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Relationships between Conformation of Antigen and Specificity of Antibody in Radioimmunoassay for Steroid Hormone. III. Highly Specific Cortisol-Antibody against 6α- and 6β-Hydroxycortisol 6-Hemisuccinate-Bovine Serum Albumin Conjugates

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anti-6α- and *anti*-6β-Hydroxycortisol 6-hemisuccinate-BSA antisera(*anti*-F-6α-HS and *anti*-F-6β-HS) were obtained by immunizing rabbits. The specificity of each antiserum was assessed by determining the cross-reactivities with 37 kinds of steroids and was compared with that of *anti*-cortisol 21-hemisuccinate-BSA conjugate antiserum(*anti*-F-21-HS).

anti-F-6β-HS had higher specificity than that of *anti*-F-6α-HS for the steroids structurally different to cortisol in ring A. *anti*-F-21-HS had an obviously lower specificity than those of *anti*-F-6α-HS and *anti*-F-6β-HS for the steroids differing structurally from cortisol only in the 17-side chain.

Keywords——radioimmunoassay; *anti*-6α-hydroxycortisol 6-hemisuccinate-BSA conjugate antiserum; *anti*-6β-hydroxycortisol 6-hemisuccinate-BSA conjugate antiserum; percentage cross-reactivity; specificity

As an approach to obtain a highly specific antibody in radioimmunoassay for steroid hormone, it has been suggested that the steroid molecule should be conjugated to the carrier protein at suitable positions as remote as possible from functional groups.[2,3)] Under the consideration, we have already reported the preparation of 6α- and 6β-hydroxycortisol 6-hemisuccinates as a hapten group for cortisol antigen.[4)] In this paper, we report the high specificity of rabbit antisera against these BSA conjugates and discuss the effect of the stereochemistry of hapten group causing the specificity, which was measured by the percentage cross-reaction of these antisera with 37 kinds of steroids, comparing with the known antiserum against cortisol 21-hemisuccinate-BSA conjugate (F-21-HS).

Experimental

Preparation of Antigen——The 6α- and 6β-hydroxycortisol 6-hemisuccinates were conjugated to BSA by the mixed anhydride method.[5)] Calculation of the cortisol number per mol of BSA was carried out entirely according to the method of Erlanger *et al.*[5)] The number of molecules of 6α- and 6β-hydroxycortisol 6-hemisuccinates bonded to a BSA molecule were 26 and 21, respectively.

Immunization of Rabbits——Each group of three rabbits was immunized with 2 mg of cortisol-BSA conjugate in complete Freund's adjuvant, then after two weeks, rabbits received injection of 1 mg of the conjugate, and thereafter injections of 1 mg of the conjugate were made at monthly intervals. The rabbits were bled 10 to 14 days after each injection, and the titre of antisera was checked. The titre of each antiserum which shows binding ability of over 50% with tritiated cortisol, attained a maximum 7 to 8 months after immunization. The antisera used in the present study were obtained 7 and 5.5 months after immunization against 6α- and 6β-hydroxycortisol 6-hemisuccinate-BSA conjugates (F-6α-HS and F-6β-HS), respectively. Each titre of *anti*-F-6α-HS was 1:5400, 1:9400, and 1:13000 and that of *anti*-F-6β-HS was 1:5400, 1:6200, and 1:8300 at the final dilution. The titre of *anti*-F-21-HS (1:12000) which was obtained from Daiichi Radioisotope Lab. Co., Ltd. was also determined by the same method as described above.

1) Location: *Maidashi 3-1-1, Higashi-ku, Fukuoka 812, Japan.*
2) H.R. Lindner, E. Perel, A. Friedlander, and A. Zeitlin, *Steroids*, **19**, 357 (1972).
3) A. Weinstein, H.R. Lindner, A. Friedlander, and S. Bauminger, *Steroids*, **20**, 789 (1972).
4) H. Sone, M. Kojima, and H. Ogawa, *Yakugaku Zasshi*, **95**, 185 (1975).
5) B.F. Erlanger, F. Borek, S.M. Beiser, and S. Lieberman, *J. Biol. Chem.*, **228**, 713 (1957).

