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Specificity of Antisera raised against Cortisol-4-Bovine Serum Albumin Conjugates in Radioimmunoassay[1)]

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In order to obtain specific antisera for use in radioimmunoassay and enzyme immunoassay of cortisol, new hapten-carrier conjugates were prepared from 4-hemisuccinoyloxycortisol, 4-(carboxymethylthio)cortisol, 4-(2-carboxyethylthio)cortisol and 4-(2-hemisuccinoyloxyethylthio)cortisol by coupling with bovine serum albumin (BSA) employing the N-hydroxysuccinimide ester method or mixed anhydride method. The specificity of anti-cortisol 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 cortisol in urine specimens by means of radioimmunoassay. Comparison of the specificity of these antisera with that of other antisera prepared with BSA conjugates of cortisol 3-(O-carboxymethyl)oxime and 21-hemisuccinoylcortisol was also carried out. The results showed that the antisera obtained were sufficiently specific and thus that the C-4 position was suitable for the attachment of a carrier protein for use in the production of antibodies.

Keywords——radioimmunoassay; cortisol; cortisol-4-BSA conjugate; N-hydroxysuccinimide ester method; anti-cortisol antisera; specificity; cross-reactivity

Anti-cortisol antisera for use in radioimmunoassay have been prepared by immunizing animals with haptenic derivatives linked through C-3,[3)] C-6[3a,4)] and C-21[3a,5)] to a carrier protein. Enzyme immunoassays have also been developed using antiserum raised against 21-hemisuccinoylcortisol-bovine serum albumin (BSA) conjugate.[6)] As for antisera elicited by immunization with cortisol 3-(O-carboxymethyl)oxime-BSA conjugate, satisfactory results have been obtained for use in radioimmunoassay, but not in enzyme immunoassay.[6b,7)] 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 cortisol 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 cortisol. It is also advantageous that several types of haptenic derivatives linked at this position are readily available, since the combination of antibody and enzyme-labeled antigen is an important factor determin-

1) Part CLXII of "Studies on Steroids" by T. Nambara; Part CLXI: H. Hosoda, H. Yoshida, Y. Sakai, S. Miyairi, and T. Nambara, *Chem. Pharm. Bull.*, **28**, 3035 (1980).
2) Location: *Aobayama, Sendai 980, Japan*; *a*) To whom inquiries should be addressed.
3) *a*) T. Nishina, A. Tsuji, and D.K. Fukushima, *Steroids*, **24**, 861 (1974); *b*) D. Fahmy, G.F. Read, and S.G. Hillier, *ibid.*, **26**, 267 (1975); *c*) R.J. Dash, B.G. England, A.R. Midgley, Jr., and G.D. Niswender, *ibid.*, **26**, 647 (1975).
4) H. Sone and M. Kojima, *Chem. Pharm. Bull.*, **26**, 2819 (1978).
5) H.J. Ruder, R.L. Guy, and M.B. Lipsett, *J. Clin. Endocrinol. Metab.*, **35**, 219 (1972); G.E. Abraham, J.E. Buster, and R.C. Teller, *Anal. Lett.*, **5**, 757 (1972).
6) *a*) S. Comoglio and F. Celada, *J. Immunol. Methods*, **10**, 161 (1976); T. Ogihara, K. Miyai, K. Nishi, K. Ishibashi, and Y. Kumahara, *J. Clin. Endocrinol. Metab.*, **44**, 91 (1977); Y. Kobayashi, T. Ogihara, K. Amitani, F. Watanabe, T. Kiguchi, I. Ninomiya, and Y. Kumahara, *Steroids*, **32**, 137 (1978); G. Holder, "Enzyme labelled Immunoassay of Hormones and Drugs," ed. by S.B. Pal, Walter de Gruyter, Berlin, 1978, p. 221; *b*) H. Arakawa, M. Maeda, and A. Tsuji, *Anal. Biochem.*, **97**, 248 (1979).
7) B.G. Joyce, A. Türkes, A. Ozoran, G.F. Read, and D.R. Fahmy, "Enzyme labelled Immunoassay of Hormones and Drugs," ed. by S.B. Pal, Walter de Gruyter, Berlin, 1978, p. 247.

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ing the sensitivity of enzyme immunoassay and hence a "bridge" heterologous system is often required.[8] In a previous paper of this series, we reported the synthesis of the cortisol haptens having different bridges at C-4.[9] The present paper deals with the specificity of antisera raised in rabbits against these haptens in the radioimmunoassay procedure.

