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Regulation of Phenylethanolamine *N*-Methyltransferase Synthesis and Degradation

II. Control of the Thermal Stability of the Enzyme by an Endogenous Stabilizing Factor

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SUMMARY

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The levels of rat adrenal phenylethanolamine *N*-methyltransferase fall dramatically following hypophysectomy and can be restored to normal values by the administration of adrenocorticotrophin or glucocorticoids. Studies using combined radiolabeling and immunochemical techniques reveal that hypophysectomy accelerates degradation of the enzyme and that proteolysis of the enzyme *in vivo* is under regulation by glucocorticoids. After hypophysectomy the stability of phenylethanolamine *N*-methyltransferase at 50° is profoundly reduced, suggesting that concomitant with the increased susceptibility of the enzyme to proteolysis *in vivo* is an enhanced vulnerability to thermal denaturation *in vitro*. The thermal stability of the enzyme seems to be regulated by a freezing-thawing-labile, dialyzable substance present in the adrenal glands of normal rats. This material, termed stabilizing factor, is lost after hypophysectomy and can be restored by the administration of ACTH or dexamethasone. The stabilizing factor appears to act by binding to phenylethanolamine *N*-methyltransferase and can be dissociated from the immunoadsorbed enzyme by washing. The partially purified stabilizing factor has an absorption maximum at 264 nm; preliminary results indicate that it may be *S*-adenosylmethionine. Thus it is possible that binding of the enzyme to *S*-adenosylmethionine confers stability against proteolysis *in vivo* and thermal denaturation *in vitro*.

INTRODUCTION

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

Hypophysectomy appears to cause a decline in phenylethanolamine *N*-methyltransferase levels by accelerating its proteolysis *in vivo*. The accelerated degradation of the enzyme is partially reversed by dex-

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amethasone administration; dexamethasone appears to have no stimulatory effect on enzyme synthesis (6). The ineffectiveness of dexamethasone or ACTH in elevating phenylethanolamine *N*-methyltransferase levels in normal rats suggests that, under basal steady-state conditions, glucocorticoid inhibition of enzyme degradation is at maximal activity and that additional administration of hormones cannot inhibit the degradation process further. These results are described in the preceding publication (6).

In the course of these studies, we undertook an inquiry into the mechanism of phenylethanolamine *N*-methyltransferase degradation *in vivo*. We were interested in studying the biochemical and biophysical properties of this enzyme in hypophysectomized and normal rats. The preceding report showed that the K_m of the enzyme for phenylethanolamine and *S*-adenosylmethionine is the same in both normal and hypophysectomized rats, but that the stability of the enzyme to heating at 50° is profoundly reduced by hypophysectomy. Thus the increased susceptibility of the enzyme to intracellular proteolysis *in vivo* is reflected by an increased susceptibility to heat denaturation *in vitro*.

In this report we show that the thermal stability of phenylethanolamine *N*-methyltransferase appears to be controlled by a freezing-thawing labile substance present in control (normal) rats that is lost after hypophysectomy. The concentration of this substance is partially restored by dexamethasone or ACTH treatment of hypophysectomized rats. This substance, termed stabilizing factor, appears to be a dialyzable molecule that acts by binding to the enzyme but can be dissociated from it. This stabilizing factor appears to protect the enzyme against thermal denaturation *in vitro*. Our preliminary speculation is that this same stabilizing factor may also bind to the enzyme in the adrenal medullary cell and protect the enzyme against proteolysis *in vivo*.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats weighing 160–200 g were obtained from 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. Hypophysectomized animals were given a commercial orange juice–water mixture.

Enzyme assays. Animals were killed by cervical dislocation. The adrenals were removed, cleaned of fat, and 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. Phenylethanolamine *N*-methyltransferase activity was assayed in 100-μl portions of the supernatant by a previously described method, using phenylethanolamine as substrate (6).

Thermal denaturation studies. Studies on the thermal stability of phenylethanolamine *N*-methyltransferase were performed on the crude supernatants as follows: 100-μl portions of the supernatant were added to tubes containing 100 μl of 50 mM potassium phosphate buffer, pH 7.9. The tubes were heated at 50° in a water bath for various periods of time, then removed from the water bath and plunged into ice. Residual phenylethanolamine *N*-methyltransferase activity was measured by adding 2 nmoles of *S*-adenosyl[^{14}C]methionine and 5 μg of phenylethanolamine and incubating the mixture at 37° for 30 min. All determinations were performed on duplicate or triplicate samples at each of five to nine time points. Most studies were done using seven time points with triplicate determinations at each point. The times usually used were 0 (no heating) 2, 3, 4, 5, 7, and 10 min for the samples from hypophysectomized rats and 0, 2, 4, 6, 8, 10, and 20 min for the samples from normal (control) rats. The residual phenylethanolamine *N*-methyltransferase activity was plotted as the natural logarithm of enzyme activity vs. time of heating (see Fig. 1). Such a plot yields a straight line, the slope of which is the apparent first-order rate constant, *k*, of thermal denaturation. The points were fitted to the best line by least-squares analysis, using a computer program for linear regressions. From this line the slope and its standard deviation were computed. The

