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Studies on Radioimmunoassay of Metanephrine

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Antigens of 3-O-methylcatecholamines (metanephrine, normetanephrine, 3-methoxytyramine) were prepared, keeping the methoxyl and hydroxyl groups of the benzene ring and the side chain intact. Rabbits were immunized with the metanephrine antigen and sufficient antiserum to develop a specific radioimmunoassay of metanephrine was obtained. The detection limit of metanephrine in the radioimmunoassay was 0.03 pmol. The radioimmunoassay was useful for the measurement of urinary metanephrine and of rat liver catechol-O-methyltransferase activity.

Keywords—catecholamine; catechol-O-methyltransferase; epinephrine; metanephrine; normetanephrine; 3-methoxytyramine; antigen; antiserum; radioimmunoassay; human urine

Epinephrine, one of the catecholamines, plays a significant role as a hormone in the body. The hormone is metabolized to inactive metanephrine by catechol-O-methyltransferase (COMT) (EC 2.1.1.6). The determination of metanephrine level is relevant to studies of the biosynthesis and metabolism of epinephrine. The determination methods so far developed for metanephrine are based on colorimetry,²⁾ fluorometry,³⁾ high performance liquid chromatography,⁴⁾ gas chromatography,⁵⁾ and gas chromatography-mass spectrometry.⁶⁾ However, these methods involve tedious pretreatment, such as extraction with organic solvents.

To overcome such difficulties, Lam *et al.* developed⁷⁾ a radioimmunoassay method for urinary metanephrine utilizing the Grota antiserum,⁸⁾ raised against a synephrine conjugated with BSA, which had high affinity for metanephrine. Though their method is simple and highly sensitive, the Grota antiserum also reacts with synephrine itself, which is contained in some fruits,⁹⁾ nuts,¹⁰⁾ and Chinese medicinal drugs,¹¹⁾ and epinephrine.

To obtain a more specific antiserum for metanephrine, we attempted to extend our prepar-

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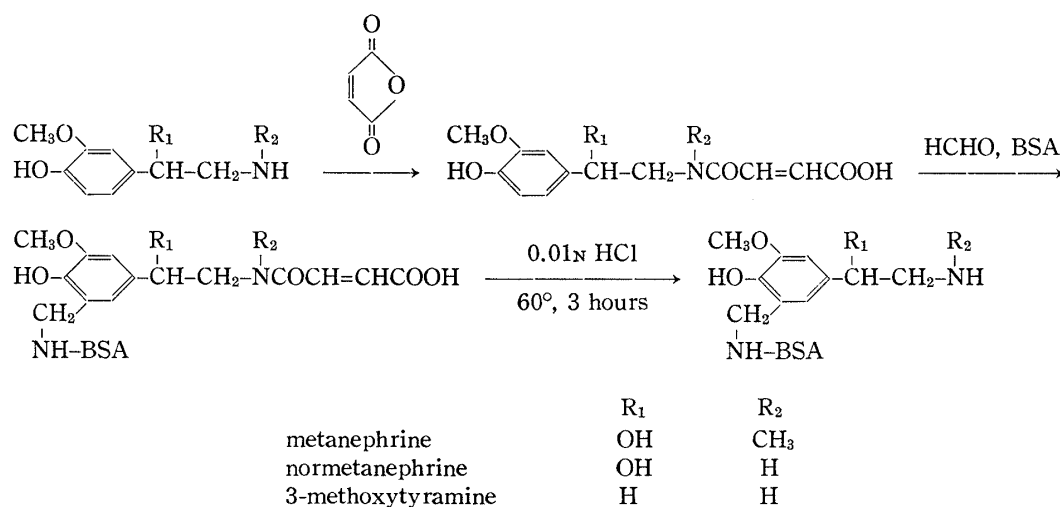


Chart 1. Procedure for the Conjugation of 3-O-Methylcatecholamines to BSA

ation method¹²⁾ for antigens, which had been successfully developed for catecholamines and L-DOPA, to 3-O-methylcatecholamines, as shown in Chart 1.

