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Effects of Sodium Chloride on Phenylethanolamine *N*-Methyltransferase Activity

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SUMMARY

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NaCl and KCl at the concentrations present in body fluids competitively inhibited phenylethanolamine *N*-methyltransferase activity whether octopamine, normetanephrine, or norepinephrine was used as substrate. This inhibitory effect was more pronounced at low concentrations of *S*-adenosylmethionine. Both salts reduced the inhibition of enzyme activity observed at high *N*-methyl acceptor concentrations. This salt effect was dependent on the buffer employed, the pH of the assay, and the *S*-adenosylmethionine concentration. Similar effects were produced by equal ionic strength solutions of NaF, NaBr, CsCl, LiCl, K_2SO_4, Na_2SO_4, $MgSO_4$, and $CaCl_2$. In addition, an increase in the ionic strength of the buffer (Tris-HCl or phosphate) reduced the enzyme activity at subsaturating amine concentrations but enhanced the reaction velocity at high substrate concentrations. The optimum pH varied, depending on the amine concentration employed. The present results suggest that an increase in the ionic strength of the salt solution might reduce binding of substrate molecules to the enzyme, which would be manifested by a decrease in the reaction velocity at low amine concentrations and by a reduction of the inhibitory effect produced by saturating amine concentrations.

INTRODUCTION

Phenylethanolamine *N*-methyltransferase (EC 2.1.1.28) catalyzes the conversion of norepinephrine to epinephrine and the *N*-methylation of a number of primary and secondary derivatives (1-5). This enzyme utilizes *S*-adenosylmethionine as the methyl donor in *N*-methylation reactions (4, 6).

Although much has been learned about the distribution of phenylethanolamine *N*-methyltransferase in mammalian tissues (7), regulation of this enzyme activity is less well understood. In a previous study from this laboratory (8) it was observed that human cerebrospinal fluid markedly inhibited the activity of a purified phenylethanolamine *N*-methyltransferase preparation obtained from bovine adrenals. The inhibition was shown to be competitive with the substrate octopamine. The high thermal stability and readily dialyzable nature of the inhibitor(s) suggested that this effect could be mediated by ions. In fact, when cerebrospinal fluid was dialyzed against 5 mM Tris-HCl containing a NaCl concentration equivalent to that present in human spinal fluid, inhibition persisted (8).

The present experiments were undertaken to investigate the mechanism by

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which NaCl modifies phenylethanolamine *N*-methyltransferase activity and to determine whether other ions normally present in the enzyme environment could play a role in regulation of the synthesis of epinephrine and other phenylethanolamines. An analysis of buffer and pH requirements for the enzyme was also performed. In addition, the use of a highly sensitive method for estimation of small quantities of norepinephrine (9) was modified to determine phenylethanolamine *N*-methyltransferase activity, employing norepinephrine as the *N*-methyl acceptor.

METHODS

Purification of phenylethanolamine N-methyltransferase. Bovine phenylethanolamine *N*-methyltransferase was partially purified by a modification of the method of Connett and Kirshner (4). The entire purification procedure was carried out at 4°. Fresh bovine adrenal glands were obtained from recently slaughtered cattle and transported to the laboratory on ice. The adrenal medullae were dissected free of cortical tissue, weighed, and homogenized in 2 volumes of 0.3 M sucrose in a commercial blender. The homogenate was subjected to differential centrifugation. The protein from the 100,000 × *g* supernatant which precipitates at an ammonium sulfate concentration of 30–55%, was taken up in 1 mM Tris-HCl buffer, pH 7.4. After dialysis against the same buffer, the pH was adjusted to 5.0 with 1.0 N acetic acid under continuous stirring. The precipitate was removed by centrifugation and discarded. After dialysis for 24 hr against 1 mM Tris-HCl, pH 7.4, aliquots of the protein preparation were applied to a Sephadex G-200 column (5 × 90 cm). The column was eluted with 0.05 M Tris-HCl buffer, pH 7.4. The peak activity fractions were pooled and concentrated with an Amicon pressure dialysis cell. The final protein concentration was 34 mg/ml. Protein was measured as described by Lowry *et al.* (10).

