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Specificity of Antisera raised against Estradiol using New Hapten-Carrier Conjugates[1]

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Three new hapten-carrier conjugates were prepared from the 6α-, 6β-, and 7α-hydroxyestradiol monohemisuccinates by coupling with bovine serum albumin employing the mixed anhydride technique. The specificity of antiestradiol antisera elicited in the rabbit by immunization with each of these antigens was assessed by testing the cross-reaction with the closely related steroids. The results indicated that highly specific antisera to estradiol would be produced by antigen whose steroidal moiety is coupled to a protein through the 6α, 6β or 7α position remote from the inherent functional groups.

In recent years, determination of estrogen and other steroid hormones in biological fluids has been greatly facilitated by the use of radioimmunoassay. The antisera required for this purpose are usually obtained by immunization with the hapten–carrier conjugate. Although numerous attempts have been made to prepare antisera used for the assay of estradiol, the satisfactory results have not yet been attained with respect to the specificity. In some cases, the hapten–carrier conjugate whose steroidal moiety is coupled to a protein through derivatization at the C-3 or C-17 substituent, has been employed.[3] However, it is sufficiently substantiated that the immunologic specificity is less dependent on the individual steroid than on the particular functional groups occupied.[4] The 2- and 4-*p*-phenylazo derivatives which are linked to a protein through the carboxyl group, have been also devised as haptens.[5] In addition, several workers attempted to couple the steroid to a carrier protein at site remote from the functional groups through a linkage such as 11α-hemisuccinate[6] and 6-O-carboxymethyloxime.[7] It is supposed that the substitution of a bulky group at the sterically hindered

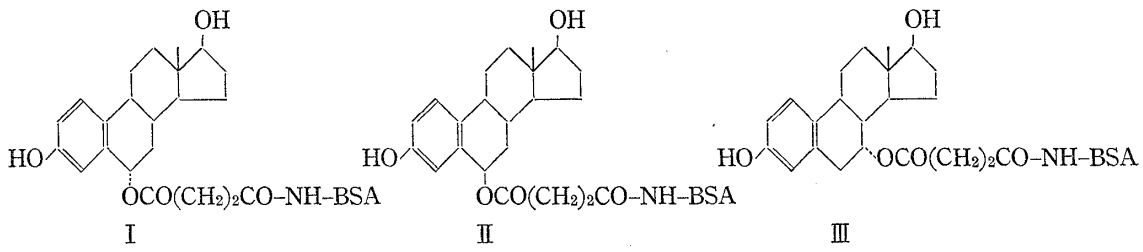

Chart 1

1) This paper constitutes Part LXXIV of the series entitled "Analytical Chemical Studies on Steroids"; Part LXXIII: T. Nambara and M. Nokubo, *Chem. Pharm. Bull.* (Tokyo), submitted.
2) Location: *Aobayama, Sendai.*
3) G. Mikhail, C.H.Wu, M. Ferin, and R.L. Vande Wiele, *Steroids*, **15**, 333 (1970).
4) S.J. Gross, "Immunologic Methods in Steroid Determination," ed. by F.G. Peron and B.V. Caldwell, Appleton-Century-Crofts, Meredith Co., New York, 1970, p. 63.
5) S.J. Gross, D.H. Campbell, and H.H. Weetall, *Immunochem.*, **5**, 55 (1969).
6) F.C. Hollander and A.H.W.M. Schuurs, *Scand. J. Clin. Lab. Invest.*, Suppl. **29**, 126 (1972).
7) P.D.G. Dean, D. Exley, and M.W. Johnson, *Steroids*, **18**, 593 (1971); S.L. Jeffcoate and J.E. Searle, *ibid.*, **19**, 181 (1972); E. Kuss and R. Goebel, *ibid.*, **19**, 509 (1972); K. Wright, D.C. Collins, and J.R.K. Preedy, *ibid.*, **21**, 755 (1973).

position and the alteration of C-6 position to trigonal carbon may cause the distortion of the fused-ring system resulting in the reduced specificity.

For the purpose of obtaining much more specific antisera we have prepared the new haptens, 6α-, 6β-, and 7α-hydroxyestradiol monohemisuccinates, which are capable of coupling to a carrier protein without influencing any disturbance on the functional groups and steroidal skeleton inherent to estradiol.[8] The present paper deals with the properties of anti-estradiol antisera elicited by immunization with these new hapten-bovine serum albumin (BSA) conjugates (I, II, and III) and the influence of the position coupled to a protein on the specificity of raised antibodies.

Experimental

Animals——Domestic strain male albino rabbits weighing 2.5—3.0 kg were used.

