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Preparation of Specific Antibodies to Catecholamines and L-3,4-Dihydroxyphenylalanine.[1] I.[2] Preparation of the Conjugates

AKIRA MIWA, MASANORI YOSHIOKA, AKIRA SHIRAHATA, and ZENZO TAMURA

Faculty of Pharmaceutical Sciences, University of Tokyo[3]

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A method for preparation of antigens of catecholamines and L-3,4-dihydroxyphenylalanine as haptans was developed. These haptens were conjugated with carrier proteins by the Mannich reaction after N-maleylation of the haptens for protecting the amino groups of the side chain, then the maleyl groups were removed by acid hydrolysis under a mild condition. By immunization of rabbits with these conjugates antibodies were produced. In a cross-reactivity test by Ouchterlony's method the antibodies to each one of the conjugates selectively reacted with the conjugate of that hapten even if the carrier protein was bovine serum albumin or rabbit serum albumin. These results show that the present method for preparation of the conjugates is a desirable one.

Keywords——catecholamine; epinephrine; norepinephrine; dopamine; 3,4-dihydroxyphenylalanine; antibody; antigen; albumin; N-maleyl-catecholamine; N-maleyl-DOPA

Catecholamines (L-epinephrine, L-norepinephrine and dopamine) and L-DOPA have the important roles in a living body. The quantities of them are so small that the accurate and rapid determination is difficult. The determination of these compounds in body fluid has been usually performed by gas chromatography[4] or fluorometry.[5] These methods, however, have a number of technical problems in parctical measurements. For the routine determination of these compounds, immunoassays would be desirable.

Since the compounds, having catechol moiety in their structure, are extremely unstable in an alkaline solution and in the presence of oxygen, the preparation of their conjugates with proteins is difficult, and the antibodies to these compounds reported[6] have not been sufficiently specific to them.

In this paper we developed a method of conjugation of catecholamines and L-DOPA with carrier proteins such as BSA[1] through the Mannich reaction,[7] and of preparation of antibodies to these compounds by immunization of rabbits with these conjugates.

The validity of the conjugates was tested by the production of antibodies which were specific to these conjugates.

Experimental

Reagents——General reagents commercially obtained were guaranteed reagents. L-DOPA was a gift from Daiichi Seiyaku Co., Ltd. L-Epinephrine was obtained from Fluka AG and Merck AG Darmstadt.

1) Abbreviation—L-DOPA: L-3,4-dihydroxyphenylalanine; BSA: bovine serum albumin; RSA: rabbit serum albumin; DMF: N,N-dimethyl formamide; PBS: phosphate buffered saline (0.15 M NaCl, 0.01 M KH_2PO_4, pH 7.4).
2) This report is a part of the work, the outline of which was communicated to the editor in *Chem. Pharm. Bull.* (Tokyo), **24**, 1422 (1976).
3) Location: *Hongo, 7-3-1, Bunkyo-ku, Tokyo.*

4) M-T. Wang, K. Imai, M. Yoshioka, and Z. Tamura, *Clin. Chim. Acta*, **63**, 13 (1975).
5) H. Weil-Malherbe, "Method of Biochemical Analysis," ed. by D. Glick, Suppl. Vol., Interscience Publishers, New York, 1971, p. 119.
6) *a*) S. Spector, U.S. Patent 3704282 (1972) [*C.A.*, **28**, 41465j (1973)]; *b*) S. Spector, C. Dalton, and A.M. Felix, *Biochem. Pharmacol.* Suppl., 263 (1974); *c*) S. Went and L. Kesztyus, *Arch. Exptl. Pathol. Pharmakol.*, **193**, 609 (1939); *d*) L.J. Grota and G.M. Brown, *Endocrinol.*, **98**, 615 (1976).
7) F.F. Blicke, "Organic Reactions," Vol. I, John Wiley and Sons, Inc., New York, 1942, p. 303.

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L-Norepinephrine and dopamine hydrochloride were obtained from Nakarai Chemicals Co., Ltd. BSA and RSA[1] were obtained from Miles Laboratories Inc. Maleic anhydride was obtained from Tokyo Organic Chemicals Co., Ltd. ^{3}H-L-Norepinephrine[7-^{3}H(N)] was obtained from New England Nuclear Inc. Complete and incomplete Freund's adjuvants were obtained from Difco Laboratories.

