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Sensitivity and Specificity in Enzyme Immunoassay of Testosterone[1)]

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The effects of the combination of antiserum and enzyme-labeled steroid and of the molar ratio of steroid to enzyme in the preparation of the conjugate on sensitivity and specificity in enzyme immunoassay of testosterone have been investigated. The enzyme labeling of testosterone was carried out by the N-succinimidyl ester method. Six testosterone derivatives were covalently linked to β-galactosidase at various molar ratios. The anti-testosterone antiserum used was that raised against the 4-O-hemiglutaroyl 4-hydroxytestosterone-bovine serum albumin conjugate. Immunological properties of the conjugate in both homologous and heterologous systems were examined. The number of steroid molecules incorporated per enzyme molecule markedly influenced the sensitivity in enzyme immunoassay. Cross-reactivity was also tested with 10 kinds of closely related steroids. The effect of site heterology on specificity was more significant than that of bridge heterology.

Keywords——enzyme immunoassay; enzyme labeling of testosterone; N-succinimidyl ester method; testosterone-β-galactosidase conjugate; site heterology; bridge heterology; immunoreactivity; sensitivity; specificity; cross-reaction

In recent years a number of papers on enzyme immunoassay of steroid hormones have been reported. Much effort has been made to obtain a high sensitivity comparable to that of radioimmunoassay. The effects of the combination of antibody and enzyme-labeled antigen on sensitivity have been investigated for estrogens by van Weemen and Schuurs.[3)] It was described that homologous assays, where the same haptenic derivative is used for the immunogen as well as for the enzyme-labeled antigen, were relatively insensitive, and more sensitive assays were likely to be obtained by employing a "site" or "bridge" heterologous system. In contrast, many workers have obtained sufficiently high sensitivity in assay employing homologous systems. For the purpose of clarifying this problem, reproducibility of the method for coupling steroid molecules to an enzyme is a prerequisite. The enzyme labeling of steroids has usually been accomplished by condensation of carboxyl groups of the antigen molecules with the amino groups of lysine residues in an enzyme. The mixed anhydride and carbodiimide methods are commonly used, but these are not always satisfactory with respect to reproducibility. In a previous paper of this series, we reported the enzyme labeling of steroids by the N-succinimidyl ester method.[4)] The activated ester method appears to be useful for investigation of factors influencing the sensitivity and specificity of enzyme immunoassay. This paper described the immunological properties of testosterone-β-galactosidase (EC 3.2.1.23) conjugates in both homologous and heterologous combination systems.

Materials and Methods

Reagents——β-Galactosidase from *E. coli* (grade VI, 290 units per mg protein) was obtained from Sigma Chemical Co. (St. Louis, Mo.) and *o*-nitrophenyl β-D-galactopyranoside from Nakarai Chemical, Ltd. (Kyoto).

1) Part CLXI of "Studies on Steroids" by T. Nambara; Part CLX: T. Nambara, K. Shimada, M. Numazawa, H. Ohta, and Y. Sawada, *J. Pharm. Dyn.*, **3**, 407 (1980).
2) Location: *Aobayama, Sendai 980, Japan*; *a*) To whom inquiries should be addressed.
3) B.K. Van Weemen and A.H.W.M. Schuurs, *Immunochemistry*, **12**, 667 (1975).
4) H. Hosoda, Y. Sakai, H. Yoshida, and T. Nambara, *Chem. Pharm. Bull.*, **27**, 2147 (1979).

The N-succinimidyl esters of 4-O-hemiglutaroyl 4-hydroxytestosterone, 4-O-hemisuccinoyl 4-hydroxytestosterone, testosterone 3-(O-carboxymethyl)oxime, 17-O-hemisuccinoyl testosterone, 4-(carboxymethylthio)-testosterone and 4-(2-carboxyethylthio)testosterone were prepared by the methods previously established in these laboratories.[5] Anti-testosterone antiserum was produced by immunization in the rabbit using the conjugate of 4-O-hemiglutaroyl 4-hydroxytestosterone with bovine serum albumin (BSA) in the manner reported in the previous paper.[6] The serum was diluted with 0.05 M phosphate buffer (pH 7.3) (PB) containing 0.1% gelatin, 0.9% NaCl, and 0.1% NaN_3 (buffer A). Goat anti-rabbit IgG antiserum and normal rabbit serum were obtained from Daiichi Radioisotope Labs., Ltd. (Tokyo).