With the metanephrine antigen thus prepared, we obtained a specific antiserum suitable for developing a method for radioimmunoassay of metanephrine which was hardly affected by synephrine and epinephrine.

The present radioimmunoassay method was useful for the measurement of urinary metanephrine and of rat liver COMT activity.

Experimental

Materials and Reagents—DL-Metanephrine hydrochloride, DL-epinephrine, DL-synephrine hydrochloride, and S-adenosylmethionine hydrochloride were obtained from Sigma Inc. L-Epinephrine was obtained from Merck AG, Darmstadt. D-Epinephrine bitartrate was a gift from Winthrop Laboratories. L-Norepinephrine, DL-normetanephrine hydrochloride, 3-methoxytyramine hydrochloride, and serotonin creatine sulfate were obtained from Nakarai Chemical Co., Ltd. (Kyoto). Homovanillic acid, vanilmandelic acid, and mescaline sulfate were obtained from Tokyo Chemical Industry Co., Ltd. Bovine serum albumin (BSA) was obtained from Miles Lab., Inc. Complete and incomplete Freund's adjuvant were obtained from Difco Lab. DL-Metanephrine[7-³H] (15.0 Ci/mmol) was obtained from New England Nuclear Inc. Other chemicals (of reagent grade) were obtained from Kanto Kagaku Co., Ltd. (Tokyo) and Nakarai Chemical Co., Ltd. Millipore filters were obtained from Millipore Co., Ltd. Cellulose acetate membrane was obtained from Fuji Photo Film Co., Ltd. Silica gel and cellulose plates (SPOTFILM) for thin-layer chromatography were obtained from Tokyo Chemical Industry Co., Ltd.

N-Hemimaleoylation of 3-O-Methylcatecholamines—3-Methoxytyramine: One mmol (203 mg) of 3-methoxytyramine hydrochloride was suspended in 5 ml of methanol, then 200 mg of pulverized maleic anhydride and 0.4 ml of triethylamine were added. The solvent was removed under reduced pressure after allowing the mixture to stand for 5 min at room temperature. Completion of the reaction was confirmed by electrophoresis.^{12b)} The residue was dissolved in 10 ml of 0.5 M NaHCO₃. Under stirring with a magnetic stirrer, the solution was carefully acidified to pH 3 with 1 N HCl and the resulting pink scale crystals (170 mg) were filtered off. mp 152 (dec.). *Anal.* Calcd for C₁₃H₁₅N₁O₅: C, 58.84; H, 5.71; N, 5.28. Found: C, 59.04; H, 5.74; N, 5.25.

¹H-NMR (10% solution in D₂O) δ:¹³⁾ 2.74 (2H, t, J=4.2 Hz, -CH₂-), 3.44 (2H, t, J=4.2 Hz, -CH₂-), 3.84 (3H, s, -OCH₃), 5.85 (1H, d, J=7.2 Hz, -CH=), 6.32 (1H, d, J=7.2 Hz, -CH=), 6.80 (3H, m, Ar-H).

Metanephrine and Normetanephrine: Since N-hemimaleoyl compounds of DL-metanephrine and DL-normetanephrine did not crystallize, these were prepared as aqueous solutions. One-tenth mmol of DL-

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metanephrine hydrochloride (23.3 mg) or DL-normetanephrine hydrochloride (22.2 mg) was suspended in 1 ml of methanol, then 20 mg of pulverized maleic anhydride and one drop of triethylamine were added. The solvent was removed under reduced pressure after allowing the mixture to stand for 5 min at room temperature. Completion of the reaction was confirmed by electrophoresis. Excess maleic anhydride was removed by washing three times with 3 ml of ether. The residue was kept under reduced pressure to remove the ether, then dissolved in 0.7 ml of chilled water. In order to remove a small quantity of remaining DL-metanephrine or DL-normetanephrine, the solution was passed through a cation exchange column (Dowex 1 $\times$ 8, Na⁺ form, 0.5 $\times$ 0.5 cm). The column was washed with a small volume of chilled water. The effluent and washing were combined, neutralized with NaHCO₃ and made up to 1.8 ml with chilled water.

