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The Binding Order of Substrates to Phenylethanolamine N-Methyltransferase

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SUMMARY

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We have studied the substrate binding order of *S*-adenosylmethionine and norepinephrine to phenylethanolamine *N*-methyltransferase via the use of two inhibitors of this enzyme. 2-(2,5-Dichlorophenyl)cyclopropylamine hydrochloride (SK&F 9208) competitively inhibited norepinephrine as the variable substrate but was an uncompetitive inhibitor with respect to *S*-adenosylmethionine. *S*-Adenosylhomocysteine, however, was a competitive inhibitor with respect to *S*-adenosylmethionine but noncompetitive as an antagonist of norepinephrine. These studies are interpreted to suggest a kinetically ordered pattern of substrate binding to the enzyme, with *S*-adenosylmethionine being initially bound.

INTRODUCTION

Phenylethanolamine *N*-methyltransferase (EC 2.1.1) is an enzyme, highly localized in the adrenal medulla of mammals, which catalyzes the final step in epinephrine biosynthesis. This reaction requires no cofactor and involves the transfer of a methyl group from *S*-adenosylmethionine to the terminal nitrogen of norepinephrine (1) (Scheme 1).

Recent studies from two laboratories have indicated that the "receptor" sites for the two substrates on the phenylethanolamine *N*-methyltransferase molecule are probably different, since chemical (drug) inhibition of the binding for one may not inhibit that of the other (2, 3). Indirect evidence has also been obtained for the presence of a ternary complex between the enzyme and the two substrates as an obligatory step in epinephrine formation, although the existence of a "ping-pong" mechanism as opposed to the formation of a true ternary complex was not ruled out (3).

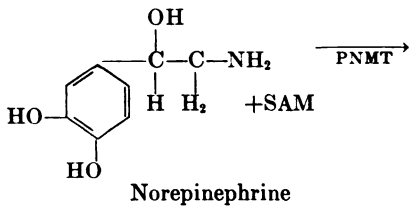
Opinions differ as to the order of binding of the two substrates for the enzyme prior to initiation of the reaction. Connett and Kirshner (4) have proposed, based on data derived from initial substrate-velocity curves, a random order of norepinephrine and *S*-adenosylmethionine binding, with the latter being the kinetically preferred substrate. Pohorecky and Baliga (3), on the other hand, have recently shown that norepinephrine, but not *S*-adenosylmethionine, forms at least a transiently stable bond with phenylethanolamine *N*-methyltransferase at 37° and postulated that norepinephrine is the preferred first substrate for the enzyme.

The objective of our study is to shed some light on this problem via the use of two pharmacological tools, *S*-adenosylhomocysteine and 2-(2,5-dichlorophenyl)cyclopropylamine hydrochloride (SK&F 9208). The former is a product of the phenylethanolamine *N*-methyltransferase-catalyzed reaction and has been shown to inhibit the enzyme

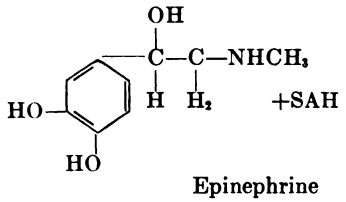
via competition with *S*-adenosylmethinoine for its receptor site (5). SK&F 9208, on the other hand, is a compound from our laboratories, previously reported as a phenylethanolamine *N*-methyltransferase antagonist (6), which inhibits the enzyme by a competitive mechanism with respect to norepinephrine. This report presents the effects of these two drugs upon the kinetics of the phenylethanolamine *N*-methyltransferase reaction. The results are subsequently interpreted according to the concepts of Cleland (7) for predicting substrate binding order. The structures of these inhibitors are shown in Scheme 2.

