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FORMATION OF PHENYLETHANOLAMINE *N*-METHYL TRANSFERASE COMPLEXES AS AN INTERMEDIATE OF THE METHYLATION OF NOREPINEPHRINE

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Abstract—The *N*-methylation of norepinephrine to form epinephrine involves the transient formation of a ternary complex involving *S*-adenosylmethionine, norepinephrine and *N*-methyl transferase (PNMT). Finding this complex suggests the mechanism and sequence of methylation of norepinephrine.

EPINEPHRINE synthesis involves the transfer of a methyl group from *S*-adenosylmethionine to the amino nitrogen of norepinephrine.[1] The reaction is catalyzed by the enzyme phenylethanolamine-*N*-methyl transferase (PNMT).[2] This enzyme is found in the supernatant fraction of adrenal homogenates.[1] Little is known as yet about the mechanism by which PNMT transfers a methyl group from donor to substrate. Connett and Kirshner[3] have reported that PNMT is a two-substrate enzyme with random binding. They also found that PNMT preferred *S*-adenosylmethionine as the first substrate to bind, since the enzyme was most efficient with high *S*-adenosylmethionine and low norepinephrine concentrations.

These experiments attempted to develop a method for the detection of a possible complex of PNMT and its substrates, and to examine the necessary conditions for complex formation. We used the millipore filter assay on purified PNMT from rat adrenal medulla to study the binding reaction between PNMT and its two substrates.

We found that PNMT forms a ternary complex with *S*-adenosylmethionine and norepinephrine, which is retained by the millipore filter. Binary complexes between the individual substrates and PNMT can also be formed. Inhibitors of the methylation of norepinephrine revealed that *S*-adenosylmethionine and norepinephrine bind to two different sites on the enzyme molecule. This led us to postulate a possible sequence for the methylation reaction.

EXPERIMENTAL

Materials and methods

PNMT was isolated and purified by modification of a procedure previously described.[2] The adrenals from 40–50 rats were removed and the capsule was excised. The tissues were homogenized in 0·32 M sucrose in 0·05 M phosphate buffer, pH 7·9. After centrifugation at 10,000 *g* for 1 hr, the pellet was discarded. The supernatant

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was adjusted to pH 5 with sodium bicarbonate and centrifuged at 10,000 *g* for 20 min to remove the precipitated proteins. The pH of the supernatant was first adjusted to 7·0, and the supernatant then layered on a Sephadex G-150 column which was eluted with 0·05 M phosphate buffer, pH 6·8, containing 1 mM dithiothreitol (DTT) and 5% glycerol. The active fractions were pooled and concentrated with a UM-10 membrane in an Amicon concentrator. The resultant protein solution was passed through a Whatman DE-52 anion exchange column, equilibrated with 0·05 M Tris–HCl buffer, pH 7·4, containing 1 mM DTT and 5% glycerol. The proteins were eluted with a linear gradient of NaCl from 0 to 0·75 M in the same buffer. After being pooled, the active eluates were concentrated as before. This procedure resulted in over 100-fold purification of the enzyme. The enzyme was free of contaminating proteins as evident from gel electrophoresis of the purified preparation. A more detailed description of the purification procedure will be published elsewhere.* ^{14}C-*S*-adenosylmethionine (specific activity 30 μc/m-mole) and ^{3}H-norepinephrine (specific activity 10 c/m-mole) were purchased from New England Nuclear Corp., Boston, Mass. *S*-adenosylmethionine was obtained from the Sigma Chemical Co., St. Louis, Mo., and norepinephrine from the Regis Chemical Co., Chicago, Ill. *p*-Chloromercuribenzoate (PCMB) was purchased from Nutritional Biochemical Corp., Cleveland, Ohio. 5,6-Dimethyl-2-aminobenzimidazole was a gift from Dr. Harry Green of Smith, Kline & French Laboratories. All other chemicals used in this study were reagent grade. Millipore filters, HA 0·45 μ pore size, were from the Millipore Filter Corp., Bedford, Mass.

