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Regulation of Synthesis and Degradation of Rat Adrenal Phenylethanolamine *N*-methyltransferase

III. Stabilization of PNMT Against Thermal and Tryptic Degradation by S-Adenosylmethionine

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SUMMARY

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The regulation of phenylethanolamine *N*-methyltransferase degradation *in vivo* is mediated via adrenal glucocorticoids. The glucocorticoid inhibition of PNMT degradation is mediated via an endogenous adrenal stabilizing factor whose levels are controlled by glucocorticoids. A partially purified preparation of the endogenous stabilizing factor has an absorption maximum of 265 nm and migrates with S-adenosylmethionine and 3',5'-cyclic AMP on paper chromatography. S-adenosylmethionine is highly effective in stabilizing PNMT against both thermal and tryptic degradation, while cyclic AMP is ineffective as a stabilizing agent. S-adenosylhomocysteine, the demethylated product formed from S-adenosylmethionine during transmethylation stabilizes PNMT against tryptic but not thermal degradation. S-adenosylmethionine administration to hypophysectomized rats partially restores PNMT levels *in vivo* and enzyme thermal stability *in vitro*.

INTRODUCTION

Rat adrenal PNMT[1] is subject to dual regulation by adrenal glucocorticoids and by splanchnic innervation to the adrenal medulla (1). Our laboratory has recently published evidence that these two regulatory systems operate by different biochemical mechanisms. Adrenal glucocorticoids appear to control steady-state PNMT levels by inhibiting enzyme proteolysis, whereas splanchnic nerve impulses induce the *de novo* synthesis of PNMT molecules. When rats are hypophysectomized, PNMT degradation is markedly accelerated and *in vivo* enzyme levels fall. Administration of glucocorticoids or ACTH to hypophysectomized rats inhibits the acceleration of PNMT proteolysis and results in a restoration of enzyme levels to control values (2).

Subsequent studies from our laboratory have shown that hypophysectomy not only

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[1] The abbreviation used is: PNMT, phenylethanolamine *N*-methyltransferase.

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accelerates PNMT proteolysis *in vivo*, but increases the rate of PNMT denaturation *in vitro* as well. When adrenal gland extracts from hypophysectomized rats are heated to 50°, the half-life of PNMT is markedly reduced compared to that seen in control rat preparations. Hypophysectomy then, results in a PNMT molecule which is more vulnerable both to *in vivo* proteolysis and to *in vitro* denaturation (2).

Inquiry into the mechanism of these findings disclosed the existence of a substance which was present in normal rat adrenals, and which conferred thermal stability on PNMT. This material was lost after hypophysectomy, but could be restored by administration of ACTH or glucocorticoids to hypophysectomized rats. This substance, termed "endogenous stabilizing factor," appeared to act by binding directly to the PNMT molecule and, in so doing, stabilized the enzyme against thermal denaturation. Preliminary attempts to characterize this substance showed it to be a dialyzable, freeze-thaw labile material, a partially purified preparation of which had an absorption maximum at 265 nm (3).

In this report we describe our studies on the preliminary characterization of the "endogenous PNMT stabilizing factor" and its mechanism of action. The partially purified stabilizing factor migrated on paper chromatography with an R_f similar to those of S-adenosylmethionine and 3′,5′-cyclic AMP, two purine-containing compounds of potential importance in PNMT regulation. When these were tested for stabilization of PNMT against thermal denaturation, however, only S-adenosylmethionine exerted a significant stabilizing effect.

The proteolysis of PNMT by trypsin was also examined in an attempt to design a model system for understanding *in vivo* PNMT proteolysis. The endogenous adrenal stabilizing factor which stabilizes PNMT against thermal denaturation also protects the enzyme against tryptic proteolysis. Similarly, addition of S-adenosylmethionine to PNMT preparations markedly stabilizes the enzyme against tryptic attack. Thus the data from the thermal denaturation and tryptic proteolysis studies are consistent, and support the view that S-adenosylmethionine may be the "endogenous stabilizing factor" previously described and may play a role in the regulation of *in vivo* PNMT levels by controlling the proteolysis of the enzyme.