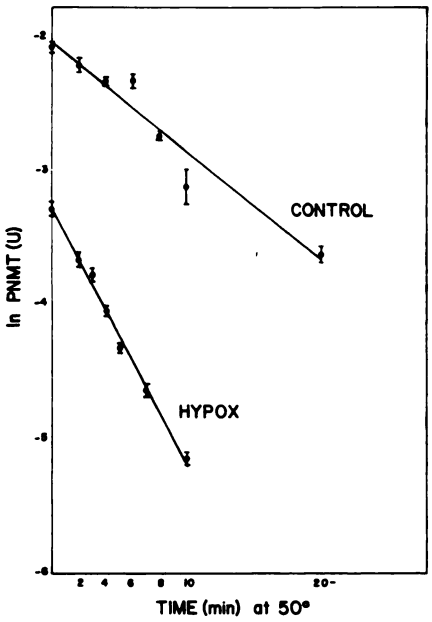

FIG. 1. *Thermal stability of phenylethanolamine N-methyltransferase (PNMT) from hypophysectomized and control rats*

Adrenal supernatants were prepared from hypophysectomized or control rats as described in the text. Samples at each time point shown were run in triplicate. All except the zero-time tubes were heated to 50°. At the designated time, thermal denaturation was terminated by plunging the tubes into cracked ice. All samples were analyzed for residual enzymatic activity. These values, expressed as the natural logarithm of the enzyme activity in units (nanomoles of *N*-methylphenylethanolamine per hour) per tube, were plotted against time of heating. Linear regression analysis was performed, and for each set of points a curve was obtained whose coefficient of correlation, r, was highly significant. The y intercept and its standard deviation, and the slope and its standard deviation, were obtained as well. The latter figures were used to calculate $t_{1/2}$. The following regression statistics were obtained. For the control animals, $k = -0.0347 \pm 0.0025$ min^{-1}; $r^2_{(24)} = 0.948$ ($p < 0.0001$); $t_{1/2} = 19.97$ min. For the hypophysectomized rats, $k = -0.2452 \pm 0.0131$ min^{-1}; $r^2_{(21)} = 0.974$ ($p < 0.0001$); $t_{1/2} = 2.83$ min. The rate constants of thermal denaturation were significantly different between the hypophysectomized and control curves at $p \ll 0.0001$, using the F-test of Sokal and Rohlf (7). These curves are typical of those used throughout this report to investigate the thermal denaturation of the enzyme.

half-life of the enzyme at 50° was computed according to the equation $t_{1/2} = \ln 2/k$.

Statistical analysis. Differences in the slopes of individual regression lines were analyzed for statistical significance from the rate constant (slope) of each regression line and its standard deviation. The method used was that of Sokal and Rohlf (7). All tests of significance were performed using the rate constant and its standard deviation. Both the rate constant and the half-life derived from it are presented in the first few tables of this report to illustrate the rate constant data on which statistical analysis was performed. In subsequent tables only the half-life data are shown, although it must be kept in mind that all tests of significance were performed on the rate constants. When groups of animals were used, the mean and standard error of the mean of the rate constants were determined and compared for statistical significance by the two-tailed Student's t-test.

RESULTS

Comparison of kinetic and thermal stability properties of phenylethanolamine N-methyltransferase from hypophysectomized and control rats. There was no difference in the K_m of the enzyme from hypophysectomized or control rats for either phenylethanolamine or *S*-adenosylmethionine (6). However, the stability of the enzyme at 50° was profoundly reduced by hypophysectomy (Fig. 1). This pilot experiment was repeated in a larger series of animals, with confirmation of the preliminary results (Table 1).

Effect of freezing and thawing of adrenal supernatants on thermal stability of phenylethanolamine N-methyltransferase. Freezing and thawing had a marked effect on the thermal stability, but not the catalytic activity, of the enzyme. When the thermal half-life of the transferase from hypophysectomized or control rats was compared in fresh adrenal supernatants with portions of the same supernatants that had been stored frozen, then thawed, and analyzed for thermal stability the next day, the results in Table 2 were obtained. Freezing and thawing caused a marked decrease in thermal stability of the transferase from the control but not the hypophysectomized rats. Freezing and thawing had no effect on enzymatic activity in any of the preparations, suggesting that the thermal half-life

of the enzyme was independent of the amount of its activity. These results were repeated in two additional sets of experiments.

Mixing experiments. The preliminary results indicated that the thermal stability, but not the activity, of phenylethanolamine N-methyltransferase from control rats was reduced by freezing and thawing. In contrast, freezing and thawing had no effect on the already reduced thermal stability of the enzyme from hypophysectomized rats. It thus appeared that control rats possessed a labile substance that protected the enzyme against thermal denaturation. If this were true, addition of adrenal supernatants from control rats to preparations from hypophysectomized rats might confer stability on enzyme preparations from the hypophysectomized animals. Several such mixing experiments were carried out. The results (Table 3) support the hypothesis that control rats indeed possess a labile substance that stabilizes the transferase against thermal denaturation. In each mixing experiment, addition of control rat adrenal supernatant to preparations from hypophysectomized rats completely restored the thermal half-life of the enzyme to control values. Since phenylethanolamine N-methyltransferase activity is independent of its thermal half-life, it is unlikely that these results could be explained by postulating that the increased stability of the mixed samples was due solely to the addition of a greater amount of enzyme from the control rats. Succeeding experiments amplify this point.