METHODS

Standard enzyme assay. A partially purified, lyophilized phenylethanolamine *N*-methyltransferase preparation derived from rabbit adrenals was obtained commercially from Gallard-Schlesinger Company, where it was prepared according to the method of Saelens *et al.* (8). The enzyme was solubilized in cold potassium phosphate buffer (167 mM). The 300-μl reaction mixture contained enzyme, 280 μg; phosphate buffer (pH 7.4), 50 μmoles; *l*-norepinephrine (Sigma), 9 nmoles; and *S*-adenosyl[*methyl*-^{14}C]methionine (approximately 20,000 dpm, New England Nuclear), 9 nmoles. The reaction mixture was contained in ice prior to initiation of the assay; norepinephrine was the last substituent added. The label was localized on the reactive methyl group of the *S*-adenosylmethionine molecule. The reaction was carried out for 30 min at 37°, after which it was terminated with 1 N HCl (200 μl). Approximately 1 g of solid NaCl was then added, and the solution was extracted with 6 ml of acid-washed, NaCl-saturated butanol. Then 1 ml of the butanol layer, containing labeled epinephrine, was added to 10 ml of an aqueous 2,5-bis[2′(5′-*tert*-butylbenzoxazolyl)]thiophene phosphor, counted in a Packard model 3003 liquid scintillation spectrometer for 10 min, and quantitated in terms of nanomoles of epinephrine. A correction was made for the small amount of *S*-adenosyl[^{14}C]methionine (approximately 60 dpm) coming through the extraction procedure. Control samples extracted from norepinephrine (30 μM)-containing assay tubes had approximately 700 dpm exclusive of the blank value; the minimal detectable ($p < 0.05$) level of radioactivity above the blank was approximately 25 dpm. The radioactive reaction product extracted into the butanol phase was identified, after acetylation, as epinephrine, using silica gel thin-layer plates (Merck, F254) with a chloroform–acetic acid–ethanol



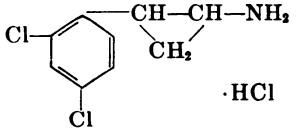
Norepinephrine



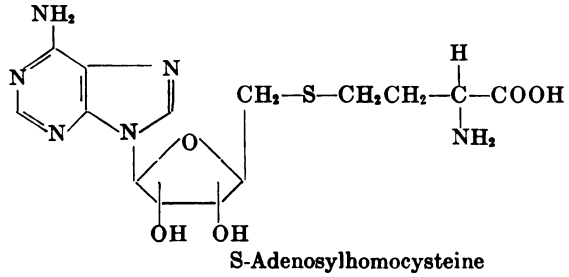
Epinephrine

SCHEME 1

The abbreviations used are: SAM, *S*-adenosylmethionine; PNMT, phenylethanolamine *N*-methyltransferase; SAH, *S*-adenosylhomocysteine.



SK&F 9208



S-Adenosylhomocysteine

SCHEME 2

(95:5:5) solvent system (9). The recovery of epinephrine in the butanol phase in this assay was 85%; all results are appropriately corrected.

The standard conditions used in the above assay are shown in Fig. 1. The substrate concentrations used were near, but not over, the peak of the substrate-dependent portion of the respective substrate-velocity curve—a necessary condition for the proper assay of competitive inhibitors. An additional consideration in selecting the *S*-adenosylmethionine concentration was the maintenance of as low a radioactive blank value as possible. Although the period of enzyme incubation was long (30 min), the enzyme has been shown to remain stable at 39° for at least 45 min (4). In kinetic studies the concentration of the variable substrate was systematically altered as shown in the figures accompanying this report. Each assay was performed in quadruplicate. Regression lines used in the Lineweaver-Burk plots were determined by a computerized method of least squares (10). Apparent K_i values were graphically determined by methods described by Dixon (11) and Rose (12). All concentrations referred to are final medium levels, and each variability term refers to the standard error of the mean (13).

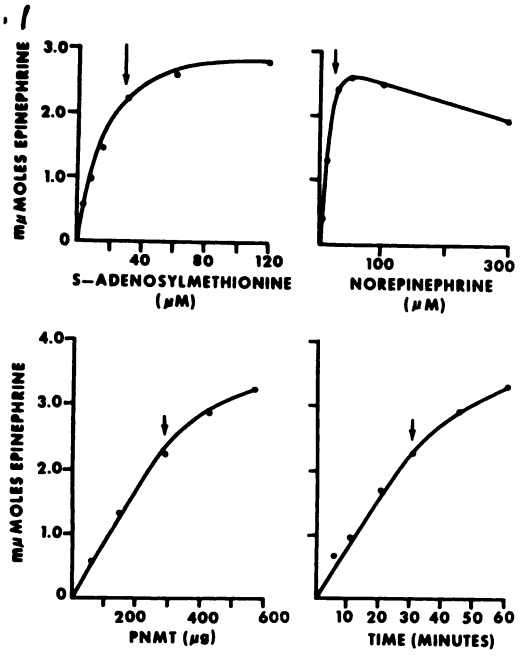


FIG. 1. *Conditions of assay*

The graphs show the effects of time, enzyme (protein) concentration, and substrate concentration on reaction velocity. Arrows indicate the conditions selected for the standard assay. In analyzing any one parameter, the standard conditions were utilized for all other parameters. Each point is the mean of four determinations. PNMT = phenylethanolamine *N*-methyltransferase.