Millipore filter assay

The reaction mixture contained in a total volume of 0·23 ml: 50 μmoles phosphate buffer (pH 7·9), 1 μmole DTT, 12 μg of purified PNMT protein. In addition, *S*-adenosylmethionine and norepinephrine, either radioactive or cold, were added as detailed in the legends of the figures and tables. *S*-adenosylmethionine was purified before use by the procedure of Shapiro and Ehninger.[4] The reaction was initiated by addition of radioactive substrate. After incubation for 3 min at 0° or 37° the reaction was terminated by the addition of 3 ml of 0·05 M cold phosphate buffer (pH 7·9). The diluted reaction mixture was immediately passed through a millipore filter under low suction. The filter was washed three times with 3 ml of the same buffer, dried and counted with toluene phosphore. Counting efficiencies were 21 per cent for ^{3}H and 75 per cent for ^{14}C.

RESULTS

When ^{3}H-norepinephrine, *S*-adenosylmethionine and PNMT are incubated in buffer and filtered through a millipore filter membrane, the radioactivity is retained on the filter. The results in Table 1 show that PNMT binds norepinephrine and *S*-adenosylmethionine to form a ternary complex. Maximal binding of ^{3}H-norepinephrine requires the presence of *S*-adenosylmethionine. However, an excess of *S*-adenosylmethionine, above 30 nmoles, decreases the ^{3}H-norepinephrine retained on the filter. Sulfhydryl reagents, such as DTT or mercaptoethanol, did not stimulate formation of this complex. The retained radioactivity in the presence of excess DTT

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is actually lower than the control. In the absence of enzyme, retained ^{3}H-norepinephrine is negligible. All the values in the tables and figures have been corrected for background radioactivity.

TABLE 1. REQUIREMENTS FOR THE BINDING OF ^{3}H-NOREPINEPHRINE TO PNMT*

Incubation media	^{3}H-norepinephrine bound (counts/min)
Complete system	57,600
Minus SAM	26,300
Excess SAM	38,800
Plus DTT	7500
Plus Mg^{2+}	55,000

* Purified PNMT (24 μg) was incubated in 50 mM phosphate buffer (pH 7·9), 30 nmoles of *S*-adenosylmethionine (SAM), 12 nmoles of ^{3}H-norepinephrine and 1 μmole of DTT. When specified, excess S-adenosylmethionine concentration was 60 μM and excess DTT was 10 mM; the concentration of Mg^{2+} was 10 μmoles. After 3 min of incubation at 37°, the reaction mixture was immediately passed through a millipore filter. The filter was washed with phosphate buffer, dried and counted with toluene phosphore.

The time course of ^{3}H-norepinephrine binding to PNMT in the absence of *S* adenosylmethionine is illustrated in Fig. 1. Most of the binding of ^{3}H-norepinephrin occurs in 5 min at 37°. Thereafter, the binding reaction proceeds at a slower rate. Binding also occurs at 0°. However, the rate and total extent of binding is more than twice as great at 37° than at 0°.

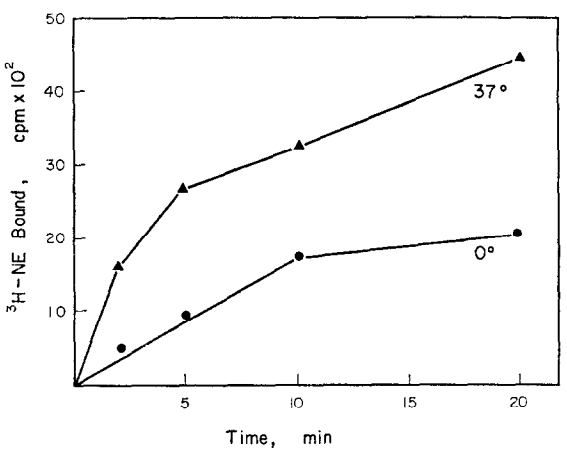

FIG. 1. Purified enzyme was incubated for various periods of time as indicated at either 0° or 37° The concentration of ^{3}H-norepinephrine was 12 $\times$ 10^{-6} M. Samples were processed as described in Table 1.