TABLE 1

Thermal stability of phenylethanolamine N-methyltransferase at 50° in hypophysectomized and control rats

Numbers in parentheses are group sizes. Ten days after hypophysectomy the rats were killed and adrenals were prepared as described in the text. Thermal denaturation studies on the enzyme from each rat were then carried out. From linear regression plots of residual enzymatic activity vs. time for each animal, the rate constant of thermal denaturation, k, was obtained. The thermal half-life for the enzyme from each animal was then calculated. The results are the means ± standard errors of the thermal half-life and rate constant of each group of animals. The level of statistical significance was obtained by the two-tailed Student's t-test.

Group	k	$t_{1/2}$ at 50°
	min^{-1}	*min*
Hypophysectomized (5)	−0.1540 ± 0.0012	4.50 ± 0.03
Control (6)	−0.0611 ± 0.0023	11.53 ± 0.43

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

Effects of dialysis on thermal stability of

TABLE 2

Effect of freezing and thawing on thermal stability of phenylethanolamine N-methyltransferase

Adrenal gland supernatants from one hypophysectomized and one control rat were prepared as described in the text. Thermal denaturation studies on triplicate samples at each of seven time points were carried out on portions of the fresh supernatant. The remaining supernatant was then frozen overnight. The next day the samples were thawed and the thermal denaturation studies were repeated. The half-life results were obtained from linear regression analysis of the thermal denaturation curves. The slopes ± standard deviations of the estimates of the slopes were obtained and converted to the half-life estimates. Statistical analysis to determine the level of significance was carried out by F-test on the slope ± its standard deviation (7). The two experiments (I and II) were performed several weeks apart on rats from different groups obtained from the supplier. The enzyme activity data are expressed as units per adrenal pair, where 1 unit is the formation of 1 nmole of [*N-methyl*-^{14}C]phenylethanolamine per hour.

Expt.	Preparation	Hypophysectomized			Control		
		Enzyme activity	$t_{1/2}$	k	Enzyme activity	$t_{1/2}$	k
		units/pr	*min*	min^{-1}	*units/pr*	*min*	min^{-1}
I	Fresh	1.64	2.83 ± 0.15	−0.2452 ± 0.0131[a]	6.76	19.73 ± 1.43	−0.0347 ± 0.0025
	Freeze-thaw	1.84	2.47 ± 0.15	−0.2807 ± 0.0170	7.18	9.13 ± 0.47	−0.0759 ± 0.0039[a]
II	Fresh	1.59	3.74 ± 0.26	−0.1854 ± 0.0130[a]	6.14	8.43 ± 1.13	−0.0822 ± 0.0100
	Freeze-thaw	1.41	3.25 ± 0.14	−0.2135 ± 0.0093	5.35	4.76 ± 0.10	−0.1455 ± 0.0030[a]

[a] $p < 0.0001$ compared with fresh control.

TABLE 3

Mixing experiments

Thermal stability experiments were performed on 100-μl portions of rat adrenal supernatants from hypophysectomized or control animals, or on mixtures of 50-μl portions from both preparations. Enzyme preparation from one hypophysectomized or one control rat was used in each experiment. Samples were assayed in triplicate at seven time points per curve. Results are the thermal half-lives ± standard deviations as calculated from the regression estimate of the slope. Levels of statistical significance were obtained by *F*-test (7).

Expt.	Preparation	$t_{1/2}$ at 50°	k
		min	min^{-1}
I	Hypophysectomized	3.05 ± 0.18	−0.2268 ± 0.013[a]
	Control	7.23 ± 0.23	−0.0958 ± 0.003
	Mixed	8.02 ± 0.65	−0.0864 ± 0.007[b]
II	Hypophysectomized	2.73 ± 0.05	−0.2538 ± 0.005[a]
	Control	10.86 ± 0.53	−0.0638 ± 0.003
	Mixed	11.08 ± 0.90	−0.0625 ± 0.005[b]
III	Hypophysectomized	2.34 ± 0.14	−0.2965 ± 0.015[a]
	Control	10.06 ± 0.46	−0.0689 ± 0.003
	Mixed	7.02 ± 0.64	−0.0987 ± 0.009[b,c]
IV	Hypophysectomized	3.55 ± 0.16	−0.1953 ± 0.009[a]
	Control	14.44 ± 0.90	−0.0480 ± 0.003
	Mixed	13.67 ± 1.29	−0.0507 ± 0.005[b]

[a] $p < 0.0001$ compared with control preparation.
[b] $p < 0.0001$ compared with hypophysectomized preparation.
[c] $p < 0.01$ compared with control preparation.

phenylethanolamine N-methyltransferase. We next sought to determine whether this labile factor that controlled the thermal stability of the enzyme could be removed from control rat supernatant preparations by dialysis. Accordingly, supernatant preparations from control rats were subjected to dialysis in the cold against 50 mM Tris buffer, pH 7.4 (Table 4 and Fig. 2). In the first experiment, dialysis was carried out for 3 hr. The thermal stability of the enzyme was determined at the beginning and end of dialysis. In addition, the ability of the dialyzed preparation to restore the thermal stability of the enzyme from hypophysectomized rats was examined. Dialysis for 3 hr resulted in a marked decline of thermal stability of the transferase from the control preparations and a sharp decrease in the ability of this preparation to stabilize the enzyme from hypophysectomized rats against thermal denaturation.