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 Lab Chow in a facility with automatic temperature and lighting regulation (12:12, light:dark, lights on at 6:00 a.m.). Hypophysectomized animals were given a commercial orange juice-water mixture *ad lib.*

Enzyme assays. Animals were killed by cervical dislocation. The adrenals were removed, cleaned of fat and homogenized in 5 ml per pair 50 mM Tris-HCl buffer, pH 7.4. The homogenates were centrifuged at 37,000 × *g* for 30 minutes. The supernatants were then dialyzed overnight at 4° against 400 volumes of Tris buffer to remove endogenous "stabilizing factors" (3). PNMT activity was assayed using 100 μl portions of the supernatant with phenylethanolamine as substrate (1).

Thermal denaturation studies. Studies on the thermal stability of PNMT were performed on the crude supernatants as previously described (3). At varying times after heating at 50°, PNMT preparations were removed and plunged into ice. Residual enzyme activity was determined and plotted as the natural logarithm of enzyme activity versus time of heating. 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 (Fig. 1). From this line, the slope and its standard deviation were derived. The thermal half-life of PNMT at 50° was computed by $t_{1/2} = \ln 2/k$.

Tests of stabilization of PNMT by various compounds. Two test systems were employed to study the effect of various compounds on PNMT thermal stability. Since hypophysectomy resulted in the loss of endogenous stabilizing factors *in vivo* (3),

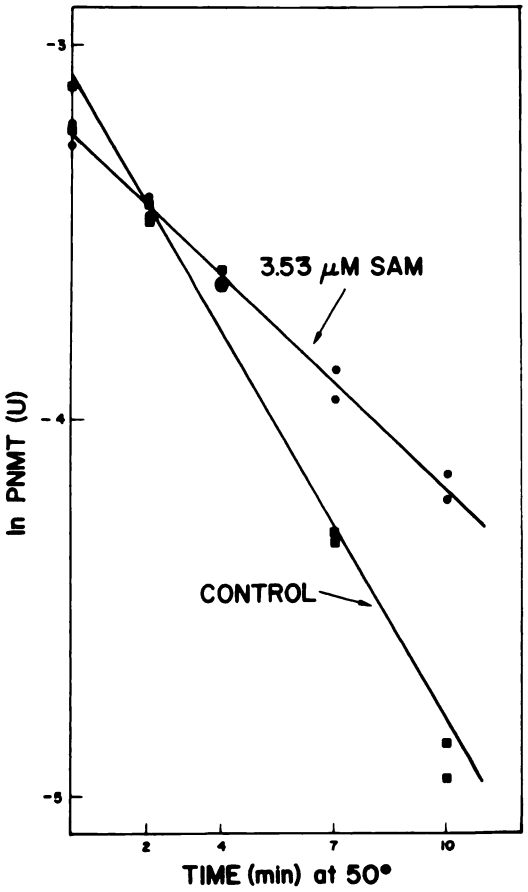

FIG. 1. *Thermal stability of dialyzed PNMT with and without SAM preincubation*

Dialyzed rat adrenal supernatants were prepared and subjected to thermal denaturation as described in METHODS. One set of samples was incubated at 50° in the presence of S-adenosylmethionine at a final concentration of 3.53 μM. At the conclusion of heating, residual PNMT activity was determined. Linear regression analysis was then performed. The following regression statistics were obtained. For the control (no SAM) group: $k = -0.1757 \pm .0094$ min^{-1}; $r^2_{(10)} = .977$ ($p < .0001$); $t_{1/2} = 3.95$ min. For the PNMT plus SAM group: $k = -0.936 \pm .0032$ min^{-1}; $r^2_{(10)} = .991$ ($p < .0001$); $t_{1/2} = 7.41$ min. The difference between the two curves is significant at $p <<<< .0001$. These curves are typical of those used throughout this report to investigate the effect of S-adenosylmethionine on the thermal denaturation of dialyzed PNMT.

a supernatant preparation of PNMT from hypophysectomized rats was used in the early experiments. In later experiments PNMT from normal rats was used after removing endogenous stabilizing factors by overnight dialysis. Dialysis reduced the thermal half-life of PNMT to that seen in hypophysectomized rats (3), although this procedure had no effect on the activity of the enzyme.

