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Radioimmunoassay of 11-Deoxycortisol. Specificity of Antisera raised against 11-Deoxycortisol-[C-4]-Bovine Serum Albumin Conjugates[1)]

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In order to obtain specific antisera for use in immunoassays of 11-deoxycortisol, new hapten-carrier conjugates were prepared from 4-hemisuccinoyloxy-11-deoxycortisol, 4-(carboxymethylthio)-11-deoxycortisol, 4-(2-carboxyethylthio)-11-deoxycortisol and 4-(2-hemisuccinoyloxyethylthio)-11-deoxycortisol by coupling with bovine serum albumin employing the *N*-succinimidyl ester method. The specificity of anti-11-deoxycortisol antisera elicited in rabbits by immunization with these antigens was tested by cross-reaction studies with closely related steroids and by measuring the amount of 11-deoxycortisol in plasma specimens by means of radioimmunoassay. The results showed that the antisera obtained were reasonably specific and useful for the determination of plasma 11-deoxycortisol levels in metyrapone tests. The assay can be done on methylene chloride extracts of plasma.

Keywords——radioimmunoassay; 11-deoxycortisol; 11-deoxycortisol-[C-4]-BSA conjugate; *N*-succinimidyl ester method; anti-11-deoxycortisol antisera; specificity; cross-reactivity; plasma 11-deoxycortisol level; metyrapone test

Immunoassays of 11-deoxycortisol in human plasma are useful in the metyrapone test,[2)] assessment of pituitary-adrenal reserve. Anti-11-deoxycortisol antisera have been prepared by immunizing animals with haptenic derivatives linked through C-3,[3)] C-6,[3)] C-7[4)] and C-21[5)] to a carrier protein. Radioimmunoassays of the steroid hormone have been developed using some of these antisera. The specificity of antibodies is significantly influenced by the position on the steroid molecule used for conjugation to the carrier and also by the stereochemistry of the steroid hapten. The position C-4 in the 11-deoxycortisol molecule appears to be an attractive site for attachment of the carrier because the trigonal carbon provides the characteristic stereochemistry, and hydroxylation at this position has not yet been reported in the metabolism of 11-deoxycortisol. For the purpose of developing enzyme immunoassay systems for 11-deoxycortisol, the availability of various haptens possessing different bridges at this position is convenient, since the combination of antibody and enzyme-labeled antigen is an important factor determining the sensitivity of enzyme immunoassay.[6)] In a previous paper of this series, we reported the synthesis of 11-deoxycortisol derivatives carboxylated at C-4.[7)] The present paper deals with the specificity of antisera raised in rabbits against these haptens in the radioimmunoassay procedure.

Materials and Methods

Materials——[1,2-^{3}H]-11-Deoxycortisol (50 Ci/mmol) was supplied by Amersham (England) and used without purification. Bovine serum albumin (BSA) (crystallized) and complete Freund's adjuvant were purchased from Sigma Chemical Co. (U.S.A.) and Iatron Laboratories (Tokyo), respectively. 4-Hemisuccinoyloxy-11-deoxycortisol (HS), 4-(carboxymethylthio)-11-deoxycortisol (CMT), and 4-(2-carboxyethylthio)-11-deoxycortisol (CET) were prepared by the methods previously established in these laboratories.[7)] 4-(2-Hemisuccinoyloxyethylthio)-11-deoxycortisol (HST) was synthesized in the manner described below. All solvents and chemicals were of analytical-reagent grade.

Synthesis of the Hapten HST——A solution of 4,5-epoxy-11-deoxycortisol 21-tetrahydropyranyl ether[7)]