Doty's Reagent: Doty's reagent[8] was prepared with a minor modification. One and a half grams of ferrous sulfate ($FeSO_4 \cdot 7H_2O$) and 1 g of sodium bisulfite ($NaHSO_3$) were dissolved in 10 ml of 0.1 N HCl, then 10 g of sodium citrate, 36.3 g of tris(hydroxymethyl)aminomethane and 30 ml of water were added, and adjusted the pH to 8.5 with 6 N HCl and added with water to make 100 ml.

Diluted Doty's Reagent: One milliliter of Doty's reagent was diluted with water to 25 ml.

Ponceau 3R Solution: Six grams of trichloroacetic acid and 0.8 g of ponceau 3R were dissolved in water to make 100 ml.

Ninhydrin Solution: In 100 ml of methanol, 0.2 g of ninhydrin was dissolved.

Cellulose Acetate Strip Electrophoresis——Cellulose acetate strips were purchased from Fuji Photo Film Co., Ltd. The electrolyte was 0.07 M veronal-sodium veronal buffer, pH 8.6. Electrophoresis was carried out for 10—20 min at 2 mA/cm. The detection of catechol moiety was carried out by spraying with Doty's reagent and of the conjugate was by staining with ponceau 3R solution for protein.

Thin-Layer Chromatography (TLC)——Silica gel (Tokyo Chemical Industry Co., Ltd.) and cellulose plates (Merck AG Darmstadt) were used as adsorbents. Solvents for developing were methanol/acetic acid (100: 1) and *n*-butanol/acetic acid/water (3: 1: 1). Detection was carried out by spraying with Doty's reagent and ninhydrin solution.

N-Maleylation——L-Epinephrine: One millimole (183.2 mg) of L-epinephrine was suspended in 5 ml of methanol and 200 mg of maleic anhydride was added. After standing for 10 min at room temperature, the solvents was removed under reduced pressure. The residue was washed with 5 ml of benzene at least 3 times to remove the excess maleic anhydride. The reaction was monitored by TLC or cellulose acetate strip electrophoresis. After the benzene was removed under reduced pressure the residue was dissolved in 5 ml of cold water, then a small quantity of remaining L-epinephrine was removed with a small volume of cation exchange resin (Dowex 50WX-8, H^+ form). The solution was lyophilized and stored in a desiccator.

L-Norepinephrine: One millimole (169.2 mg) of L-norepinephrine and 200 mg of maleic anhydride were mixed in 5 ml of methanol. The reaction was performed under the same condition of L-epinephrine. After washing with benzene, the reaction product was dried under reduced pressure. Slightly yellowish powder was obtained, mp 230—231° (decomp.). *Anal.* Calcd. for $C_{12}H_{13}O_6N$: C, 53.93; H, 4.90; N, 5.24. Found: C, 53.81; H, 5.02; N, 5.16.

NMR (10% solution in DMSO^{d-6}) δ: 3.30 (2H, m, $-CH_2-NH$) 4.53 (1H, m, Ar-CH-) 5.35 (1H, Ar-C-OH) 6.23 (1H, d, J=12 Hz, OC-CH=C) 6.54 (1H, d, J=12 Hz, =CH-CON) 6.68, 6.80 (3H, Ar-H) 8.80 (2H, Ar-OH) 9.28 (1H, NH).

Dopamine: One millimole (189.6 mg) of dopamine hydrochloride was dissolved in 2 ml of methanol. Then 0.4 ml of triethylamine and 360 mg of maleic anhydride were added. The reaction was run under the same condition of L-epinephrine, and the solvent was removed under reduced pressure. The residue was washed with 5 ml of benzene at least 3 times after 5 ml of water was added. The reaction mixture was allowed to stand in the cold place to obtain slightly yellowish needles, mp 187—188° (uncorr.). *Anal.* Calcd. for $C_{12}H_{13}O_5N$: C, 57.37; H, 5.22; N, 5.57. Found: C, 56.95; H, 5.34; N, 5.55.

L-DOPA: One millimole (197.2 mg) of L-DOPA was suspended in 2 ml of DMF[1] and 200 mg of maleic anhydride was added to dissolve. The mixture was stood for 30 minutes at 60—65°, then DMF was removed under reduced pressure. The residue was washed with 5 ml of benzene at least 3 times to remove the unreacted maleic anhydride. After benzene was removed under reduced pressure, 3 ml of cold water was added to dissolve the residue, then immediately the solution was cooled in an ice bath and cold 0.1 N $NaHCO_3$ was added to neutralize. The solution was lyophilized and dissolved in a small volume of methanol, and a large volume of ethylacetate was added. The precipitates yielded were collected by centrifugation and dried under reduced pressure.

