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Isolation and Characterization of Multiple Forms of Phenylethanolamine N-Methyltransferase

TONG HYUB JOH AND MENEK GOLDSTEIN[1]

Department of Psychiatry, Neurochemistry Laboratory, New York University Medical Center, New York, New York 10016

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SUMMARY

JOH, TONG HYUB, AND GOLDSTEIN, MENEK: Isolation and characterization of multiple forms of phenylethanolamine *N*-methyltransferase. *Mol. Pharmacol.* **9**, 117–129 (1973). Phenylethanolamine *N*-methyltransferase (EC 2.1.1) was purified from the supernatant and particulate fractions of bovine adrenal medulla. The bulk of the enzymatic activity was associated with the supernatant fraction, but 15–20% of the activity was found to be associated with the particulate fraction (100,000 × *g* sediment). The enzyme from the particulate fraction was solubilized and partially purified. The enzyme from the supernatant fraction was isolated in pure form and has a molecular weight of approximately 40,000. The enzyme also occurs in two higher molecular forms, with molecular weights of approximately 80,000 and 160,000. Differently charged isozymes were separated from the low molecular weight form of the enzyme by DEAE-Sephadex chromatography and polyacrylamide disc gel electrophoresis. Amino acid analysis revealed a relatively high content of dicarboxylic acids or their amides and the presence of hexosamine. It is possible that the charge isozymes may arise by deamidation, or that a difference in the carbohydrate residues exists. The two major charge isozymes (designated here as B_1 and B_2) are indistinguishable from each other on immunoelectrophoresis. The immunochemical analyses revealed heterogeneity of adrenal phenylethanolamine *N*-methyltransferase among different species. It appears that the corticoid-inducible, mammalian adrenal enzyme is immunologically distinguishable from the uninducible, frog adrenal enzyme.

INTRODUCTION

The enzyme phenylethanolamine *N*-methyltransferase (EC 2.1.1) catalyzes the conversion of norepinephrine to epinephrine (1, 2). It accepts as substrates various phenylethylamines which possess a β-hydroxyl group at the side chain (2). The enzyme is present in the soluble fraction of the adrenal medulla, and *S*-adenosylmethionine serves as methyl donor (1, 2).

The present report describes the purification and characterization of multiple forms of bovine adrenal phenylethanolamine *N*-methyltransferase. Partial characterization of the major molecular species was accomplished by means of physicochemical and immunological analyses. The interaction of rabbit anti-enzyme antiserum with enzyme from tissues of different species was also

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investigated. Preliminary reports on these studies have been presented (3, 4).

EXPERIMENTAL PROCEDURE

Materials and Methods

[^{14}C]*S*-Adenosylmethionine with a specific activity of 45 μCi/μmole was obtained from New England Nuclear Corporation. Bio-Gel P-100 was obtained from Bio-Rad Laboratories, and Sephadex and DEAE-Sephadex were obtained from Pharmacia. All other compounds were obtained from various commercial sources.

Enzyme assay. Enzyme activity was assayed as previously described (2, 5), using phenylethanolamine as substrate if not otherwise stated. In some experiments the enzymatically formed products were separated from the substrate as acetylated derivatives by paper chromatography (6). Under the standard assay conditions the incubation was carried out at 37° for 10 min. One unit of phenylethanolamine *N*-methyltransferase is defined as the amount which catalyzes the formation of 1 μmole of product in 60 min under the standard assay conditions. Specific activity is expressed as units per milligram of protein.

Protein was assayed by the method of Lowry *et al.* (7), except in monitoring column effluents, when the optical density at 280 nm was taken as a measure of protein concentration.

Polyacrylamide gel electrophoresis was performed by the method of Davis (8) and Ornstein (9). Electrophoresis was carried out in 0.4 M glycine-Tris buffer, pH 8.2, at 3 mamp/tube at room temperature, using 7.0% gel if not otherwise stated. In some experiments the electrophoresis was carried out in 0.05 M Tris-HCl buffer, pH 8.2. Amido black was used to stain the protein on the gel. To test enzyme activity gels were sliced into pieces, and each piece was dispersed in 0.5 ml of 4 mM potassium phosphate buffer, pH 7.3. The activity was tested in these mixtures by the standard assay.

Electrophoresis on polyacrylamide gel containing sodium dodecyl sulfate was carried out according to the method of Shapiro *et al.* (10). For molecular weight determinations by polyacrylamide gel electrophoresis standard proteins and the purified enzyme were incubated for 4 hr at 37° in 0.1 M sodium phosphate buffer, pH 7, containing 0.1% SDS[2] and 0.1% mercaptoethanol. The mixture was then dialyzed for 14 hr against 0.01 M sodium phosphate buffer, pH 7, containing 0.01% SDS and 0.1% mercaptoethanol.

To distinguish between a size isomer family of proteins and a charge isomer family of proteins the disc gel electrophoretic procedure described by Hedrick and Smith (11) was used. Isoelectric focusing in polyacrylamide gels was performed by a modified procedure of Righetti and Drysdale (12).[3]

Analytical ultracentrifugation. Centrifugation was performed in a Spinco model E analytical ultracentrifuge equipped with ultraviolet absorption optics and a photoelectric scanner. For the determination of molecular weight the column height and motor speed were adjusted to enable use of the meniscus depletion method developed by Yphantis (13).