Measurement of enzyme activity. For the substrates octopamine and normetanephrine, phenylethanolamine *N*-methyltransferase activity was determined by a modification of the procedure described by Axelrod (3). This assay employs *S*-adenosyl[*methyl*-^{3}H]methionine, from which an *N*-methyl radioactive amine is formed in the presence of the enzyme. The product is separated from the ^{3}H-SAM[1] by solvent extraction at alkaline pH. The samples are dried, and the residue is dissolved in a mixture of toluene–methanol–2,5-diphenyloxazole (1000:150:5, v/v/w) and counted by liquid scintillation spectrometry (for details, see the legend to Fig. 1).

For norepinephrine, the procedure of Henry *et al.* (9) was modified to obtain optimal linear relationships for both enzyme concentration and time of incubation. In this assay norepinephrine is *N*-methylated to form [^{3}H]epinephrine in the presence of [^{3}H]SAM and the enzyme. The [^{3}H]epinephrine formed is separated from the [^{3}H]SAM by alumina adsorption, precipitation of the SAM with phosphotungstic acid, and ion pair solvent extraction with a mixture of toluene and diethylhexylphosphoric acid (10:1, v/v). The radioactivity present in an aliquot of the organic phase was determined by liquid scintillation spectrometry (for details, see the legend to Fig. 3).

Both procedures maintained linear relationships for enzyme concentration and time of incubation.

Materials. *S*-Adenosylmethionine chloride, Trizma base, octopamine, normetanephrine, norepinephrine, and epinephrine were purchased from Sigma Chemical Company. All other reagents were of the highest purity obtainable from commercial sources.

Calculations. Means and standard errors of the mean were calculated by conventional procedures. Ionic strength calculations for buffer and salt solutions were performed as described by Long (11).

RESULTS

Effects of NaCl on phenylethanolamine N-methyltransferase activity: substrate requirements. Substrate-velocity relationships were determined for octopamine, normetanephrine, and norepinephrine. The apparent K_m values for the amines varied with SAM concentration (Fig. 1). The apparent K_m values for octopamine

[1] The abbreviation used is: SAM, *S*-adenosylmethionine.

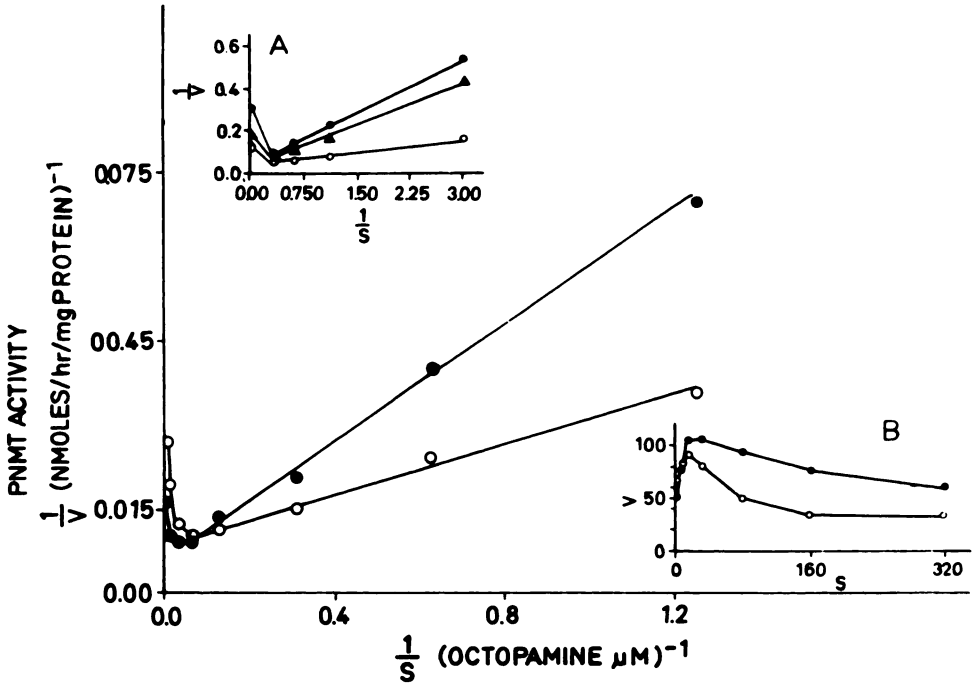

FIG. 1. *Effect of NaCl on phenylethanolamine N-methyltransferase (PNMT) activity with octopamine as the variable substrate*