Preparation of Conjugates——Conjugation: Conjugation was carried out according to the method for phenols proposed by Fraenkel-Conrat and Olcott.[9] N-Maleyl-catecholamines (0.05 mmol) and BSA (100 mg) were dissolved in 1 ml of 0.3 M $NaHCO_3$. In the case of N-maleyl-L-DOPA, its sodium salt (0.3 mmol) and BSA (100 mg) were dissolved in 1 ml of water. Each solution was added with an adequate volume of 3 M sodium acetate to adjust the pH 6.0, although the yields of the conjugates are almost the same at the pH 5.5 to 7.6. Then 1 ml of 7.5% formaldehyde was added and stirred thoroughly. After substituting the air phase with nitrogen gas the reaction tubes were tightly closed with ground stoppers. The reaction mixture was allowed to stand at 18—23° in the dark for 3 days.

Removal of Maleyl Group by Acid Hydrolysis: N-Maleyl-hapten-BSA obtained was dialyzed against 0.01 N HCl for two days and the dialysate was transferred to a test tube with a glass stopper. After the

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9) H. Fraenkel-Conrat and H.S. Olcott, *J. Biol. Chem.*, **174**, 827 (1948).

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substitution with nitrogen gas, the tube was warmed at 60°. The removal of maleyl groups from N-maleyl-hapten-BSA was monitored by cellulose acetate strip electrophoresis. The solution was, further, dialyzed against water or PBS[1] for 3 days, and lyophilized to be stored.

Determination of the Molar Ratio of Hapten to BSA in the Conjugates: The conjugate thus obtained was dissolved in water to make an exact volume. A portion of the solution was pipetted and added with 10-fold volumes of diluted Doty's reagent. The reaction solution was thoroughly stirred and the optical density at 550 nm of the solution was measured. The concentration of the hapten was determined from the working curve, obtained with standard hapten solutions, then the molar ratio of the hapten to BSA was calculated.

The molar ratio in N-maleyl-L-norepinephrine-BSA was also determined by a radioactivity method, which was performed as follows. Fifty one milligrams of L-norepinephrine was suspended in 2 ml of methanol, and 50 µl of ^{3}H-L-norepinephrine solution (1.3×10^{-4} M, 3.8 Ci/mmol) and 60 mg of maleic anhydride were added. The reaction was performed by the same procedure previously described and ^{3}H-N-maleyl-L-norepinephrine (^{3}H-MNE) was obtained. Then the conjugation of ^{3}H-MNE with BSA was performed according to the method previously described. An adequate volume of the conjugate solution was mixed with Bray's solution[10] and counted by a scintillation counter, then the concentration of the hapten was calculated from the standard activity of ^{3}H-MNE.

Amino Acid Analysis of L-Epinephrine-BSA——L-Epinephrine-BSA, formaldehyde treated BSA and BSA itself were dissolved in 6 N HCl and hydrolyzed in sealed tubes at 110° for 24 hours. These hydrolysates were dried under reduced pressure, and dissolved in 0.2 M sodium citrate buffer, pH 2.2. They were analyzed according to the procedure of Moore and Stein.[11] And the relative molar quantities of the amino acid residues were calculated according to the data of Spahr and Edsall.[12]

Preparation of Antisera——Ten milligrams of each conjugate were dissolved in 1 ml of PBS or water, mixed with 1 ml of complete Freund's adjuvant and stirred vigorously to prepare a w/o emulsion. The emulsion, containing 3 mg of the conjugate, was injected into the foot pads of a rabbit. Four weeks later 1 mg of conjugate was boosted intravenously as a PBS solution or subcutaneously as an emulsion obtained by mixing with incomplete Freund's adjuvant. The booster injections were given at each 2 weeks and one week after each boost the rabbit was bled and these sera were examined on the presence and specificity of antibody to the conjugate by Ouchterlony's method.[13]

The antibody to BSA was removed by absorption of antiserum with BSA solution in the following procedure. The antiserum was mixed with an equal volume of BSA solution (1.0 mg/ml), and the mixture was allowed to stand at 37° for 1 hr and at 4° overnight, then the precipitates were removed by centrifugation.

Results

N-Maleylation of Haptens

The completion of N-maleylation was examined by TLC and electrophoresis. The data are shown in Table I and II respectively.