The putative stabilizing factors tested in this series of studies were S-adenosylmethionine, S-adenosylhomocysteine and 3',5' cyclic AMP. These compounds were added to tubes containing the enzyme preparation prior to incubation at 50°. The concentration ranges employed were: S-adenosylmethionine and S-adenosylhomocysteine, 0–4 μM, and cyclic AMP, 0–40 μM. When the stabilizing effects of S-adenosylmethionine were being studied the final concentration of each tube was adjusted to 4 μM before assaying for PNMT to ensure that each sample was assayed at a constant S-adenosylmethionine concentration. Adjustments in the S-adenosylhomocysteine concentration prior to assay were found to be unnecessary.

Whenever stabilization was observed by a given compound, an attempt was made to determine both an EC_{50} and a stabilization constant, K_s, for the compound under investigation. The EC_{50} was that concentration required to produce a half-maximal degree of stabilization. The stabilization constant, K_s, was determined by plotting $1/k$, the reciprocal rate constant of thermal or tryptic (see below) denaturation versus the reciprocal concentration of stabilizing agent, as described by Borchardt *et al.* (4). This was done because, when stabilization by a given compound was seen, a plot of the rate constant versus stabilizer concentration gave a rectangular hyperbola with the appearance of Michaelis-Menten kinetics. Accordingly the analogy was extended further and double-reciprocal plots of the Lineweaver-Burke type were made. From the regression line the k_{max}, analogous to V_{max}, and the stabilization constant, K_s, analogous to K_m were calculated. This procedure was used to determine K_s in both the thermal and tryptic experiments.

Tryptic stability. Studies on the tryptic stability of PNMT were performed on crude rat adrenal supernatants from which endogenous stabilizing factor(s) had been removed by dialysis. In the basic design,

100 μl portions of supernatant were added to tubes containing 190 μl of 50 mM Tris buffer, pH 8.6 and 1 μg of purified bovine trypsin (Sigma, St. Louis) in a volume of 10 μl. The proteolysis was then carried out in a temperature-controlled waterbath at 25°. Duplicate determinations at from 5 to 7 time points were carried out. At the appropriate intervals, tryptic degradation was terminated by transfer of the incubation mixture to a 200 μl solution containing 2 μg soybean trypsin inhibitor (Sigma) in 50 mM potassium phosphate buffer, pH 7.9. Residual PNMT activity was determined in the usual manner and was plotted as the natural logarithm of enzyme activity versus time of exposure to trypsin. This plot yields a straight line whose slope corresponds to the apparent first-order rate constant, k, of tryptic proteolysis. The line was determined by linear regression analysis. From this line the slope and its standard deviation were computed. The half-life of PNMT in trypsin was computed by $t_{1/2} = \ln 2/k$.

The effects of S-adenosylmethionine or S-adenosylhomocysteine on PNMT tryptic stability was determined by adding varying concentrations of each compound to enzyme preparations in the presence of trypsin. The concentration range for both compounds was 0–4 μM. Residual PNMT activity was determined in the usual manner when S-adenosylmethionine was being studied, with the final S-adenosylmethionine concentrations being adjusted to 4 μM before assaying for PNMT. S-adenosylhomocysteine is a potent competitive inhibitor of PNMT activity (5). Therefore when S-adenosylhomocysteine was present, activity of PNMT was determined by adjusting the final S-adenosylmethionine concentration of each tube to 8 μM before assaying for the enzyme.

Paper chromatography of the endogenous stabilizing factor. The endogenous adrenal stabilizing factor was partially purified by IgG immunoadsorption chromatography as previously described (3). A pair of adrenals from a male Sprague-Dawley rat was homogenized in 1.0 ml of 50 mM Tris buffer, pH 7.4. The homogenate was centrifuged at 37,000 × g for 30 minutes at 4°. The supernatant was applied to a 1-cc column of Sepharose 4B to which purified anti-PNMT IgG had been covalently bound (2). Following sample application, the column was washed with 20 ml of 150 mM NaCl-5 mM K_2HPO_4 buffer, pH 7.4 and 1 ml fractions were collected.