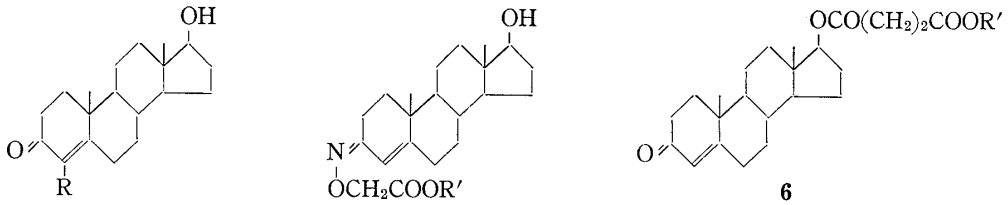
Preparation of Testosterone-β-Galactosidase Conjugate——A solution of the N-succinimidyl ester (**1b**—**6b**) (28, 56, 112 nmol) in dioxane (0.1 ml) was added to β-galactosidase (1 mg) in PB (0.5 ml) at 0°, and the mixture was gently stirred at 4° for 2 hr. The resulting solution was dialyzed against cold PB (1 liter) for 2 days. The solution was stored at 4° at a concentration of 200 μg per ml, adjusted with PB containing 0.1% gelatin and 0.1% NaN_3 (buffer B). For the immunoassay procedure this was diluted with the buffer solution containing 0.5% normal rabbit serum.

Inhibition of Binding by Addition of 1 ng of Steroids——A solution of testosterone or a related steroid (1 ng) in Buffer B (0.1 ml) and diluted antiserum (0.1 ml) were added to testosterone-enzyme conjugate (0.1 μg, 0.1 ml) in the buffer, and the mixture was incubated at 4° for 4 hr. Goat anti-rabbit IgG antiserum (0.1 ml) diluted 1:30 with Buffer A containing 0.3% EDTA was added to the incubation mixture, and the solution was vortex-mixed then allowed to stand at 4° for 16 hr. After addition of Buffer B (1 ml), the resulting mixture was centrifuged at 3000 rev./min for 15 min. The supernatant was removed and the immune precipitate was used for measurement of the enzymic activity. At the same time, the procedure without addition of steroid or first antibody was carried out to provide B_0 and blank values, respectively.

Measurement of β-Galactosidase Activity of the Immune Precipitate——The immune precipitate was diluted with buffer A (1 ml) containing 0.2% $MgCl_2$ and 0.7% 2-mercaptoethanol, vortex-mixed, and pre-incubated at 37° for 3 min. *o*-Nitrophenyl β-D-galactopyranoside (0.06%, 1 ml) in PB was added to the resulting solution, and the mixture was incubated for 30—90 min. The reaction was terminated by addition of 1 M Na_2CO_3 (2 ml). The absorbance was then measured at 420 nm with a Hitachi Perkin-Elmer 139 UV-VIS spectrophotometer.

Results and Discussion

The purpose of this work was to investigate the effects of the combination between antiserum and enzyme-labeled steroid on sensitivity and specificity, and of the molar ratio of steroid to enzyme in the preparation of the conjugate on sensitivity in the enzyme immunoassay of steroid hormones by using testosterone as a model substance. It has previously been demonstrated that these are critical factors for the assessment of an assay.[3,7,8] Systematic studies are necessary to obtain further detailed information as a basis for the development of enzyme immunoassay. The N-succinimidyl ester method seemed suitable for such studies because of its excellent reproducibility and simplicity.



1: R=$OCO(CH_2)_3COOR'$
2: R=$OCO(CH_2)_2COOR'$
3: R=SCH_2COOR'
4: R=$S(CH_2)_2COOR'$

5

6

a: R'=H

b: R'=

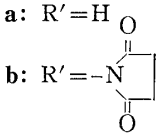


Chart 1

5) H. Hosoda, Y. Sakai, H. Yoshida, S. Miyairi, K. Ishii, and T. Nambara, *Chem. Pharm. Bull.*, **27**, 742 (1979).
6) H. Hosoda, K. Tadano, S. Miyairi, and T. Nambara, *J. Steroid Biochem.*, **10**, 513 (1979).
7) S. Comoglio and F. Celada, *J. Immunol. Methods*, **10**, 161 (1976).
8) B.G. Joyce, G.F. Read, and D. Riad-Fahmy," International Symposium on Radioimmunoassay and Related Procedures in Medicine," Berlin (West), Vol. 1, IAEA, 1978, p. 289.

