in adrenal protein synthesis[2] without affecting the rate of adrenal protein degradation. This was demonstrated by the proportionate decrease in both ^{14}C and tritiated glutamic acid incorporated into adrenal protein. In contrast, there was no difference in the amount of [^{14}C]glutamate incorporated into phenylethanolamine *N*-methyltransferase, indicating that hypophysectomy had no effect on the rate of enzyme synthesis. A marked decrease in tritiated enzyme was seen in the hypophysectomized animals, however, suggesting that hypophysectomy lowered enzyme levels by accelerating the rate of enzyme degradation. The decline in enzyme activity seen in hypophysectomized animals was compatible with the decreased tritium activity seen in the immunochemically isolated enzyme.

Effects of dexamethasone on phenyleth-

[2] The effect of hypophysectomy on adrenal protein synthesis is a time-dependent process. In our previous studies (4) we found that adrenal protein synthesis was unaffected 5 days after surgery but was nearly always reduced 10–12 days after hypophysectomy. In the studies shown in Table 3 a reduction in adrenal protein synthesis was seen 10 days after hypophysectomy, but the small sample size and high variability made this change nonsignificant. In the experiment shown in Table 4, however, larger group sizes were used and a significant decrease in adrenal protein synthesis was seen 10 days after hypophysectomy, in confirmation of our previous results (4).

TABLE 3

Double-label glutamic acid incorporation in hypophysectomized and control rats

Three control and four hypophysectomized rats were used. Nineteen days after hypophysectomy, the rats received 250 μCi of L-[^{3}H]glutamic acid intraperitoneally, followed 24 hr later by an injection of 25 μCi of L-[^{14}C]glutamic acid. The animals were killed 2 hr after the second injection and the adrenals were prepared as described in the text. The volume of antiserum used removed 96–98% of the total enzyme; disintegrations per minute in the enzyme were not corrected for recovery. All radioactivity values are net after subtraction of appropriate "NCS" in "PCS" blanks. In the hypophysectomized rats there is a significant reduction in the incorporation of glutamic acid into adrenal protein, but no evidence of accelerated protein breakdown. PNMT synthesis is not affected by hypophysectomy, but its degradation is accelerated. The tritiated enzyme remaining compares favorably with the reduction in enzyme level (44% vs. 41%). The ratio of ^{14}C to tritium in the enzyme is markedly different from the ^{14}C:^{3}H ratio in protein, suggesting little contamination of the enzyme precipitate by labeled protein.

Group	Enzyme activity	Radioactivity in soluble protein			Radioactivity in PNMT		
		^{14}C	^{3}H	^{14}C:^{3}H	^{14}C	^{3}H	^{14}C:^{3}H
	units/pr	*dpm/ml*			*dpm/pr*		
Control	8.78 ± 1.57	845 ± 138	1443 ± 400	0.65 ± 0.13	27 ± 3	375 ± 59	0.076 ± 0.012
Hypophysectomized	3.54 ± 0.22	530 ± 70	948 ± 268	0.77 ± 0.27	37 ± 3	166 ± 15	0.229 ± 0.028
p	<0.02	NS[a]	NS	NS	NS	<0.02	<0.005

[a] Not significantly different from control.

anolamine N-methyltransferase synthesis and degradation. Dexamethasone caused an increase in phenylethanolamine *N*-methyltransferase activity and in immunotitratable enzyme levels in hypophysectomized rats. This effect could be due to induction of enzyme synthesis or to inhibition of the accelerated enzyme degradation caused by hypophysectomy. To investigate these possibilities further, the double-label isotope incorporation technique was employed in hypophysectomized rats treated with dexamethasone (Table 4). These data show that hypophysectomy causes a decrease in adrenal protein synthesis but has no effect on the rate of protein degradation. Dexamethasone did not reverse the effects of hypophysectomy on adrenal protein synthesis. In contrast, hypophysectomy caused accelerated degradation of the enzyme, which was reversed by dexamethasone. Neither hypophysectomy nor dexamethasone administration had any effect on the rate of enzyme synthesis. These results were taken as evidence that dexamethasone reverses the accelerated degradation of phenylethanolamine *N*-methyltransferase caused by hypophysectomy. These findings were confirmed in a second experiment of the same design.