Our previous studies had shown that the endogenous stabilizing factor was eluted from the immunoadsorbent column after 4–6 ml of buffer (3). Accordingly these fractions were assayed for endogenous stabilizing factor activity by mixing with an adrenal supernatant preparation from hypophysectomized rats. Samples were heated at 50° for varying periods of time and the ability of each column fraction to protect PNMT against thermal denaturation was determined. As previously described (3), the bulk of endogenous stabilizing factor was found in IgG column fraction 5. This fraction was chromatographed in a descending system on Whatman #3 paper. Two solvent systems, acetic acid:butanol:water (1:2:1) and isopropranol:hydrochloric acid:water (68:17.6:14.4) were used. S-adenosylmethionine, 3′,5′-cyclic AMP, S-adenosylhomocysteine and a variety of purine and pyrimidine nucleosides and bases were also run as standards.

Statistical analysis. Differences in the slopes of individual regression lines were analyzed for statistical significance by the method of Sokal and Rohlf (6), as previously described (2, 3). All tests of significance were done using the rate constant and its standard deviation.

RESULTS

Paper chromatography of the endogenous stabilizing factor. Paper chromatography was performed in an effort to characterize the endogenous stabilizing factor. Several purine and pyrimidine ribosides, ribotides and bases were tested in the paper chromatographic system described earlier.

Of the standards tested, only S-adenosylmethionine and cyclic AMP had an R_f similar to that of the endogenous stabilizing factor. The R_f of S-adenosylhomocysteine was widely separated from that of the stabilizing factor. When S-adenosylmethionine was tested for its effect on the thermal half-life of PNMT, a nearly two-fold in-

crease in thermal stability of the enzyme was seen. Cyclic AMP, on the other hand, gave barely significant degrees of stabilization at concentrations up to 40 μM, levels substantially in excess of those usually reported in the adrenal (7). Despite the fact that S-adenosylhomocysteine did not migrate with the endogenous stabilizing factor on the paper chromatographic system, the effect of this compound on PNMT thermal stability was also tested: S-adenosylhomocysteine had no effect on PNMT thermal stability at any concentration tested.

Dose response effects of S-adenosylmethionine on PNMT thermal stability. Two preliminary experiments had shown that S-adenosylmethionine stabilized PNMT against thermal denaturation. To explore the stabilizing effects of S-adenosylmethionine further, a series of dose-response studies were undertaken. PNMT was prepared from adrenals of hypophysectomized animals or from control rats after dialysis. Eight separate dose response experiments were performed; the results of these experiments are summarized in Fig. 2. At maximally effective concentrations, S-adenosylmethionine caused a greater than two-fold increase in the thermal half-life of PNMT (2.09 ± .06-fold increase, eight experiments). A plot of 1/k versus 1/[SAM] for each experiment yielded a straight line from which the stabilization constant, K_s, could be determined. The mean K_s value was estimated to be 0.48 ± .05 μM; this compares with the K_m of SAM for PNMT of 2.75 μM in unpurified preparations from rat adrenals (2).

Stability of PNMT to tryptic proteolysis. While the effects of S-adenosylmethionine, S-adenosylhomocysteine and cyclic AMP on PNMT thermal stability are of considerable interest, their meaning and significance lies in the role these compounds may or may not play in regulating *in vivo* PNMT proteolysis. The ideal way to study this is to incubate these compounds with purified PNMT and purified specific PNMT protease and determine their effect on PNMT degradation. Since we have not yet isolated the PNMT protease, the use of trypsin as a proteolytic enzyme seemed a valid alternative model system in which we could test our hypotheses. Accordingly, we examined the tryptic proteolysis of PNMT along the same parameters used in our thermal de-