Amino acid analysis. A solution of enzyme containing 1 mg/ml of protein was dialyzed exhaustively against distilled water in the cold. The samples were then hydrolyzed under vacuum with 6 N HCl for 24 hr. The acid hydrolysates were analyzed with a Beckman model 120 C instrument according to the procedure of Spackman *et al.* (14).

Immunochemical Studies

Immunization of rabbits. Purified phenylethanolamine *N*-methyltransferase (after the DEAE-Sephadex purification step) in 0.9% NaCl was emulsified with an equal volume of complete Freund's adjuvant. Immunization was performed by injecting 1.0 mg of enzyme in 1 ml of adjuvant in divided doses at four intramuscular sites. The injections were repeated every 2 weeks for a period of 3 months, and the rabbits were bled 1 week later. In some experiments about 1 mg of purified enzyme was subjected to disc gel electrophoresis, loading 100 μg of protein on each of 10 gels. The protein bands

[2] The abbreviation used is: SDS, sodium dodecyl sulfate.

[3] S. Lee-Huang, unpublished data.

(3–4 mm wide) were cut out of the gels, extruded through an 18-gauge needle, and allowed to stand in an equal volume of 0.9% NaCl for a few hours in the cold. An equal volume of complete Freund's adjuvant was added and thoroughly mixed. The entire mixture was injected subcutaneously into rabbits, and the immunization procedure was repeated at the same time intervals as described above. The antibodies in the serum were tested against the antigen by Ouchterlony immunodiffusion analyses (15) and microimmunoelectrophoresis (16). Immunoglobulin was precipitated from the serum at 40–50% saturation with ammonium sulfate, or in some experiments was further purified on DEAE-cellulose (17). The active sera were stored at −20°.

RESULTS

Purification of Bovine Adrenal Phenylethanolamine N Methyltransferase

All steps were carried out at 0–4° unless otherwise specified. Fresh or fresh frozen adrenal medulla (about 200 g) was suspended in 800 ml of 0.25 M sucrose and homogenized in a Waring Blender for 30 sec. The homogenate was then centrifuged for 10 min at 700 × *g*, and the sediment was discarded. The supernatant fraction was diluted to 1800 ml with 0.25 M sucrose and then decanted through four layers of cheesecloth to remove the fat. The supernatant fraction was then centrifuged at 100,000 × *g* for 1 hr, and the precipitate was removed. The enzyme was purified from the supernatant and particulate fractions separately.

Supernatant fraction. A summary of the results of the purification of phenylethanolamine *N*-methyltransferase from the supernatant fraction is shown in Table 1. Following chromatography on a Sephadex G-100 column two peaks of enzymatic activity were found; much higher specific activity was present in the more slowly moving peak (Table 1), and this material was used for further purification. When the peak with the lower specific activity was rechromatographed on Sephadex G-100, the elution pattern again showed two peaks of activity, with the higher specific activity present in the slower peak. When the enzyme purified on a DEAE-Sephadex A-50 column was rechromatographed on a second DEAE-Sephadex A-50 column, five tubes with high enzymatic activity were separately collected (Table 1). The most active enzyme obtained by this procedure had a specific activity of approximately 3000 units/mg when assayed under standard conditions. At this stage the enzyme could be stored in the presence of dithiothreitol at −20° for 3 months with-

TABLE 1

Purification of phenylethanolamine N-methyltransferase obtained from supernatant fraction of bovine adrenal medulla (200 g of bovine adrenal medulla)

Purification step	Volume	Total protein	Total activity	Specific activity	Yield	Purification
	ml	*mg*	*units*	*units/mg*	%	*-fold*
$(NH_4)_2SO_4$, 80% ppt	250	8,400	198,240	23.6	100	0
$(NH_4)_2SO_4$, 35–50% ppt	50	1,050	122,640	116.8	62	5
Sephadex G-100						
Fraction 1	40	582	72,284	124.2	36.4	5.3
Fraction 2	30	152	66,394	436.8	33.5	18.5
pH 5.5 supernatant	28	98	60,642	618.8	30.6	26.2
First DEAE-Sephadex A-50	5.0	21.3	41,045	1,927.0	20.7	81.7
Second DEAE-Sephadex A-50						
Fraction 1	1.0	2.3	4,570	1,987.0	2.3	84.2
Fraction 2	1.0	3.9	10,667	2,735.0	5.4	115.9
Fraction 3	1.0	3.8	11,348	2,989.0	5.7	126.7
Fraction 4	1.0	3.0	7,389	2,463.0	3.7	104.4
Fraction 5	1.0	2.4	4,637	1,932.0	2.3	81.9

out any loss of activity. All studies, if not otherwise stated, were carried out with enzyme preparations from the supernatant fraction.

Particulate fraction. The 100,000 × *g* sediment was suspended in 400 ml of 0.25 M sucrose, homogenized gently, and then centrifuged. The pellet was washed three times with 200 ml of 0.02 M potassium phosphate buffer, pH 6.5. Aliquots of each wash were assayed for enzymatic activity, and the last wash contained less than 10% of the activity of the original wash. The pellet was suspended again in 200 ml of 0.02 M phosphate buffer, and the enzyme was solubilized with the detergent Cutscum by the same procedure as previously described for the solubilization of dopamine β-hydroxylase (18). The solubilized enzyme was purified by $(NH_4)_2SO_4$ fractionation and then further purified by chromatography on a Bio-Gel P-100 column. Chromatography on Bio-Gel P-100 yielded two peaks of activity; the higher specific activity was found in the more slowly moving peak (Table 2). When the peak with the lower specific activity was rechromatographed on Bio-Gel P-100, the elution pattern again showed two peaks of activity, with the higher specific activity present in the slower peak. The results of the purification of phenylethanolamine *N*-methyltransferase from the particulate fraction (the same preparation as presented in Table 1) are summarized in Table 2.

