plasma and the addition of toxic doses of salicylate to human serum altered the binding of MeTHF (Table 1). It is possible, however, that, if a larger number of experiments had been performed, the binding of added (±)MeTHF to frozen serum or to serum at 43° or to serum to which probenecid was added might have differed significantly from that of controls. Similar effects of salicylate on the binding of other weak carboxylic acids has been reported [7].

This study, however, documents only the binding of added MeTHF to normal fasting serum and the binding of endogenous folate when measured by the competitive binding method with β-lactoglobulin [11]. The explanation for the different values obtained for folate binding by these two methods is not clear. Moreover, extrapolation of these results to folate-depleted patients may not be warranted [11, 14].

In conclusion, added MeTHF is 60–70 per cent bound to fresh serum of normal men after an overnight fast. Albumin could account for the major portion of the binding and the binding did not appear to be stereospecific. Drugs including diphenylhydantoin, probenecid and salicylate have only a minimal, if any, effect on added MeTHF binding at therapeutic dosages (Table 1). Moreover, increasing the concentration of MeTHF in serum by over a thousand times did not alter the percentage of MeTHF bound.

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Substrate and inhibitor kinetics of bovine phenylethanolamine-*N*-methyltransferase

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The methylation of norepinephrine, the final step in the synthesis of epinephrine, is catalyzed by phenylethanolamine-*N*-methyltransferase (PNMT). This enzyme was partially purified from monkey adrenal gland [1] and was found to have an exclusive specificity for both ring-substituted and unsubstituted phenylethanolamines. Subsequent studies [2] also pointed out that β-hydroxyphenylethylamines (phenylethanolamines) are much better substrates than the unsubstituted phenylethylamine derivatives (> 100:1), although the latter are methylated to a limited extent. Using PNMT isolated from the rabbit, Fuller *et al.* [3] showed that β-keto dopamine (arterenone) and 3,4-dichloro-β-amino-phenylethanolamine are also suitable substrates, suggesting that nucleophilic groups other than hydroxyl can interact with the enzyme. Recently, Laduron [4] reported the methylation of dopamine by PNMT. This paper will report on the substrate specificity and the inhibitor kinetics of PNMT using a purer enzyme preparation than other studies have utilized.

Bovine PNMT was purified by a modification of the method of Connett and Kirshner [5], as described by Ciaranello and Axelrod [6]. Briefly, protein from adrenal medullae that precipitates at an ammonium sulfate concentration of 30–60% was taken up in 50 mM potassium phosphate buffer, pH 7·2, and made 0·5 mM in dithiothreitol. After dialysis against the same buffer, the protein solution was adjusted to pH 5 and centrifuged. The pH of the supernatant was then brought up to 7, and the material was again dialyzed as before. Afterwards, the solution was applied to a G-100 Sephadex column. The peak, corresponding to a molecular weight of 40,000 was applied to a DEAE-Sephadex A-50 ion-exchange column. When the peak fractions from this column were pooled, concentrated and subjected to sodium dodecyl sulfate-acrylamide electrophoresis [6], a single staining band was observed. PNMT was assayed as previously described [1]. Each reaction tube contained 5 μmoles potassium phosphate buffer, pH 7·9, 25 μg protein, 2 nmoles ^{14}C-*S*-adenosylmethionine (52·3 mCi/

Table 1. Michaelis–Menten constants for phenylethanolamine-*N*-methyltransferase*

	$K_m \pm$ S.D. (μM)	$V_{max} \pm$ S.D. (nmoles/min)
β-Hydroxy analogues		
L-Norepinephrine	6·4 ± 1·0	1·9 ± 0·1
L-Octopamine	11·4 ± 0·1	1·6 ± 0·0
Phenylethanolamine	82 ± 6	2·7 ± 0·1
L-Epinephrine	23·6 ± 7·2	0·15 ± 0·01
L-Normetanephrine	382 ± 38	1·4 ± 0·1
Substituted phenylethylamines		
Dopamine	800 ± 300	0·320 ± 0·039
Tyramine	2400 ± 300	0·040 ± 0·003
Phenylethylamine	2000 ± 300	0·010 ± 0·001
Epinine	Not tested	
3-Methoxytyramine†		

* Michaelis–Menten constants and V_{max} values were determined by a computer program on a Wang 700B calculator. Product yields have not been corrected for recovery from the organic solvents used in the extraction process. The product yields usually obtained in this laboratory are: L-epinephrine (from acid–butanol), L-synephrine and L-metanephrine (from toluene isoamyl alcohol, 3:2) 80–90 per cent; *N*-methylphenylethanolamine (from toluene–isoamyl alcohol, 97:3) 85–95 per cent [1].