Preparation of Antigens—In the case of 3-methoxytyramine, one-tenth mmol of the N-hemimaleoyl compound (24.1 mg) and 100 mg of BSA were dissolved in 1.8 ml of 0.3 M NaHCO₃; in the case of metanephrine or normetanephrine, 100 mg of BSA was dissolved in the solution of the N-hemimaleoyl compound. Next, 0.3 ml of 3 M sodium acetate and 0.2 ml of 37% formaldehyde were added. After replacing the air phase with nitrogen gas, the reaction tube was tightly closed with a ground glass stopper. The mixture was allowed to stand at 18–25° in the dark for three days. The reaction mixture was then dialyzed in a Visking tube against 1 liter of water for two days at least three times, and furthermore against 1 liter of 0.01 N HCl for a day. The dialysate was warmed at 60° for three hours in a test tube with a ground glass stopper to hydrolyze the N-hemimaleoyl compound. The extent of the removal of maleyl groups from the conjugate was monitored by electrophoresis as described previously.^{12b)} The solution was dialyzed twice against 1 liter of water for a day and lyophilized for storage.

Determination of Molar Ratio of 3-O-Methylcatecholamines to BSA—Before lyophilization, the concentration of 3-O-methylcatecholamine residues coupled to BSA was determined by measuring the absorbance at 280 nm of the antigen solution, taking BSA solution reacted with formaldehyde as a reference. The molar extinction coefficient, ϵ , of each 3-O-methylcatecholamine at 280 nm in water was 2400 cm⁻¹M⁻¹. The concentration of BSA was calculated from the starting weight and the final volume of the antigen solution. The molar ratio was calculated from these data.

In the case of metanephrine, the antigen was also prepared from N-hemimaleoyl-³H-metanephrine. The amount of ³H-metanephrine coupled was determined by measuring the radioactivity. The amount of BSA was determined from the weight of the antigen after lyophilization by deducing the weight of ³H-metanephrine coupled. The molar ratio was also calculated from these data.

Preparation of Anti-metanephrine-BSA-antiserum—Five rabbits were immunized with one of the antigens, metanephrine-BSA. The antigen was dissolved in water (8.0 mg/ml) and emulsified with an equal volume of Freund's complete adjuvant. A half ml of the emulsion (2 mg of the antigen) was injected into the footpads of each rabbit. Starting one month after the initial injection, a booster shot with 1 mg of the antigen emulsified with Freund's incomplete adjuvant was given subcutaneously every month. The rabbits were bled seven days after the third booster injection. The antisera were stored at -18°.

Procedure for Radioimmunoassay—A buffer solution (BBS) consisting of 0.15 M NaCl–0.05 M borate buffer, pH 8.5, was used for the preparation of diluted antisera, sample solutions and ³H-metanephrine solution. In a siliconized micro test tube, a mixture of 2000-fold diluted antiserum (200 μ l) and the sample solution (10 μ l) was added to 10 μ l of ³H-metanephrine solution, corresponding to 6000 dpm total count (*T*). The solution was shaken well and incubated at 4° for 2 hours, then it was filtered through a Millipore DAWP filter (ϕ 15 mm).^{12d)} The filter was washed with 0.3 ml of chilled BBS and dissolved in 10 ml of modified Bray's solution.^{12d)} The radioactivity of the solution was measured with a Packard 3255 Tri-carb System scintillation spectrometer on-line programmed. The radioactivity bound to the antibody, *B*, was calculated after correction for the blank value of BBS. Each assay datum is an average of duplicate assay values.