Reaction mixtures, containing 19.3 μmoles of Tris-HCl buffer (pH 8.6), 0.5 μl of enzyme preparation (17 μg of protein), 0.1 μCi of [*methyl*-^{3}H]SAM (specific activity, 12.6 Ci/mmole; New England Nuclear), 0.38 or 3.8 nmoles of SAM (4.74 or 47.4 μM, final concentration), and varying amounts of octopamine in a final volume of 80 μl, were incubated at 37° for 20 min in the presence and absence of NaCl. The reaction was stopped with 50 μmoles of borate buffer, pH 10, and the [*methyl*-^{3}H]amine formed was extracted into toluene–isoamyl alcohol (3:2, v/v). A 1.0-ml aliquot of the organic phase was dried, and the residue was dissolved and counted. Blanks averaged 101 ± 8 cpm. Large figure: the SAM concentration was 47.4 μM; ○, control; ●, 150 mM NaCl. Inset A: SAM concentration, 4.74 μM; ○, control; ▲, 100 mM NaCl; ●, 150 mM NaCl. Inset B: linear substrate-velocity relationships, where the ordinate (V) represents nanomoles of product formed per hour per milligram of protein and the abscissa is the molar concentration of octopamine. SAM concentration, 47.4 μM; ○, control; ●, 150 mM NaCl. Each point is the mean of at least three experiments. Standard errors did not exceed 12% of the means.

were 1.03 and 3.45 μM in the presence of 4.74 and 47.4 μM SAM, respectively. Similarly, the extrapolated V_{max} values increased from 26.4 (4.74 μM SAM) to 121.9 nmoles of product per hour per milligram of protein (47.4 μM SAM).

NaCl produced concentration-dependent inhibition of the enzyme activity. This effect was reversed by increasing the octopamine concentration (Fig. 1). The apparent K_m for octopamine in the presence of NaCl (150 mM) was 4.2 times the value obtained in the absence of the salt. When the concentration of NaCl was plotted against the reciprocal of velocity (Dixon plot) in the presence of 4.74 μM SAM, linear relationships were obtained. The K_i for NaCl was 49.8 mM. At octopamine concentrations above 32 μM and in the presence of 47.4 μM SAM, NaCl reduced substrate inhibition of enzyme activity. In fact, with 160 and 320 μM octopamine, the velocity of the reaction in the presence of NaCl (150 mM) was twice that observed in the absence of the salt. The effects of NaCl on phenylethanolamine *N*-methyltransferase activity were also evaluated employing SAM as the variable substrate (Fig. 2A and B). The apparent K_m for SAM and the V_{max} varied with octopamine concentration. At 1.6, 32, and 320 μM octopamine the apparent K_m values for SAM were 5.3, 23.7, and