TABLE I. Thin-Layer Chromatography of N-Maleylated Compounds of Catecholamines and L-DOPA

Compound	Rf_1[a]	Rf_2[b]
L-Epinephrine	0.45	0.35
N-Maleyl-L-epinephrine	0.52	0.70
L-Norepinephrine	0.62	0.28
N-Maleyl-L-norepinephrine	0.74	0.70
Dopamine	0.49	0.38
N-Maleyl-dopamine	0.63	0.83
L-DOPA	0.58	0.35
N-Maleyl-L-DOPA	0.53	0.74

a) Silica gel, MeOH/AcOH (100:1).
b) Cellulose, *n*-BuOH/AcOH/water (3:1:1).
Detection was performed by spraying with Doty's reagent, and N-maleylated compounds were not detected with ninhydrin solution.

10) G.A. Bray, *Anal. Biochem.*, **1**, 279 (1960).
11) S. Moore and W.H. Stein, "Method in Enzymology," Vol. VI. ed. by S.P. Colowick and N.O. Kaplan, Academic Press, New York, 1963, p. 819.
12) P.F. Spahr and J.T. Edsall, *J. Biol. Chem.*, **239**, 850 (1964).
13) O. Ouchterlony and L.A. Nilsson, "Handbook of Experimental Immunology," 2nd ed., ed. by D.M. Weir, Blackwell Scientific Publications, Oxford, 1973, Chapter 19.

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Table II. The Results of Electrophoreses of N-Maleylated Compounds of Catecholamines and L-DOPA

Compound	Relative mobility[a]
N-Maleyl-L-epinephrine	0.68
N-Maleyl-L-norepinephrine	0.71
N-Maleyl-dopamine	0.71
N-Maleyl-L-DOPA	1.53

a) Relative mobility to the anode of each compound was expressed to Bromcresol Green.

Conjugation of Haptens with BSA

Conjugation procedures of the haptens with BSA were illustrated in the previous paper.[2] The conjugates with RSA[1] were prepared by the same procedure of the conjugates with BSA. The molar ratios of conjugated hapten to BSA were estimated as 15—30 by the colorimetry with diluted Doty's reagent. The similar value was obtained by the radioactivity method, *e.g.* 27 *vs.* 26 by the colorimetry for MNE-BSA.

The optimal reaction temperature was between 18 and 23°. Above 25°, the conjugates obtained became less soluble in PBS and water, and at 4°, the reaction time longer than 10 days was required to obtain the value larger than 10. It was noted that the conjugates of the molar ratios over 20 were less soluble in PBS but soluble in water.

The electrophoresis of hydrolyzed N-maleyl-L-DOPA (Fig. 1) showed that the maleyl group was completely removed from the compound during 3 hours under the hydrolysis condition.

Furthermore the electrophoreses of N-maleyl-L-epinephrine-BSA and the formaldehyde treated BSA, which are shown in Fig. 2A and 2B respectively, demonstrated that the hydrolysis of the maleyl group was similarly completed even in the conjugate molecule, while the protein itself remained partially modified with formaldehyde.

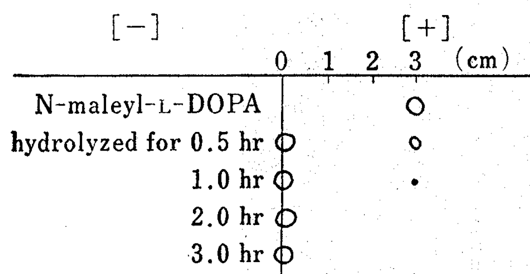

Fig. 1. Electrophoretic Pattern of Hydrolyzed N-Maleyl-L-DOPA

N-Maleyl-L-DOPA(5×10^{-3}M) in 0.01N HCl was hydrolyzed at 60° for 0—3 hr. An aliquot of 0.2 μl each was spotted on a cellulose acetate strip and carried out electrophoresis under the condition described in the text. The location was detected by spraying with Doty's reagent.