Preparation of Urine Sample—After the collection of urine (100 ml) from a normal young man (26 years old) in the morning, the pH of the urine was adjusted to 1.0 with 6 N HCl. Ten ml of the urine was heated in a sealed glass tube at 100° for 1 hour to hydrolyze conjugated metanephrine and to racemize L-metanephrine. The solution was neutralized with NaHCO₃ and was used as a hydrolyzed urine sample.

In the radioimmunoassay, a half ml of the urine before hydrolysis was diluted with BBS to make 100 ml and used as the buffer solution to dilute antiserum.

Preparation of COMT—Enzyme solution was prepared from the liver of rats (Wistar ♀ 150–200 g) by a modification of Gulliver's method.¹⁴⁾ Rat livers were homogenized in twice their weight of 0.135 M KCl–0.01 M phosphate buffer, pH 8.0, containing 2 mM dithiothreitol. The homogenate was centrifuged at 50000 $\times g$ for 1 hour and the supernatant was divided into small volumes and stored at -18°. Protein concentration was determined by Lowry's method modified to be suitable for use in the presence of an SH compound.¹⁵⁾

Assay of COMT Activity—DL-Epinephrine was used as the substrate, as no stereospecificity has been

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reported.¹⁶⁾ DL-Metanephrine produced by the enzyme reaction was determined by the present radioimmunoassay.

A mixture consisting of 1 mM MgCl₂, 2 mM dithiothreitol, 0.5 mM S-adenosylmethionine, 2 mM DL-epinephrine and 100 μ l of the enzyme solution was made up to 250 μ l with 0.05 M phosphate buffer, pH 8.0. After shaking the mixture at 37°, 1 ml of 0.15 M borate buffer (BB), pH 8.5, was added to stop the reaction. The reaction mixture was diluted with BB in order to bring the concentration of produced metanephrine within the range measurable by the radioimmunoassay. In the radioimmunoassay, BB was used instead of BBS to eliminate the cross-reaction of a large amount of epinephrine.

Results

N-Hemimaleoylation of 3-O-Methylcatecholamines

The product of 3-O-methylcatecholamine gave a single spot in thin-layer chromatography and electrophoresis (Table I). The spot was negative in the ninhydrin reaction. In electrophoresis at pH 8.6, the products moved towards the anode, while the corresponding 3-O-methylcatecholamines moved towards the cathode. These results, together with ¹H-NMR data and elemental analysis, demonstrated that the products were N-hemimaleoyl compounds.

TABLE I. Behavior of N-Hemimaleoyl-3-O-methylcatecholamines in Thin-Layer Chromatography and Electrophoresis

Compound	<i>R</i> _f ^{a)}	<i>R</i> _f ^{b)}	<i>R</i> _m ^{c)}
DL-Metanephrine	0.68	0.56	-1.5
N-Hemimaleoyl-DL-metanephrine	0.85	0.71	0.5
DL-Normetanephrine	0.71	0.61	-1.3
N-Hemimaleoyl-DL-normetanephrine	0.91	0.78	0.5
3-Methoxytyramine	0.71	0.58	-1.8
N-Hemimaleoyl-3-methoxytyramine	0.93	0.84	0.5

a) Silica gel, MeOH/AcOH (100:1).

b) Cellulose, BuOH/AcOH/H₂O (3:1:1).

c) Cellulose acetate membrane electrophoresis was carried out for 15 min at 1 mA/cm. The electrolyte was 0.07 M veronal-sodium veronal buffer, pH 8.6. The mobility towards the anode of each compound is expressed relative to that of Bromocresol Green.

Detection was performed by spraying with diazotized reagent.¹⁷⁾

Preparation of Antigens

Antigens of metanephrine, normetanephrine and 3-methoxytyramine were prepared. The molar ratios of 3-O-methylcatecholamine to BSA determined by the absorbance method ranged between 18 and 27. The value with the antigen using tritiated metanephrine was quite similar to that obtained by the radioactivity method (24 and 23, respectively).