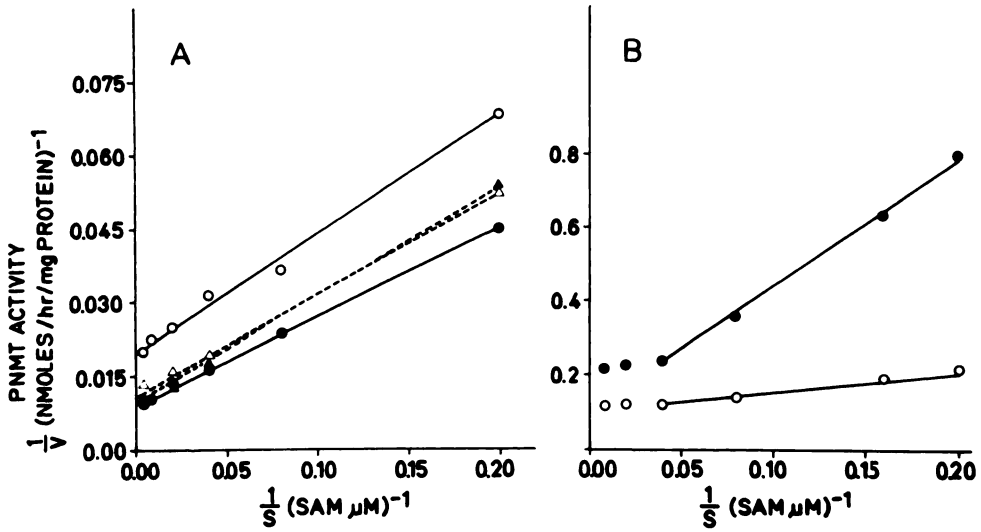

FIG. 2. *Effect of NaCl on phenylethanolamine N-methyltransferase (PNMT) activity with SAM as the variable substrate*

Reaction mixtures, containing 19.3 μmoles of Tris-HCl buffer (pH 8.6), 0.5 μl of enzyme preparation (17 μg of protein), 0.1 μCi of [*methyl*-^{3}H]SAM, either 1.6, 2.56, or 25.6 nmoles of octopamine, and varying amounts of unlabeled SAM, were incubated at 37° for 20 min in the presence and absence of NaCl. Blanks averaged 109 ± 12 cpm for 4.74 μM SAM and 96.5 ± 7 cpm for 47.4 μM SAM. A. ——, 320 μM, octopamine: ○, control; ●, 150 mM NaCl. - - -, 32 μM octopamine: △, control; ▲, 150 mM NaCl. B. Octopamine concentration, 1.6 μM. ○, control; ●, 150 mM NaCl. Each point is the mean of four experiments. Standard errors did not exceed 10% of the means.

11.8 μM, respectively.

Throughout the range of SAM concentrations (4.7–237 μM) and in the presence of 320 μM octopamine, NaCl produced a considerable increase in the velocity of the reaction. The magnitude of the enhancement was greater at higher SAM concentrations. However, for subsaturating octopamine concentrations, the degree of inhibition of enzyme activity produced by NaCl was reduced at higher SAM concentrations (Fig. 2B).

To determine whether the effects of NaCl on phenylethanolamine *N*-methyltransferase activity were dependent on the amine employed, experiments were performed in which normetanephrine or norepinephrine was used as *N*-methyl acceptor. With 47.4 μM SAM, NaCl (150 mM) increased the apparent K_m of normetanephrine (78 μM) and norepinephrine (8.3 μM) 4.3 and 4.8 times, respectively (Fig. 3A and B). In addition, the high substrate inhibition of enzyme activity was markedly reduced by NaCl (Fig. 3). As with octopamine, the effects of NaCl on the reaction using normetanephrine and norepinephrine were dependent on SAM concentration; i.e., greater inhibition was obtained at lower SAM concentrations (not shown).

Effects of NaCl on phenylethanolamine N-methyltransferase activity: buffer and pH requirements. At similar substrate concentrations the velocities obtained with sodium and potassium phosphate buffers were lower than those observed with Tris-HCl (Fig. 4A and B). An inverse relationship between enzyme activity and buffer concentration was observed at pH 8.6 in the presence of 0.8 μM octopamine. At this amine concentration, NaCl inhibited the enzyme activity. This effect was observed with both buffer systems over a wide pH range (7.0–9.4) and was enhanced when less concentrated buffers were employed (Fig. 4A).

On the other hand, at high amine concentrations an increase in the enzyme activity was observed at higher pH and with greater buffer concentrations (Fig. 4B). Under saturating amine conditions, NaCl