RESULTS

Inhibition of phenylethanolamine N-methyltransferase. The relative activities of *S*-adenosylhomocysteine and SK&F 9208 as inhibitors of the enzyme in our standard assay (Fig. 2) indicate that the compounds are essentially equipotent ($I_{50} \cong 10$ μM). The curves for each inhibitor are sigmoid, suggesting a reversible mode of enzyme inhibition in which the inhibitor concentrations are in excess of that of the enzyme (14).

In subsequent studies SK&F 9208 was found not to be [^{14}C]methylated when placed in our standard enzyme assay, in lieu of norepinephrine, at concentrations ranging from 1 to 100 μM. The drug itself was completely extracted into the butanol phase in our extraction procedure (determined spectrophotometrically). Since the *N*-methylated derivative should be even more lipid (butanol)-soluble than the parent compound, it is unlikely that the above result was artificial because of the nonextractability of methylated SK&F 9208.

Similarly, when *S*-adenosylhomocysteine at 30 μM was incubated for 30 min with ^{14}C-labeled epinephrine (30 μM), no radioactivity, corresponding on the thin-layer chromatography plates to norepinephrine, was found above that seen in the controls, indicating that the addition of *S*-adenosylhomocysteine to the incubation medium does not reverse the reaction (Table 1). This finding is in keeping with the observation of others that the phenylethanolamine *N*-methyltransferase-catalyzed reaction is irreversible (16). It should be noted that the concentration of *S*-adenosylhomocysteine used in this experiment was the highest level used in subsequent kinetic studies; also, the amount of epinephrine present was in excess of that formed in the latter studies.

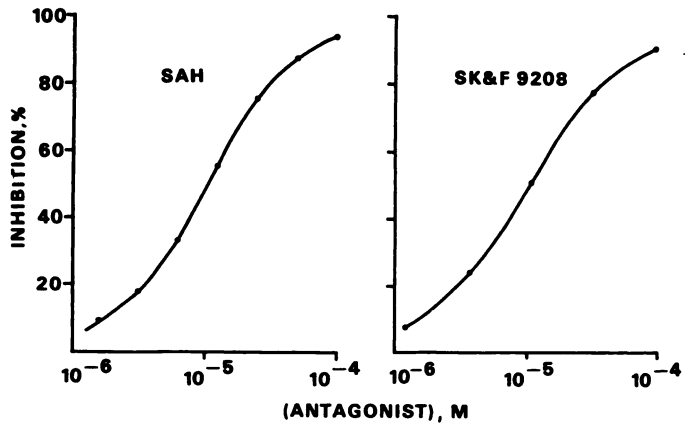


FIG. 2. *Dose-response curves showing effects of S-adenosylhomocysteine (SAH) and SK&F 9208 as inhibitors of phenylethanolamine N-methyltransferase*

The standard assay conditions were used. Each point is the mean of four values.

TABLE 1

Irreversibility of phenylethanolamine N-methyltransferase reaction

Conditions	Radioactivity	
	Epinephrine	Norepinephrine
	dpm	*dpm*
[^{14}C]Epinephrine (30 μM) + enzyme	14,684 ± 32	331 ± 49
[^{14}C]Epinephrine (30 μM) + *S*-adenosylhomocysteine (30 μM) + enzyme	14,901 ± 440	330 ± 10

[7-^{14}C]Epinephrine (20,926 dpm), with and without *S*-adenosylhomocysteine, was added to our standard incubation medium containing 280 μg of enzyme in a final volume of 300 μl. The mixture was then incubated for 30 min at 37° in a metabolic shaker. The reaction was terminated with 0.2 ml of 1 N HCl and 1.5 ml of 0.2 N acetic acid containing 1 mg each of epinephrine and norepinephrine. The catecholamines were subsequently acetylated with acetic anhydride, separated on silica gel thin-layer chromatography plates, and assayed for radioactivity (15). Each reaction was run in quadruplicate. Experiments using labeled epinephrine alone, without incubation, revealed a similar percentage of the label to be present at the norepinephrine locus on the thin-layer plates to that observed above (2%).