Preparation of Anti-metanephrine-BSA-antiserum

Rabbits were immunized with metanephrine-BSA having a molar ratio of 26. Ouchterlony test¹⁸⁾ of the antisera thus obtained demonstrated the production of antibody specific to the metanephrine moiety, as shown in Fig. 1.

Radioimmunoassay of Metanephrine

One of highest titer antisera was adopted. The antiserum was diluted with BBS (1:2000) to make the *B/T* ratio in the radioimmunoassay about 0.5. The blank value of BBS was equal to that of 2000-fold diluted normal serum. As the optimum pH for binding was between

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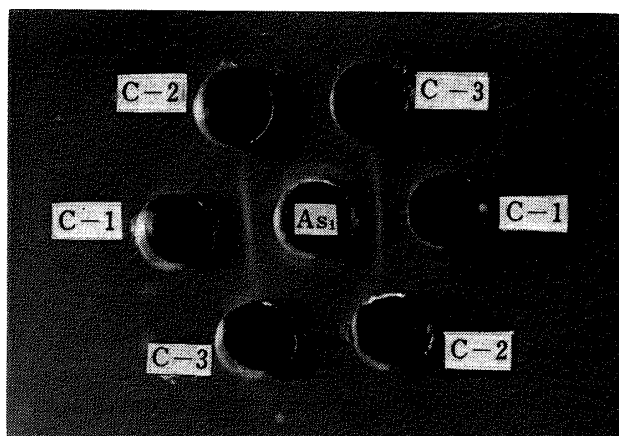


Fig. 1. Precipitin Reaction of an Antiserum with Antigens

As₁: anti-metanephrine-BSA-antiserum, C-1; metanephrine-BSA; C-2; normetanephrine-BSA, C-3; 3-methoxytyramine-BSA.

The concentration of the antigens used in this test was 1.0 mg/ml. As₁ was used after absorption of antibody to BSA, as described in the text.¹⁸⁾

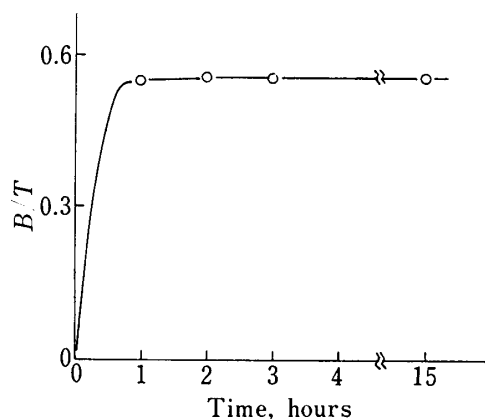


Fig. 3. Time Course of Binding

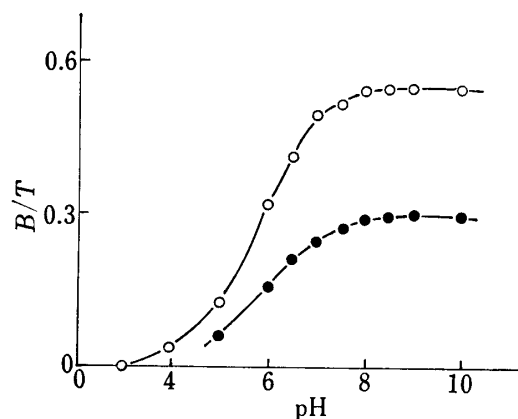


Fig. 2. Influence of the pH of the Assay Buffer on B/T

The B/T value was determined in the absence (○) or presence (●) of 1 pmol/tube of cold DL-metanephrine. Buffers of 0.05 M citrate (pH 3.0–6.5), 0.01 M phosphate (pH 6.5–8.0), and 0.05 M borate (pH 8.0–10.0) containing 0.15 M NaCl were used in place of BBS.