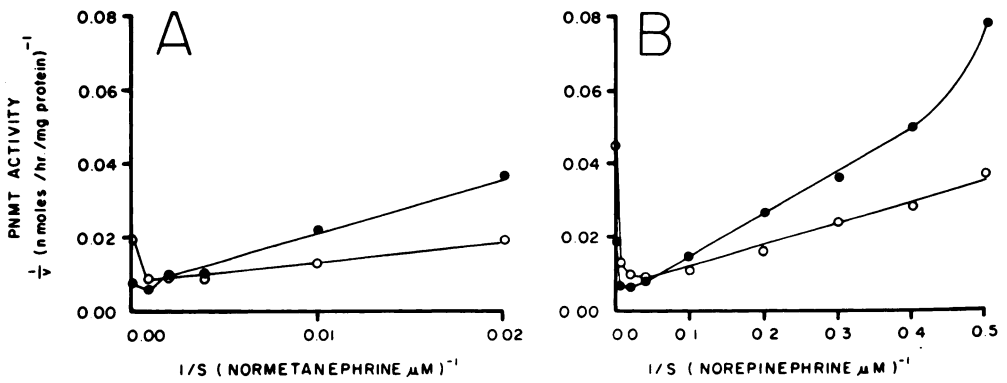

FIG. 3. *Effects of NaCl on phenylethanolamine N-methyltransferase (PNMT) activity with normetanephrine and norepinephrine as the variable substrates*

A. Assays were performed as described for Fig. 1, using an SAM concentration of 47.4 μM. The normetanephrine concentration ranged from 50 μM to 5 mM. Blanks averaged 80 ± 7 cpm. ○, control; ●, 150 mM NaCl.

B. Reaction mixtures, containing 19.3 μmoles of Tris-HCl buffer (pH 8.6), 0.5 μl of enzyme preparation, 0.5 μCi of [*methyl*-^{3}H]SAM, 3.8 nmoles of unlabeled SAM (47.4 μM, final concentration), and varying amounts of norepinephrine in a final volume of 80 μl, were incubated at 37° for 8 min. The reaction was stopped by adding 2 ml of sodium phosphate, pH 10, containing 5% EDTA and 0.1 mM dithiothreitol. Aluminum oxide (150 mg) was added to each tube. The samples were vortexed for five 5-sec periods and centrifuged at 600 × *g* for 20 sec. The supernatant was discarded, and the alumina was washed three times with 2 ml of distilled water. Then 1 ml of $HClO_4$ was added, and the tubes were vortexed as above. Acetic acid (50 μl, 0.2 N) containing 100 μg of SAM chloride and 25 μg of epinephrine was added to each tube, followed by 100 μl of a saturated solution of 25% phosphotungstic acid. After a 5-min rest on ice, the tubes were centrifuged at 600 × *g* for 5 min. A 1-ml aliquot of the supernatant was transferred to a tube containing 10 ml of 1% (v/v) diethylhexylphosphoric acid-toluene and 1 ml of 1.0 M phosphate buffer, pH 7.15. After shaking, the samples were centrifuged and 8 ml of the organic phase were counted by liquid scintillation spectrometry. Blanks averaged 47 ± 10 cpm, and 25 μM norepinephrine yielded 4394 ± 376 cpm. ○, control; ●, 150 mM NaCl.

accelerated the reaction at the concentrations of Tris-HCl employed (12.5–250 mM), while with phosphate buffer (2.5–125 mM) had only a negligible effect (10–20% increase) (Fig. 4B). Moreover, the degree of enhancement of enzyme activity by NaCl was pH-dependent; i.e., while no effect was obtained at pH 7.0, a 95.0% ± 8.2% increase was observed at pH 8.9.

Effects of KCl on phenylethanolamine N-methyltransferase activity. Because of the intracellular localization of the enzyme and the rather low concentrations of NaCl in this compartment (0.1 K_i), it appeared interesting to investigate whether KCl, the main intracellular monovalent ion salt, could modify the *N*-methylating activity of the enzyme. Like NaCl, KCl inhibited the enzyme activity at low substrate concentrations and increased the reaction velocity at high amine concentrations. Similar effects were obtained when norepinephrine, normetanephrine, or octopamine was used as *N*-methyl acceptor. The results obtained in the presence of octopamine are shown in Fig. 5.

Linear relationships were observed when the concentration of KCl was plotted against the reciprocal of velocity (Dixon plot) in the presence of 4.74 μM SAM (Fig. 5). These results are compatible with competitive kinetics. The K_i for KCl was 63 μM.