Electrophoretic Studies

Polyacrylamide disc gel electrophoresis of the purified enzyme preparations obtained from the supernatant (after the first DEAE-Sephadex chromatography) and particulate (after Bio-Gel P-100 chromatography) fractions revealed several protein-staining bands. The distribution of enzyme activity among the gel sections after disc gel electrophoresis of the supernatant enzyme preparation is shown in Fig. 1. The enzymatic activity was associated with several protein bands and seemed to be proportional to the density of the stained proteins. The activity pattern obtained after elution of protein from disc gel electrophoresis of the particulate fraction was similar to that of the supernatant preparation. The enzymatic activity was associated mainly with protein bands located in the middle of the gel.

Polyacrylamide disc gel electrophoresis patterns of the five enzymatically active fractions obtained after the second DEAE-Sephadex chromatography are shown in Fig. 2. Each fraction showed one or two major protein-staining bands. Fractions 3 and 4 each exhibited a major protein band with different electrophoretic mobility.

The polyacrylamide disc gel electrophoresis pattern of the combined fractions 3 and 4 is shown in Fig. 3. Electrophoresis of the combined fractions gave two major protein bands (B_1 and B_2), both enzymatically active.

When band B_1 was eluted from the first electrophoresis gel and subjected again to electrophoresis, small amounts of band B_2 (approximately 20% of the amount of band B_1) were generated. When band B_2 was subjected to second electrophoresis, no new protein bands were generated.

The pattern given by electrofocusing of the combined fractions 3 and 4 in a wide pH

TABLE 2

Purification of phenylethanolamine N-methyltransferase obtained from particulate fraction of bovine adrenal medulla (200 g of bovine adrenal medulla)

Purification step	Volume	Total protein	Total activity	Specific activity	Yield	Purification
	ml	*mg*	*units*	*units/mg*	%	*-fold*
$(NH_4)_2SO_4$, 80% ppt	250	2,380	41,174	17.3	100	0
$(NH_4)_2SO_4$, 35–50% ppt	25	396	23,482	59.3	57.0	3.4
Bio-Gel P-100						
Fraction 1	20	262	15,799	60.3	38.4	3.5
Fraction 2	2	7.9	4,500	569.6	10.9	32.9

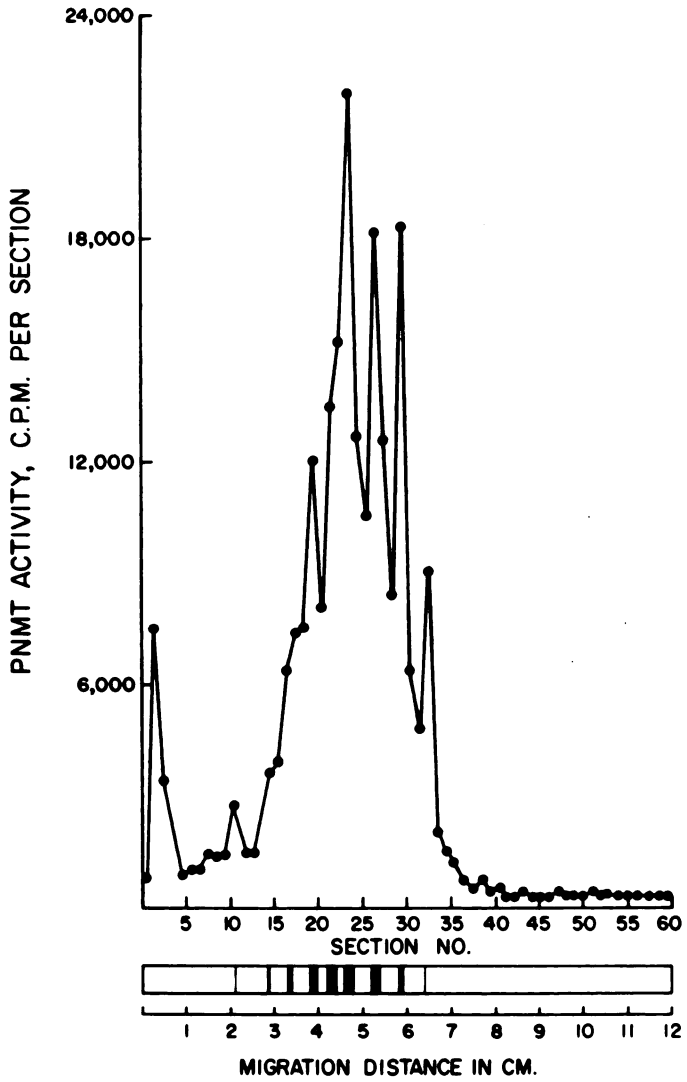

FIG. 1. *Distribution of phenylethanolamine N-methyltransferase (PNMT) activity after polyacrylamide gel electrophoresis*

A 25-μl aliquot (50 μg of protein) of enzyme preparation from the supernatant fraction obtained after the first DEAE-Sephadex A-50 purification stage was layered onto the gel (120-mm length). The sample was run for 120 min. The unstained gel was sliced into 2-mm sections as described in the text. The proteins were stained on a gel run in parallel with the gel shown to determine the distribution of enzyme activity, and the positions of the bands are depicted above.

range (pH 3–10) was somewhat similar to the conventional electrophoretic pattern. Two protein-staining bands, though poorly resolved, were visible, with isoelectric points between approximately pH 5.6 and 6.1. Isoelectric focusing in polyacrylamide gel of the transferase obtained from the combined five enzymatically active fractions after chromatography on the second DEAE-Sephadex column revealed a third protein band, with an isoelectric point between approximately pH 8.0 and 8.5.