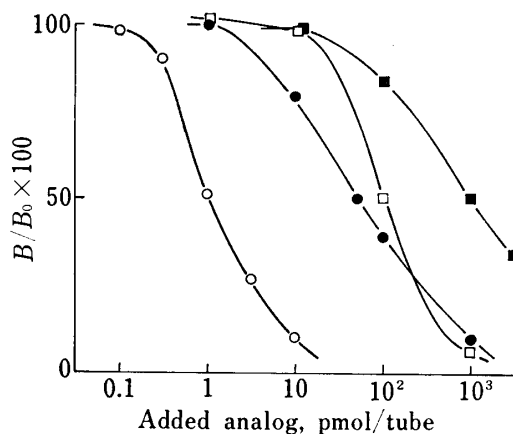


Fig. 4. Dose-response Curves for DL-Metanephrine and the Analogs

DL-Metanephrine (○), DL-Normetanephrine (□), DL-Synephrine (●), D- or L-Epinephrine (■).

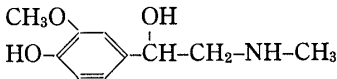
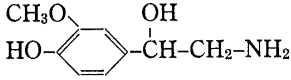
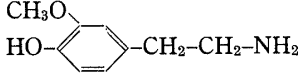
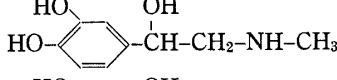
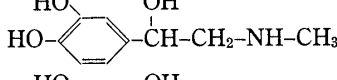
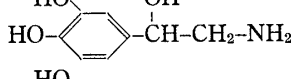
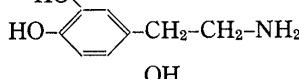
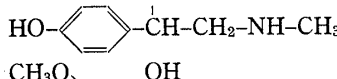
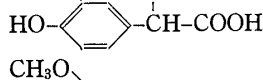
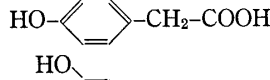
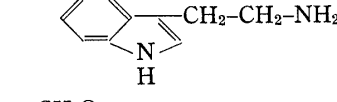
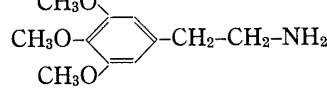
B_0 is B at zero dose.

8.0 and 10.0, as shown in Fig. 2, the pH of BBS was adjusted to 8.5. Under these conditions, incubation for 2 hours at 4° was sufficient to attain binding equilibrium (Fig. 3).

The specificity of the radioimmunoassay was examined by drawing dose-response curves for various analogs to metanephrine (Fig. 4), and the cross-reactivity with metanephrine was calculated as shown in Table II. These data show that the specificity of the antiserum was high enough for practical radioimmunoassay of biological samples.

A log-logit plot for standard DL-metanephrine was drawn in the range of 0.03–5.0 pmol/tube (Fig. 5). The detection limit, taken as the amount of DL-metanephrine which caused a decrease of twice the standard deviation of B_0 (B at zero dose) was 0.03 pmol/tube. The coefficients of variation of B_0 and B 's at 0.1, 0.3, and 1.0 pmol/tube were 2.2, 5.5, 3.6, and 5.5%, respectively ($n=4$; mean, 4.1%).

TABLE II. Cross-reactivity of Analogs to DL-Metanephrine

Compound	Structure	Cross-reactivity (%) ^{a)}
DL-Metanephrine		100
DL-Normetanephrine		1.0
3-Methoxytyramine		<0.02
L-Epinephrine		0.1
D-Epinephrine		0.1
L-Norepinephrine		<0.02
Dopamine		<0.02
DL-Synephrine		1.9
Vanilmandelic acid		<0.02
Homovanillic acid		<0.02
5-Hydroxytryptamine		<0.02
Mescaline		<0.02

$$a) \text{ Cross-reactivity} = \frac{\text{amount for 50\% inhibition of DL-metanephrine}}{\text{amount for 50\% inhibition of the analog}} \times 100$$