Radioimmunoassay——A given amount of unlabeled steroid was dissolved in a test tube with 0.1 ml of 0.1 M borate buffer (pH 8.2) containing 0.03% gelatine. To the solution were added 0.1 ml of diluted antiserum binding approximately 50% of tritiated cortisol and 0.1 ml of buffer solution of tritiated cortisol (*ca.* 2×10^4 dpm, 80 pg). The mixture was incubated overnight at 4°, then saturated ammonium sulfate (0.3 ml) was added to the solution. The mixture was further incubated for 30 minutes at 4°. Free steroid was separated at 4° by centrifugation for 30 minutes at 2000 ***g***. The radioactivity of 0.3 ml of the supernatant was determined. The specificity of the antiserum was determined by measuring the cross-reaction with various steroids. The percentage cross-reaction was calculated from the relative amounts required to displace 50% of the ^{3}H-cortisol bound to the antibody. All solvents were of analytical grade and were used without further purification.

Steroids for Cross-reaction——Cortisol-1,2-^{3}H (40Ci/mmol) was obtained from New England Nuclear Co., Ltd. Thirty-seven kinds of steroids were used to measure the percentage cross-reaction. All the steroids except 11β,17α,21-trihydroxy-4-pregnen-20-one (II), 11β,17α,21-trihydroxy-5α-pregnane-3,20-dione (III), 11β,17α,21-trihydroxy-5β-pregnane-3,20-dione (IV), and 11α,17α,21-trihydroxy-4-pregnene-3,20-dione (VIII) were obtained from Sigma Chemical Co.

11β,17α,21-Trihydroxy-4-pregnen-20-one (II)——II was prepared according to the modified Broome's procedure.[6] Lithium aluminum hydride ($LiAlH_4$) (0.5 g) was added to a solution of 17α,20,20,21-bismethylenedioxy-11β-hydroxy-4-pregnen-3-one[7] (0.5 g) in dry ether (25 ml). After the mixture was refluxed for 40 min, $AlCl_3$ (1.0 g) was added under cooling and the mixture was refluxed again for 2 hr. The excess $LiAlH_4$ and hydride complex was carefully decomposed by adding ethyl acetate dropwise and the insoluble material was collected by filtration. A suspended solution of the collected material in ethyl acetate was refluxed for 4 hr. After filtration of the reaction mixture, the filtrate was evaporated *in vacuo* to dryness and the residue was chromatographed on silica gel eluting with chloroform to give 0.4 g of 17α,20,20,21-bismethylenedioxy-4-pregnen-11β-ol as crystals, mp 158—160°. *Anal.* Calcd. for $C_{23}H_{34}O_5$: C, 70.74; H, 8.78. Found: C, 70.77; H, 8.80. IR ν_{max}^{Nujol} cm^{-1}: 3560 (O–H), 1110, 1085, 950 (BMD). The bismethylenedioxy compound was dissolved in 100 ml of 50% aqueous acetic acid and heated for 6 hr at 100° under a nitrogen atmosphere. After removal of the solvent *in vacuo*, the residual oil was chromatographed on silica gel eluting with chloroform–methanol (95:5) to yield 0.1 g of II as colorless needles. The melting point was 190—193° after recrystallization from benzene. $[\alpha]_D^{20.5}$ +58.7° (c=1.2, pyridine). *Anal.* Calcd. for $C_{21}H_{32}O_4$: C, 72.38; H, 9.26. Found: C, 72.58; H, 9.01. IR ν_{max}^{Nujol} cm^{-1}: 3520, 3440 (O–H), 1720 (C=O).

11β,17α,21-Trihydroxy-5α-pregnane-3,20-dione (III) and 11β,17α,21-Trihydroxy-5β-pregnane-3,20-dione (IV)——III and IV were prepared according to the modified Harnik's procedure.[8] Cortisol (5 g) was hydrogenated under the presence of Pd/C (5 g) in ethanol (150 ml) for 4 hr. After removal of the catalyst by filtration through a thin-layer of Celite, the filtrate was evaporated *in vacuo*. A mixture of benzene and ethyl acetate was added slowly to the residual oil to give a powder (1.4 g). Recrystallization of the powder from ethanol gave colorless plates of III, mp 248—250° (lit.[8] mp 230—233°). $[\alpha]_D^{24}$ +54° (c=0.94, pyridine) (lit.[8] $[\alpha]_D$ +63°). *Anal.* Calcd. for $C_{21}H_{32}O_5$: C, 69.20; H, 8.85. Found: C, 69.06; H, 8.86. IR ν_{max}^{Nujol} cm^{-1}: 3500 (O–H), 1720, 1710 (C=O).