To test whether this stabilizing factor could be completely removed, a longer course of dialysis was performed. Dialysis dramatically reduced the thermal half-life of phenylethanolamine *N*-methyltransferase from control rats. By 24 hr the thermal half-life had reached a level similar to that seen in hypophysectomized rats (Fig. 2). Figure 2 also shows that the activity of the enzyme, as contrasted with its thermal stability, remained unchanged by dialysis. When enzyme from control rat supernatants that had been dialyzed for 24 hr was mixed with enzyme from hypophysectomized rats, no stabilization of the latter preparation was seen. These results support the previous findings, and suggest that the stabilizing factor was indeed being removed by the dialysis procedure (Table 5).

Dialysis could have removed the stabilizing factor from solution, or the stabilizing factor could be unstable to even relatively brief storage in the cold. To test the latter possibility, a supernatant preparation from control rats was prepared and stored in ice. At various times portions of this preparation were withdrawn and the thermal stability and activity of the enzyme were measured. The results showed no significant change in either thermal stability or activity of the enzyme over a time course of cold storage similar to that used in the dialysis

TABLE 4

Effect of dialysis on thermal stability of phenylethanolamine N-methyltransferase

An adrenal gland supernatant from a control rat was prepared. A portion of this supernatant was tested directly for thermal stability of the enzyme. Another portion was mixed with adrenal supernatant from a hypophysectomized rat (50-μl aliquots of each). The remaining supernatant from the control rat was dialyzed for 3 hr in the cold against 50 mM Tris buffer, pH 7.4. The thermal stability of the dialyzed preparation was then determined. The dialyzed material was also mixed with enzyme from a hypophysectomized animal, and the thermal stability of the mixture was tested. Dialysis caused a significant decrease in thermal stability of enzyme from the control preparation, and a marked decline in the ability of the control supernatant to alter the thermal half-life of the enzyme from the hypophysectomized preparation.

Preparation	Thermal half-life at 50°		
	Hypophysectomized (undialyzed)	Control	Mixed
	min	*min*	*min*
Fresh	3.34 ± 0.14	10.06 ± 0.46	7.02 ± 0.64
Dialyzed (3 hr)		7.44 ± 0.16[a]	5.04 ± 0.33[b,c]

[a] $p < 0.0001$ compared with undialyzed control.
[b] $p < 0.01$ compared with dialyzed control.
[c] $p < 0.01$ compared with fresh mixed values.

experiments. This suggested that the stabilizing factor was dialyzable rather than unstable in the cold.

Effects of ACTH and dexamethasone. Both ACTH and dexamethasone effectively restore phenylethanolamine *N*-methyltransferase values after hypophysectomy. This process is thought to reflect a restoration of the normal rate of enzyme degradation by glucocorticoids (6). To test the effect of hormone administration on the thermal stability of the enzyme, hypophysectomized animals were treated with either dexamethasone or ACTH. The results of three separate experiments (Table 6) show that restoration of enzyme levels by hormone administration was accompanied by an increase in thermal half-life toward control values. In the third experiment, brief dexamethasone treatment failed to increase enzyme levels significantly; in that experiment the thermal $t_{1/2}$ of dexamethasone-treated rats was not significantly different from that of untreated hypophysectomized animals. In experiment II, and in several other experiments in which dexamethasone effectively increased enzyme levels, a concomitant increase in thermal $t_{1/2}$ was seen.

Dose-response curve for ACTH. ACTH was very effective in restoring both the levels and the thermal half-life of the enzyme in hypophysectomized rats. To test whether these phenomena were dose-related, hypophysectomized animals were treated for 3 or 7 days with various doses of ACTH. The results (Fig. 3) show a clear-cut relationship between ACTH dose and thermal $t_{1/2}$, which was maximal at 4 IU of ACTH per day. These results further verify the effectiveness of ACTH in restoring the thermal stability of the transferase.

IgG immunoadsorption studies. The prolonged time required to dialyze the stabilizing factor completely suggested that this substance might be macromolecule-bound, perhaps to adrenal protein or to the transferase itself. To test whether the sta-

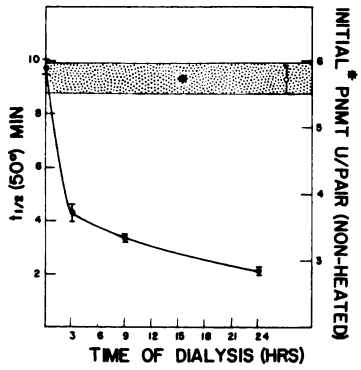

FIG. 2. *Decline of thermal half-life of phenylethanolamine N-methyltransferase (PNMT) from normal rats as a result of dialysis*

The thermal half-life of enzyme from normal rat adrenal supernatants dialyzed for various times is plotted against time of dialysis. The figure shows the precipitous decline in thermal half-life caused by dialysis; at 24 hr the half-life was reduced to a level comparable to that seen in hypophysectomized rats. Enzyme *activity,* however, remained constant and unaffected by dialysis; the shaded section of the figure is the mean and standard error of enzyme activity values obtained from the dialyzed or zero-time preparations and is intended to show that the activity of the transferase, in contrast to its thermal stability, was unaffected by dialysis.