TABLE III. Recovery of DL-Metanephrine added to Urine before Hydrolysis

Added MN ^{a)} (pmol/tube)	Final MN (pmol/tube)	Found MN (pmol/tube)	Recovery (%)
0	—	0.30	—
0.10	0.40	0.42	120
0.30	0.60	0.59	97
0.50	0.80	0.82	104
1.0	1.3	1.3	100
3.0	3.3	3.0	90
		Mean	102 ± 11

a) The amount of DL-metanephrine (MN) added to 2 μ l of urine.
Ten μ l of hydrolyzed urine which had been 5-fold diluted with BBS was used as a sample in the assay.

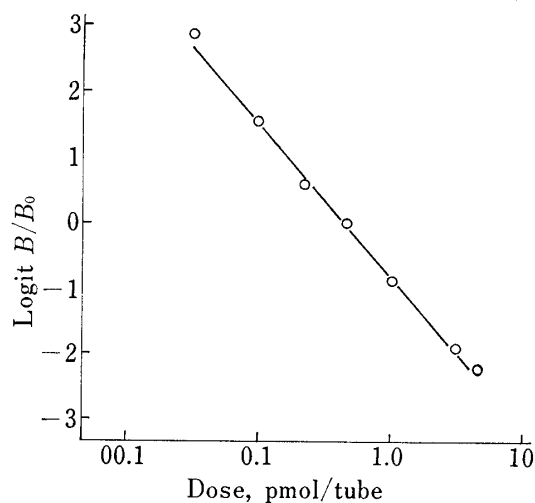


Fig. 5. Standard Curve for DL-Metanephrine

B_0 is B at zero dose. Antiserum finally diluted to 1/4400 was used. $B/T=0.34$.

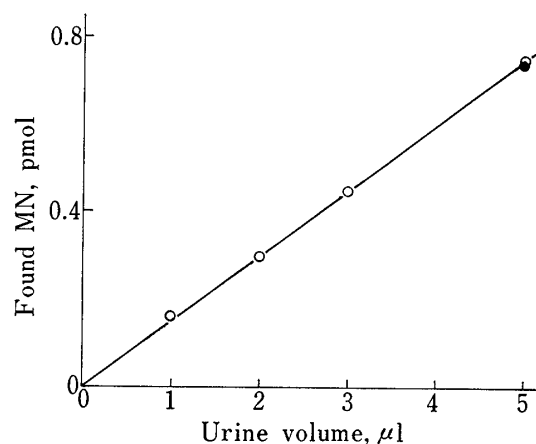


Fig. 6. Relation between the Amount of Found DL-Metanephrine and Urine Volume

Urine hydrolyzed for 1 hour (○) or 1.5 hours (●) was used.

Assay of Urine Sample

A working curve for DL-metanephrine similar to the standard curve was obtained. With urine hydrolyzed at 100° for 1 hour, the amount of DL-metanephrine obtained by using the working curve was proportional to the urine volume (Fig. 6). The value was not affected by further heating during hydrolysis. The concentration of endogeneous metanephrine was estimated from the slope and was $30 \mu\text{g/l}$ of urine. The average recovery of DL-metanephrine added to the urine before hydrolysis was $102 \pm 11\%$, as shown in Table III.

Assay of COMT Activity

COMT fraction was prepared as a crude protein solution. The DL-metanephrine produced increased in proportion to the protein concentration (Fig. 7) and the reaction time (Fig. 8).