Materials and Methods

Materials——[1,2,6,7-^{3}H]-Cortisol (90 Ci/mmol) was supplied by CEA (Gif-Sur-Yvette, France) and used without purification. BSA (crystallized) and complete Freund's adjuvant were purchased from Sigma Chemical Co. (St. Louis, Mo.) and Iatron Laboratories (Tokyo), respectively. 4-Hemisuccinoyloxycortisol, 4-(carboxymethylthio)cortisol, 4-(2-carboxyethylthio)cortisol, and 4-(2-hemisuccinoyloxyethylthio)cortisol were prepared by the methods previously established in these laboratories.[9] All solvents and chemicals were of analytical-reagent grade.

Preparation of Antigens——i) Tri-*n*-butylamine (8 μl) and isobutyl chlorocarbonate (4 μl) were added to a solution of **1** (15 mg) in dry dioxane (0.3 ml) at 11°, and the resulting solution was stirred for 30 min. BSA (50 mg) in water (1.2 ml)-dioxane (0.7 ml) containing 1 N NaOH (40 μl) was then added under ice-cooling and the mixture was stirred for 3 hr. 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 completely. 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.* 50 mg) as a fluffy powder.

ii) 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 (**2**—**4**) (0.18 mmol) in 95% dioxane (0.35 ml), and the resulting solution was stirred at room temperature for 2 hr. 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 filtered rapidly through an Al_2O_3 (4 g) layer on a sintered-glass funnel, and the filtrate was evaporated down to give the N-hydroxysuccinimide ester of cortisol. BSA (90 mg) in 0.05 M phosphate buffer (pH 7.4) (1 ml)-pyridine (1 ml) was then added and the whole was stirred overnight at 4°. Dialysis, acetone treatment and lyophilization were carried out in the manner described in i). Similarly, the N-hydroxysuccinimide esters of **5** and **6**[10] were coupled to BSA at a molar ratio (steroid to protein) of 60.

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 **1** 12300, for **2**, **3**, **4** 11000, for **5** 24000, and for **6** 16000. The number of steroid molecules linked to a BSA molecule was determined to be 21, 21, 25, 33, 19 and 27, respectively.

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 a week for 3 weeks and then once every fortnight. Two rabbits were used for each conjugate. Blood was collected 6 months after the initial injection and centrifuged at 3000 rev./min for 10 min. After addition of NaN_3 (0.1%), the antiserum was stored at 4°.

Assay Procedure——All dilutions of the standard, tracer and antiserum were performed in 0.05 M phosphate buffer (pH 7.4) containing 0.1% gelatin, 0.9% NaCl, and 0.01% NaN_3. [^{3}H]-Cortisol (*ca.* 18000 dpm, 0.5 ml) and diluted antiserum (0.1 ml) were added to a series of standard solutions (0, 20, 40, 100, 200, 400, and 1000 pg of cortisol) or urine samples in buffer (0.1 ml) and the mixtures were incubated overnight at 4°. After addition of dextran (0.06%)-charcoal (0.5%) (0.5 ml), the suspension was vortex-mixed, allowed to stand at 0° for 20 min, and then centrifuged at 4° (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.

Extraction of Urine Cortisol——A solution of [^{3}H]-cortisol (*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. Urine (0.1 ml) was added to this residue, and the solution was vortex-mixed, then allowed to stand at 4° for 1 hr. After addition of water (0.5 ml), the urine sample was extracted with an organic solvent (diethyl ether, ethyl acetate or

8) B.K. Van Weemen and A.H.W.M. Schuurs, *Immunochemistry*, **12**, 667 (1975); H. Hosoda, H. Yoshida, Y. Sakai, S. Miyairi, and T. Nambara, *Chem. Pharm. Bull.*, **28**, 3035 (1980).
9) H. Hosoda, S. Miyairi, and T. Nambara, *Chem. Pharm. Bull.*, **28**, 1294 (1980).
10) H. Hosoda, Y. Sakai, H. Yoshida, S. Miyairi, K. Ishii, and T. Nambara, *Chem. Pharm. Bull.*, **27**, 742 (1979).

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methylene chloride) (1 ml). The 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.

Cross-Reaction Study——The specificity of antisera raised against the cortisol-BSA conjugates was tested by cross-reaction studies with 15 kinds of steroids related to cortisol. The relative amounts required to reduce the initial binding of [^{3}H]-cortisol by half, where the mass of unlabeled cortisol was arbitrarily taken as 100%, were calculated from standard curves.