Kinetic studies. The kinetic studies performed with SK&F 9208 are shown graphically in Figs. 3 and 4. The substrate-velocity curves as well as the Lineweaver-Burk plots indicate that the inhibition produced by this drug at two concentrations was surmountable by increasing concentrations of norepinephrine, which is indicative of a competitive mode of inhibition. However, when *S*-adenosylmethionine was the variable substrate, the antagonism was not surmountable, and the parallelism of the Lineweaver-Burk plots is demonstrative of an uncompetitive mode of inhibition. Appropriate secondary replots of the primary kinetic data (slopes or intercepts with respect to inhibitor concentration) were linear, which indicates that SK&F 9208 is a complete inhibitor of the enzyme as opposed to a partial antagonist (17).

On the other hand, *S*-adenosylhomocysteine is a competitive inhibitor of *S*-adenosylmethionine at concentrations essentially equivalent to those of SK&F 9208 for producing inhibition in our standard enzyme assay (Fig. 5). These results are similar to those reported previously by Deguchi and Barchas (5), showing that *S*-adenosylhomocysteine was a competitive inhibitor of *S*-adenosylmethionine as the variable substrate for phenylethanolamine *N*-methyltransferase when normetanephrine was the fixed substrate. Our data also show that *S*-adenosylhomocysteine, at the same concentrations employed above, is a noncompetitive inhibitor of the enzyme when norepinephrine is employed as the variable substrate (Fig. 6). As with SK&F 9208, the

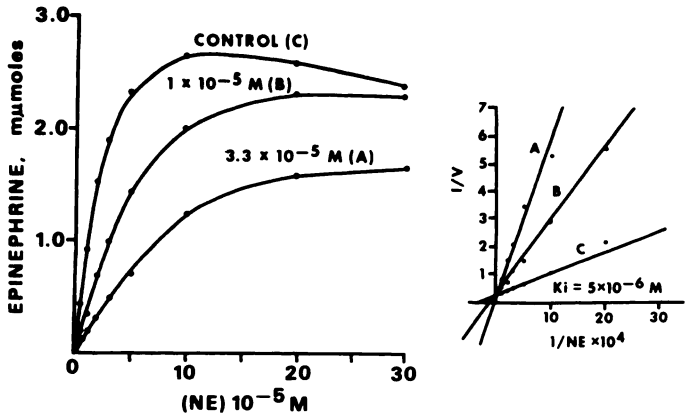


FIG. 3. *Kinetic analysis of phenylethanolamine N-methyltransferase inhibition produced by SK&F 9208 at two concentrations*

Norepinephrine (NE) was the variable substrate; the fixed concentration of *S*-adenosylmethionine was 30 μM. The reaction was conducted for 30 min; velocity is expressed as nanomoles of epinephrine produced per assay period.

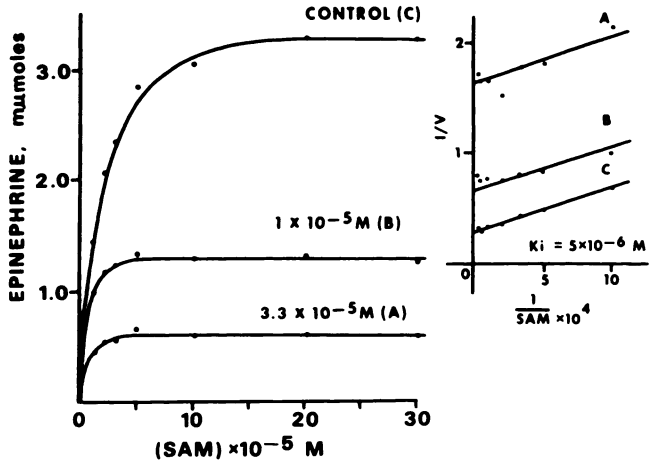


FIG. 4. *Kinetic analysis of phenylethanolamine N-methyltransferase inhibition produced by SK&F 9208 at two concentrations*

S-Adenosylmethionine (SAM) was the variable substrate; the fixed concentration of norepinephrine was 30 μM. The reaction was conducted for 30 min; velocity is expressed as nanomoles of epinephrine produced per assay period.

secondary replots (17) of the kinetic data were linear.