The rate of complex formation of ^{14}C-*S*-adenosylmethionine and PNMT is shown in Fig. 2. This binding occurs only at 0°. Approximately half of the total ^{14}C-*S*-adenosylmethionine–PNMT complex is formed in 2–5 min of incubation. Addition of cold norepinephrine had no significant stimulatory effect on this complex. When the

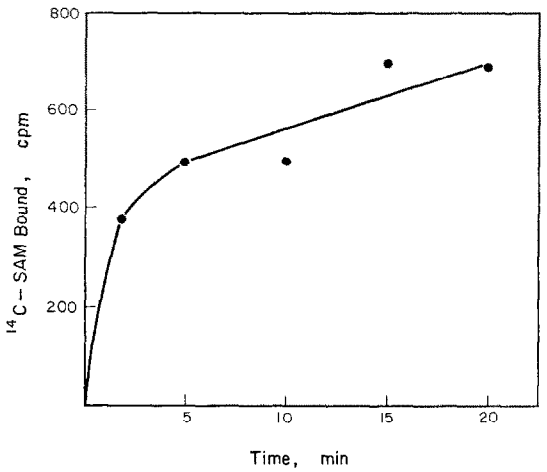

FIG. 2. Purified PNMT was incubated at 0° with 30 nmoles of ^{14}C-*S*-adenosylmethionine for various periods of time. Samples were processed as described under Table 1.

reaction mixture was incubated at 37°, very little ^{14}C-*S*-adenosylmethionine radioactivity was retained on the millipore filter. Addition of sulfhydryl reagents such as DTT, or of cold norepinephrine did not stabilize the complex at 37°. This suggested that the binding of ^{14}C-*S*-adenosylmethionine to the enzyme is more labile than the binding of norepinephrine.

TABLE 2. EFFECT OF ^{3}H-NOREPINEPHRINE CONCENTRATION ON COMPLEX FORMATION*

Norepinephrine concn (nmole)	^{3}H-norepinephrine bound (counts/min)
6	18,800
18	37,400
36	48,600

* PNMT was incubated with various concentrations of ^{3}H-norepinephrine. At the end of 3 min of incubation at 37°, the samples were processed as described in Table 1.

The experiments shown in Table 2 examine the relationship between the concentration of ^{3}H-norepinephrine and complex formation. The amount of ^{3}H-norepinephrine binding to the enzyme at 37° increased when the concentration of norepinephrine was increased. The concentration of norepinephrine for half-maximal complex formation was calculated as 24 nmoles for 12 μg of enzyme protein.

The effect of the PNMT concentration on the binding of ^{3}H-norepinephrine is

illustrated in Table 3. In the presence of a fixed amount of ^{3}H-norepinephrine, complex formation was linearly dependent on enzyme concentration.

TABLE 3. EFFECT OF PNMT CONCENTRATION ON COMPLEX FORMATION*

Enzyme protein (μg)	^{3}H-norepinephrine bound (counts/min)
4	704
8	1910
16	3230

* Varying amounts of purified PNMT were incubated for 3 min at 37° with 6 nmoles of ^{3}H-norepinephrine. Samples were processed as described in Table 1.

We have also examined the influence of a number of agents which may inhibit the *N*-methylation of norepinephrine. PNMT has been shown to be a sulfhydryl enzyme.[3] We tested the effect of a sulfhydryl blocking agent, *p*-chloromercuribenzoate, on the binding of ^{3}H-norepinephrine and ^{14}C-*S*-adenosylmethionine to PNMT. As shown in Table 4, PCMB inhibited binding of ^{3}H-norepinephrine and ^{14}C-*S*-adenosylmethionine to the enzyme. Inhibition of binding by PCMB at 1×10^{-4} M was 34 per cent for ^{3}H-norepinephrine at 37° and 45 per cent for ^{14}C-*S*-adenosylmethionine at 0°. Addition of 5,6-dimethyl-2-aminobenzimidazole, an inhibitor of the *N*-methylation *in vitro*[5] at 1×10^{-5} M concentration, blocked the binding of ^{14}C-*S*-adenosylmethionine to PNMT to the extent of 63 per cent. However, 5,6-dimethyl-2-aminobenzimidazole did not inhibit ^{3}H-norepinephrine binding.