The anti-testosterone antiserum elicited by the conjugate of 4-hydroxytestosterone 4-hemiglutarate (**1a**) with BSA was used because its specificity in radioimmunoassay[6] was comparable to those of antisera raised against [C-3]-, [C-6]- and [C-7]-BSA conjugates, and several haptenic derivatives possessing different bridges were available at the same C-4 position. The carboxylated testosterone derivatives (**1a**—**6a**) were covalently linked to β-galactosidase by the activated ester method. Enzyme labeling with molar ratios of 15, 30 and 60 was carried out by mixing the N-succinimidyl esters (**1b**—**6b**) with the enzyme in phosphate buffer-dioxane, where no significant loss of enzymic activity occurred. Although the number of steroid molecules incorporated into an enzyme molecule was not determined, it is expected to range from 1 to 10.[7]

The assay using the conjugate of 4-O-hemiglutaroyl 4-hydroxytestosterone with the enzyme (4-HG) is a homologous combination. The conjugates of 4-(carboxymethylthio)-testosterone (4-CMT), 4-(2-carboxyethylthio)testosterone (4-CET) and 4-O-hemisuccinoyl 4-hydroxytestosterone (4-HS) were used for bridge heterology, while testosterone 3-(O-carboxymethyl)oxime (3-CMO) and 17-O-hemisuccinoyl testosterone (17-HS) were used for site heterology. The bound and free enzyme-testosterone conjugates were separated by a double antibody method, and the enzymic activity of immune precipitate was determined colorimetrically using *o*-nitrophenyl β-D-galactopyranoside as a substrate.

The binding abilities of a definite amount (0.1 μg) of enzyme-steroid conjugates were investigated at 1 : 500 dilution of the anti-testosterone antiserum (Fig. 1). The immunoreactivity increased with increasing molar ratio. It seems likely that the immunoreactivity of the homologous label is not always higher than those of other systems. The homologous and bridge heterologous systems exhibited similar patterns of binding ability and reached a plateau at a ratio of 30,[9] whereas lower binding ability was observed in the case of site heterology,

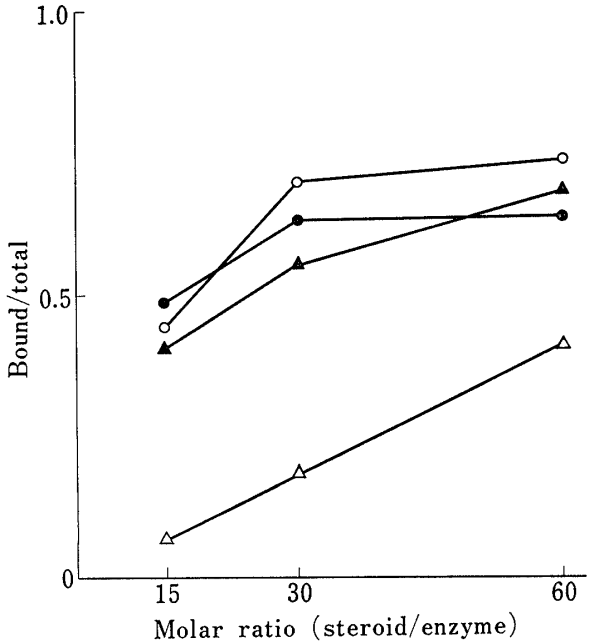


Fig. 1. Binding Abilities of the Testosterone-β-Galactosidase Conjugate of 4-CMT (○—○), 3-CMO (▲—▲), 17-HS (△—△) and 4-HG (●—●) at Molar Ratios of 15, 30 and 60

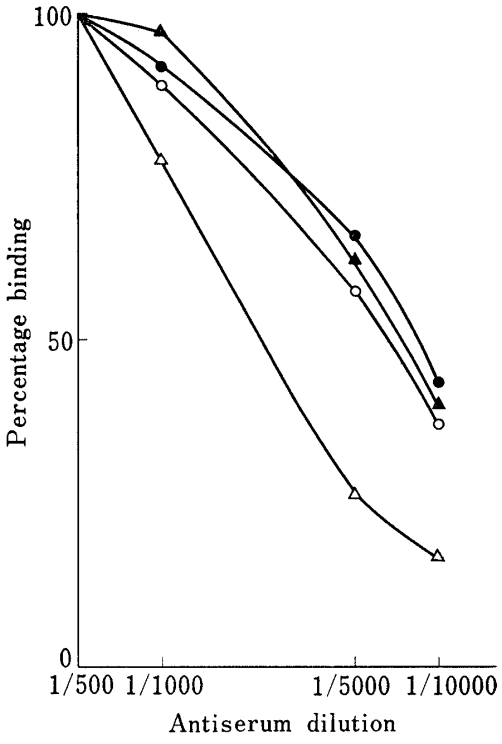


Fig. 2. Antibody Dilution Curves with the Conjugates prepared at a Molar Ratio of 60

Symbols are the same as in Fig. 1.