Effects of sucrose on phenylethanolamine N-methyltransferase activity. The effects of 150 and 300 mM sucrose on the enzyme activity were studied at two octopamine concentrations (1.6 and 320 μM) and at 4.74, 23.7, and 118.5 μM SAM. Under these conditions sucrose failed to modify the activity.

Effects of salts of monovalent and divalent ions on phenylethanolamine N-methyltransferase activity. To determine whether

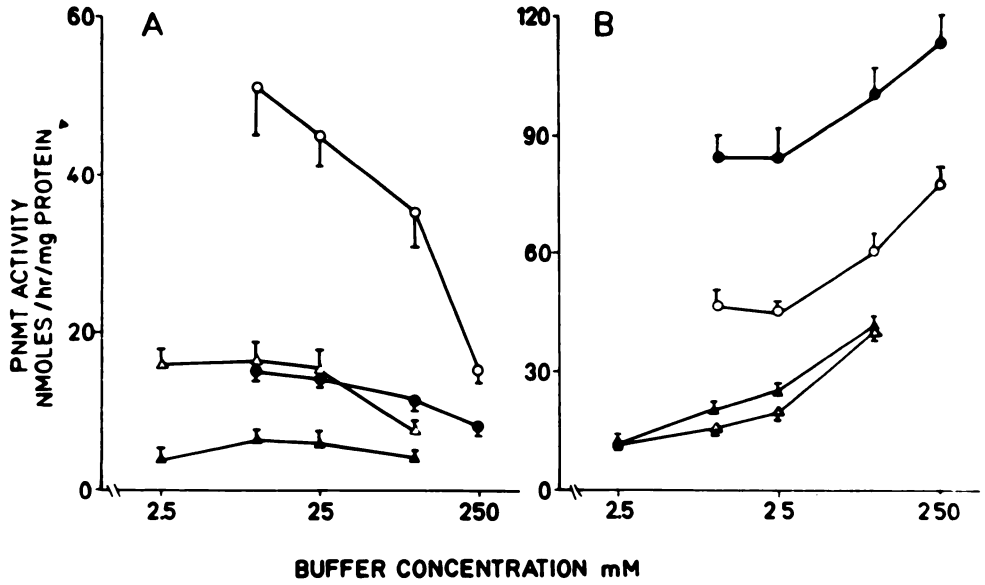

FIG. 4. *Effect of NaCl on phenylethanolamine N-methyltransferase (PNMT) activity: buffer requirements*
Enzyme activity was measured at constant pH (8.6) in the presence of several concentrations of sodium phosphate or Tris-HCl buffer. Results were the same with sodium and potassium phosphate buffers. ○, Tris-HCl, no NaCl; ●, Tris-HCl, 150 mM NaCl; △, phosphate, no NaCl; ▲, phosphate, 150 mM NaCl. A. Octopamine, 0.8 μM; SAM, 47.4 μM. The pH 8.6:7.9 activity ratios were 0.77, 1.0, and 1.16 for 12.5 mM phosphate, 125 mM phosphate, and 250 mM Tris-HCl, respectively. B. Octopamine, 320 μM; SAM, 47.4 μM. The pH 8.6:7.9 activity ratios were 1.23, 1.50, and 1.78 for 12.5 μM phosphate, 125 mM phosphate, and 250 mM Tris-HCl, respectively.

the effects of NaCl and KCl on the enzyme activity were dependent on the cationic or anionic moiety of the salt, experiments were undertaken in which the effects of several salts of monovalent and divalent ions on phenylethanolamine *N*-methyltransferase activity were evaluated. As shown in Table 1, equimolar concentrations of LiCl, CsCl, NaF, and NaBr produced effects on the enzyme activity similar to those observed with NaCl and KCl, i.e., inhibition at low and "activation" at high octopamine concentrations. The observed increase in the reaction velocity was dependent on the octopamine concentration employed (Table 1).

The possibility that these salt effects were dependent on the ionic strength of the solutions was investigated by comparing the effects of monovalent and divalent ion salts under identical ionic strength conditions. The results of these experiments are shown in Table 1. At subsaturating octopamine concentrations, inhibition of enzyme activity was observed in the presence of K_2SO_4, Na_2SO_4, $MgCl_2$, and $CaCl_2$. In addition, at high octopamine concentrations an increase in the velocity was found with the divalent ion salts.