Evidence for Charge Isozymes

It was shown above that the enzyme activity is associated with several protein

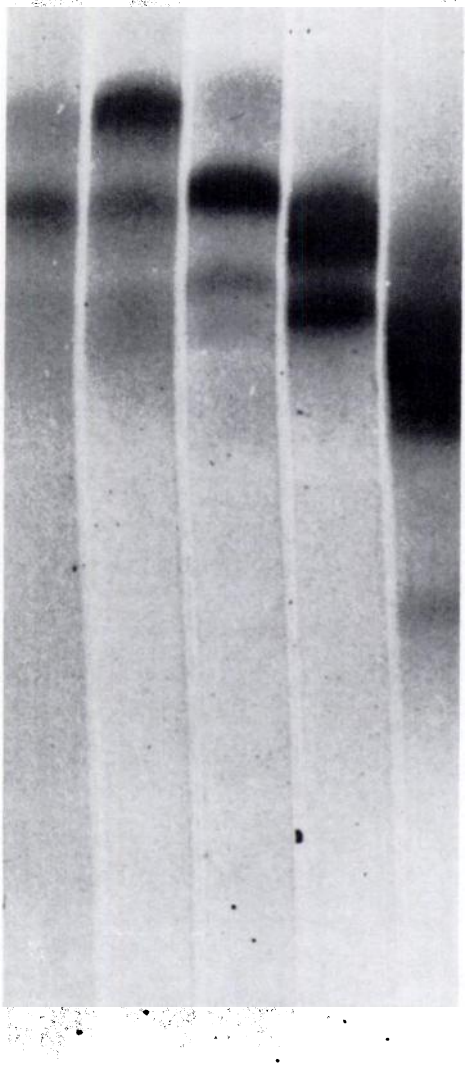

FIG. 2. *Photograph of polyacrylamide gel electrophoresis of the five enzymatically active fractions obtained after second chromatography on DEAE-Sephadex A-50*

The samples (100 μg of protein in each gel) were run for 90 min. The anode is at the bottom. Gels were developed as described in the text.

bands obtained by polyacrylamide gel electrophoresis (Fig. 1). The enzyme preparation obtained after the first DEAE-Sephadex chromatography was subjected to electrophoresis on gels with different percentages of acrylamide. The results presented in Fig. 4 show that when the logarithm of protein mobility (relative to dye front) was plotted with respect to acrylamide gel concentration, a family of parallel lines was obtained. Proteins which differ in charge but not in size give such a pattern.

Molecular Weights of Different Forms of the Enzyme

Chromatography on Sephadex G-200. Sephadex gel filtration experiments were performed to estimate the molecular weight of different forms of phenylethanolamine *N*-methyltransferase. Sephadex G-200 chro-

FIG. 3. *Photograph of polyacrylamide gel electrophoresis of combined fractions 3 and 4 obtained after second chromatography on DEAE-Sephadex A-50*

The enzyme was subjected to electrophoresis as described in Fig. 2.

matography of the enzyme preparation obtained after the $(NH_4)_2SO_4$ fractionation step revealed three active forms of the enzyme with different sizes (Fig. 5). Based on calibration of the Sephadex column with proteins of known molecular weight (Fig. 6), it can be estimated that the enzymatic activity peaks correspond to molecular weights of approximately 40,000, 80,000, and 160,000.

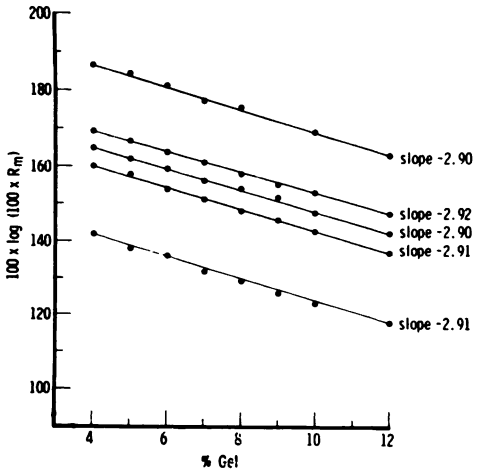

FIG. 4. *Effect of differing acrylamide concentrations on migration of phenylethanolamine N-methyltransferase isozymes during electrophoresis on polyacrylamide gels*

A 25-μl aliquot (50 μg of protein) of enzyme preparation from the supernatant fraction obtained after the first DEAE-Sephadex purification stage was layered onto the gels with different acrylamide concentrations. See the text for details.

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate. When the purified enzyme (after the first DEAE-Sephadex purification step) was subjected to gel electrophoresis in sodium dodecyl sulfate, a single band was obtained (Fig. 7). A molecular weight of approximately 38,000 was estimated from the migration of this band relative to those of several of other proteins of known polypeptide chain molecular weight (Fig. 8).