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(1 g) in ethanol (15 ml) was added to 2-mercaptoethanol (200 mg) in 5% KOH (0.4 ml), and the resulting solution was stirred at room temperature under an N_2 gas stream for 18 h. The reaction mixture was diluted with ethyl acetate, washed with water, dried over anhydrous Na_2SO_4, and evaporated down. The residue was treated with succinic anhydride (600 mg) in pyridine (2 ml) overnight at 35°C. After addition of water, the resulting mixture was extracted with ethyl acetate. The organic layer was washed with water, dried over anhydrous Na_2SO_4, and evaporated down. The crude product obtained was chromatographed on silica gel (16 g). Elution with hexane–ethyl acetate (1: 3) gave 4-(2-hemisuccinoyloxyethylthio)-11-deoxycortisol 21-tetrahydropyranyl ether (760 mg) as colorless semi-crystals. These were dissolved in 50% acetic acid (10 ml) and allowed to stand at room temperature for 5 h. The reaction mixture was diluted with ethyl acetate, washed with water, and dried over anhydrous Na_2SO_4. Upon removal of the solvent, HST (630 mg) was obtained as semi-crystals. ^{1}H-NMR ($CDCl_3$) δ: 0.71 (3H, s, 18-CH_3), 1.24 (3H, s, 19-CH_3), 2.65 (4H, s, $COCH_2CH_2CO$), 2.90 (2H, m, 4-$SC\underline{H}_2CH_2O$), 3.66 (1H, m, 6α-H), 4.11 (2H, m, 4-$SCH_2C\underline{H}_2O$), 4.52 and 4.66 (each 1H, d, J=19 Hz).

Preparation of Antigen——1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl (0.27 mmol) and *N*-hydroxysuccinimide (0.27 mmol) were added to a solution of steroid carboxylic acid (HS, CMT, CET, HST) (0.22 mmol) in 95% dioxane (0.7 ml), and the resulting solution was stirred at room temperature for 2 h. The reaction mixture was diluted with water and extracted with ethyl acetate. The organic layer was washed with water and dried over anhydrous Na_2SO_4. The solution was passed quickly through an aluminum oxide (4 g) layer on a sintered-glass funnel, and the filtrate was evaporated down to give the *N*-succinimidyl ester of 11-deoxycortisol. BSA (90 mg) in 0.05 M phosphate buffer (pH 7.3) (1 ml)–pyridine (1 ml) was then added and the whole was stirred overnight at 4°C. The resulting solution was dialyzed against cold running water overnight. After addition of acetone and a small amount of NaCl, the suspension was centrifuged at 3000 rev./min for 20 min. This procedure was repeated until free steroid was removed. The precipitate was dissolved in 20% pyridine and dialyzed in the manner described above. Lyophilization of the resulting solution afforded the steroid-BSA conjugate (*ca.* 90 mg) as a fluffy powder.

Determination of the Molar Ratio of Hapten to BSA in the Conjugate——Ultraviolet spectra were measured in 0.05 M phosphate buffer (pH 7.4) with a Hitachi model 124 spectrophotometer. Spectrometric analysis was carried out by comparing the absorbance at 250 nm of the conjugate with those of BSA and hapten as controls in the same buffer and by using the following constants: molecular weight of BSA, 65000; ε values for HS 12300, for the thioethers 11000. The number of steroid molecules linked to a BSA molecule was determined to be 19, 21, 20, and 25 respectively, for HS, CMT, CET and HST.

Immunization of Rabbits——The antigen (1 mg) was dissolved in sterile isotonic saline (0.5 ml) and emulsified with complete Freund's adjuvant (0.5 ml). The emulsion was injected into domestic male albino rabbit subcutaneously at multiple sites along the back. This procedure was repeated once every fortnight. Blood was collected 4 months after the initial injection and centrifuged at 3000 rev./min for 20 min. After addition of NaN_3 (0.1%) the antiserum was stored at 4°C. The abbreviations HS, CMT, CET, and HST are also used for the antisera, which were prepared by the use of the corresponding carboxylated derivatives. Two preparations (1 and 2) were elicited in two rabbits by immunization with the same hapten-BSA conjugate.

Assay Procedure——All dilutions of the standard, tracer and antiserum were performed in 0.05 M phosphate buffer (pH 7.3) containing 0.1% gelatin, 0.9% NaCl, and 0.01% NaN_3. [^{3}H]-11-Deoxycortisol (*ca.* 18000 dpm, 0.5 ml) and diluted antiserum (0.1 ml) were added to a series of standard solutions (0, 20, 40, 70, 100, 200, 300, and 600 pg of 11-deoxycortisol) or plasma samples in buffer (0.1 ml), and the mixtures were incubated overnight at 4°C. After addition of dextran (0.06%)–charcoal (1%) (0.5 ml), the suspension was vortex-mixed, allowed to stand at 0°C for 20 min, and then centrifuged at 4°C (2000 rev./min for 10 min). The supernatant was transferred by decantation into a vial containing a scintillation cocktail (10 ml), and the radioactivity was measured with a Beckman LS-7000 liquid scintillation spectrometer. Radioimmunoassay of cortisol was carried out in the manner described previously.[8)]