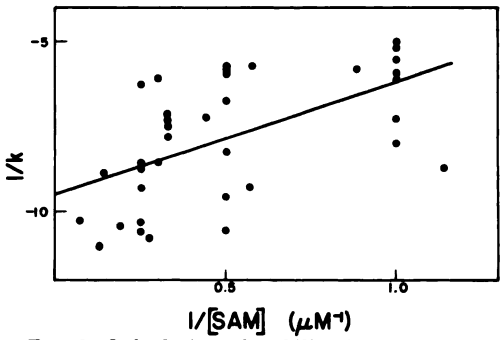

FIG. 2. *Calculation of stabilization constants for S-adenosylmethionine in the PNMT thermal denaturation system*

Over the course of several months eight separate experiments were carried out testing the ability of various concentrations of SAM to stabilize PNMT against thermal denaturation. In each experiment, SAM significantly stabilized PNMT in a manner suggestive of Michaelis-Menten kinetics, i.e., a plot of the rate constant, k, versus SAM concentration gave a rectangular hyperbola, while a plot of $1/k$ versus 1/[SAM] gave a straight line. We used this analogy to Lineweaver-Burk kinetics, described by Borchardt (4), in the following way: The intersection of the line with the ordinate gives $1/k_{max}$, where k_{max} is the maximum rate constant of stabilization (analogous to V_{max} in the Michaelis-Menten system). The slope of the line is k_s/k_{max}, where K_s is the stabilization constant, analogous to K_m. The maximum predicted stabilization may then be calculated by $t_{1/2\,max}/t_{1/2^\circ}$ where $t_{1/2^\circ}$ is the PNMT half-life in the absence of SAM (3.46 ± .11 min, N = 8).

For each experiment k_{max}, $t_{1/2\,max}$, and K_s were derived. The mean ± SEM of these measures was then obtained. The above figure shows the plot of all the thirty-six rate constants obtained from the eight experiments graphed as $1/k$ versus 1/[SAM]. The data have not been corrected for assay variation or changes in baseline half-life. The line was obtained by linear regression analysis ($r^2_{36} = .557$, $p < .0003$). K_s, k_{max}, $t_{1/2\,max}$ and the maximum stabilization for the composite curve were calculated and compared with the mean values obtained from the eight experiments and are shown below:

	Calculated (from figure)	Mean ± SEM of eight experiments
K_s	0.434 μM	0.480 ± 0.054 μM
k_{max}	$-.1165\ min^{-1}$	$-.1017 \pm .0043\ min^{-1}$
$t_{1/2\,max}$	6.57 min	6.81 ± .29 min
Maximum stabilization	1.9-fold	2.09 ± .06-fold

TABLE 1

Effect of S-adenosylmethionine and S-adenosylhomocysteine on PNMT tryptic stability

The tryptic stability of PNMT was determined in the presence of S-adenosylmethionine and S-adenosylhomocysteine. The experiments were performed as described in METHODS; different enzyme preparations were used, and the experiments were carried out on different days. When S-adenosylhomocysteine was present, residual PNMT activity was measured in the presence of 8 μM SAM; this was sufficient to overcome competitive inhibition of the enzyme by SAH.

Stabilizing compound	Concentration	k	$t_{1/2}$
	(μM)	(m^{-1})	(min)
S-Adenosylmethionine	0	−.2983 ± .0343	2.32 ± .27
	8	−.2098 ± .0225*	3.30 ± .35
	16	−.1993 ± .0185**	3.48 ± .32
S-Adenosylhomocysteine	0	−.2461 ± .0127	2.82 ± .15
	0.5	−.1572 ± .0091***	4.41 ± .26

* $p < .025$ greater than control.
** $p < .01$ greater than control.
*** $p < .001$ greater than control.

naturation experiments.

Effects of dialysis on PNMT tryptic stability. Dialysis of adrenal preparations from control rats removes endogenous stabilizing factors which stabilize PNMT against thermal denaturation. To test whether this same procedure alters the tryptic stability of PNMT, adrenal preparations from control rats were dialyzed against 50 mM Tris buffer, pH 7.4. Dialysis reduced the tryptic half-life of PNMT from 16 to 4 minutes, indicating that the stability of PNMT to tryptic proteolysis, like the stability of the enzyme to thermal denaturation could be reduced by the removal of endogenous stabilizing factors. As has been previously reported (2, 3), dialysis had no effect on PNMT catalytic activity.