The effects of $MgCl_2$ and $CaCl_2$ were quantitatively different from those observed with the other salts. In fact, in the presence of 32 μM octopamine, both salts inhibited rather than increased the reaction velocity (Table 1). Complete inhibition of enzyme activity was observed with $CuSO_4$ and $CuCl_2$. Under equal ionic strength conditions, the degree of enhancement of enzyme activity both for mono- and divalent ion salts was much lower with phosphate than with Tris-HCl buffer (Table 1).

DISCUSSION

Phenylethanolamine *N*-methyltransferase activity is commonly measured with substrates other than norepinephrine (12–15). Modification of a highly sensitive method for the determination of small quantities of norepinephrine (9) allowed us

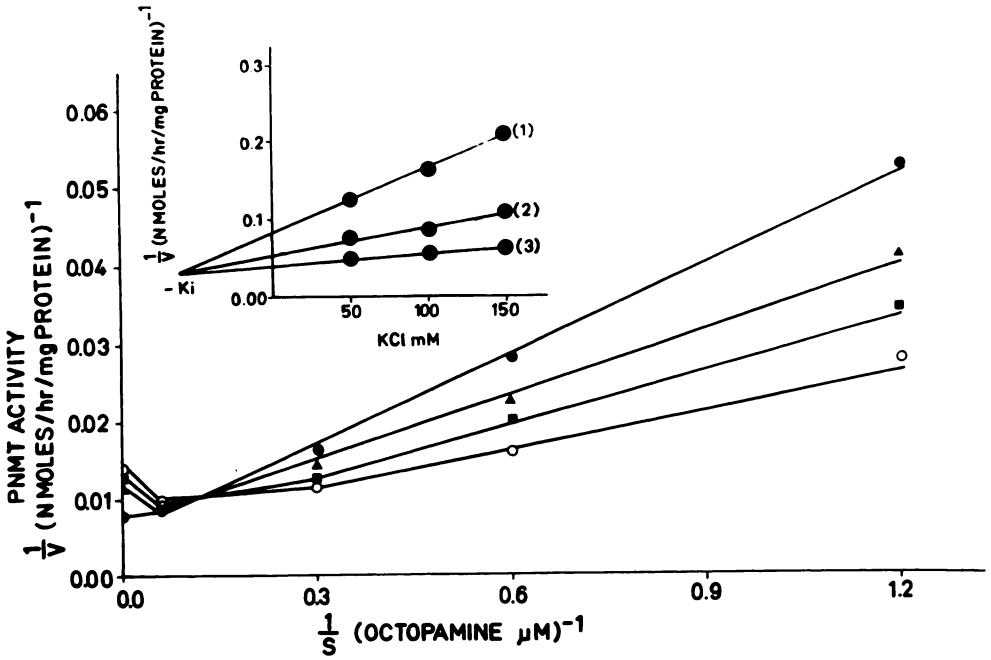

FIG. 5. *Effect of KCl on phenylethanolamine N-methyltransferase (PNMT) with octopamine as the variable substrate*

Assays were performed as described for Fig. 1, using 47.4 μM SAM, in the absence (○) and presence of several concentrations of KCl, ■, 50 mM; ▲, 100 mM; ●, 150 mM. Points are the means of four experiments. Standard errors did not exceed 12% of the means. Inset: assays were performed as for Fig. 1, using 4.74 μM SAM. Curve *1*, 0.8 μM octopamine; *2*, 1.6 μM; *3*, 3.2 μM. Points are the means of four experiments. Standard errors did not exceed 8% of the means.

to carry out studies with the natural substrate. This study shows that NaCl and KCl, at the concentrations present in body fluids, inhibited the enzyme activity in the presence of either norepinephrine, octopamine, or normetanephrine. In addition, at saturating amine concentrations, both salts enhanced the reaction velocity.

Although our studies provide no direct proof that the activity of phenylethanolamine *N*-methyltransferase could be modified *in vivo* by high intracellular concentrations of KCl, this possibility seems worthy of consideration, particularly since the salt effect was seen with the natural substrate, norepinephrine.