Ultracentrifugation. The purified enzyme (after the first DEAE-Sephadex purification step) was analyzed in a model E analytical ultracentrifuge. The patterns observed showed a homogeneous, symmetrical peak which slightly widened as it moved down the cell (Fig. 9). A sedimentation coefficient ($s_{20,w}$) with a value of 3.5 S was calculated.

Molecular weights were estimated by recording sedimentation equilibrium data at various concentrations of the transferase. The values seemed to be independent of concentration in the range from 0.7 to 1 mg of protein per milliliter. At these protein concentrations a minimum molecular weight of 38,500 and a maximum molecular weight of 39,600 were calculated.

Amino Acid Analyses

Amino acid analyses were carried out on enzyme preparations from fractions 3 and 4 obtained after the second DEAE-Sephadex chromatography. The data in Table 3 show that the amino acid composition of phenylethanolamine *N*-methyltransferase is similar

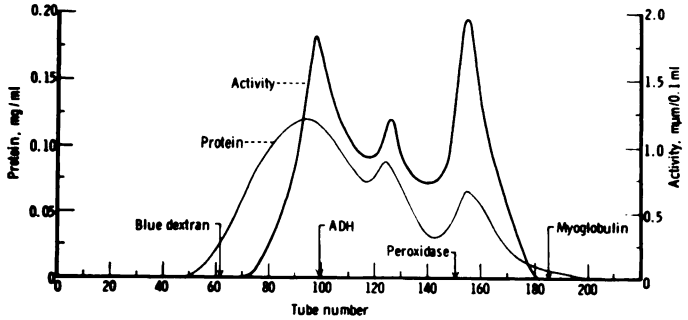

FIG. 5. *Sephadex G-200 column chromatography of phenylethanolamine N-methyltransferase*

Protein (approximately 30 mg) obtained after the $(NH_4)_2SO_4$ fractionation step was applied to a column of Sephadex G-200 (2.5 × 95 cm) and eluted with 0.01 M Tris-HCl, pH 7.3. Proteins of known molecular weight were co-chromatographed on the same column. The elution peaks for protein standards are indicated by arrows. ADH, alcohol dehydrogenase.

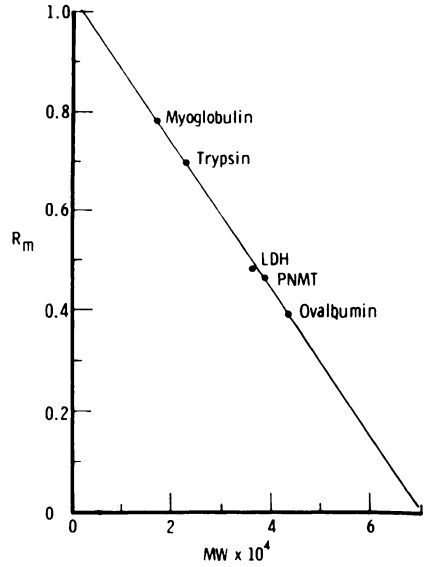

FIG. 6. *Determination of molecular weight of phenylethanolamine N-methyltransferase (PNMT) by Sephadex G-200 column chromatography*

The transferase and proteins of known molecular weight were chromatographed on the column described in Fig. 5. Elution volumes were determined for the three forms of phenylethanolamine *N*-methyltransferase and for the protein standards, and these values were used to calculate their Stokes radii by the method of Ackers (19). Values of 4.70 nm for isozyme 1, 2.70 nm for isozyme 2, and 1.28 nm for isozyme 3 were obtained. For protein standards the values were similar to those reported by Ackers (19). ADH, alcohol dehydrogenase.

in fractions 3 and 4. The major discrepancy between the two fractions is the content of threonine and serine, which are known to be labile under the conditions of acid hydrolysis. The transferase is especially rich in the dicarboxylic acids, glutamic acid and aspartic acid, or their amides, as well as in arginine and leucine. The analysis also revealed the presence of 1% hexosamine per total content of protein in both fractions.

Immunochemical Characterization

Three samples of antisera were prepared. One was prepared with the enzyme preparation obtained after the first DEAE-Sephadex column chromatography (enzyme antiserum I). The other two samples were prepared with enzyme preparations after separate elutions from the gels of protein bands B_1 and B_2 following polyacrylamide disc gel electrophoresis (enzyme antisera B_1 and B_2). Figure 10 shows that antiserum I gave a single long precipitin arc with purified bovine adrenal phenylethanolamine *N*-methyltransferase on immunoelectrophoresis. Higher quantities of antigen produced two precipitin arcs with the same antigenic mobility but with different diffusion rates.