Effects of S-adenosylmethionine on PNMT tryptic stability. The ability of S-adenosylmethionine to stabilize PNMT against tryptic proteolysis was examined. The presence of S-adenosylmethionine markedly stabilized the enzyme to tryptic attack (Table 1). Stabilization was observed as a typical rectangular hyperbola dose-response relationship, similar to those seen in the thermal denaturation experiments. These and a number of additional experiments were analyzed by the double-reciprocal method described earlier. The results of one such experiment are shown in Fig. 3. In this experiment, S-adenosylmethionine stabilized PNMT about 3.2-fold compared to control. The stabilization constant, K_s, for S-adenosylmethionine was estimated to be 0.49 μM, very close to that estimated for S-adenosylmethionine in the thermal denaturation system.

Effects of S-adenosylhomocysteine on PNMT tryptic stability. When S-adenosylhomocysteine was tested for its effect on PNMT tryptic stability (Table 1), it exerted a significant stabilizing effect which closely resembled that observed with S-adenosylmethionine. This finding was in marked contrast to the lack of a stabilizing effect seen with S-adenosylhomocysteine in the thermal denaturation system. The observed tryptic stabilization was 1.6-fold at the rather low S-adenosylhomocysteine concentration chosen for study. A dose response study was conducted in order to assess the relative binding affinities of S-adenosylmethionine and S-adenosylhomocysteine with respect to PNMT tryptic stabilization and the maximal level of stabilization exerted by both compounds (Fig. 4). It is clear that the maximal levels of stabilization as determined from the Lineweaver-Burke plot are nearly identical for the two compounds. The stabilization constants of the two differed somewhat, however, with S-adenosylhomocysteine having a greater affinity for PNMT than S-adenosylmethionine. This is not surprising in view of the work of Deguchi and Barchas (5), who showed the K_i of S-adenosylhomocysteine to be considerably lower than the K_m of S-adenosylmethionine for PNMT and a variety of other methyltransferases.

Effect of S-adenosylmethionine on in

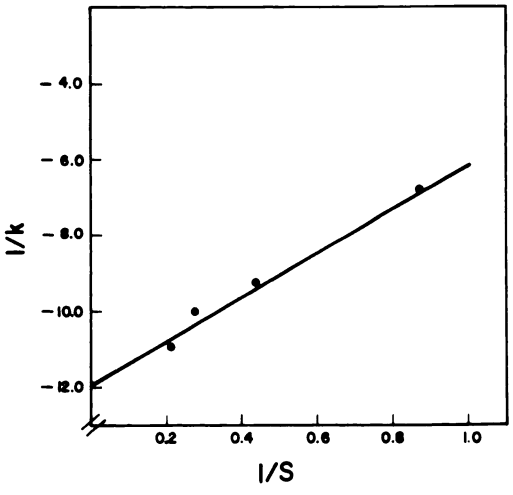

FIG. 3. *Lineweaver-Burke plot of SAM dose-response curve with respect to PNMT tryptic stability*

The tryptic degradation of PNMT follows Michaelis-Menten kinetics. Accordingly the effect of varying concentrations of SAM on PNMT tryptic stability was tested. The reciprocal rate constant for each tryptic digestion curve was plotted against reciprocal SAM concentration, as described in Fig. 4. In the tryptic system, as in the thermal studies, K_s, k_{max} and $t_{1/2\,max}$ were obtained.

In the study shown here, adrenal glands from control rats were pooled and homogenized in 5 ml per pair 50 mM Tris buffer, pH 7.4. To remove endogenous stabilizing factors, the adrenal supernatants were dialyzed overnight against 4 l of 50 mM tris buffer, pH 7.4. To portions of the dialyzed supernatants, SAM in varying concentrations were added to samples which were then subjected to tryptic digestion. Linear regression analysis gave the following: $k_{max} = -0.0836$, $t_{1/2\,max} = 8.28$ min., K_s SAM = 0.491 μM. These values are typical of those seen for SAM with respect to stabilization of PNMT against tryptic degradation.

vivo PNMT levels. The results of the studies described here all suggested that S-adenosylmethionine might play a significant role in regulating *in vivo* PNMT levels. To test this proposition further, hypophysectomized rats were treated with S-adenosylmethionine or with dexamethasone. This latter drug is a potent synthetic glucocorticoid which restores PNMT levels in hypophysectomized rats by inhibiting the proteolysis of the enzyme (3). The results of this study (Table 2) showed that S-adenosylmethionine was effective in elevating PNMT levels in hypophysectomized rats. The thermal stability of PNMT was determined at the same time; here too SAM administration caused a significant increase in the thermal half-life of the enzyme. Thus the results we have been obtaining *in vitro* are also seen *in vivo* when SAM is administered to hypophysectomized rats.

