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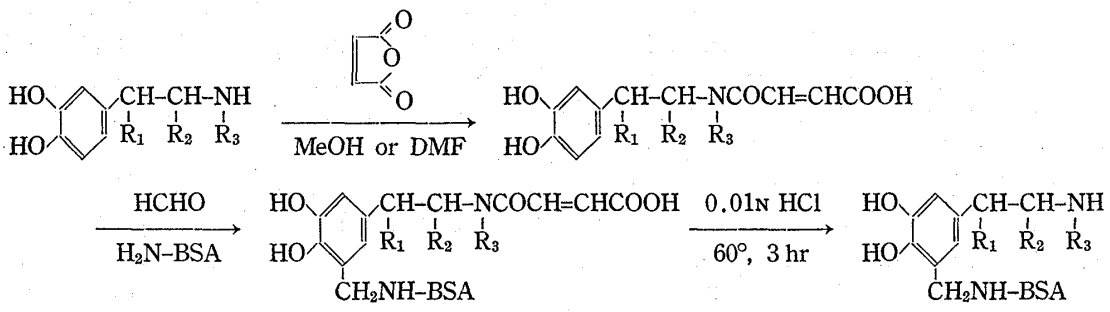
Preparation of a Specific Antibody to Each One of Catecholamines and L-DOPA[1]

A method was developed for conjugation of catecholamines or L-DOPA with BSA leaving the catechol moiety and side chain intact. The specificity of the antibody, obtained by immunization of rabbits with the conjugate, was demonstrated by Ouchterlony method and by equilibrium dialysis.

Catecholamines and their related compounds have usually been determined by gas chromatography[2] or fluorometry.[3] These methods, however, have a number of technical problems in practical measurement of biological samples.

Development of radioimmunoassay is desirable for studies on catecholamines. Preparations of antibodies to catecholamines have been reported by Spector and others.[4] This paper describes a method of conjugation of catecholamines (L-epinephrine, L-norepinephrine and dopamine) or L-DOPA with BSA by Mannich reaction and of preparation of the specific antibodies to the haptens by immunization with these conjugates.

Conjugation procedure of the haptens with BSA are shown in Chart 1.



	R_1	R_2	R_3
L-epinephrine:	OH	H	CH_3
L-norepinephrine:	OH	H	H
dopamine:	H	H	H
L-DOPA:	H	COOH	H

Chart 1. Procedure for Conjugation of the Haptens with BSA

Since the side chain of the hapten cyclizes with formaldehyde by Mannich reaction,[5] the amino group of the side chain was tentatively protected by maleyl group. N-Maleylation of catecholamines was performed in methanol at room temperature, and that of L-DOPA in DMF at 60°. The reactions were monitored by thin-layer chromatography and paper or cellulose acetate membrane electrophoreses.

The coupling of N-maleyl-hapten to BSA was carried out according to the method for phenols proposed by Fraenkel-Conrat, *et al.*[6] with minor modifications. A few hundred-fold moles of N-maleyl-hapten was added to an aqueous BSA solution. The solution was adjusted

1) Abbreviation····L-DOPA: L-3,4-dihydroxyphenylalanine; BSA: bovine serum albumin; RSA: rabbit serum albumin; DMF: N,N-dimethylformamide; PBS: phosphate buffered saline.
2) M-T. Wang, K. Imai, M. Yoshioka and Z. Tamura, *Clin. Chim. Acta*, **63**, 13 (1975).
3) C.R. Creveling and J.W. Daly, "Method of Biological Analysis," ed. by D. Glick, Suppl. Vol., Interscience Publishers, New York, 1971, p. 153.
4) S. Spector, U.S. Patent 3704282 (1972) [*C.A.*, **28**, 41465*j* (1973)]; S. Spector, C. Dalton and A.M. Felix, *Biochem. Pharmacol. Suppl.*, 263 (1974); S. Went and L. Kesztyus, *Arch. Exptl. Pathol. Pharmakol.*, **193**, 609 (1939).
5) G. Cohen and M. Collins, *Science*, **167**, 1749 (1970).
6) H. Fraenkel-Conrat and H.S. Olcott, *J. Biol. Chem.*, **174**, 827 (1948).

to pH 5.5—7.6 with sodium acetate and added with an appropriate quantity of formaldehyde. After standing at 20—23° for 3—5 days, the mixture was dialysed against 0.01N HCl and heated at 60° for 3 hours to remove the maleyl group. The removal of the maleyl group from the conjugate by acid hydrolysis was monitored by cellulose acetate membrane electrophoresis. Finally the solution was dialysed against PBS (0.15M NaCl, 0.01M KH_2PO_4, pH 7.4).