DISCUSSION

An enzyme inhibitor (*I*) increases the slope of the Lineweaver-Burk reciprocal plot relationship when it and the variable substrate (*S*) either compete for the same form of the enzyme or react with forms in the reaction sequence which are separated by reversible steps, such that the effect of *I* may be decreased by increasing the concentrations of *S*. If such a mechanism occurs alone, a competitive pattern of enzyme inhibition results, in which the effects of the inhibitor may be eliminated at saturating substrate concentrations (17, 18). On the other hand, the intercept of the reciprocal plot is changed when an inhibitor combines with a form of the enzyme with which the substrate does

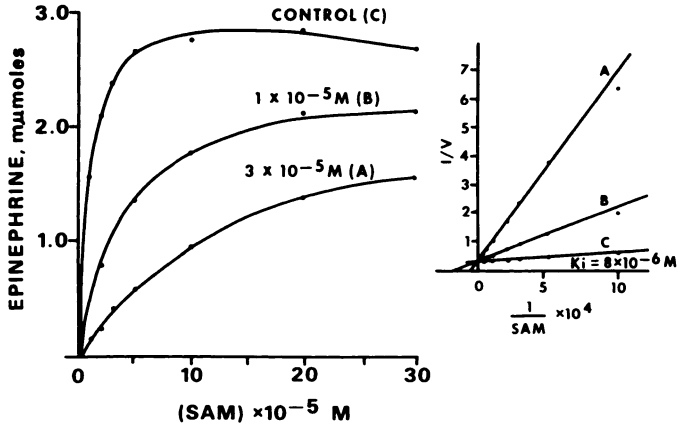


FIG. 5. *Kinetic analysis of phenylethanolamine N-methyltransferase inhibition produced by S-adenosylhomocysteine*

S-Adenosylmethionine (SAM) was the variable substrate; the fixed concentration of norepinephrine was 30 μM. The reaction was conducted for 30 min; velocity is expressed as nanomoles of epinephrine produced per assay period. Concentrations listed above the curves are those of *S*-adenosylhomocysteine.

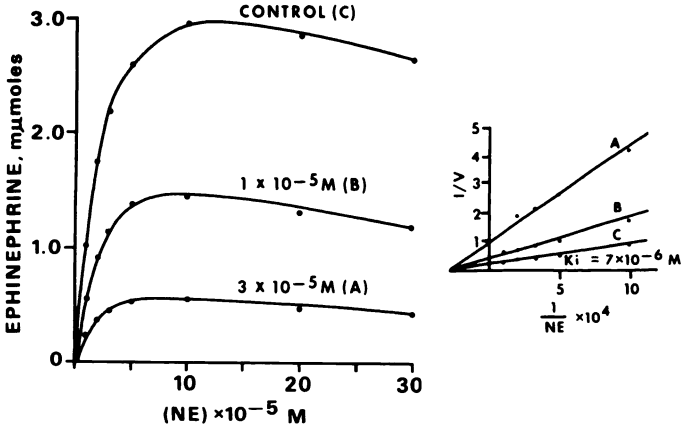
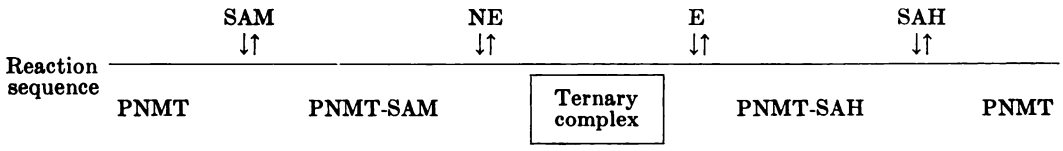


FIG. 6. *Kinetic analysis of phenylethanolamine N-methyltransferase inhibition produced by S-adenosylhomocysteine*

Norepinephrine (NE) was the variable substrate; the fixed concentration of *S*-adenosylmethionine was 30 μM. The reaction was conducted for 30 min; velocity is expressed as nanomoles of epinephrine produced per assay period. Concentrations listed above the curves are those of *S*-adenosylhomocysteine.