Materials——Estradiol-6,7-^{3}H (56 Ci/m mole) was supplied from the Radiochemical Centre, Amersham. 6α-, 6β-, and 7α-Hydroxyestradiol monohemisuccinates were synthesized in these laboratories by the methods previously reported.[8] Other steroids were kindly donated from Teikoku Hormone Mfg. Co., Tokyo. BSA, bovine gamma-globulin (Miles Laboratories, Inc., Kankakee), and Freund's complete adjuvant (Difco Laboratories, Detroit) were purchased, respectively. All solvents and chemicals used were Analytical Reagent grade.

Conjugation of Estradiol Derivatives to BSA

Estradiol-6α-BSA Conjugate (I)——i) To a mixed solution of 6α-hydroxyestradiol 6-hemisuccinate (65 mg) in dioxane (10 ml) and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (65 mg) in H_2O (5 ml) was added dropwise a solution of BSA (400 mg) in 1/15 M Sörensen buffer (pH 7.9) (10 ml) and stirred at room temperature for 24 hr. The resulting solution was dialyzed against cold running water for 3 days. The suspension was centrifuged at 3000 rpm for 10 min and the supernatant was lyophilized to give I (390 mg) as a fluffy powder.

ii) To a stirred solution of 6α-hydroxyestradiol 6-hemisuccinate (65 mg) in dry dioxane (5 ml) were added $(n\text{-}C_4H_9)_3N$ (0.08 ml) and then isobutylchlorocarbonate (0.02 ml) under ice-cooling. Thirty min later to this was added dropwise a solution of BSA (200 mg) in H_2O (9 ml)–dioxane (6 ml) containing 1 N NaOH (0.2 ml) under ice-cooling and stirred maintaining a pH of about 7 for 4 hr. The resulting solution was dialyzed against cold running water overnight and the turbid protein solution was brought to pH 4.6 with 1 N HCl. After allowing to stand at 0°, the suspension was centrifuged at 3000 rpm for 10 min. The precipitate was dissolved in 5% $NaHCO_3$ and dialyzed as before. Lyophilization of the solution afforded I (220 mg) as a fluffy powder.

Estradiol-6β-BSA Conjugate (II)——i) Prepared from 6β-hydroxyestradiol 6-hemisuccinate (50 mg) and BSA (400 mg) by the carbodiimide method in the manner as described above. Yield 370 mg.

ii) Prepared from 6β-hydroxyestradiol 6-hemisuccinate (50 mg) and BSA (200 mg) by the mixed anhydride method in the manner as described above. Yield 200 mg.

Estradiol-7α-BSA Conjugate (III)——Prepared from 7α-hydroxyestradiol 7-hemisuccinate (75 mg) and BSA (200 mg) by the mixed anhydride method in the manner as described above. Yield 220 mg.

Number of steroid molecules linked to each BSA molecule was calculated by measuring the absorbance at 390 nm with the solutions of hydroxyestradiol monohemisuccinate (20 μg/ml), BSA (200 μg/ml), and hapten-BSA conjugate (220 μg/ml) in 0.1 N NaOH, whereby the following results were obtained.[9]

Method	6α	6β	7α
Carbodiimide	14	16	—
Mixed anhydride	35	20	26

Radioactivity Measurement——The samples were counted in a Packard Tri-Carb Model 3380 liquid scintillation spectrometer employing Bray's scintillant,[10] composed of 2,5-diphenyloxazole (4 g), 1,4-bis-[2-(5-phenyloxazolyl)]benzene (200 mg), naphthalene (60 g), MeOH (100 ml), ethylene glycol (20 ml), and sufficient dioxane to make the total volume 1 liter. For quenching corrections the channel ratio and external standard methods were employed.

8) T. Nambara, M. Takahashi, and M. Numazawa, *Chem. Pharm. Bull.* (Tokyo), **22**, 1167 (1974).
9) L. Goodfriend and A.H. Sehon, *Can. J. Biochem. Physiol.*, **36**, 1177 (1958).
10) G.A. Bray, *Anal. Biochem.*, **1**, 279 (1960).

Immunization of Rabbits——Groups of 2—3 rabbits were immunized with each of the estradiol-BSA conjugates (I, II, and III). The antigen (2 mg) was dissolved in sterile isotonic saline (0.5 ml) and emulsified with Freund's complete adjuvant (0.5 ml). This emulsion was injected into rabbits subcutaneously at multiple sites on the back. This procedure was repeated once a week for further 3 weeks and then once a fortnight for the following month. Bleedings (10 ml) were begun 10 days after the final injection. The antisera against the 6α-, 6β-, and 7α-BSA conjugates (E_2-6α, E_2-6β, and E_2-7α-1) were raised 2.5 months after the primary injection. As a separate run the estradiol-7α-BSA conjugate (III) was injected into a rabbit in the similar manner twice with 2 weeks interval and subsequently once a month. The anti-7α-BSA conjugate anti-serum (E_2-7α-2) was harvested 2 months after the initial injection. These antisera were separated by centrifuging at 3000 rpm for 10 min, stored at $-80°$, and used in the assay at an initial dilution of 1 : 5000—6000.