Use of the antienzyme immunoadsorbent in turnover studies. One of the major problems in turnover work using immunochemical techniques is the nonspecific coprecipitation of radioactive protein during the course of the antigen-antibody reaction. Since phenylethanolamine *N*-methyltransferase accounts for a minuscule portion of the total adrenal protein, it is important to ensure that the immunoprecipitates are free of contamination by labeled soluble adrenal protein. This can be done by using a labeling amino acid such as glutamate, in which phenylethanolamine *N*-methyltransferase is especially rich (12). Other techniques that are often useful for minimizing nonspecific coprecipitation include carrying out the precipitation reaction in solutions of high bovine serum albumin concentration, and extensive washing of the immunoprecipitate. Despite these precautions, we seemed to have no good way to ensure the purity of the phenylethanolamine *N*-methyltransferase-containing immunoprecipitate. Moreover, the results of the studies described in Table 4 suggested the possibility of considerable contamination of the immune precipitate by labeled protein.

Accordingly, an anti-phenylethanolamine *N*-methyltransferase immunoadsorbent gel was employed. After passage over the IgG column, the enzyme from the adrenal supernatants is completely adsorbed from solution and remains tightly bound to

TABLE 4

Double-label glutamic acid incorporation after dexamethasone administration

Each group contained six animals. Ten days after hypophysectomy some of the hypophysectomized rats received 5 mg/kg of dexamethasone intraperitoneally daily for 4 days. Three days after beginning dexamethasone administration, all animals received 250 μCi of L-[^{3}H]glutamic acid, followed 24 hr later by 25 μCi of L-[^{14}C]-glutamic acid. Hypophysectomy caused a reduction in protein synthesis in the soluble fraction, but did not affect enzyme synthesis. Degradation of enzyme, but not of soluble protein, was accelerated by hypophysectomy. Dexamethasone administration reversed the effect of hypophysectomy on enzyme degradation but did not affect its synthesis. Dexamethasone had no effect on the rate of soluble protein synthesis or degradation. Dexamethasone restored enzyme degradation to control rates, but did not completely restore enzyme activity. This probably reflects some contamination of the immune precipitate by labeled soluble protein.

Group	Enzyme activity	Radioactivity in soluble protein			Radioactivity in PNMT		
		^{14}C	^{3}H	^{14}C:^{3}H	^{14}C	^{3}H	^{14}C:^{3}H
	units/pr		*dpm/pr*			*dpm/pr*	
Control	8.41 ± 0.37	1180 ± 44	3111 ± 190	0.38 ± 0.02	61 ± 10	183 ± 39	0.33 ± 0.02
Hypophysectomized	2.64 ± 0.33[a]	884 ± 68[a]	1931 ± 67[a]	0.46 ± 0.03	60 ± 10	88 ± 17[b]	0.68 ± 0.04[c]
Hypophysectomized + dexamethasone	4.16 ± 0.22[d]	944 ± 64[e]	1773 ± 252[e]	0.45 ± 0.04	78 ± 4	192 ± 19[f]	0.41 ± 0.03

[a] $p < 0.001$ compared with control.
[b] $p < 0.02$ compared with control.
[c] $p < 0.01$ compared with control.
[d] $p < 0.0001$ compared with untreated hypophysectomized rats.
[e] $p < 0.02$ compared with untreated hypophysectomized rats.
[f] $p < 0.01$ compared with untreated hypophysectomized rats.

the immobilized antibody. The antigen-antibody complex can then be exhaustively washed until all nonspecific radioactive protein is removed, ensuring the purity of the enzyme remaining bound. The enzyme can then be eluted from the antibody using urea or other agents.

The IgG immunoadsorbent gel used bound 98–100% of the phenylethanolamine *N*-methyltransferase present in the rat adrenal supernatants. The bulk of the labeled adrenal protein was removed from the column after 5 ml of wash. None of the bound enzyme was removed by washing, despite the use of considerable volumes of buffer. The column was then eluted in three 1-ml fractions of 6 M urea. The first 1 ml of urea eluate was discarded; it contained no phenylethanolamine *N*-methyltransferase activity and very little radioactivity. The second 1 ml had all the phenylethanolamine *N*-methyltransferase activity that could be eluted from the gel (about 20% of the applied activity). This fraction was kept for double-label counting. The third 1 ml of the urea eluate contained no enzyme activity or radioactivity and was discarded. The purity of the eluted enzyme was checked by polyacrylamide disc gel electrophoresis, and it was found to consist of a single radioactive peak that comigrated with authentic bovine phenylethanolamine *N*-methyltransferase.