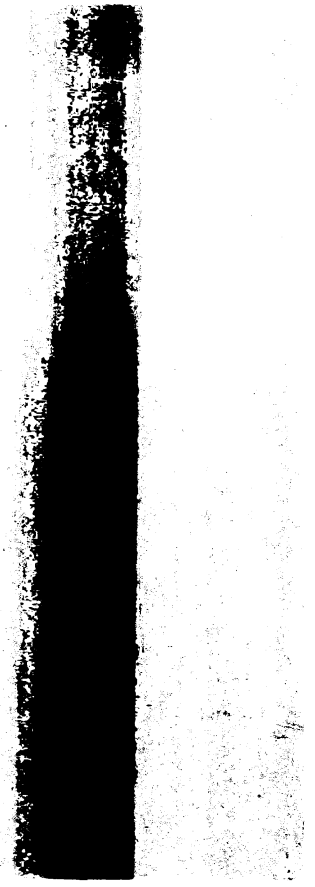

FIG. 7. *Electrophoresis of phenylethanolamine N-methyltransferase on sodium dodecyl sulfate-polyacrylamide gel*

A 10% acrylamide–0.30% bis-gel in 0.1% SDS was employed; the direction of migration is from top to bottom. Enzyme preparation (0.05 mg) obtained from the pooled fractions after the second DEAE-Sephadex A-50 column chromatography, in 0.05 ml of sodium phosphate buffer (pH 7.1), 0.1 ml of glycerol, 0.005 ml of bromphenol blue, and 0.005 ml of mercaptoethanol, was applied to a 9-cm SDS-gel. Electrophoresis was carried out for 1.5 hr at 4 mamp/gel.

Figure 11 shows that antisera B_1 and B_2 gave a single precipitin arc with purified bovine phenylethanolamine *N*-methyltransferase. When antisera B_1 and B_2 were mixed together, or individually with antiserum I, a single arc was also obtained on immunoelectrophoresis.

Cross-reactivity

Interaction of antibody with phenylethanolamine *N*-methyltransferase from other sources was investigated. In double-diffusion reactions with the antibody, lines of precipitation were obtained with phenylethanolamine *N*-methyltransferase from rat

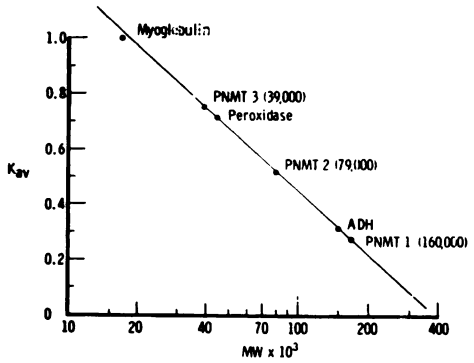

FIG. 8. *Electrophoretic mobility of phenylethanolamine N-methyltransferase (PNMT) and protein standards on sodium dodecyl sulfate–polyacrylamide gel*

A 10% acrylamide–0.20% bis-gel in 0.1% SDS was employed. The gel buffer was 0.05 M sodium phosphate, pH 7.1. Samples were subjected to electrophoresis for 1.5 hr at 8 mamp/gel at 25°. The relative migration (R_m) of the proteins was estimated as described by Rutner (20). LDH, actate dehydrogenase.

TABLE 3

Amino acid composition of bovine phenylethanolamine N-methyltransferase

All figures are averages of values from two analyses. The number of residues was calculated by assuming a molecular weight of 40,000 for the protein.

Amino acid	Nearest integer	
	Fraction 3	Fraction 4
Lysine	14	13
Histidine	6	8
Arginine	31	29
Cystine	—[a]	—[a]
Aspartic acid[b]	25	30
Threonine	6	10
Serine	13	19
Glutamic acid[b]	50	50
Proline	29	25
Glycine	33	33
Alanine	43	41
Valine	33	30
Methionine sulfone	1	1
Isoleucine	15	14
Leucine	50	47
Tyrosine[c]	6	6
Phenylalanine	6	6
Tryptophan[c]	12	12

[a] Present.

[b] These figures include both free and amidated residues of aspartic and glutamic acids.

[c] Determined spectrophotometrically.

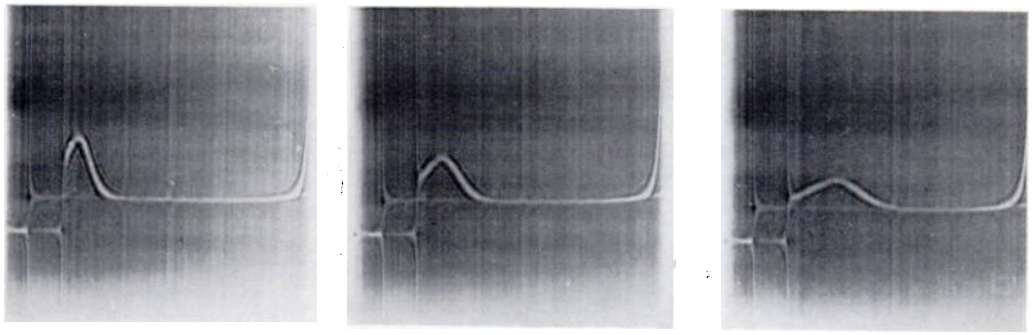

FIG. 9. *Schlieren pattern of purified phenylethanolamine N-methyltransferase obtained in Spinco model E ultracentrifuge*

Enzyme obtained after the first DEAE-Sephadex column chromatography was centrifuged at 52,600 rpm (3.3 mg of protein per milliliter, double-sector cell) at 5°. Photographs, from left to right, were taken at 8, 32, and 64 min.