† No detectable activity was seen with this compound.

m-mole, New England Nuclear Corp.) and substrate in a final volume of 210 μl.

The substrate kinetics for several phenylethylamines and phenylethanolamines are shown in Table 1. Substitution of 4-OH or 3,4-OH on the ring increases affinity of the enzyme for phenylethanolamines. 3-*O*-methylation, a step in catecholamine metabolism, decreases the affinity of the substrate. While phenylethanolamines are clearly the preferred substrates, phenylethylamine, tyramine and dopamine are also substrates for PNMT. The physiologically occurring substrate with the lowest K_m is generally considered to be the natural substrate for the enzyme. The data indicate the order of preference for the adrenal catecholamines to be: norepinephrine > epinephrine ≫ dopamine. The observation that dopamine is a substrate for PNMT has led to the proposal that conversion of dopamine to epinine, followed by β-hydroxylation, is the principal route of epinephrine biosynthesis *in vivo* [4]. The kinetic analysis performed here has made this conclusion unlikely for the following reasons: (a) norepinephrine has a much higher (125-fold) affinity for PNMT than does dopamine; (b) norepinephrine is *N*-methylated at a rate at least six times faster than dopamine; and (c) adrenal dopamine concentration is extremely low relative to the norepinephrine content [7].

Table 2. Inhibitor constants for phenylethanolamine-*N*-methyltransferase*

	K_i (μM)	Type of apparent inhibition
L-Octopamine	6·4	Mixed
L-Norepinephrine	5·9	Non-competitive
Phenylethylamine	3600	Competitive
Tyramine	6100	Competitive
3-Methoxytyramine		No inhibition
Dopamine	8000	Competitive

* K_i values were determined graphically by the method of Lineweaver–Burk or by the method of Dixon and Webb [9]. Phenylethanolamine was used as the substrate for K_i determinations at saturating concentrations (0·15 mM).

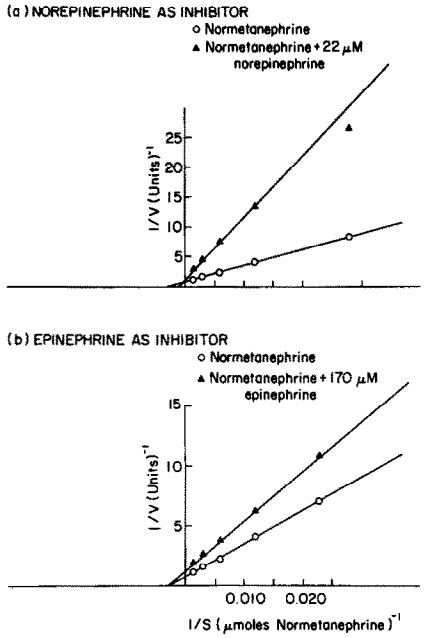

Fig. 1. Inhibition of PNMT by norepinephrine and epinephrine. Lineweaver–Burk plot of the inhibitory effect of: (a) norepinephrine, and (b) epinephrine on the *N*-methylation of normetanephrine. Units are nmoles of product/hr. These plots indicate that norepinephrine appears to inhibit competitively, while epinephrine appears to inhibit non-competitively.

Norepinephrine, octopamine, phenylethanolamine and normetanephrine inhibit PNMT at high substrate concentrations [8]. Both phenylethylamines and phenylethanolamines inhibit the methylation of phenylethanolamine. Using a single concentration of inhibitor, Fuller and Hunt [8] reported that phenylethylamines are generally better inhibitors than are phenylethanolamines. Kinetic studies shown in Table 2, however, demonstrate that K_i values for phenylethanolamines are 1000 times lower than the K_i values for phenylethylamines. With the exception of dopamine, the K_i values for these amines are approximately equal to their K_m values. PNMT, therefore, exhibits both substrate (norepinephrine) and product (epinephrine) inhibition. Dopamine, an earlier compound in the metabolic pathway of epinephrine synthesis, has no appreciable inhibitory effect on PNMT.

When normetanephrine is used as a substrate for PNMT, norepinephrine acts as a competitive inhibitor, while epinephrine is a non-competitive inhibitor (Fig. 1). Fuller and Hunt [10] also reported that epinephrine inhibits norepinephrine in a non-competitive manner. These observations indicate that epinephrine and norepinephrine might have separate binding sites on the PNMT molecule, although both can be methylated using *S*-adenosylmethionine as the methyl donor.

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