TABLE 5

Effect of dialysis on thermal stability of phenylethanolamine N-methyltransferase

Adrenal supernatant from a control rat was prepared. A portion was dialyzed for 24 hr against 50 mM Tris, pH 7.4. The remainder ("fresh") was tested for thermal stability of the enzyme, and for stabilizing factor activity by mixing with adrenal supernatant from a hypophysectomized rat (50-μl portions of each supernatant). The samples were then heated to 50° for various times and frozen immediately thereafter. The next day, a similar set of experiments was carried out on the dialyzed material. At the conclusion of the experiment, all tubes were assayed for enzyme activity, which is expressed as milliunits per tube (100 μl of supernatant) ± standard error; this estimate was obtained from the triplicate determination of activity in the unheated preparations. Thermal half-life is expressed in the usual fashion, with the estimate of dispersion reflecting the standard deviation.

Group	Enzyme activity	$t_{1/2}$ at 50°
	munits/tube	*minutes*
Hypophysectomized	35.1 ± 0.3	2.41 ± 0.28[a]
Fresh control	94.3 ± 6.4	10.93 ± 0.64
Dialyzed control	109.8 ± 3.4	6.08 ± 0.60[a]
Hypophysectomized + fresh control	71.9 ± 1.5	8.82 ± 1.75[b]
Hypophysectomized + dialyzed control	71.7 ± 1.9	2.74 ± 0.13[a,c]

[a] $p < 0.0001$ compared with fresh control.

[b] Not significantly different from fresh control.

[c] Not significantly different from hypophysectomized preparation.

bilizing factor was bound to the enzyme, the antienzyme immunoadsorbent described in the preceding publication was employed (6). The a priori assumption was that adsorption of enzyme from the supernatant solution by the immobilized antibody should remove both the enzyme and the enzyme-bound stabilizing factor. Subsequent washing of the antibody-bound transferase might then dissociate the bound stabilizing factor from the enzyme and permit its recovery in partially purified form.

Accordingly, an adrenal supernatant preparation from control rats was passed over an antienzyme IgG immunoadsorbent column. The effluent, containing the bulk of the non-enzyme adrenal protein, was collected. The column was then washed, and fractions of the wash were collected. Portions of the effluent and the wash were tested for protein and phenylethanolamine *N*-methyltransferase activity. The bulk of the adrenal protein was removed by the fourth wash fraction; no enzyme was washed from the antibody column. When portions of the effluent and wash were tested for stabilizing factor activity by mixing with enzyme from hypophysectomized rats, a sharp peak of stabilizing factor activity was observed to emerge from the column in a single fraction (fraction 5). Addition of this fraction to enzyme from hypophysectomized rats restored the thermal half-life of the enzyme to control values (Fig. 4). These results, replicated twice additionally, strongly suggested that the stabilizing factor was bound to the transferase and could be dissociated from the antibody-bound enzyme.

While our results tend to confirm the existence of an endogenous stabilizing factor, there is no evidence that the increased susceptibility to thermal denaturation seen in enzyme from hypophysectomized animals is related to its accelerated proteolysis *in vivo*. The single most important experimental proof that the phenomena *in vitro* and *in vivo* are related requires the demonstration that purified stabilizing factor, when added to a mixture of purified phenylethanolamine *N*-methyltransferase protease and transferase, slows the degradation of the latter.

At present such proof is lacking. Nonetheless, additional criteria can be established that must also be satisfied before a relationship between thermal stability and proteolytic vulnerability *in vivo* can be proposed: (a) the stabilizing factor activity recovered from the antienzyme immunoadsorbent should be present in adrenal supernatants from control rats and absent from supernatant preparations from hypophysectomized rats; (b) dexamethasone or ACTH treatment of hypophysectomized rats, which inhibits proteolysis of the enzyme *in vivo* and restores its thermal stability, should also restore the stabilizing factor activity recovered from the immunoadsorbent columns.

To study these issues further, several experiments using the IgG immunoadsorbent columns were carried out. In the first set of

TABLE 6

Effects of ACTH or dexamethasone on thermal stability of phenylethanolamine N-methyltransferase in hypophysectomized rats

Animals received either ACTH (4 IU/day subcutaneously) or dexamethasone (1 mg/day intraperitoneally) for 3 days, beginning 10 days after hypophysectomy. In experiment I two hypophysectomized rats were used, one of which received ACTH. In experiment II rats (group sizes in parentheses) received ACTH or dexamethasone. In both drug-treated groups enzyme levels and thermal stability were elevated significantly. In experiment III brief dexamethasone treatment did not increase enzyme levels, and thermal stability was similarly unaffected. ACTH was effective in restoring both enzyme levels and thermal stability. Levels of statistical significance were obtained by the *F*-test comparison of the thermal rate constant and its standard deviation (experiment I) or by the *t*-test on the means and standard errors of the rate constants for each group (experiments II and III).