Sample Preparation——Plasma specimens were provided by Dr. Sasaki, Tohoku University School of Medicine. In metyrapone tests, patients received 0.5 g of metyrapone every 4 h for a total of six doses. Blood was withdrawn 24 h and 48 h after the initial administration and plasma was immediately obtained. A solution of [^{3}H]-11-deoxycortisol (*ca.* 1800 dpm) in ethanol (0.1 ml) was transferred to a test tube, and the solvent was removed with the aid of an N_2 gas stream. Plasma (0.1 ml) was added to this residue, vortex-mixed, and allowed to stand overnight at 4°C. This solution was diluted with water (1 ml) and extracted once with methylene chloride (3 ml). The organic solvent was evaporated off under an N_2 gas stream, and the residue was redissolved in the assay buffer. An aliquot of this solution was used for radioactivity counting to estimate the recovery rate. Plasma samples extracted with carbon tetrachloride and methylene chloride for determination of 11-deoxycortisol and cortisol, respectively, were also prepared according to the method of Spark.[9)] The denatured sample was prepared by heating the plasma diluted with the buffer at 60°C for 30 min.

Cross-Reaction Study——The specificity of antisera raised against the 11-deoxycortisol-BSA conjugates was tested by cross-reaction studies with six kinds of steroids related to 11-deoxycortisol. The relative amounts required to reduce the initial binding of [^{3}H]-11-deoxycortisol by half, where the mass of unlabeled 11-deoxycortisol was arbitrarily taken as 100%, were calculated from the standard curves.

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Results and Discussion

The steroid derivatives used as haptens in this study were the ester HS and the thioethers CMT, CET and HST. The *N*-succinimidyl esters prepared from the carboxylated derivatives by condensation with *N*-hydroxysuccinimide in the presence of a water-soluble carbodiimide were covalently linked to BSA.[10] Measurement of the ultraviolet absorption due to the α,β-unsaturated ketone structure revealed that satisfactory numbers of steroid molecules were incorporated into each BSA molecule in all the conjugates. Two rabbits were used for immunization with each conjugate.

In radioimmunoassays, [^{3}H]-11-deoxycortisol was used as a labeled antigen, and the separation of bound and free fractions was carried out by a dextran-coated charcoal method. The dilution of antiserum which was capable of binding 50% of the label was defined as a titer. The sera obtained from the rabbits immunized with the antigens for four months showed significantly increased response to 11-deoxycortisol. The dose-response curves were obtained by incubating 20—600 pg of unlabeled 11-deoxycortisol and a fixed amount of the labeled steroid with appropriately diluted antisera. When logit transformation was used to construct the

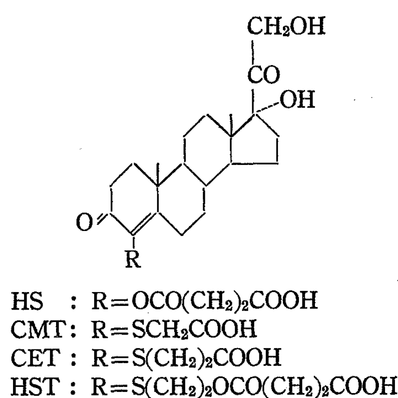

Chart 1

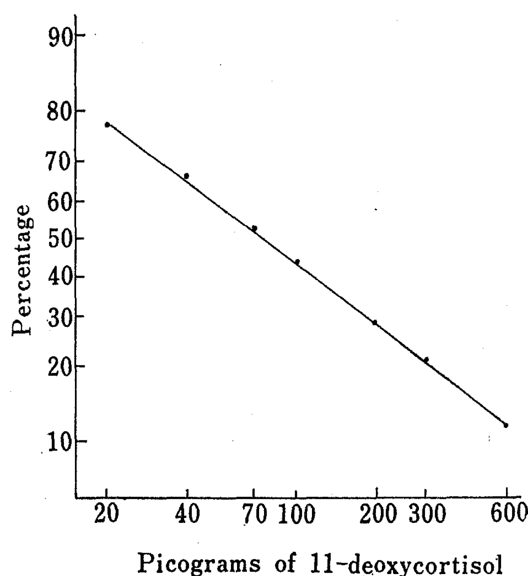

Fig. 1. Dose–Response Curve for Radioimmunoassay of 11-Deoxycortisol using the Antiserum CET-2