The additional oil was obtained from the mother liquor (benzene–ethyl acetate) after evaporation *in vacuo*, and crystallized on trituration with ethyl acetate. Recrystallization from ethyl acetate gave colorless plates of IV, free from 5α-isomer (III). mp 197—200° (lit[8] mp 206—208°). $[\alpha]_D^{24}$ +55° (c=1.2, pyridine) (lit[8] $[\alpha]_D$ +63°). *Anal.* Calcd. for $C_{21}H_{32}O_5$: C, 69.20; H, 8.85. Found: C, 69.00; H, 8.94. IR ν_{max}^{Nujol} cm^{-1}: 3500 (O–H), 1720, 1710 (C=O).

11α,17α,21-Trihydroxy-4-pregnene-3,20-dione (VIII)——VIII was prepared according to the method of Bernstein *et al.*[9] mp 204—206° (lit[9] mp 214—216°). *Anal.* Calcd. for $C_{21}H_{30}O_5$: C, 69.59; H, 8.34. Found: C, 69.55; H, 8.49. IR ν_{max}^{Nujol} cm^{-1}: 3440 (O–H), 1720, 1670 (C=O).

Results

Table I shows the percentage cross-reactivities of *anti*-F-6α-HS, *anti*-F-6β-HS, and *anti*-F-21-HS with various steroids. *anti*-F-21-HS shows the highest specificity for 11β,17α,21-trihydroxy-4-pregnen-20-one (II), 5α-dihydrocortisol (III), 5β-dihydrocortisol (IV), 3α,11β,17α,21-tetrahydroxy-5β-pregnan-20-one (V), and 3β,11β,17α,21-tetrahydroxy-5-pregnen-20-one (VI) which differ structurally from cortisol only in ring A. Moreover, for these steroids, *anti*-F-6β-HS showed higher cross-reactivities than *anti*-F-6α-HS. On the other hand, the

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7) D.K. Fukushima and S. Daum, *J. Org. Chem.*, **26**, 520 (1961).
8) M. Harnik, *Israel J. Chem.*, **1**, 158 (1963) [*Chem. Abstr.*, **60**, 6904*g* (1964)].
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TABLE I. Percentage Cross Reaction of *anti*-Cortisol Antisera