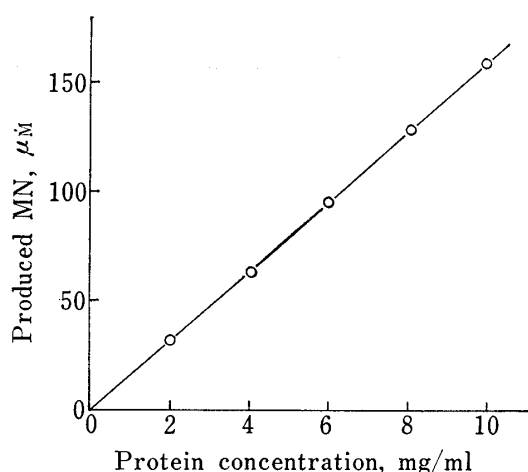


Fig. 7. Relation between Protein Concentration of the Enzyme Solution and DL-Metanephrine produced, determined by the Present Radioimmunoassay Method

Reaction was carried out for 30 min.

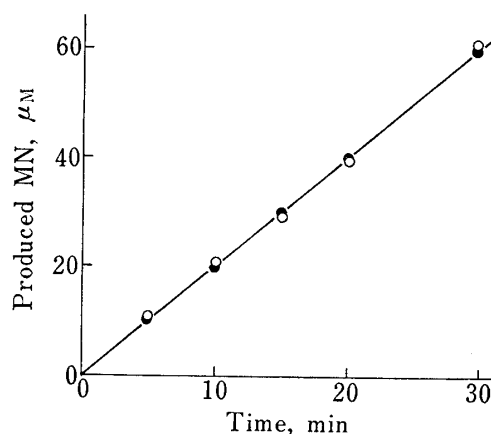


Fig. 8. Time Course of Formation of DL-Metanephrine determined by the Radioimmunoassay Method (○) and the Amberlite XAD-4 Method (●)

Four mg/ml enzyme solution was used.

The time course for the same reaction was also compared by a radiometric method¹⁹⁾ using an Amberlite XAD-4 column. The results obtained by the two methods were compatible (Fig. 8).

Discussion

Since side reaction was prevented by the maleyl group, no reaction other than the condensation with BSA (*e.g.* cyclization in the 3-O-methylcatecholamine molecule and condensation of the side chain with BSA or with another 3-O-methylcatecholamine molecule) should occur under the reaction conditions used. The position of condensation in the benzene ring with BSA was estimated to be position 5 from the ¹H-NMR data for a model compound²⁰⁾ prepared by the condensation of metanephrine with 3-(4-benzyloxyphenyl)propaneamine. The expected structure of each antigen is shown in Chart 1.

As shown in Table II, the present radioimmunoassay of metanephrine was scarcely affected by DL-synephrine, which showed substantial cross-reactivity in Grota's method (Grota *et al.*, 12.5%; Lam *et al.*, more than 50%).²¹⁾ Lam *et al.* adopted a complicated pretreatment to eliminate the cross-reaction of Grota antiserum with epinephrine. In a preliminary experiment, the cross-reactivity of our antiserum using phosphate buffer was about 10% in the presence of an antioxidant, ascorbic acid, and was only 1.0% in its absence. The interference of epinephrine can be practically removed by chelate formation with borate.²²⁾

For the determination of L-metanephrine in biological samples, the working curve should be obtained with L-metanephrine. However, as L-metanephrine is not commercially available and our attempt at optical resolution of DL-metanephrine was unsuccessful, we had to make a working curve with the DL-form. Accordingly, in a preliminary measurement of urinary metanephrine, the quantity found was not proportional to the volume of urine, with or without hydrolysis at pH 2.0 and 80° for 30 min, in contrast to the case of Fig. 6. This difficulty was overcome by the complete racemization of DL-metanephrine, as in the case of L-epinephrine.²³⁾

If necessary, the levels of free and conjugated form of metanephrine can be determined by the present radioimmunoassay method after separation of the two forms through a column of Amberlite XAD-4.¹⁹⁾

The present radioimmunoassay method is useful for the screening of pheochromocytoma, and the application data will be described elsewhere.²⁴⁾

It was reported²⁵⁾ that changes of the activity of COMT are associated with a number of disease states. The present assay method for COMT activity is highly sensitive and specific, and can be used with a large number of samples. Thus, it could be useful in clinical and biochemical studies.

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