Table I. Affinity Constant and Titer of Antisera raised against 11-Deoxycortisol-[C-4]-BSA Conjugates

Antiserum	Final dilution	K_a ($M^{-1} \times 10^{-10}$)
HS-1	1:210000	3.3
HS-2	1:140000	3.5
CMT-1	1:35000	2.5
CMT-2	1:140000	3.5
CET-1	1:49000	3.2
CET-2	1:105000	2.1
HST-1	1:49000	1.8
HST-2	1:70000	2.9

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standard curves, plots of logit per cent bound radioactivity *vs.* logarithm of the amount of unlabeled 11-deoxycortisol showed a linear relationship. A typical dose-response curve is shown in Fig. 1. Affinity constants obtained by the Scatchard analysis[11] and the final dilutions of the antisera are listed in Table I. It is obvious that these antisera possess high binding affinity for 11-deoxycortisol.

The specificity of antisera was assessed by ascertaining the ability of various related steroids to compete with [^{3}H]-11-deoxycortisol for binding to antibody. The per cent cross-reaction of antisera was determined according to the method of Abraham.[12] The cross-reactions of anti-11-deoxycortisol antisera with six kinds of related steroids are listed in Table II. All the antisera were reasonably specific. The results obtained with cortisol and cortisone show that these antisera are capable of discriminating the functional groups at the 11-position. In the case of steroids having a monodeoxygenated side chain, such as 11-deoxycorticosterone and 17α-hydroxyprogesterone, rather high cross-reactions were observed. We found less than 1.4% cross-reaction with progesterone, and negligible values with corticosterone. The cross-reactivities of the antisera elicited by antigens having a linkage through C-3,[3] C-6,[3] C-7,[4] or C-21[5] are listed in Table III. It is evident from the data in Tables II and III that the specificity of the antisera prepared in this study is higher than that of 21-HS and is comparable to those of 3-CMO, 6-HS and 7-CM.

TABLE II. Per Cent Cross–Reaction of Antisera raised against 11-Deoxycortisol-[C-4]-BSA Conjugates with Selected Steroids

Steroid	% cross-reactivity (50%)							
	HS-1	HS-2	CMT-1	CMT-2	CET-1	CET-2	HST-1	HST-2
11-Deoxycortisol	100	100	100	100	100	100	100	100
Cortisol	1.5	0.35	0.59	0.58	0.75	0.32	0.45	0.34
Cortisone	0.85	0.77	0.26	0.86	0.005	1.3	0.36	2.1
Corticosterone	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
11-Deoxycorticosterone	9.4	4.8	2.2	6.4	8.7	11	11	2.6
17α-Hydroxyprogesterone	12	3.9	5.3	4.1	0.90	15	7.7	5.1
Progesterone	1.4	0.31	0.13	0.33	0.24	0.75	1.2	0.62

TABLE III. Per Cent Cross–Reaction of Antisera raised against Various 11-Deoxycortisol-BSA Conjugates with Selected Steroids

Steroid	% cross-reactivity (50%)					
	3-CMO[3]	6α-HS[3]	6β-HS[3]	7α-CM[4]	7β-CM[4]	21-HS[5]
11-Deoxycortisol	100	100	100	100	100	100
Cortisol	1.0	1.0	2.7	0.66	1.5	1.7
Cortisone	4.8	2.1	2.4	2.80	10.4	—
Corticosterone	0.04	0.05	0.4	<0.05	<0.05	<0.01
11-Deoxycorticosterone	0.6	4.2	2.9	1.32	1.20	7.1
17α-Hydroxyprogesterone	75.0	0.2	4.0	0.7	4.6	100
Progesterone	11.6	0.07	3.1	0.10	0.40	5.7

Abbreviations for antisera: 3-CMO=anti-11-deoxycortisol 3-(*O*-carboxymethyl)oxime-BSA, 6α-HS=anti-6α-hemisuccinoyloxy-11-deoxycortisol-BSA, 6β-HS=anti-6β-hemisuccinoyloxy-11-deoxycortisol-BSA, 7α-CM=anti-7α-carboxymethyl-11-deoxycortisol-BSA, 7β-CM=anti-7β-carboxymethyl-11-deoxycortisol-BSA, and 21-HS=anti-21-hemisuccinoyl-11-deoxycortisol-BSA.