Experiment	Preparation	Enzyme activity	$t_{1/2}$ at 50°
		units/pr	*min*
I	Hypophysectomized	0.93	3.29 ± 0.30
	Hypophysectomized + ACTH	1.83	5.18 ± 0.56[a]
II	Control (2)	3.79 ± 0.01	21.69 ± 2.55
	Hypophysectomized (3)	0.75 ± 0.04[b]	5.78 ± 0.69[c]
	Hypophysectomized + ACTH (3)	1.94 ± 0.13[d]	12.64 ± 0.90[e]
	Hypophysectomized + dexamethasone	1.25 ± 0.08[f]	9.35 ± 0.95[g]
III	Control (3)	1.77 ± 0.06	13.22 ± 1.12
	Hypophysectomized (3)	0.44 ± 0.05[h]	4.10 ± 0.24[h]
	Hypophysectomized + ACTH (3)	0.81 ± 0.05[i]	8.18 ± 1.84[j]
	Hypophysectomized + dexamethasone	0.63 ± 0.06	4.27 ± 0.21

[a] $p < 0.001$.
[b] $p < 0.002$ compared with control.
[c] $p < 0.01$ compared with control.
[d] $p < 0.001$ compared with hypophysectomized.
[e] $p < 0.01$ compared with hypophysectomized.
[f] $p < 0.002$ compared with hypophysectomized.
[g] $p < 0.02$ compared with hypophysectomized.
[h] $p < 0.001$ compared with control.
[i] $p < 0.005$ compared with hypophysectomized.
[j] $p < 0.03$ compared with control.

experiments, adrenal supernatant preparations from control or hypophysectomized rats were passed over antienzyme IgG columns, which then were assayed for stabilizing factor activity (Fig. 5). The stabilizing factor peak present in control supernatants (Fig. 5A) was absent from supernatants from hypophysectomized rats (Fig. 5B). When hypophysectomized rats were treated with dexamethasone or ACTH, substantial increases in the levels and thermal stability of the enzyme were seen (Table 6). Treatment with these drugs also restored the stabilizing factor peak eluted from the antienzyme IgG columns (Fig. 5C and D). These experiments were replicated four additional times, with identical results.

Preliminary characterization of stabilizing factor. The stabilizing factor could usually be recovered in a single fraction from the IgG column. Spectrophotometric analysis of this fraction showed it to possess a sharp absorption maximum between 262 and 270 nm (Fig. 6), with a peak at 265 nm. The A_{260}:A_{280} ratio of the fraction was 1.23 and was higher than that seen for any other fraction.

These results, taken with those from the

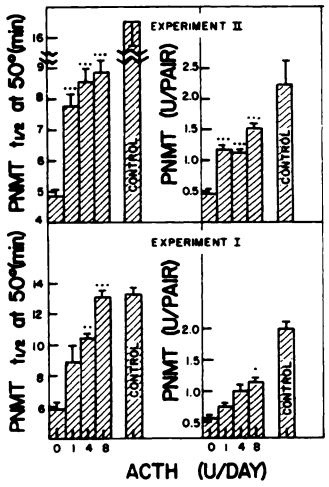

FIG. 3. Beginning 10 days after hypophysectomy, rats were treated for 3 (experiment I) or 7 (experiment II) days with various doses of ACTH subcutaneously. At the conclusion of the experiment the animals were killed and their adrenals were removed. Thermal denaturation studies were performed on the supernatants prepared in the usual way. The results showed a dose-dependent response in the thermal stability of phenylethanolamine *N*-methyltransferase (PNMT) which paralleled the increase in enzyme activity.

* Significantly greater than untreated hypophysectomized; $p < 0.01$.

** Significantly greater than untreated hypophysectomized; $p < 0.02$.

*** Significantly greater than untreated hypophysectomized; $p < 0.001$.

dialysis studies, suggest that the stabilizing factor is a small molecular weight substance that has an absorption maximum at 265 nm and binds to the transferase. The purine and pyrimidine ribonucleosides have absorption maxima in this region, and among these the compounds known to be involved in phenylethanolamine *N*-methyltransferase activity are cyclic 3',5'-AMP and *S*-adenosylmethionine. Cyclic AMP may be effective in increasing levels of this enzyme in hypophysectomized rats (8), while *S*-adenosylmethionine is the methyl donor in the phenylethanolamine *N*-methyltransferase reaction. *S*-Adenosylmethionine markedly increased the thermal stability of enzyme from hypophysectomized rats (Table 7). These results suggested that *S*-adenosylmethionine should be studied further to determine its possible role as the stabilizing factor for the enzyme. An extensive series of experiments, to be described in a subsequent report, was accordingly begun.

DISCUSSION

The studies described in this report demonstrate the existence of an endogenous compound that stabilizes phenylethanolamine *N*-methyltransferase against thermal denaturation. This compound appears to be a freezing-thawing labile, dialyzable substance that binds to the enzyme. The stabilizing factor is lost after hypophysectomy and can be restored by treatment of hypophysectomized rats with ACTH or dexamethasone.