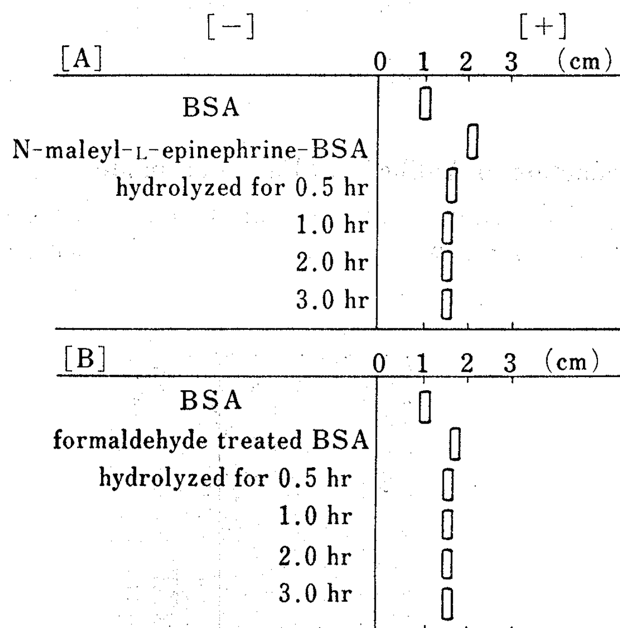

Fig. 2. Electrophoretic Patterns of Hydrolyzed N-Maleyl-L-epinephrine-BSA and Formaldehyde Treated BSA

N-Maleyl-L-epinephrine-BSA, formaldehyde treated BSA and BSA were dialyzed against or dissolved in 0.01 N HCl, then hydrolyzed at 60° for 0—3 hr. These solutions were diluted to 4 mg/ml with water and each 1 μl of the solutions was applied to the cellulose acetate strip. The protein was detected with ponceau 3R solution.

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Since the number of lysine residue of L-epinephrine-BSA was smaller than that of the BSA and the formaldehyde treated BSA (Table III), the ε-amino groups of lysine of BSA probably made a covalent bond with epinephrine. The decrease in tyrosine was more distinct in the formaldehyde treated BSA, which suggested the possibility that tyrosine would play like the hapten under the condition for conjugation.

TABLE III. Amino Acid Composition of L-Epinephrine-BSA[a]

Amino acid	BSA[b]	Formaldehyde treated BSA[c]	L-Epinephrine-BSA[d]
Lysine	58	48	27
Histidine	16	16	15
Arginine[e]	22	22	22
Aspartic acid[e]	54	54	54
Threonine[f]	33	32	32
Serine[f]	25	27	26
Glutamic acid	86	89	89
Proline	25	25	27
Glycine	17	16	17
Alanine	44	47	43
Half-cystine[f]	32	28	25
Valine	34	32	32
Methionine	4	4	3
Isoleucine	13	12	12
Leucine	60	57	59
Tyrosine[f]	20	3	11
Phenylalanine	28	27	25

a) The number of amino acids were expressed as nearest integrals.
b) The BSA was used for conjugation with haptens.
c) Formaldehyde treated BSA was hydrolyzed under the same condition as in the conjugates.
d) The molar ratio of hapten to BSA in the conjugate was 23.
e) The relative molar quantities of the amino acid residues were calculated by assuming that the moles of arginine and aspartic acid were 22.0 and 54.0 respectively.[12]
f) No correction was applied for decomposition of amino acids in hydrolysis.

Production of Antibody to Each Conjugate

The serum of rabbits immunized with L-epinephrine-BSA reacted with both the conjugate and BSA, while after the absorption with BSA, the serum reacted with conjugate alone as shown in Fig. 3.

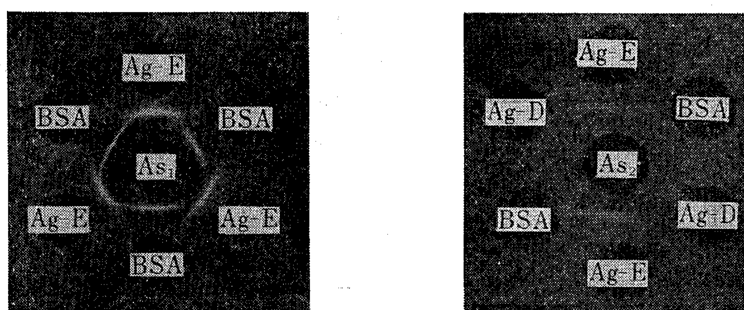

Fig. 3. Precipitin Reaction of an Antiserum with Conjugates and BSA

As_1: antiserum to L-epinephrine-BSA.
As_2: antiserum to L-epinephrine-BSA absorbed with BSA.
Ag-E: L-epinephrine-BSA.
Ag-D: L-DOPA-BSA.