Results and Discussion

The steroid haptens used in this study were 4-hemisuccinoyloxycortisol (**1**), 4-(carboxymethylthio)cortisol (**2**), 4-(2-carboxyethylthio)cortisol (**3**), and 4-(2-hemisuccinoyloxyethylthio)cortisol (**4**). For comparative studies on the specificity of antisera obtained with these four haptens, cortisol 3-(O-carboxymethyl)oxime (**5**) and 21-hemisuccinoylcortisol (**6**) were also used for the preparation of antisera. The steroid carboxylic acids were covalently linked to BSA to provide cortisol-BSA conjugates by the N-hydroxysuccinimide ester method previously developed in these laboratories[10] or by the mixed anhydride method.[11] Measurement of the ultraviolet absorption due to the α,β-unsaturated ketone structure revealed that satisfactory numbers of steroid molecules were covalently bound to each BSA molecule in all the conjugates. Two rabbits were used for immunization with each conjugate. The dilution of antiserum which was capable of binding 50% of the [^{3}H]-cortisol was defined as a titer. The sera obtained from the rabbits immunized with these antigens for six months showed

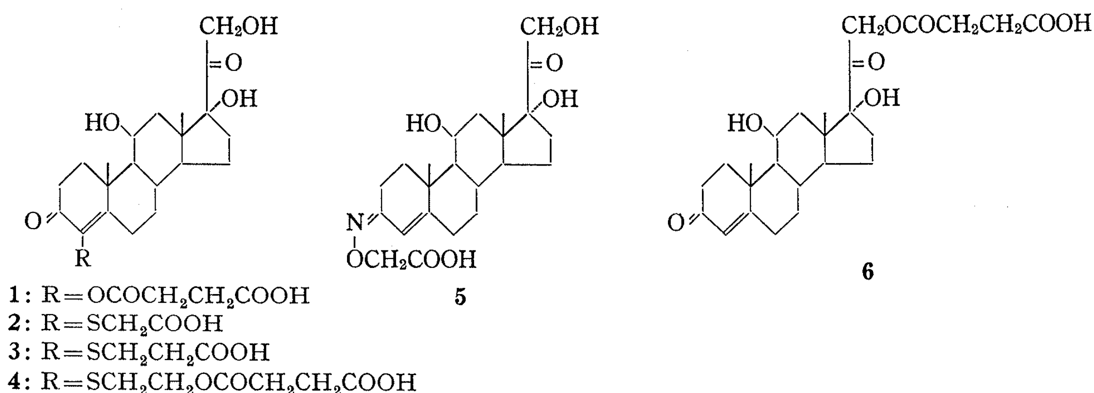

Chart 1

TABLE I. Affinity Constant and Titer of Antisera raised against Cortisol-4-BSA Conjugates

Antiserum[a)]	Final dilution	K_a ($M^{-1} \times 10^{-9}$)
4-HS-1	1 : 3500	3.6
4-HS-2	1 : 3500	2.3
4-CMT-1	1 : 14000	2.4
4-CMT-2	1 : 70000	5.7
4-CET-1	1 : 7000	3.4
4-CET-2	1 : 14000	2.1
4-HST-1	1 : 7000	2.9
4-HST-2	1 : 14000	8.8

a) Abbreviations for antisera: 4-HS=anti-4-hemisuccinoyloxycortisol-BSA, 4-CMT=anti-4-(carboxymethylthio)-cortisol-BSA, 4-CET=anti-4-(2-carboxyethylthio)cortisol-BSA, and 4-HST=anti-4-(2-hemisuccinoyloxyethylthio)cortisol-BSA.

11) B.F. Erlanger, S.M. Beiser, and S. Lieberman, *J. Biol. Chem.*, **228**, 713 (1957).

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significantly increased response to cortisol. The dose-response curves were obtained by incubating 20—1000 pg of unlabeled cortisol and a fixed amount of the labeled steroid with appropriately diluted antisera. When logit transformation was used to construct the standard curves, plots of logit per cent bound radioactivity *vs.* logarithm of the amount of unlabeled cortisol showed a linear relationship. The Scatchard plots[12] showed that these antisera exhibited high binding affinity for cortisol. The results obtained with anti-cortisol[C-4]-BSA antisera are listed in Table I.