DISCUSSION

This report describes the stabilization of PNMT against thermal and tryptic denaturation by S-adenosylmethionine and S-adenosylhomocysteine. Both compounds markedly stabilize the enzyme against tryptic proteolysis, while SAM is highly effective as a stabilizer against thermal denaturation as well.

The significance of these findings is that they may provide clues as to how PNMT is

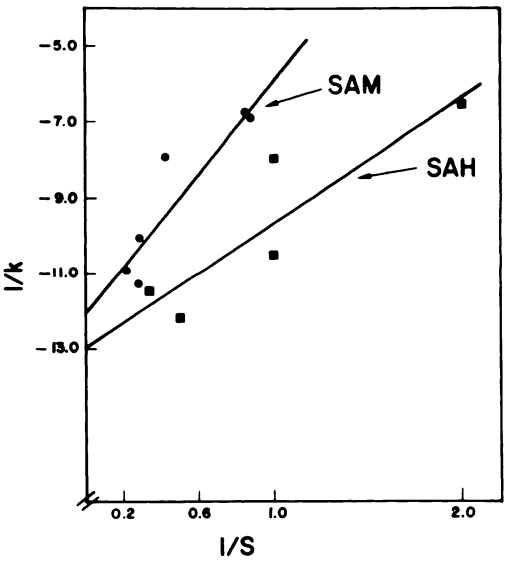

FIG. 4. *Lineweaver-Burke plot of SAM and SAH dose-response curves with respect to PNMT tryptic stability*

Dialyzed rat adrenals were prepared as described in METHODS. To portions of the dialyzed supernatants, SAM, or SAH was added in varying concentrations; samples were then subjected to tryptic digestion as described in METHODS. Residual PNMT activity and rate constants, k, were determined as described in METHODS. As shown in Figs. 2 and 3, the data may be transformed to a Lineweaver-Burke plot. From such a plot, K_s, k_{max}, and $t_{1/2\,max}$ may be obtained. In this study the following parameters were determined: SAH: $k_{max} = -0.0774\ m^{-1}$, $K_s = 0.257$ μM, $t_{1/2\,max} = 8.95$ min. SAM: $k_{max} = -0.0830\ m^{-1}$, $K_s = 0.520$ μM, $t_{1/2\,max} = 8.35$ min.

TABLE 2

Effect of SAM administration on PNMT levels and thermal half-life

Group	PNMT	Thermal Stability (50°)	
		k	$t_{1/2}$
	(units/pr)	*(m^{-1})*	
Hx	0.409 ± .056	−0.2437 ± .0367	3.13 ± .49 min
Hx + SAM	0.627 ± .047*	−0.1563 ± .0136[a]	4.64 ± .50 min
Hx + DEX	0.950 ± .079**	−0.1200 ± .0071[b]	5.85 ± .39 min
Control	2.039 ± .134	−0.0979 ± .0078[c]	7.30 ± .55 min

* $p < .02$ differs from hypophysectomized (Hx).
** $p < .001$ differs from hypophysectomized.
[a] $p < .025$ differs from hypophysectomized.
[b] $p < .008$ differs from hypophysectomized.
[c] $p < .003$ differs from hypophysectomized.

regulated *in vivo*. Previous studies have shown the existence of two distinct modes of regulation of adrenal PNMT: 1) induction of *de novo* enzyme synthesis by splanchnic nerve impulses and 2) inhibition of enzyme degradation by adrenal glucocorticoids (1, 2), acting, apparently, through an endogenous stabilizing factor. Our subsequent work has been directed at determining the nature of this stabilizing factor, its mechanism of *in vitro* action, whether it plays a role in *in vivo* PNMT regulation, and if more than one "stabilizing factor" exists.