The IgG immunoadsorbent gel was then used in double-label turnover studies. The results of this inquiry (Table 5) confirmed our previous findings that the degradation of the enzyme was accelerated by hypophysectomy, with no effect of surgical manipulation on enzyme synthesis. Dexamethasone caused partial restoration of enzyme activity by reversing the hypophysectomy-mediated increase in enzyme degradation. Dexamethasone had no apparent effect on synthesis of the enzyme.

Kinetic properties of phenylethanolamine N-methyltransferase in hypophysectomized and control rats. Hypophysectomy might accelerate enzyme degradation by altering its structure or conformation, rendering it more susceptible to intracellular proteolysis. Such an altered structure or conformation might be detected as a change in the affinity of the enzyme for its

TABLE 5

Dual-label turnover of phenylethanolamine N-methyltransferase in hypophysectomized rats using antienzyme immunoadsorbent

Each group contained four rats. Beginning 10 days after surgery, the animals received dexamethasone, 1 mg/rat intraperitoneally, daily for 3 days. All animals were then given 250 μCi of L-[^{3}H]glutamic acid, followed 24 hr later by 25 μCi of L-[^{14}C]glutamic acid. Two hours later the animals were killed and the adrenals were removed. Adrenal supernatants were applied to 1-ml columns of anti-enzyme IgG immunoadsorbent. The column effluents were counted for isotope incorporation into protein. Enzyme was eluted from the columns with 6 M urea, and the urea eluates were counted for isotope incorporation. Dexamethasone had no effect on protein or enzyme synthesis. Enzyme degradation was significantly increased by hypophysectomy; this process was partly reversed by dexamethasone administration.

Treatment	Radioactivity in protein		Radioactivity in enzyme	
	^{3}H	^{14}C	^{3}H	^{14}C
	dpm/pr		*dpm/pr*	
Control	16,087 ± 887	173 ± 32	118 ± 8	41 ± 1
Hypophysectomy	9,510 ± 819	237 ± 44	71 ± 2[a]	35 ± 1
Hypophysectomy + dexamethasone	8,926 ± 241	195 ± 45	84 ± 5[b]	38 ± 1

[a] Significantly different from control; $p < 0.001$.

[b] Significantly different from untreated hypophysectomized rats; $p < 0.002$.

substrate, or in the thermal stability of the enzyme. Accordingly, the kinetic properties and thermal stability of phenylethanolamine *N*-methyltransferase from hypophysectomized and normal rats were investigated.

There was no difference in the kinetic properties of the enzyme between hypophysectomized and control rats for either phenylethanolamine or *S*-adenosylmethionine. However, there was a profound decrease in the thermal stability (estimated from the half-life at 50°) of the enzyme from hypophysectomized rats. These studies suggested that the increased susceptibility of the enzyme to proteolysis *in vivo* caused by hypophysectomy was also reflected in the stability of the enzyme to heating *in vitro*. Thus an independent confirmation that hypophysectomy accelerates the degradation of the enzyme was obtained (Table 6).

The increased thermal lability of phenylethanolamine *N*-methyltransferase after hypophysectomy has been extensively investigated and is detailed in the accompanying report (14). The results of that investigation show that the thermal stability of the enzyme is dependent on a dialyzable stabilizing factor present in control rats that is lost after hypophysectomy and restored by dexamethasone or ACTH treatment. This factor appears to bind to enzyme *in vivo*, but can be dissociated from it. The action of this stabilizing factor *in vivo* may regulate the proteolysis of the enzyme *in vivo* (14).

DISCUSSION

This report describes the mechanism of hormonal control of phenylethanolamine *N*-methyltransferase in rat adrenal glands. The fall in enzyme levels seen after hypophysectomy is associated with a decline in the number of immunotitratable enzyme molecules. These molecules retain the kinetic properties of the normal enzyme, but are degraded at a more rapid rate both *in vivo* and *in vitro*. Enzyme activity is partially restored with dexamethasone, which acts by inhibiting the accelerated rate of enzyme breakdown.

Our previous studies on dopamine β-hydroxylase have demonstrated that glucocorticoids play a similar role in regulating the steady-state levels of this enzyme. Dopamine β-hydroxylase levels fall dramatically after hypophysectomy; this is caused by a marked increase in the rate of enzyme degradation. Administration of either ACTH or glucocorticoids results in partial restoration of enzyme levels by inhibiting the accelerated breakdown of the enzyme (4).