Assay Procedure——The antisera were thawed and diluted as needed with 0.05M borate buffer (pH 8.0) containing 0.06% BSA and 0.05% bovine gamma-globulin. To a test sample containing estradiol were added estradiol-^{3}H (*ca.* 10^4 dpm) and diluted antisera (0.25 ml), and incubated at room temperature for 30 min. After addition of 50% $(NH_4)_2SO_4$ (0.25 ml) the resulting mixture was allowed to stand at room temperature for 10 min and centrifuged at 3000 rpm for 10 min, and 0.2 ml aliquot of the supernatant was used for counting. The percentage of bound estradiol in the sample was determined with the following equation.

$$\% \text{ bound estradiol} = \frac{A - P \times \frac{0.5}{0.2}}{A} \times 100$$

where A=count/min added to each sample, P=count/min recovered.

Cross-Reaction Study——The specificity of antisera raised against the estradiol-BSA conjugates was tested by cross-reaction studies with 19 kinds of steroids related to estradiol (see Table II). The relative amounts required to reduce the initial binding of estradiol-^{3}H by half, where the mass of non-labeled estradiol was arbitrarily chosen as 100%, were calculated by the standard curves.

Results and Discussion

The steroid hemisuccinates were covalently linked to BSA by the carbodiimide method[11] and the mixed anhydride technique.[12] The latter procedure by which satisfactory number of steroid molecules were jointed to each BSA, proved to be more favorable. The serum samples obtained from the immunized rabbits showed an increased binding activity to estradiol, though there was considerable individual variation. After two or three months of injection several samples showed a significant increase in the binding activity.

The standard curves were obtained with 1: 5000—6000 dilution of the rabbit sera raised against the 6α-, 6β-, and 7α-BSA conjugates and used for replicate analyses and cross-reaction studies. As illustrated in Fig. 1 the plot of per cent bound radioactivity versus the logarithm of inert estradiol showed a liner relationship. The precision and accuracy of the method using these antisera were then examined with various amounts of authentic estradiol ranging from 20

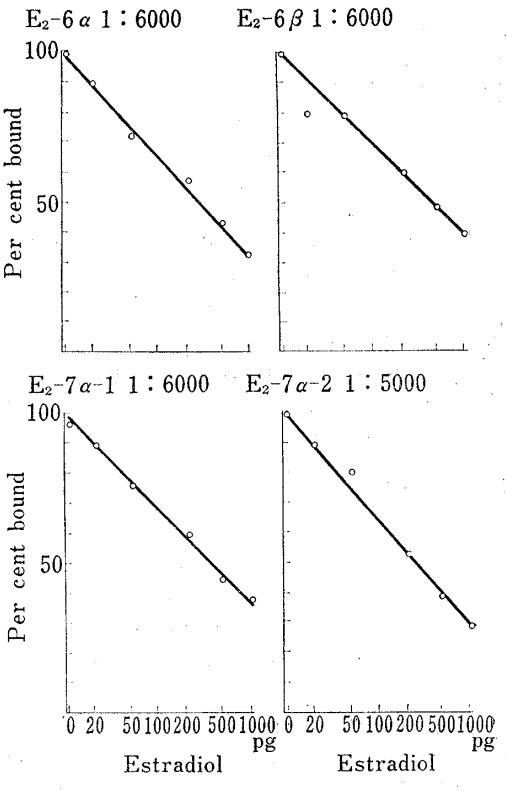

Fig. 1. Standard Curves for Estradiol

11) J.C. Sheehan and J.J. Hlavka, *J. Org. Chem.*, **21**, 439 (1956); H.G. Khorana, *Chem. Rev.*, **53**, 145 (1959).
12) S.A. Berson and R.S. Yalow, *J. Clin. Invest.*, **36**, 873 (1957); G. Mikhail, C.H.Wu, M. Ferin, and R.L. Vande Wiele, *Acta Endocrinol.*, **64**, Suppl., 147, 347 (1970).

TABLE I. Replicate Analyses of Authentic Estradiol added to Deionized Distilled Water[a)]