1. *Nature of the stabilizing factor.* Immunoabsorption chromatography, in which anti-PNMT antibody was coupled to Sepharose 4B (3), was used to isolate the stabilizing factor. Paper chromatography with a variety of nucleotides, nucleosides and purine and pyrimidine bases showed the endogenous stabilizing factor ran with cyclic 3′,5′-AMP and with S-adenosylmethionine. Of these compounds, only S-adenosylmethionine significantly stabilized the enzyme against thermal denaturation. In an effort to construct a model system more appropriate to *in vivo* enzyme proteolysis, we began studying the stabilization properties of S-adenosylmethionine against tryptic destruction of PNMT. The results were identical to those seen in the thermal denaturation system, and suggested a potentially important *in vivo* regulatory role for S-adenosylmethionine in maintenance of intracellular PNMT levels.

2. *Mechanism of SAM stabilization of PNMT.* There seemed to be two possible mechanisms whereby SAM could stabilize PNMT against *in vivo* proteolysis. In the first case binding of SAM to PNMT would bring about a change in the tertiary conformation of the enzyme, making it a less vulnerable substrate for intracellular proteolysis. The second possibility involves formation of a methylated enzyme, and would be brought about by transfer of the methyl group from S-adenosylmethionine to PNMT, possibly by esterification of free carboxyl groups (8), again resulting in a more stable enzyme configuration against proteolysis.

The experiments with S-adenosylhomocysteine were undertaken to test these possibilities. If a methylated enzyme was being formed, then S-adenosylhomocysteine would not be expected to be an effective stabilizing agent. On the other hand, if binding without enzyme methylation was the mechanism, S-adenosylhomocysteine might be an even more effective stabilizer, since it has a higher affinity for PNMT than does S-adenosylmethionine (5). The results of these experiments showed S-adenosylhomocysteine was a highly effective stabilizer against tryptic denaturation of PNMT, but not against thermal denaturation. From these data, then, it seems reasonable to make the following conclusions: a) both S-adenosylmethionine and S-adenosylhomocysteine are effective stabilizers; b) the mechanism of stabilization probably does not involve formation of a methylated enzyme intermediate, and may involve a conformational change in the enzyme induced by direct binding of the stabilizer; c) since S-adenosylhomocysteine does not stabilize PNMT against thermal denaturation,

it is probably not the "endogenous stabilizing factor" described in our earlier reports. However, this does not rule out a potential role for SAH in PNMT regulation *in vivo*.

3. *Does S-adenosylmethionine play a role in regulating PNMT levels in vivo?* If this were the case, then administration of S-adenosylmethionine to hypophysectomized rats should increase PNMT levels by inhibiting the accelerated proteolysis of the enzyme. We have obtained confirmation of the first part of this prediction (Table 2) by showing that S-adenosylmethionine administration partially restores PNMT levels, and also elevates the thermal half-life of the enzyme in hypophysectomized rats. We are now testing the second part of the hypothesis by measuring PNMT turnover in hypophysectomized rats given S-adenosylmethionine.

4. *Role of other compounds as potential stabilizers of PNMT degradation.* While it seems likely that SAM and the stabilizing factor are identical, additional evidence to confirm this point is needed. Moreover, we have not yet established that SAM is the sole endogenous stabilizing factor. Other compounds which bind to PNMT and whose levels fall after hypophysectomy are also appropriate candidates for consideration. The most logical group of compounds to test in this capacity are the biogenic amine substrates of PNMT, and the reaction product, epinephrine. Both the substrates and the product bind to PNMT, presumably at different sites (9). The levels of epinephrine, but not norepinephrine, fall after hypophysectomy (10).

We have carried out an extensive series of studies on the role of biogenic amine substrates in the stabilization of PNMT. Our results, which are described in the accompanying report (11) point to a probable regulatory role for substrate, but not for product, as well as for SAM, and indicate that both substrate and SAM may work together in regulating PNMT degradation.

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