The specificity of antisera was assessed by ascertaining the ability of various related steriods to compete with [^{3}H]-cortisol for binding to antibody. The per cent cross-reaction of antisera was determined according to the method of Abraham.[13] The cross-reactions of the anti-cortisol antisera with fifteen kinds of related steroids are listed in Table II. The antisera except for 4-CMT-2 and 4-HST-2 were satisfactorily specific, although 5α-dihydrocortisol showed considerable cross-reaction. With 4-CET-2, for example, 11-deoxycortisol, corticosterone, and 21-deoxycortisol exhibited less than 23% cross-reaction. We found 3.6% cross-reaction with cortisone, 0.6% with 6β-hydroxycortisol, 1.4% with 17α-hydroxyprogesterone, and a very low or negligible value with progesterone, 11-deoxycorticosterone, and tetrahydrocortisol. It is of interest that the cross-reactivity of 4-HS-2 with 5α-dihydrocortisol was very low (8.1%) and most of the antisera were capable of discriminating tetrahydrocortisol clearly from cortisol despite the use of a linkage through the site involved in the Δ^4-3-oxo structure for the preparation of the hapten-carrier conjugates.

Table II. Per Cent Cross-Reaction of Antisera raised against Cortisol-4-BSA Conjugates with Selected Steroids

Steroid	% Cross-reactivity (50%)							
	4-HS-1[a]	4-HS-2	4-CMT-1	4-CMT-2	4-CET-1	4-CET-2	4-HST-1	4-HST-2
Cortisol	100	100	100	100	100	100	100	100
11-Deoxycortisol	25	7.8	18	33	25	8.2	18	49
Corticosterone	19	18	1.6	2.1	11	1.9	6.5	1.2
21-Deoxycortisol	10	5.0	4.0	1.6	29	23	3.3	1.9
Cortisone	19	0.4	9.4	130	0.2	3.6	3.2	880
17α-Hydroxyprogesterone	13	0.3	0.9	0.3	7.5	1.4	0.5	6.3
11-Deoxycorticosterone	5.0	1.3	<0.001	0.4	3.2	0.2	1.3	6.9
Progesterone	1.7	0.7	<0.001	<0.001	0.4	<0.001	<0.001	<0.001
6β-Hydroxycortisol	1.7	0.8	6.4	5.4	0.7	0.6	2.2	40
Tetrahydrocortisol	<0.001	0.5	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Tetrahydrocortisone	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Tetrahydro-11-deoxycortisol	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
5α-Dihydrocortisol	21	8.1	53	56	29	46	38	320
5β-Dihydrocortisol	5.3	8.9	13	8.5	8.4	7.5	6.8	64
20α-Dihydrocortisol	9.7	3.4	<0.001	<0.001	0.1	<0.001	<0.001	1.7
20β-Dihydrocortisol	1.1	1.7	<0.001	<0.001	0.5	<0.001	<0.001	<0.001

a) Abbreviations for antisera are the same as in Table I.

The cross-reactivities of 4-CET-2 and 4-HS-2 with selected steroids were compared with those of antisera elicited by antigens having a linkage through C-3, C-6, or C-21. It is evident from the data in Table III that the specificity of the antisera prepared in this study is higher than those of 6-HS and 21-HS and is comparable to that of 3-CMO. Because of the limitation of test steroids available, specificity data obtained from the cross-reaction study are not always sufficient for the assay of the biological materials such as urine and plasma. It is well recog-

12) G. Scatchard, *Ann. N.Y. Acad. Sci.*, **51**, 660 (1949).
13) G.E. Abraham, *J. Clin. Endocrinol. Metab.*, **29**, 866 (1969).

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nized that in practice, when various antisera and purification procedure are compared using biological samples, the assay result yielding the lowest estimation can usually be assumed to be closest to the true value.[14] This is applicable to the assessment of specificity of antisera. Therefore, the specificity was ascertained by measuring cortisol in biological fluids. In view of the metabolism of cortisol, the use of urine was thought to be more suitable than that of plasma for this purpose. Employing 4-CET-2, 4-HS-2, 4-CMT-1 and 4-HST-1, together with 3-CMO and 21-HS, we carried out radioimmunoassays of cortisol with normal human urine specimens. Three kinds of solvents, diethyl ether, ethyl acetate, and methylene chloride, were tested for the extraction of urinary cortisol. The observed values were corrected on the basis of the recovery rate of [^{3}H]-cortisol added to each urine sample. The results of the assays with and without extraction are listed in Table IV. All the direct assays (without extraction) were found to overestimate the amount of cortisol. It seems likely that the interfering substances are extracted more readily with ethyl acetate or methylene chloride than with diethyl ether

Table III. Per Cent Cross–Reaction of Antisera raised against Various Cortisol–BSA Conjugates with Selected Steroids