The importance of this material, of course, is that it may play a role in regulating proteolysis of phenylethanolamine *N*-methyltransferase *in vivo*. While there is as yet no direct evidence in support of this hypothesis, several indirect and correlative associations make the idea tenable. First, transferase levels fall markedly after hypophysectomy; this is because the enzyme is degraded at a more rapid rate (6). Associated with the increased susceptibility of enzyme from hypophysectomized rats to proteolysis *in vivo* is an increased susceptibility to denaturation at 50°. ACTH or dexamethasone treatment, which restores enzyme levels by inhibiting proteolysis, also restores its thermal stability toward normal levels. Analysis of the ACTH dose-response curves shows an almost exact parallel between the restoration of enzyme levels and the restoration of thermal stability.

There is some evidence, albeit preliminary, that the stabilizing factor is a nucleotide-like compound, perhaps *S*-adenosylmethionine. When added to phenylethanolamine *N*-methyltransferase from hypophysectomized animals, *S*-adenosylmethionine stabilizes the enzyme against thermal denaturation. Little is known about the regulation of *S*-adenosylmethionine levels in the adrenal *in vivo*. Currently we are studying the effects of hypophysectomy and hormone administration on *S*-adenosylmethionine levels; obviously a necessary requisite is that these levels must be hormonally controlled if this compound is to be implicated as a stabilizing factor for the transferase.

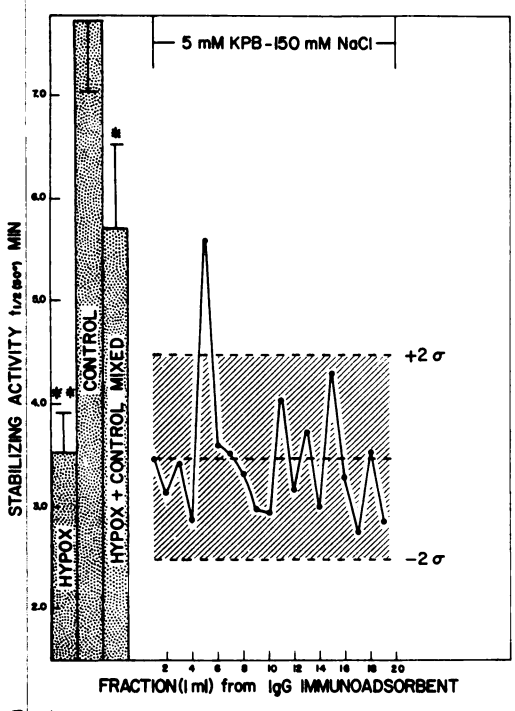

FIG. 4. *Stabilizing factor activity and thermal stability of fractions from rat adrenal supernatant applied to anti-phenylethanolamine N-methyltransferase immunoadsorbent gel*

Freshly prepared adrenal supernatant from control rats was applied to the antienzyme immunoadsorbent gel. Portions (50 μl) of the column effluent and wash (1-ml fractions) were then assayed for stabilizing factor activity by mixing with 50-μl portions of supernatant from hypophysectomized rats. Thermal stability curves at 50° were then obtained for each fraction. The thermal half-life was determined and plotted for each fraction. The three bars show the thermal half-life of enzyme from hypophysectomized rats, from control rats before application to the column, and a mixture of 50-μl portions of the two supernatants. Hypophysectomy significantly reduced the thermal stability of the enzyme relative to controls; mixing the control and hypophysectomized preparations partially restored thermal stability. The shaded portion of the IgG chromatogram is the mean ±2 SD range of thermal half-lives for all the fractions. Column fractions whose thermal half-lives lay outside the 2σ limits were considered to differ significantly (p at least < 0.05) from the remaining fractions, and were therefore presumed to contain stabilizing factor activity. Of the IgG column fractions, only fraction 5 lay outside this 2 σ limit. When a 50-μl portion of enzyme preparation from hypophysectomized rats was mixed with a 50-μl portion of fraction 5, the thermal half-life of the mixture was nearly identical with that obtained for the mixed control-hypophysectomized preparation (third bar). KPB, potassium phosphate buffer.

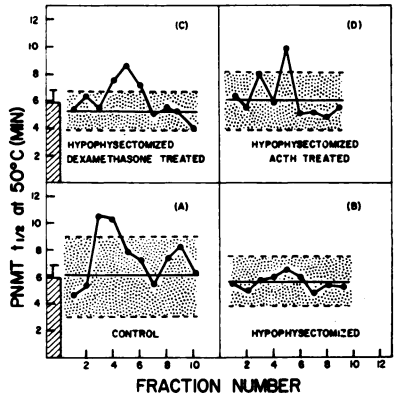

FIG. 5. *Stabilizing factor activity and thermal stability of control and treated and untreated hypophysectomized rats*