The 15—30 hapten molecules combined with a BSA molecule were determined by a modified method of Doty.[7] The structure of the conjugate is presumed as in Chart 1 from the report on Mannich reaction of catechols by Burckhalter, *et al.*,[8] although it is under investigation by infra-red spectrometry and other techniques.

The amino acid composition of L-epinephrine-BSA was determined by an amino acid analyser. The number of lysine in the conjugate was smaller than that of BSA. It suggests that the hapten is incorporated into the ε-amino group of lysine residue.

Two to three mg of the conjugate suspended in complete Freund's adjuvant was injected to foot pads of a rabbit. Four weeks later the rabbit was boosted with 1 mg of the conjugate intravenously or subcutaneously, and bled one week later.

Table I. Reaction of Antiserum with Each Conjugate observed by Ouchterlony Method

Antigen	Antiserum[a] to the BSA conjugate of			
	L-DOPA	L-Epinephrine	L-Norepinephrine	Dopamine
L-DOPA-BSA	##	−	−	−
L-DOPA-RSA[b]	##	−	−	−
L-Epinephrine-BSA	−	##	±	±
L-Norepinephrine-BSA	±	±	##	±
Dopamine-BSA	±	±	±	##

a) Antibody to BSA in antiserum was removed by absorption with BSA.
b) L-DOPA-RSA was obtained by the same procedure of L-DOPA-BSA.

Table II. Binding Rate of ^{3}H-Epinephrine[a] with Antibody[b] to L-Epinephrine obtained by Equilibrium Dialysis

Analogue compound[c]	Binding rate (%)[d]
None	100
L-Epinephrine	0
L-Norepinephrine	55
Dopamine	88
L-DOPA	91
DL-Metanephrine	82
DL-Normetanephrine	97
3,4-Dihydroxyphenylacetic acid	84
4-Hydroxy-3-methoxyphenylacetic acid	92
L-Isoproterenol	73

a) ^{3}H-DL-epinephrine (3.4×10^{-7}M, 11 Ci/mmole)
b) Gamma globulin (0.94 mg/ml) was obtained from the antiserum to L-epinephrine.
c) The concentration of each analogue was 3.4×10^{-5}M.
d) The binding rate of the labeled epinephrine with gamma-globulin in the absence of the unlabeled was expressed 100% (45000 cpm) as a control.

7) J. Doty, *Anal. Chem.*, **20**, 1166 (1948).
8) J.H. Burckhalter, F.H. Tendick, E.M. Jones, W.F. Holcomb and A.L. Rawlins, *J. Am. Chem. Soc.*, **68**, 1894 (1946).

The sera obtained were tested for the antibody production by Ouchterlony method.[9] Since a serum showed precipitin reactions with both BSA and the conjugate, the antibody to BSA was absorbed with BSA. The specificity of the antiserum to each conjugate was demonstrated as shown in Table I.

It was probable that each antibody recognized the hapten moiety of each conjugate, since the antibody to L-DOPA-BSA reacted with L-DOPA-RSA as well as L-DOPA-BSA.

The binding activity of antibody to L-epinephrine was further tested by equilibrium dialysis.[10] The results are shown in Table II.

These data demonstrated that the antibody recognized not only the catechol moiety but also the side chain of L-epinephrine molecule.

The present method will be suitable to obtain an antibody specific to each catecholamine or L-DOPA.

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10) H.N. Eisen, E.S. Simms and M. Potter, *Biochemistry*, 7, 4126 (1968).