Steroid	% Cross-reactivity (50%)						
	4-HS-2	4-CET-2	3-CMO[a]	3-CMO[b]	6α-HS[a]	6β-HS[c]	21-HS[a]
Cortisol	100	100	100	100	100	100	100
11-Deoxycortisol	7.8	8.2	58	3.8	26	20.86	68
Corticosterone	18	1.9	23.4	12.5	11.6	7.83	27.8
21-Deoxycortisol	5.0	23	58	3.7	70	18.04	88
Cortisone	0.4	3.6	54	6.6	1.1	23.78	65
6β-Hydroxycortisol	0.8	0.6	1.4	0.05	7.0	—	0.2
17α-Hydroxyprogesterone	0.3	1.4	25	0.06	10	5.96	34

a) Reference 3*a*; b) reference 3*c*; c) reference 4.
Abbreviations for antisera: 3-CMO=anti-cortisol 3-(O-carboxymethyl)oxime-BSA, 6α-HS=anti-6α-hemisuccinoyloxycortisol-BSA, 6β-HS=anti-6β-hemisuccinoyloxycortisol-BSA, 21-HS=anti-21-hemisuccinoylcortisol-BSA. Other abbreviations are the same as Table I.

Table IV. Urinary Cortisol Levels obtained by Radioimmunoassay using Various Antisera (μg/day)

Solvent for extraction	Antiserum[a]								
	4-CET-2	4-HS-2	4-CMT-1	4-HST-1	21-HS-1	4-CET-2	3-CMO-1	3-CMO-2	21-HS-2
Urine I						Urine III			
Diethyl ether	105	138	180	157	120	80	169	113	107
Ethyl acetate	103	164	292	190	128	94	200	130	127
Methylene chloride	113	200	262	204	187	82	182	120	106
Direct	301	450	424	320	288	296	402	264	280
Urine II						Urine IV			
Diethyl ether	103	117	173	171	141	75	142	103	91
Ethyl acetate	103	165	255	195	135	88	205	130	126
Methylene chloride	115	143	210	195	200	69	152	99	101
Direct	255	482	372	306	261	234	408	252	216

a) Abbreviations for antisera are the same as in Tables I and III.

14) S.Z. Cekan, *J. Steroid Biochem.*, 11, 135 (1979).

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and hence, if an antiserum is less specific, the use of the former two solvents results in higher values. On the other hand, a specific antiserum should give the same value for the extracts with different solvents. As can be seen in Table IV, the observed values varied with both antisera and extracting solvents in most cases. With all the urine samples, 4-CET-2 gave the lowest value for each of the three extracts. Furthermore, this antiserum showed similar results with these extracts. Other antisera gave different values; extractions with ethyl acetate and methylene chloride gave higher values than with diethyl ether. Thus, 4-CET-2 is considered to possess the highest specificity among the antisera tested. It should be noted that a separate experiment using the antiserum 4-CET-1 gave a result similar to that obtained with 4-CET-2. Other antisera prepared with the hapten-4-BSA conjugates gave unsatisfactory results, which could not have been anticipated from the results in Table I. The antisera raised against 3- and 21-BSA conjugates were also less specific than 4-CET-2.

Relatively specific anti-cortisol antisera could be prepared by using the [C-4]-haptens. The present results, together with the previous findings on the radioimmunoassay of testosterone,[15] show that the C-4 position is a suitable site for the attachment of a carrier protein in immunoassays of Δ^4-3-oxo steroids. Good agreement of the values obtained with 4-CET-1 and -2 with those reported by Odagiri[16] suggests that these antisera may be practically useful for the radioimmunoassay of urine cortisol without chromatographic purification. Recently, Fukushima *et al.* obtained a highly specific antiserum by the immunization of ducks with cortisol 3-(O-carboxymethyl)oxime-BSA.[17] The use of duck may be advantageous in the preparation of specific antisera for corticosteroids. Applications of the antisera obtained here to the radioimmunoassay of cortisol in biological fluids are being conducted in these laboratories. A sensitive enzyme immunoassay has been successfully developed by the appropriate combination of antibody and enzyme-labeled steroid using these antisera and haptenic derivatives (**1**—**4**), and the details will be reported elsewhere in the future.

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15) H. Hosoda, K. Tadano, S. Miyairi, and T. Nambara, *J. Steroid Biochem.*, **10**, 513 (1979).
16) E. Odagiri, *Tokyo Joshi Ika Daigaku Zasshi*, **45**, 494 (1975).
17) D.K. Fukushima, T. Nishina, R.H.K. Wu, L. Hellman, and J.W. Finkelstein, *Clin. Endocrinol.*, **10**, 367 (1979).

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