not react, and saturation with the variable substrate cannot eliminate the inhibition. Under conditions in which only the intercept is altered, the degree of inhibition is independent of the substrate concentration, and the Lineweaver-Burk plots for the control and inhibited reactions are parallel (17, 18). This mode of enzyme inhibition is called uncompetitive and is characteristically produced by combination of the inhibitor with an enzyme form that occurs after combination of the variable substrate (19). If the above mechanisms occur together, or if enzyme inhibition is produced in such a way that the effect of the inhibitor is only partially dependent on the concentration of variable substrate, then noncompetitive inhibition results, in which *both* the slope and the intercept of the reciprocal plot are altered (17, 18).

Based on these considerations and the previous evidence for a ternary phenyletha-



SCHEME 3

The abbreviations used are: SAM, *S*-adenosylmethionine; PNMT, phenylethanolamine *N*-methyltransferase; SAH, *S*-adenosylhomocysteine; NE, norepinephrine; E, epinephrine.

nolamine *N*-methyltransferase complex (3), the inhibition patterns produced by SK&F 9208 and *S*-adenosylhomocysteine are compatible with an ordered system of substrate binding to the enzyme, with *S*-adenosylmethionine being the first substrate bound, as is shown in Scheme 3 in the graphic shorthand notation due to Cleland (17). All steps are considered reversible except the interconversion of the ternary complex (norepinephrine–enzyme–*S*-adenosylmethionine → epinephrine–enzyme–*S*-adenosylhomocysteine). Both *S*-adenosylhomocysteine and SK&F 9208 are considered to be "dead end" inhibitors (17), since neither is apparently chemically altered as a result of interacting with the enzyme.

According to this scheme, the competition by *S*-adenosylhomocysteine with *S*-adenosylmethionine for the free enzyme would also be *partially* reversible by norepinephrine as the variable substrate, because, by increasing the rate of conversion of the enzyme–*S*-adenosylmethionine complex to the ternary complex, norepinephrine would tend to accelerate the binding of *S*-adenosylmethioine to the enzyme by a mass action effect. Therefore *S*-adenosylhomocysteine should produce noncompetitive inhibition of the transferase when norepinephrine is the variable substrate, as is in fact the case (17, 18).

Conversely, SK&F 9208 inhibits phenylethanolamine *N*-methyltransferase in this scheme by competing with norepinephrine for the enzyme–*S*-adenosylmethionine form of the transferase. The reaction would not be reversed by the addition of *S*-adenosylmethionine, since this would also provide additional enzyme–*S*-adenosylmethionine complex with which the inhibitor could combine. Thus SK&F 9208 would be predicted to inhibit the enzyme uncompetitively when *S*-adenosylmethionine is the variable substrate, as is found experimentally (17, 18).

Alternatively, if *S*-adenosylmethionine and norepinephrine reacted randomly with the enzyme, one might expect competitive inhibitors for either substrate to inhibit the other by at least the same kinetic mechanism. This is obviously not the case. Furthermore, the observation that SK&F 9208 inhibits *S*-adenosylmethionine uncompetitively is totally incompatible with a random substrate-binding pattern (17, 18).

A "ping-pong" mechanism, in which *S*-adenosylmethionine would bind, donate a methyl group to the enzyme, and then leave the reactive site (as *S*-adenosylhomocysteine) prior to the binding of norepinephrine, is also incompatible with our data. For this to occur, *S*-adenosylhomocysteine should show competitive kinetics with respect to norepinephrine (but not with *S*-adenosylmethionine) while epinephrine should compete with *S*-adenosylmethionine for the free form of the enzyme (17, 18). The former has been shown not to be true in this report, while epinephrine has previously been shown to inhibit *S*-adenosylmethionine in an uncompetitive manner (9).

These observations, in general, support the hypothesis of Connett and Kirshner (4) that *S*-adenosylmethionine is the kinetically preferred first substrate for phenylethanolamine *N*-methyltransferase. However, the observations do not deny the possibility that norepinephrine may also bind independently to the enzyme under certain conditions. The kinetic data simply suggest that the *rate* of reaction of *S*-adenosylmethionine with the free enzyme is greater than with norepinephrine, and therefore it is the preferred first substrate to be bound.

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