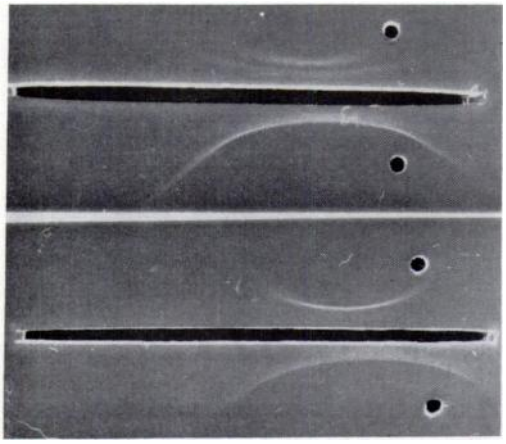

FIG. 10. *Immunoelectrophoretic analyses of purified phenylethanolamine N-methyltransferase against 100 μl of rabbit antiserum I*

The buffer was sodium barbital, pH 8.6, ionic strength 0.1. Upper: the upper well contained 10 μg of enzyme; the lower well contained 7.5 μg. Lower: the upper well contained 5 μg of enzyme; the lower well contained 7.5 μg.

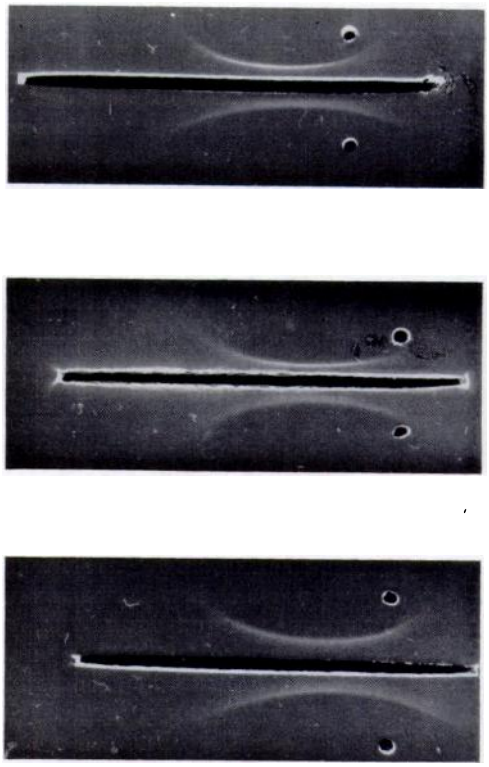

FIG. 11. *Immunoelectrophoretic analyses of purified phenylethanolamine N-methyltransferase against rabbit antisera B_1 and B_2*

Conditions were the same as described in Fig. 10. Upper: 100 μl of antiserum B_1; the upper well contained 5 μg of enzyme, and the lower well contained 7.5 μg. Middle: 100 μl of antiserum B_2; the upper well contained 5 μg of enzyme, and the lower well contained 7.5 μg. Lower: 50 μg of antiserum B_1 and 50 μg of antiserum B_2; the upper well contained 5 μg of enzyme, and the lower well contained 7.5 μg.

adrenals and human pheochromocytoma. Figure 12 shows that the enzyme antiserum gave a single short arc with the antigen obtained from the adrenals of rats. The precipitin arc obtained with rat adrenal enzyme had a different antigenic mobility from that obtained with the bovine adrenal preparation.

An immunoelectrophoretic analysis of antiserum I against a mixture of antigens from rat and bovine adrenals shows two arcs with fusion (one spur), indicating some common determinants of the antibody reaction with both enzymes.

Cross-reactivity to phenylethanolamine *N*-methyltransferase was also investigated by enzyme inhibition studies. As shown in Table 4, an amount of antiserum which inhibited homologous antigen by 95–100% inhibited the enzyme from human pheochromocytoma, mouse neuroblastoma, and rat adrenals by 45–60%. No inhibition, but rather a slight enhancement of activity, was observed upon addition of the antiserum to enzyme preparations from adrenals of frogs and olfactory bulbs of rats.

DISCUSSION

While this study was in progress (3, 4) another report described the purification of phenylethanolamine *N*-methyltransferase from the supernatant fraction of bovine adrenal medulla (21). Although both procedures are similar, one difference deserves to be pointed out. The purified enzyme (after the Sephadex G-100 purification step) became unstable, and the addition of dithiothreitol during the purification procedure resulted in stabilization of enzyme activity. The purified enzyme in the presence of dithiothreitol does not lose enzymatic activity during the ultracentrifugation studies or upon storage for several months at −20°, as previously reported (21).

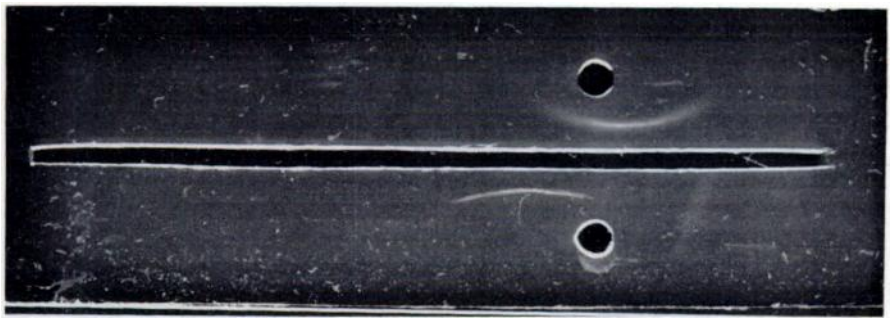

FIG. 12. *Immunoelectrophoretic analyses of rat adrenal phenylethanolamine N-methyltransferase against 100 μl of rabbit antiserum I*

Conditions were the same as described in Fig. 10. The upper well contained 5 μg of enzyme; the lower well contained 5 μg of rat adrenal enzyme. Rat adrenal phenylethanolamine *N*-methyltransferase was prepared as described in Table 4.