9) 4-HS and 4-CET are not shown in Fig. 1.

particularly in the 17-HS system. This may be ascribable to the affinity of the antibody used, and not to the difference in reactivity of the activated esters with the enzyme in enzyme labeling, since the 17-hemisuccinyl derivative coupled most readily with the ε-amino groups of lysine residues in a protein.[5] In order to determine an appropriate dilution of antiserum (40—60% initial binding ability) for use in immunoassay, antibody dilution curves were then constructed for each conjugate. The results obtained with the conjugates prepared at a molar ratio of 60 are illustrated in Fig. 2. The binding ability was expressed for convenience as a percentage of the ability obtained with 1: 500 dilution. In all the systems, the percentage binding obtained with the conjugates prepared at molar ratios of 15 and 30 was found to be nearly equal to that shown in Fig. 2. at a given dilution of antiserum.

Table I. Percentage Inhibition of Bound Enzymic Activity by 1 ng of Testosterone[a]

Molar ratio	Conjugate[b]					
	4-HG (1:10000)	4-HS (1:4000)	4-CMT (1:5000)	4-CET (1:5000)	3-CMO (1:10000)	17-HS (1:2000)
15	76	84	64	63	59[c]	—
30	43	—	44	45	46	85
60	27	38[d]	37	37	40	87

a) Incubation time for the enzymic reaction was 90 min, and 0.1 μg of conjugate was used. —: not carried out.
b) Abbreviations are described in the text. Antiserum dilution in parentheses.
c) O.D. of $B_0 < 0.2$.
d) The value obtained with a molar ratio of 50.

The sensitivity attainable with these conjugates was then investigated. If one expects a sensitivity[10] of 20 pg and range of measurement of 20—1000 pg, the inhibition of enzymic activity by 1 ng of antigen should be over 60%. Therefore, inhibition of bound enzymic activity by the addition of 1 ng of testosterone per test tube at an appropriate dilution of antiserum was examined, and the results are listed in Table I. Sufficient inhibition was observed at a molar ratio of 15 in the homologous and bridge heterologous systems with an adequate optical density for B_0 during a limited incubation period. In fact, desirable dose–response curves with these conjugates could be constructed with a satisfactory sensitivity and range of measurement.[4] In the case of site heterology, a higher molar ratio was required to give sufficient optical density. Molar ratios of 60 and 30 were most suitable for 17-HS and 3-CMO, respectively. Nevertheless, the sensitivity of the latter was less. In any case, the site heterologous system could offer no advantage according to our criteria. The homologous and bridge heterologous conjugates obtained at a molar ratio of 60 were further assessed by varying the conditions of the immune reaction, and the results are listed in Table II. The use of 0.1 μg of 4-HG at 1: 10000 dilution of the antiserum showed 27% inhibition and at 1: 20000 dilution showed no increased inhibition. Furthermore, the use of a smaller amount of enzyme label (0.05 μg) was not effective. Similarly, sufficient inhibition was not obtained with 4-CMT, although slight increases were observed. It is evident from these data that a satisfactory result cannot be obtained at a molar ratio of 60 even when the dilution of antiserum and the amount of enzyme-steroid conjugate are varied. Thus, in the homologous system, for example, the sensitivity with the use of conjugate prepared at a molar ratio of 15 may be 10—100 times higher than those with molar ratios of 30 and 60. These results indicate that the sensitivity is markedly influenced by the number of steroid molecules incorporated per enzyme molecule. This effect is not dependent upon the enzyme used, since a similar phenomenon has recently been observed with horseradish peroxidase.[8]

10) H. Kaiser and H. Specker, *Z. Anal. Chem.*, **149**, 46 (1956).

Table II. Effects of the Amount of Conjugate and the Antiserum Dilution on Percentage Inhibition by 1 ng of Testosterone[a]

Amount of conjugate (μg)	Antiserum dilution	Conjugate[b]	
		4-HG	4-CMT
0.1	1 : 5000	—	37(0.58)
	1 : 10000	27(0.34)	34(0.22)
	1 : 20000	23(0.11)	—
0.05	1 : 5000	—	46(0.30)
	1 : 10000	30(0.21)	45(0.20)

a) A molar ratio of 60 was used. Incubation time for the enzymic reaction was 90 min. O.D. of B_0 in parentheses. —: not carried out.
b) Abbreviations are described in the text.