The concentrations of the conjugates and BSA solutions used in this test were 1.0 mg/ml. As_1 was used without dilution but As_2 was used after the absorption of the antibody to BSA as described in the text. The respective wells (3 mm, diameter × 2 mm, depth) were filled up with these solutions and antisera (As_1 and As_2).

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The cross-reactivities of the antisera to every conjugates are summerized in Table IV.

Table IV. Reaction of Antisera with Conjugates observed by Ouchterlony's Method[a)]

Conjugate	Antiserum to the BSA[b)] conjugate of			
	L-Epinephrine	L-Norepinephrine	Dopamine	L-DOPA
E-BSA[c)]	++[d)]	±[e)]	±	−[f)]
E-RSA[g)]	++	/	/	/
ME-BSA[h)]	−	/	/	/
NE-BSA[i)]	±	++	±	±
NE-RSA[j)]	/	++	/	/
MNE-BSA[k)]	/	−	/	/
DA-BSA[l)]	±	±	++	±
DA-RSA[m)]	/	/	++	/
MDA-BSA[n)]	/	/	−	/
L-DOPA-BSA	−	−	−	++
L-DOPA-RSA	−	−	−	++

a) The conditions were same as in Fig.4.
b) Antibody to BSA in antisera was removed by absorption with BSA.
c) L-Epinephrine-BSA.
d) The sign (++) showed a strong positive reaction, as shown in Fig. 3.
e) The sign (±) showed a weak reaction and/or a rising of a spur over the precipitin line directed to the conjugate.
f) The sign (−) showed a negative reaction.
g) L-Epinephrine-RSA.
h) N-Maleyl-L-epinephrine-BSA.
i) L-Norepinephrine-BSA.
j) L-Norepinephrine-RSA.
k) N-Maleyl-L-norepinephrine-BSA.
l) Dopamine-BSA.
m) Dopamine-RSA.
n) N-Maleyl-dopamine- BSA.

The antiserum to L-DOPA-BSA reacted with L-DOPA-RSA as well as L-DOPA-BSA. The similar results were obtained with the antisera to the catecholamine-BSA's. Further each antiserum showed a weak or no reaction with the conjugates of other haptens, including N-maleyl-hapten-BSA.

Discussion

The Mannich reaction has been widely investigated as a method of conjugation of low molecular weight compounds with proteins under mild conditions.[6d,9,14)] By the reaction, phenols[15)] and catechols[16)] conjugate with amino groups at the *ortho*-position to hydroxyl group in the aromatic ring. The substituted position in the case of catechols, however, was not confirmed. We prepared a model compound of the conjugation of catecholamines and albumin from 4-methylcatechol and ethylamine by the Mannich reaction. The probable substituted structure as shown in Fig. 4 was suggested by ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra and infrared (IR) spectra of the model. The details will be described elsewhere.

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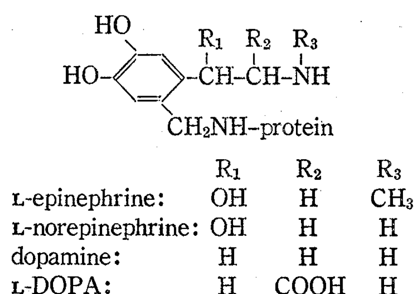

Fig. 4. Probable Structure of Conjugates

For the conjugation of these haptens with proteins by the Mannich reaction, it is necessary that the amino groups of the side chains of the haptens are previously protected to prevent the side-reactions such as cyclization.[17] As a method for the protection, N-maleylation was adopted here, for the reason that N-maleylation is readily performed and the maleyl groups are readily removed from the conjugates under a mild condition.[18]

The molar ratios of haptens to carrier proteins in the conjugates thus obtained will fit to the production of antibodies to the haptens.[19]

Table IV demonstrates that the antibodies obtained by immunization of rabbits with the conjugates recognize the structures of the haptens in the conjugates.

Therefore the conjugates prepared by the present method seem to be suitable for the production of antibodies specific to catecholamines and L-DOPA for the immunoassay. In fact, by equilibrium dialysis it was found that the antibody (gamma globulin) to L-epinephrine-BSA bound specifically L-epinephrine but not the other catecholamines and metabolites, as presented in the previous paper.[2] The standard curve of 0.043 to 1.3 nmoles of epinephrine for radioimmunoassay was obtained by the equilibrium dialysis method. The attempts to increase the sensitivity is under investigation by use of the antibody of the higher titer and a micro filtration method, and will be described in the following paper.

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