TABLE 4

Specificity of phenylethanolamine N-methyltransferase inhibition by antibody

Tissues were homogenized in 0.005 M potassium phosphate buffer, pH 7.3. The homogenates were centrifuged at 40,000 × *g* for 20 min. The volumes in the supernatant fraction were adjusted to contain approximately 0.1×10^{-3} unit of enzyme activity. Aliquots of the supernatant were incubated with 25 μl of enzyme antiserum I for 1 hr at room temperature, and bovine serum albumin was added to maintain a roughly constant protein concentration. Following the incubation period the mixtures were centrifuged at 40,000 × *g* for 30 min, and aliquots of the supernatant were assayed for enzymatic activity.

Enzyme source	Activity
	% *Control*
Bovine adrenal	0–5
Rat adrenal	40–50
Frog adrenal	120–130
Human pheochromocytoma tumor	45–50
Mouse neuroblastoma tumor	50–55
Rat olfactory bulb area	115–125

The results presented in Tables 1 and 2 show that approximately 15–20% of the enzymatic activity remains even after several washes with the particulate fraction. The particulate localization of the bovine adrenal transferase was examined further by gradient centrifugation and analysis of several enzymes and catecholamines in the isolated fractions. The bulk of the activity of the particulate enzyme seems to be associated with microsomal material.[4] It is noteworthy that the bulk of particulate tyrosine hydroxylase activity is associated with mitochondrial and microsomal material (22). It is conceivable that tyrosine hydroxylase and phenylethanolamine *N* - methyltransferase are associated with membrane fragments of cellular particles. Evidence has been obtained that the transferase, like tyrosine hydroxylase, exists in multiple forms and that both enzymes might be soluble *in situ* and tend to aggregate and become adsorbed to particulate material.

The elution pattern from Sephadex G-200 indicates that the enzyme exists in three different molecular forms, with molecular weights of approximately 40,000, 80,000, and 160,000. The finding that on immunoelectrophoresis higher quantities of antigen produced two arcs with the same antigenic mobility but with different diffusion rates indicates that the enzyme aggregates to higher molecular weight species. The molecular weight of 38,000–40,000 for the purified enzyme was also calculated from ultracentrifugation analysis and from the electrophoretic mobility of the enzyme on sodium dodecyl sulfate–polyacrylamide gel. This value is in agreement with the previously reported molecular weight of 38,000 for purified phenylethanolamine *N*-methyltransferase (21).

The electrophoretic pattern indicates that

[4] T. H. Joh, T. Ohuchi, and M. Goldstein, unpublished observations.

the purified enzyme consists of two major protein bands (which were arbitrarily designated B_1 and B_2) and several minor protein bands. This leads to two important questions. First, what is the structural and chemical basis of the multiple forms of phenylethanolamine *N*-methyltransferase and, second, what is their origin? The results presented in this paper indicate that the multiple forms are charge isozymes with the same molecular weight. However, it is not yet clear whether the isozymes are artifacts formed during the purification procedure or whether they occur naturally. It was noted that all enzyme preparations, regardless of the purification stage, showed multiple bands of activity and that these bands did not change significantly in the course of the purification. The possibility that all multiple bands were formed as artifacts during the electrophoresis does not seem likely, on the basis of the following experiments. First, each of the five enzymatically active fractions obtained after the second DEAE-Sephadex chromatography showed a different electrophoretic pattern. Second, isozyme B_1 generated only a small amount of isozyme B_2 after the second electrophoresis. Thus the rate of interconversion of the isozymes may have been accelerated during the electrophoresis although originally some isozymes may have been formed in the living cell. It is possible that the charge isozymes may have resulted from the same protein with various degrees of amidation or various carbohydrate residues. Since phenylethanolamine *N*-methyltransferase is rich in dicarboxylic acids and contains hexosamine, both possibilities deserve further investigation. We would like to postulate that, because of the difference in charge, the various isozymes may be found differently within the cell, with varying functional or catalytic activities.

It is noteworthy that not only phenylethanolamine *N*-methyltransferase but also other soluble protein constituents of the chromaffin granules, such as chromogranins (23, 24), contain large amounts of acidic amino acids. Consideration might be given to the possibility that the acidic amino acids or their amides are involved in the interaction of these proteins with the granule membrane.

Axelrod and Vesell (25) have shown that adrenal phenylethanolamine *N*-methyltransferase is heterogeneous among different species with respect to electrophoretic mobility on starch blocks. We have shown that the precipitin arc obtained with the rat enzyme has a different antigenic mobility from that obtained with the bovine adrenal transferase. The spur formation seen upon immunoelectrophoresis of enzyme antiserum against a mixture of phenylethanolamine *N*-methyltransferases from rat and bovine adrenals also indicates enzyme heterogeneity in these two species. The partial inhibition of the enzyme from human pheochromocytoma, mouse neuroblastoma, and adrenal glands of rats produced by quantities of antiserum sufficient to inhibit the bovine adrenal enzyme completely may be attributable to antibody heterogeneity, or more probably, to enzyme heterogeneity. The enzyme from frog adrenals and rat olfactory bulbs is not inhibited by antibody. It is noteworthy that phenylethanolamine *N*-methyltransferase in mammalian adrenal glands is induced by corticoids, whereas the frog enzyme probably is uninducible (26). It appears, therefore, that the antibody does not cross-react with the corticoid-uninducible form of the enzyme and that the two forms may be immunologically distinguishable.

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