Ten days after hypophysectomy, three rats were treated with either no drug, 1 mg of dexamethasone, or 4 IU of ACTH daily for 7 days. They and an unoperated control rat were killed, and the adrenals were removed and homogenized in 1 ml of 50 mM Tris. Each supernatant preparation was applied to a separate 1-ml anti-phenylethanolamine N-methyltransferase (PNMT) immunoadsorbent column. The sample effluent and 1-ml fractions of the buffer wash were collected. The first 10 fractions from each column were assayed for stabilizing factor activity by mixing 50 μl of each fraction with 50 μl of a supernatant preparation from a hypophysectomized rat (adrenals homogenized in 5 ml/pair of 50 mM Tris). Thermal denaturation at 0, 3, and 5 min was carried out for each mixture, and at 0, 3, 5, and 7 min for a 100-μl sample of supernatant from the hypophysectomized rat. The thermal half-life ±2 SD of the hypophysectomized preparation alone is shown as a bar figure. For each chromatogram the mean thermal half-life of all fractions and the 2 SD limits were determined, and are represented by the shaded areas inside the dashed lines. For the control animal (A), two fractions showed a substantially greater thermal half-life than the remaining fractions. The mean ± SD value for these fractions was above the +2σ limits of the other fractions, indicating a significant (p at least < 0.05) stabilization of the enzyme from the hypophysectomized preparation. These fractions were presumed to contain the recoverable stabilizing factor activity. The chromatogram from the hypophysectomized rat (B) showed no stabilizing factor activity, while a significant peak of stabilizing factor activity was observed in the ACTH and dexamethasone-treated rats (C and D).

* $p < 0.01$ compared with hypophysectomized.
** $p < 0.001$.

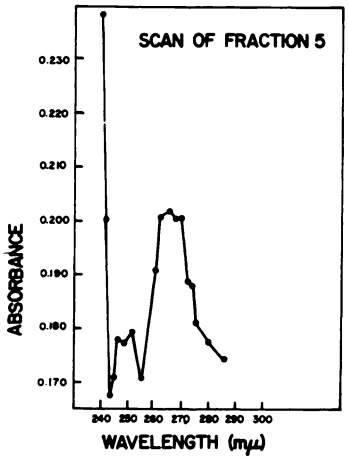

FIG. 6. *Plot of optical density vs. wavelength for stabilizing factor fraction eluted from antienzyme immunoadsorbent column*

TABLE 7

Effect of S-adenosylmethionine on thermal stability of phenylethanolamine N-methyltransferase

An adrenal supernatant preparation from hypophysectomized rats was used. *S*-Adenosylmethionine in various concentrations was added, and the tubes were heated at 50°. The samples were then assayed for residual enzymatic activity and the thermal half-lives were determined. To ensure that all samples would be assayed at a constant *S*-adenosylmethionine concentration, *S*-adenosyl[*methyl*-^{14}C]methionine was used in the heating experiments. At the conclusion of heating, enough radioactive *S*-adenosylmethionine was added so that each tube was assayed with 2 nmoles of *S*-adenosyl[*methyl*-^{14}C]methionine. Appropriate blanks and controls were tested to control for the effect of heating at 50° on *S*-adenosylmethionine. However, no increase in the blanks was seen as a result of heating the *S*-adenosylmethionine.

S-Adenosylmethionine	k	$t_{1/2}$ at 50°
μM	min^{-1}	min
0	−0.1190 ± 0.0056	5.82 ± 0.27
0.5	−0.1360 ± 0.0010	5.10 ± 0.41
1	−0.1255 ± 0.0106	5.52 ± 0.47
2	−0.0948 ± 0.0105[a]	7.31 ± 0.81
4	−0.0539 ± 0.0068[b]	12.87 ± 1.62

[a] $p < 0.03$ compared with control, by *F*-test.
[b] $p < 0.0001$ compared with control, by *F*-test.

At present, then, this work is suggestive that proteolysis of phenylethanolamine *N*-methyltransferase *in vivo* may be regulated by an endogenous substance that binds to the enzyme and stabilizes it. While little is known about control of enzyme proteolysis *in vivo*, a mechanism has been postulated (9) for the stabilization against proteolysis and also the thermal denaturation of tryptophan pyrrolase by tryptophan. Binding of tryptophan apparently alters the enzyme conformation in such a way that it is no longer a preferred substrate for proteolysis. This conformational change, if present, does not seem to involve the formation of enzyme multimers (9). Similarly, we were unable to detect any aggregate or multimer formation in phenylethanolamine *N*-methyltransferase isolated from control or hypophysectomized rats. Extensive polyacrylamide disc gel electrophoresis studies were carried out to look for such aggregates. Using the disc gel system previously described by us (10), we examined the electrophoretic migration of the transferase from control rats, hypophysectomized rats, and hypophysectomized rats treated with ACTH or dexamethasone. In every case a single, fast running peak ($R_F = 0.83$) was seen. No aggregates or subsidiary peaks were found in the control or drug-treated animals.

Currently our laboratory is investigating the several lines of inquiry that need elucidation before the control of phenylethanolamine *N*-methyltransferase degradation is understood. Among these are the regulation of *S*-adenosylmethionine levels by hormones, and the isolation and characterization of the protease(s) involved in the destruction of this enzyme. Subsequent reports in this series will deal with these topics.

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