There is a striking compatibility between the data for these two enzymes, which points to the important role glucocorticoids play in regulating their steady-state levels.

TABLE 6

Properties of phenylethanolamine N-methyltransferase from hypophysectomized and control rats

Kinetic constants were obtained by the method of Lineweaver and Burk (13). The stability of the enzyme at 50° was measured as follows. Adrenals from control or hypophysectomized rats were homogenized in 5 ml/pair of 50 mM Tris-HCl buffer, pH 7.4. The homogenates were centrifuged at 37,000 × *g* for 30 min. Then 100-μl portions of the supernatant were added to reaction tubes containing 100 μl of 50 mM potassium phosphate buffer, pH 7.9. The samples were heated to 50° in a water bath for various periods of time. After heating, the tubes were plunged into ice. Residual enzymatic activity was measured by adding 5 μg of phenylethanolamine and 2 nmoles of *S*-adenosyl-[^{14}C]methionine in a volume of 50 μl to each tube and incubating the reaction mixture for 30 min at 37°. Enzymatic activity was determined in the usual way. The thermal half-life of the enzyme was determined by plotting the natural logarithm of enzyme activity vs. time of heating at 50°. Since thermal denaturation is a first-order process, a plot of enzyme against time gives a linear curve whose slope is k, the first-order reaction rate constant of thermal decay. The half-life is then calculated by the expression $t_{1/2} = \ln 2/k$. Supernatant samples were measured in triplicate at each time point; linear regression analysis was used to determine k.

Group	K_m of phenylethanolamine	K_m of *S*-adenosylmethionine	$t_{1/2}$ at 50°
	μM	μM	min
Control	76.9	2.75	19.97 ± 0.47
Hypophysectomized	71.4	2.66	2.83 ± 0.15[a]

[a] $p \ll 0.0001$ compared with control.

It would appear from this work that glucocorticoids act *in vivo* to inhibit the proteolysis of both enzymes and that under normal conditions this inhibition is nearly total. This would explain the repeated failure of this and other laboratories to provoke an elevation in either dopamine β-hydroxylase or phenylethanolamine *N*-methyltransferase in normal rats with ACTH or dexamethasone; the enzymes do not respond because the mechanism regulating their response to hormonal stimulation, inhibition of enzyme degradation, is at or near its maximal activity under normal conditions. Increases in enzyme activity well above control levels can be readily attained in intact rats by administration of neurally active drugs such as reserpine, because these drugs act by induction of enzyme synthesis *de novo*. We (4) and others (15) have previously shown this to be true for dopamine β-hydroxylase, and studies investigating this process for phenylethanolamine *N*-methyltransferase are in progress.

Despite the similarities in the mode of regulation of dopamine β-hydroxylase and phenylethanolamine *N*-methyltransferase, it would be incorrect to conclude that the responses of the enzymes to hypophysectomy or to reserpine treatment are nonspecific in nature. Hypophysectomy causes a marked reduction in adrenal protein synthesis, but has no effect on the synthesis of either enzyme. Conversely, hypophysectomy has no effect on adrenal protein degradation, but markedly accelerates the breakdown of both enzymes. Similarly, reserpine treatment induces the synthesis of both enzymes, and this effect is abolished by adrenal denervation (4). While it is true that reserpine induces adrenal protein synthesis, this process occurs at a much later time after reserpine treatment than do the catecholamine enzyme elevations, and is not blocked by adrenal denervation (4). It is equally likely that neither adrenal medullary atrophy nor necrosis explains the effects of hypophysectomy on dopamine β-hydroxylase and phenylethanolamine *N*-methyltransferase degradation, since electron microscopic studies on adrenal medullary architecture did not reveal significant necrotic changes (16).

The role of intracellular protein degradation is becoming widely acknowledged as an important regulatory mechanism in mammalian cells. Although proteolytic enzymes have been an important tool in the structural analysis of proteins, comparatively little is known about how intracellular proteases act *in vivo*. A variety of biological insults, such as starvation (17), atrophy (18), and vitamin deficiency (19), are associated with an increase in protein degradation. Treatment with hormones such as insulin (20), cyclic 3′,5′-AMP (21), glucocorticoids (22), and ACTH (23) stimulates the synthesis of specific proteins and in some cases concurrently decreases the protein catabolic rate (18). Proteolytic cleavage is an important regulatory mech-

anism in the conversion of inactive prohormones such as proinsulin to the active form (24).