The biphasic shape of the substrate-velocity plots for the amines, plus the dual salt effect (inhibition at low and "activation" at high amine concentrations), would necessarily produce intersecting substrate-velocity curves. Whether this is responsible for the competitive kinetics of inhibition can not be ruled out.

The experiments with monovalent ion salts (LiCl, CsCl, NaF, and NaBr) and sucrose demonstrated that the NaCl and KCl effects were not determined by either the Na^+, K^+, or Cl^- moiety of the molecule or by the osmotic strength of the salt solution.

The possibility of an ionic strength-mediated effect was investigated by comparing, under identical ionic strength conditions, the effects of mono- and divalent ion salts on phenylethanolamine *N*-methyltransferase activity. Similar effects were obtained with mono- and divalent ion salts, i.e., inhibition at low and enhancement at high substrate concentrations. These results suggest that the NaCl and KCl effects are at least partly related to the ionic strength of the assay medium. The complete inhibition of enzyme activity produced by $CuCl_2$ and $CuSO_4$ at an ionic strength of 0.150 is probably related to

TABLE 1

Effects of salts of mono- and divalent ions on phenylethanolamine N-methyltransferase activity

Enzyme assays were performed at different SAM and octopamine concentrations as described in the legend to Fig. 1. At the final concentrations employed, each salt contributed an ionic strength of 0.150 to the reaction mixture, and no salt affected either reaction blanks or product extraction by more than 5%. The first four columns of results were obtained with Tris-HCl buffer, pH 8.6 (ionic strength 0.04). Results in column V were obtained in the presence of 85 mM sodium phosphate buffer, pH 8.6 (ionic strength 0.04).

Salt	I. 0.8 μM octopamine, 4.7 μM SAM	II. 32 μM octopamine, 47.4 μM SAM	III. 64 μM octopamine, 47.4 μM SAM	IV. 320 μM octopamine, 47.4 μM SAM	V. 320 μM octopamine, 47.4 μM SAM
			% control		
None (control)[a]	100	100	100	100	100
NaCl, 150 mM	25.6	119.6	140.0	190.0	110.3
KCl, 150 mM	24.6	123.3	154.0	239.1	136.3
LiCl, 150 mM	19.5	136.5	149.5	219.2	142.4
CsCl, 150 mM	29.7		158.8		
NaF, 150 mM	29.5	142.0	166.0	211.6	110.9
NaBr, 150 mM	28.2		142.0		
Na_2SO_4, 50 mM	32.4	141.0		187.3	134.3
K_2SO_4, 50 mM	31.8	136.6		202.0	123.1
$MgSO_4$, 37.5 mM	26.2	76.8		163.1	
$CuSO_4$, 37.5 mM	0				
$CuCl_2$, 50 mM	0				
$CaCl_2$, 50 mM	16.2	64.0		157.0	

[a] Phenylethanolamine *N*-methyltransferase activity was 18.2, 120.3, 90.3, 64.0, and 32.9 nmoles of octopamine formed per hour per milligram of protein in columns I–V, respectively. Values are the means of at least two experiments, in which assays were performed in triplicate. Standard errors did not exceed 12% of the means.

binding of the copper ions to sulfhydryl groups on the enzyme, with subsequent loss of enzyme activity (16). The effects produced by different buffer concentrations were similar to those observed with mono- and divalent ion salts. The present study indicates that measurements of phenylethanolamine *N*-methyltransferase activity at saturating substrate concentrations are facilitated by using more concentrated buffers, while more diluted buffers are recommended when low amine concentrations are employed.

In summary, the present study suggests that the effects of NaCl and KCl on phenylethanolamine *N*-methyltransferase activity are mediated by the ionic strength of the salt solutions. It is possible that these salt effects are related to changes in the conformational state of the enzyme molecule, rather than to specific competition by any mono- or divalent ion for the amine binding site. An increase in ionic strength would lead to reduced binding of the substrate molecules to the enzyme, which would be manifested by a decrease in the reaction velocity at subsaturating amine concentrations and by a reduction of the inhibitory effect observed at high or saturating amine concentrations.

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