Table III. Cross-Reactions ($CR_{1\ ng}$) with Selected Steroids[a]

Steroid	Conjugate[b]					
	4-HG	4-HS	4-CMT	4-CET	3-CMO	17-HS
Testosterone	100	100	100	100	100	100
5α-Dihydrotestosterone	91	89	97	86	100	91
5β-Dihydrotestosterone	63	55	59	55	50	58
Androstenedione	26	30	24	48	41	35
Epitestosterone	5	14	6	16	0	10
Dehydroepiandrosterone	13	12	13	18	19	6
Progesterone	3	6	0	0	35	24
Androstenediol	29	31	44	26	41	51
5α-Androstane-3α,17β-diol	47	52	51	34	59	43
5α-Androstane-3β,17β-diol	38	47	49	46	37	71
Estradiol	23	28	14	22	26	15

a) Molar ratios were 15 for 4-HG, 4-HS, 4-CMT and 4-CET, 30 for 3-CMO, and 60 for 17-HS.
b) Abbreviations are described in the text.

The effect of the combination on specificity was also investigated in this study. The cross-reactivities with 10 kinds of closely related compounds were tested by the $CR_{1\ ng}$ method proposed by De Lauzon *et al.*,[11] and the results are listed in Table III. Comparison of cross-reactions in the assay systems of 4-HS, 4-CMT and 4-CET with that of 4-HG revealed that the specificity pattern of the antiserum was not significantly influenced by the bridge heterology. On the other hand, the site heterology did have some effect. In the case of 17-HS, progesterone and 5α-androstane-3β,17β-diol cross-reacted markedly as compared with the homologous system, while the cross-reaction with dehydroepiandrosterone was less marked. With another site heterologous system, 3-CMO, the cross-reactions with epitestosterone and progesterone displayed a different specificity pattern. Van Weemen and Schuurs described a tendency for a decrease in the specificity of heterologous systems in the enzyme immunoassay of estrogens.[3] This does appear to be the case, because significantly increased cross-reaction were observed with progesterone and 5α-androstane-3β,17β-diol. It should be noted, however, that in the site heterologous systems, the C-3 and C-17 positions on the steroid molecule were used as the sites of enzyme labeling, where all the steroids tested for cross-reaction possess functional groups. The use of such heterologous combinations may result in the cross-reactivities of steroids commonly present in biological fluids and result in a lack of specificity of the assay, even when the antiserum employed is highly specific in a radioimmunoassay.

11) S. De Lauzon, N. Cittanova, B. Desfosses, and M.F. Jayle, *Steroids*, **22**, 747 (1973).

Specificity in the homologous and bridge heterologous systems seemed to be similar to that in the radioimmunoassay previously described,[6] although cross-reactivity was tested by different methods. Recently, enzyme immunoassays of testosterone have been reported by several groups.[12] The specificity obtained here was higher than that reported by Rajkowski *et al.*[12b] and seems comparable to those reported by others. When one employs heterology owing to insufficient sensitivity in the case of homology, the use of a bridge heterologous system rather than site heterology, if possible, is recommended. Although the effectiveness of heterology on sensitivity has previously been discussed,[3,13,14] no report has discussed the basis for selecting the heterology. Such a study requires control of the molar ratio factor. Further studies are being conducted in these laboratories.

Acknowledgement This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan, which is gratefully acknowledged.

12) *a*) K. Tateishi, H. Yamamoto, T. Ogihara, and C. Hayashi, *Steroids*, **30**, 25 (1977); *b*) K.M. Rajkowski, N. Cittanova, P. Urios, and M.F. Jayle, *ibid.*, **30**, 129 (1977); *c*) A. Turkes, A.O. Turkes, B.G. Joyce, G.F. Read, and D. Riad-Fahmy, *ibid.*, **33**, 347 (1979); *d*) U.M. Joshi, H.P. Shah, and S.P. Sudhama, *ibid.*, **34**, 35 (1979).

13) H. Arakawa, M. Maeda, and A. Tsuji, *Anal. Biochem.*, **97**, 248 (1979).

14) D. Exley, "Enzyme Labelled Immunoassay of Hormones and Drugs," ed. by S.B. Pal. Walter de Gruyter, Berlin (1978), p. 207.