TABLE 4. EFFECT OF PCMB AND 5,6-DIMETHYL-2-AMINOBENZIMIDAZOLE ON THE BINDING OF ^{14}C-*S*-ADENOSYLMETHIONINE AND ^{3}H-NOREPINEPHRINE TO PNMT*

Incubation media	^{14}C-*S*-adenosylmethionine (counts/min) 0°	^{3}H-norepinephrine (counts/min) 37°
Control	1300	37,800
PCMB (1×10^{-4} M)	575	25,000
Inhibition	(45%)	(34%)
2,5-Dimethyl-2-aminobenzimidazole (1×10^{-5} M)	290	36,800
Inhibition	(63%)	(2%)

* The purified PNMT enzyme was incubated in the presence or absence of inhibitors. The incubation mixture contained 12 nmoles of ^{3}H-norepinephrine and 30 nmoles of *S*-adenosylmethionine. Incubation was for 3 min at 0° and 37° as indicated. Samples were processed as described in Table 1.

Fuller and Hunt[6] have observed end-product inhibition of norepinephrine methylation of epinephrine. An experiment was designed to test the effect of epinephrine on ^{3}H-norepinephrine binding. PNMT was incubated with ^{3}H-norepinephrine with or without *S*-adenosylmethionine and the rate of ^{3}H-norepinephrine binding to the enzyme was measured (Fig. 3). In the absence of *S*-adenosylmethionine, binding of

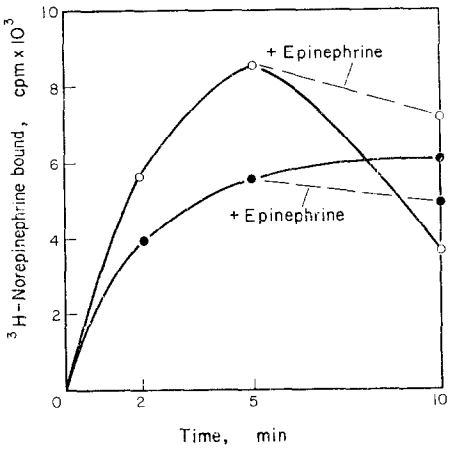

FIG. 3. PNMT was incubated at 37° for various times as indicated in the figure with 12 nmoles ^{3}H-norepinephrine and with (open circles) or without (closed circles) 30 nmoles *S*-adenosylmethionine. After 5 min, 12 nmoles epinephrine was added and the samples were further incubated for 5 min (dashed lines); thereafter samples were processed as indicated in Table 1.

^{3}H-norepinephrine to PNMT is completed in 2–5 min of incubation and remains constant thereafter. In the presence of *S*-adenosylmethionine, the binding was greater. However, there was a rapid loss of labeled complex after 5-min incubation probably due to release of ^{14}C-epinephrine from the enzyme complex. Addition of an excess of unlabeled epinephrine at this point prevents this loss of radioactivity which indicates that epinephrine in some manner stabilizes the ternary complex.

If such stabilization occurs, epinephrine should prevent the *N*-methylation of norepinephrine. This was further tested by measuring relative individual binding affinities of norepinephrine and epinephrine toward the enzyme. Table 5 shows the comparison of the binding affinity of norepinephrine and epinephrine for PNMT. The reaction was carried out in two steps. In the first step, the enzyme was preincubated for 2 min at 37° with ^{3}H-norepinephrine or ^{14}C-epinephrine to allow the respective complexes to form. After this incubation, cold norepinephrine or epinephrine was added and the

TABLE 5. BINDING AFFINITY OF NOREPINEPHRINE AND EPINEPHRINE*

Incubation media	^{14}C-epinephrine bound (counts/min)	^{3}H-norepinephrine bound (counts/min)
Buffer + enzyme	9000	38,000
Plus cold epinephrine	2900	35,900
Plus cold norepinephrine	3600	32,000
Plus *S*-adenosylmethionine	7000	55,000
Plus *S*-adenosylhomocysteine	5300	30,000

* Purified enzyme was incubated with ^{14}C-epinephrine (12 nmoles) or ^{3}H-norepinephrine (12 nmoles) and in the presence of the following additions: cold epinephrine (6 nmoles), cold norepinephrine (6 nmoles), *S*-adenosylmethionine (30 nmoles), *S*-adenosylhomocysteine (30 nmoles). Incubation was for 3 min at 37°; samples were processed as indicated in Table 1.

mixture incubated further for 3 min. The radioactivity of the ^{3}H-norepinephrine–PNMT complex could not be chased by the addition of either cold norepinephrine or epinephrine, whereas the bound ^{14}C-epinephrine was chased by both unlabele-epinephrine and norepinephrine. This points out enzyme preference for norepinephrine over epinephrine. When *S*-adenosylmethionine was added to the preformed complex of ^{14}C-epinephrine–PNMT, retained radioactivity decreased by 22 per cent. However, the radioactivity of the ^{3}H-norepinephrine–PNMT complex almost doubled with the addition of *S*-adenosylmethionine which confirms our earlier result in Fig. 3.