Compoends	*anti*-F-6α-HS[a)]	*anti*-F-6β-HS[a)]	*anti*-F-21-HS
11β,17α,21-Trihydroxy-4-pregnene-3,20-dione (Cortisol) (I)	100	100	100
11β,17α,21-Trihydroxy-4-pregnen-20-one (II)	5.16	4.71	1.17
11β,17α,21-Trihydroxy-5α-pregnane-3,20-dione (5α-Dihydrocortisol) (III)	49.36	32.12	20.33
11β,17α,21-Trihydroxy-5β-pregnane-3,20-dione (5β-Dihydrocortisol) (IV)	22.84	14.16	6.10
3α,11β,17α,21-Tetrahydroxy-5β-pregnan-20-one (V)	0.19	0.046	0.047
3β,11β,17α,21-Tetrahydroxy-5-pregnen-20-one (VI)	7.86	3.68	0.031
17α,21-Dihydroxy-4-pregnene-3,11,20-trione (Cortisone) (VII)	6.26	23.78	19.06
11α,17α,21-Trihydroxy-4-pregenne-3,20-dione (11-Epicortisol) (VIII)	0.72	0.78	0.48
17α,21-Dihydroxy-4-pregnene-3,20-dione (11-Deoxy-17α-hydroxycorticosterone) (IX)	13.48	20.86	17.43
11β,21-Dihydroxy-4-pregnene-3,20-dione (Corticosterone) (X)	7.25	7.83	11.09
11β,17α,20β,21-Tetrahydroxy-4-pregnen-3-one (XI)	1.77	1.66	2.77
18,11-Hemiacetal of 11β,21-Dihydroxy-3,20-dioxopregn-4-en-18-al (Aldosterone) (XII)	0.0027	0.0014	0.48
21-Hydroxy-4-pregnene-3,20-dione (Deoxycoritcosterone) (XIII)	1.26	1.74	6.63
21-Hydroxy-4-pregnene-3,11,20-trione (11-Dehydrocorticosterone) (XIV)	4.04	3.16	7.35
3α,17α,20α,21-Tetrahydroxy-5β-pregnan-11-one (α-Cortolone) (XV)	0.010	0.010	0.010
5β-Pregnane-3α,11β,17α,20α,21-pentaol (α-Cortol) (XVI)	0.010	0.010	0.023
5-Pregnene-3β,11β,17α,20α-tetraol (XVII)	0.030	0.068	0.024
4-Pregnene-3,20-dione (Progesterone) (XVIII)	0.15	0.31	12.39
11β-Hydroxy-4-pregnene-3,20-dione (11β-Hydroxyprogesterone) (XIX)	2.09	4.53	2.85
11α-Hydroxy-4-pregnene-3,20-dione (11α-Hydroxyprogesterone) (XX)	0.0019	0.017	0.39
4-Pregnene-3,11,20-trione (11-Ketoprogesterone) (XXI)	0.17	0.35	9.19
17α-Hydroxy-4-pregnene-3,20-dione (17α-Hydroxyprogesterone) (XXII)	1.37	5.96	18.00
20α-Hydroxy-4-pregnen-3-one (XXIII)	0.013	0.033	6.48
20β-Hydroxy-4-pregnen-3 one (XXIV)	0.015	0.012	7.31
5α-Pregnane-3,20-dione (5α-Pregnanedione) (XXV)	0.14	0.086	3.75
3α-Hydroxy-5α-androstan-17-one (Androsterone) (XXVI)	0.000020	0.000058	0.095
5α-Androstane-3,17-dione (XXVII)	0.012	0.021	0.029
5β-Androstane-3,17-dione (XXVIII)	0.0061	0.0064	0.16
5 Androstene 3β,17β diol (XXIX)	0.000020	0.000037	0.00028
17β-Hydroxy-4-androsten-3-one (Testosterone) (XXX)	0.010	0.010	1.21
5-Cholesten-3β-ol (Cholesterol) (XXXI)	0.000020	0.000042	0.0012
1,3,5(10)-Estratriene-3,17β-diol (Estradiol-17β) (XXXII)	0.000020	0.000049	0.013
1,3,5(10)-Estratriene-3,16α,17β-triol (Estriol) (XXXIII)	0.000020	0.000059	0.0092
3-Hydroxy-1,3,5(10)-estratrien-17-one (Estrone) (XXXIV)	0.00014	0.000099	0.013
5α-Androstane (XXXV)	0.000020	0.000020	0.0021
11β,17α,20α,21-Tetrahydroxy-4-pregnen-3-one (XXXVI)	0.29	0.071	17.37
11β,17α-Dihydroxy-4-pregnene-3,20-dione (XXXVII)	16.25	18.04	232.43

a) The value represents the average of three antisera.

cross-reactivities of these antisera with cortisone (VII), 11-deoxy-17α-hydroxycorticosterone (IX), corticosterone (X), deoxycorticosterone (XIII), and 11-dehydrocorticosterone (XIV), which differ structurally from cortisol only in ring C or D, decreased in the order of *anti*-F-6α-HS, *anti*-F-6β-HS, and *anti*-F-21-HS. *anti*-F-21-HS was not able to discriminate among the steroids with structural similarity to the substituents in ring D of cortisol [especially progesterone (XXII)]. As for the specificity for a given steroid among three antisera, the percentage cross-reactivities were extremely variable as follows: with *anti*-F-6α-HS, 5α-dihydrocortisol (III) (49.36±21.93%), 5β-dihydrocortisol (IV) (22.84±10.38%), 11-deoxy-17α-hydroxycorticosterone (IX)(13.48±6.77%), 11-dehydrocorticosterone (XIV) (4.04±3.97%), and 11β-hydroxyprogesterone (XIX) (2.09±2.24%); with *anti*-F-6β-HS, 5α-dihydrocortisol (III) (32.12±6.52%), cortisone (VII) (23.78±20.81%), 11-deoxy-17α-hydroxycorticosterone (IX) (20.86±10.28%), and 17α-hydroxyprogesterone (XXII) (5.96±4.45%). The steroids, 5α-dihydrocortisol (III), 5β-dihydrocortisol (IV), cortisone (VII), and 11-deoxy-17α-hydroxycorticosterone (IX), which differ structurally from cortisol only at position 5 or 11, show percentage cross-reactivities higher than 10% with *anti*-F-6α-HS and *anti*-F-6β-HS.