Application of the antisera to the determination of 11-deoxycortisol in plasma presents some problems. In most of the radioimmunoassays reported,[5,13] purification step such as extraction with organic solvents and chromatography were employed, even if excellent speci-

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ficity could be obtained in the cross-reaction study as listed in Table III. Commonly, the most abundant steroid in normal human plasma is cortisol, whose level may be several hundred times higher than that of 11-deoxycortisol. From the extent of cross-reaction of cortisol, overestimation may be inevitable in assays without a purification step. In the metyrapone test, a subject with intact pituitary-adrenal reserve shows a markedly enhanced 11-deoxycortisol level with a decrease in the cortisol level, and hence, the interference of the assay due to the latter would not be significant. However, because of the limitation of test steroids available, specificity data obtained from the cross-reaction study are not always sufficient for the assay of biological samples. It is well recognized that in practice, when various antisera and purification procedures are compared using biological samples, the assay result yielding the lowest estimation can usually be assumed to be proximate to the true value.[14] This is applicable to the assessment of the specificity of antisera. Therefore, the specificity was ascertained by measuring 11-deoxycortisol in plasma specimens obtained after metyrapone administration. Three kinds of pretreatments were employed: one of these is denaturation of corticosteroid-binding globulin by heating[13b] and the remaining two involve extraction with organic solvents, methylene chloride and carbon tetrachloride. In the extraction methods, the observed values were corrected on the basis of the recovery rate of [^{3}H]-11-deoxycortisol added to each plasma sample. Recovery rates of the tracer were found to be 90—100% with methylene chloride and *ca.* 80% with carbon tetrachloride. In the case of cortisol, the recovery rates were over 80% with methylene chloride and less than 5% with carbon tetrachloride. It was found that all the direct assays (without extraction) gave values several times higher than those of solvent extracts. On the other hand, there was no significant difference between the values obtained by the extraction methods. Thus, overestimation of 11-deoxycortisol in the direct assays is ascribable to the interference of steroid metabolites more polar than cortisol. The results of the assays with methylene chloride are listed in Table IV. It seems that the antisera CMT-2 and CET-2 are more specific than other antisera.

TABLE IV. Plasma 11-Deoxycortisol Levels obtained by Radioimmunoassay using Various Antisera (μg/l)

Plasma sample	Antiserum							
	HS-1	HS-2	CMT-1	CMT-2	CET-1	CET-2	HST-1	HST-2
I	261	222	263	200	250	207	227	250
II	140	144	161	144	159	136	159	140

TABLE V. Plasma 11-Deoxycortisol and Cortisol Levels before and after Administration of Metyrapone (μg/l)[a]

Subject	11-Deoxycortisol		Cortisol	
	Before	After	Before	After
A	2.1	165	100	39
B	2.4	171	67	38
C	2.9	108	96	58

a) The antiserum CET-2 was used.

The plasma 11-deoxycortisol levels in seven samples collected after administration of metyrapone, which were determined by the use of CET-2, ranged from 90 to 207 μg/l with a mean value of 141±39 μg/l. With the antiserum CMT-2, similar results were obtained. In the cases of normal subjects and patients before administration of metyrapone, the levels were less than 4 μg/l with a mean value of 2.5±0.8 μg/l (n=9). The direct assays exhibited

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somewhat higher values, but not over 6 μg/l. Although these values may be higher than the true amounts, they are acceptable for the purpose of metyrapone tests. This is well shown in Table V, which gives the results of measurement of 11-deoxycortisol and cortisol in the metyrapone test conducted on three patients.

Relatively specific antisera to 11-deoxycortisol could be prepared by using the [C-4]-haptens. Good agreement of the values obtained with CMT-2 and CET-2 with those reported by several workers[5,13] suggests that these antisera may be practically useful for the radioimmunoassay of plasma 11-deoxycortisol. A sensitive enzyme immunoassay has been successfully developed by the appropriate combination of antibody and enzyme-labeled steroid using these antisera and haptenic derivatives, and the details will be reported elsewhere in the future.

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