Anti-estradiol serum	Estradiol Added (pg)	Estradiol Found (pg)	Standard deviation	Standard error	Coefficient of variation (%)	Regression equation
E_2-6α	0	10	3.37	1.19	33.7	$Y=1.10X-10.0$
	20	22	2.87	1.44	13.0	
	50	45	6.08	2.48	13.5	
	200	160	4.12	1.68	2.6	
	500	470	3.87	1.58	0.8	
	1000	1100	3.92	1.60	0.4	
E_2-6β	0	5	3.77	1.54	75.4	$Y=0.98X+1.92$
	20	43	11.37	4.64	26.4	
	50	52	4.93	2.01	9.5	
	200	180	8.29	3.39	4.6	
	500	450	7.92	3.23	1.8	
	1000	1000	7.60	3.10	0.8	
E_2-7α-1	0	15	3.79	1.43	25.2	$Y=1.06X+14.04$
	20	19	5.41	2.21	28.5	
	50	50	6.16	2.52	12.3	
	200	180	5.74	2.87	3.2	
	500	560	4.89	2.00	0.9	
	1000	1000	3.72	1.52	0.4	
E_2-7α-2	0	20	2.89	1.44	14.4	$Y=1.01X+0.74$
	20	23	5.20	2.60	22.6	
	50	26	2.58	1.29	9.9	
	200	215	3.70	1.85	1.7	
	500	520	3.85	1.93	0.7	
	1000	1000	4.96	2.48	0.5	

a) number of determination (n)=4

TABLE II. Per Cent Cross-Reaction of Anti-Estradiol Sera with Selected Steroids

Steroids	E_2-6α	E_2-6β	E_2-7α-1	E_2-7α-2
Estrone	0.461	1.90	1.20	0.25
Estrone sulfate	<0.001	<0.001	<0.001	<0.001
2-Methoxyestrone	0.046	0.447	0.42	0.15
3-Deoxyestrone	0.13	0.292	0.079	0.12
Estradiol	100	100	100	100
17α-Estradiol	0.6	0.558	0.06	0.42
6-Ketoestradiol	1.30	2.11	2.33	2.45
16-Ketoestradiol	0.3	<0.001	3.20	0.03
3-Deoxyestradiol	7.143	7.60	0.42	0.93
Estriol	0.30	0.10	0.47	0.34
Estriol 16-glucuronide	<0.001	<0.001	<0.001	<0.001
16-Epiestriol	3.0	1.583	2.10	0.15
17-Epiestriol	0.111	0.38	0.06	0.14
Testosterone	0.027	0.064	0.03	0.02
Progesterone	<0.001	0.003	<0.001	<0.001
Pregnenolone	<0.001	<0.001	<0.001	<0.001
17α-Hydroxyprogesterone	<0.001	0.003	<0.001	<0.001
Cortisol	<0.001	<0.001	<0.001	<0.001
Corticosterone	<0.001	<0.001	<0.001	<0.001
Cholesterol	<0.001	<0.001	<0.001	<0.001

pg to 1 ng dissolved in deionized distilled water. The replicate determination of each sample gave the satisfactory results with the regression lines as listed in Table I.

The specificity of antisera was assessed by testing the ability of the compounds closely related to estradiol to compete for binding sites on the antibody. The results of cross-reaction studies of various steroids with four different anti-estradiol antisera are collected in Table II. These antisera were all reasonably specific for estradiol, and there was not a significant difference in the respective cross-reaction levels. The antibodies yielded with the same antigen did not differ remarkably in specificity. However, the antiserum (E_2-7α-2) raised against the 7α-BSA conjugate by injections with a longer time interval appeared to be somewhat more specific than the other (E_2-7α-1). None of the steroids tested in this system had more than 1% of the relative activity of estradiol except 6-ketoestradiol (2.45%). The cross-reactions of remaining antisera against the 7α-, 6α-, and 6β-BSA conjugates were also not significant, though 16-epiestriol, 6-ketoestradiol, and 3-deoxyestradiol reacted to a certain extent.

It is evident from these results that antigen having a linkage to the steroid molecule through C-6 or C-7 position would elicit highly specific antibody. To the best of our knowledge this is the first recorded instance of antisera produced by immunization with estradiol derivative conjugated at C-7.[13] It is also to be noted that both the 6α- and 6β-BSA conjugates yielded more specific antisera to estradiol than the 6-O-carboxymethyloxime conjugate which has so far been regarded as the most potent antigen. Several investigators have already developed specific antisera to different steroids by conjugation to a protein through a position in the B or C ring. In all cases these antisera were more specific than those obtained by antigen in which a protein was coupled with the steroid molecule through the A or D ring. From the present results together with the previous findings we arrive at the conclusion that in order to obtain specific antisera against estradiol the steroidal hapten should be conjugated to a protein in such a way that both the A and D rings are left available as antigenic determinant without suffering from any distortion of the molecular shape.

The utilization of these highly specific antisera for measurements of estradiol in the biological fluids will be the subject of a future communication.

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13) During the continuation of this work the preparation of antigenic complexes of C_{19} and C_{21} steroids by coupling to a protein through C-7 has been reported.[14]

14) A. Weinstein, H.R. Lindner, A. Friedlander, and S. Bauminger, *Steroids*, **20**, 789 (1972); R.S. Rosenfeld, B. Rosenberg, J. Kream, and L. Hellman, *Steroids*, **21**, 723 (1973).