An important question, then, is: What is the reaction sequence involved in the proteolytic destruction of phenylethanolamine *N*-methyltransferase and dopamine β-hydroxylase? The latter enzyme is a tetrameric glycoprotein that consists of subunits of mol wt 77,000 (25). It is not known whether these subunits represent a natural degradation product of the enzyme, although there is some evidence in liver enzyme systems that conversion from a multimer to the monomeric form of an enzyme is a likely first step in the degradative process (26). Phenylethanolamine *N*-methyltransferase is a monomeric protein of mol wt 40,000 (12). Both phenylethanolamine *N*-methyltransferase and dopamine β-hydroxylase are strikingly rich in glutamic acid residues and, in fact, display similar ratios of several amino acids (Table 7). Whether any of these are involved as key residues for intracellular proteolysis is, of course, not known at this time, but the possibility is worthy of further investigation.

Another important question raised by these studies is: How do glucocorticoids inhibit the intracellular degradation of phenylethanolamine *N*-methyltransferase and dopamine β-hydroxylase? One possibility might be by binding directly to the enzymes and stabilizing them in some way against degradation. Such a stabilization mechanism has been invoked to explain the elevation of tryptophan pyrrolase levels by tryptophan (28).

An alternative mechanism is that glucocorticoids promote the synthesis of a substance that binds to the enzymes, stabilizing them against proteolysis *in vivo*. Studies on the thermal stability of phenylethanolamine *N*-methyltransferase in hypophysectomized and normal rats have disclosed the existence of such a substance. These studies are described in the following report (14).

What is the biological significance of the hormonal-neuronal interregulation of dopamine β-hydroxylase and phenylethanolamine *N*-methyltransferase? Quite likely it is in the response of the animal to stress, in which the adrenal plays an important role in the "fight or flight" response. Under stress conditions, both splanchnic neural activity and pituitary ACTH outflow are increased. By acting at different sites, the neuronal and hormonal regulatory systems combine to control the availability of catecholamines in tandem. In times of stress, splanchnic nerve firing results in increased dopamine β-hydroxylase (and quite likely phenylethanolamine *N*-methyltransferase synthesis, while the increased glucocorticoid outflow from ACTH ensures maximal inhibition of degradation of these enzymes.

Under resting conditions the two systems

TABLE 7

Amino acid composition of dopamine β-hydroxylase and phenylethanolamine N-methyltransferase

There is a striking similarity in the proportional mole fractions of several residues in both enzymes. For example, the ratio of molecular weight of dopamine β-hydroxylase (subunit) to phenylethanolamine *N*-methyltransferase is 1.93; six amino acids occurs in comparable proportion. Moreover, both enzymes are extremely rich in glutamic acid. Thus proteases that cleave at any of these key residues might act on both enzymes.

Amino acid	Phenylethanolamine *N*-methyltransferase (12) (A)	Dopamine β-hydroxylase (27) (B)	B:A
	residues/ 40,000 mol wt	*residues/ 77,000 mol wt*	
Subunit mol wt	40,000	77,000	1.93
Lysine	14	56	4.00
Histidine	6	14	2.33
Arginine	31	42	1.35
Cysteine	+		
Aspartic acid	25	50	2.00
Threonine	6	14	2.33
Serine	13	54	4.15
Glutamic acid	50	142	2.84
Proline	29	68	2.34
Glycine	33	52	1.58
Alanine	43	54	1.26
Valine	33	25	0.76
Methionine sulfone	1	12	12.00
Isoleucine	15	10	0.67
Leucine	50	54	1.08
Tyrosine	6	8	1.25
Phenylalanine	6	12	2.00
Tryptophan	12		
Cysteic acid		4	

would retain their concerted action, since tonic neural activity must be present for glucocorticoids to maintain steady-state enzyme levels (7). When glucocorticoid production is reduced, inhibition of dopamine β-hydroxylase and phenylethanolamine N-methyltransferase degradation abates and enzyme levels fall. Thus the interaction of the two systems ensures that the availability of glucocorticoids and catecholamines is controlled in tandem. In view of the importance of the adrenal in the stress response, such a linkage between the production of cortical and medullary hormones would seem to be an important regulatory process.

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