Discussion

Considering the structural differences from cortisol in ring A and B, the greater dissimilarity of 5β-dihydrocortisol (IV) to cortisol compared with 5α-dihydrocortisol (III), may be expected to give rise to a low cross-reactivity of (IV) with both *anti*-F-6α-HS and *anti*-F-6β-HS. However, both (III) and (IV) show considerably high cross-reactivities with *anti*-F-6α-HS and *anti*-F-6β-HS. A possible reason for the low specificity to (III) and (IV) is that the site of attachment of BSA at the position 6 is situated in proximity of ring A and B. However, even in the case of F-21-HS having BSA at position 21 of cortisol molecule which is not near ring A and B, the relative higher cross-reactivity to (III) and (IV) was observed. The 4,5 double bond does not stand out for the A–B ring skeleton compared with the other functional groups. Therefore, this stereochemical situation may be one of the factors making the difficulty of preparation of the specific antibody against the double bond.

The fact that *anti*-F-6β-HS has higher specificity than *anti*-F-6α-HS for the steroids structurally different to cortisol in ring A led us to make the conformational consideration of cortisol-BSA conjugate investigated here. Inspection of Dreiding models suggests that a more favorable conformation for 6α-hydroxycortisol 6-hemisuccinate in F-6α-HS could exist in which the carbonyl group at position 3 of cortisol stands out toward BSA molecule, whereas 6β-hydroxycortisol 6-hemisuccinate in F-6β-HS could adopt a preferred conformation in which the carbonyl group stands out opposite to BSA. Consequently, the antibody population against ring A of cortisol could be prepared more easily for F-6β-HS than for F-6α-HS. The similar conformational consideration is also applicable for the functional groups in ring C and D of cortisol. In this case, the antibody populations against those could be prepared more easily for F-6α-HS than for F-6β-HS. The above conformational consideration is helpful for a better understanding of the relative specificity of the antibody produced against F-6α-HS and F-6β-HS. The cortisol 21-hemisuccinate-BSA molecule shuld be less rigid than the corresponding BSA conjugate at the position 6 of cortisol owing to the flexibility of the 17-side chain. Namely, cortisol 21-hemisuccinate may exist on BSA in flexible form. One of the factors influencing the great variability of specificity of an antibody raised against F-21-HS,[10] as indicated by our experimental results, seems to be the flexibility of the structure of cortisol 21-hemisuccinate-BSA conjugate molecule.

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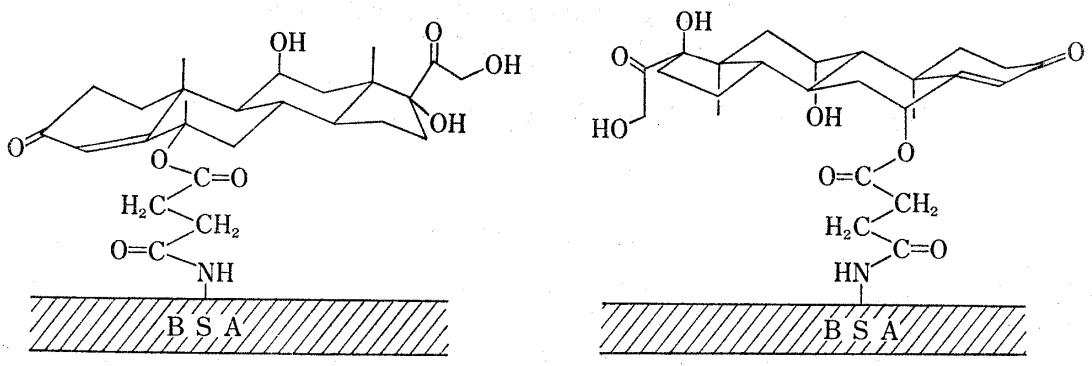
anti-F-21-HS has obviously lower specificity than *anti*-F-6α-HS or *anti*-F-6β-HS for the steroids differing structurally from cortisol only in the 17-side chain.



6α-hydroxycortisol 6-hemisuccinate-BSA conjugate 6β-hydroxycortisol 6-hemisuccinate-BSA conjugate

Fig. 1. Putative Conformation of Cortisol-BSA Conjugate

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