The effect of *S*-adenosylhomocysteine, the end product of *S*-adenosylmethionine, on the ^{14}C-epinephrine and ^{3}H-norepinephrine enzyme complexes was studied next, as shown in line 5, Table 5. *S*-adenosylhomocysteine does not affect the binding of ^{3}H-norepinephrine to the enzyme, whereas the radioactivity of ^{14}C-epinephrine–PNMT complex was lower by 45 per cent.

DISCUSSION

The mechanism of *N*-methylation of norepinephrine has not been studied in detail. A number of events must occur before the enzyme PNMT transfers the methyl group from the donor *S*-adenosylmethionine to norepinephrine. So far no models have been proposed to explain the order of events before epinephrine is synthesized and released. We have arbitrarily defined three distinct steps during the methylation of norepinephrine: (1) formation of a ternary complex between PNMT and its two substrates; 2) methylation of norepinephrine to form epinephrine and; 3) release of epinephrine. In the present report, we have focused our attention on the first two of these steps. We have previously shown that when norepinephrine was first preincubated with PNMT followed by the addition of *S*-adenosylmethionine, the rate of methylation was faster than in non-preincubated controls. This suggests that the complex between PNMT and its substrate must be an obligatory intermediate, since the methylation of norepinephrine is considerably faster when the preformed enzyme complex is used than when separate individual components are added.

A ternary complex of PNMT and its two substrates has been demonstrated for the first time in this paper. The formation of this complex is dependent upon the presence of both substrates, and the removal of any one appears to decrease the complex formed. ^{3}H-norepinephrine binds PNMT both at 37° and at 0°, while ^{14}C-*S*-adenosylmethionine–PNMT complex appears to be stable only at 0°. The data suggest that the binding of *S*-adenosylmethionine to the enzyme is less stable than that of norepinephrine. The fate of *S*-adenosylhomocysteine, the end product of *S*-adenosylmethionine, after the methylation reaction is still unknown, though now under study.

For the purpose of determining the binding sites of *S*-adenosylmethionine and norepinephrine on the enzyme, the effect of known PNMT inhibitors, PCMB and 2,5-dimethyl-1-aminobenzimidazole, was examined. Agents such as PCMB, which react specifically with the sulfhydryl groups of proteins, have been shown to inhibit the *N*-methylation by PNMT,[3] which indicates the essential role of sulfhydryl groups in enzyme function. This inhibition of *N*-methylation has been traced to an inhibition of the ability of the enzyme to bind, with its two substrates. However, 5,6-dimethyl-2-aminobenzimidazole, another inhibitor of methylation by PNMT *in vitro*,[5] primarily prevented the binding of *S*-adenosylmethionine to the enzyme, while the binding of norepinephrine was insensitive to this agent. This observation strongly suggests that

the enzyme has two distinct binding sites for *S*-adenosylmethionine and norepinephrine. The binding sites for epinephrine and norepinephrine appear to be identical. However, the binding affinity of the enzyme for norepinephrine is much greater than that for epinephrine.

Finally, epinephrine is known to inhibit methylation of norepinephrine.[6] The mechanism of this end-product inhibition of PNMT is not known. In our experiments, in the presence of *S*-adenosylmethionine, epinephrine inhibited the decrease of labeled norepinephrine–PNMT complex due to the formation and release of newly formed epinephrine. If inhibition by epinephrine occurred after the methylation of norepinephrine, the newly formed ^{14}C-epinephrine should exchange with the cold epinephrine (Table 5). This suggests that epinephrine in some manner prevents methylation by *S*-adenosylmethionine to form epinephrine and the subsequent release. This suggestion is speculative; experiments are in progress to establish this point further.

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