Sensitivity of Steroid Enzyme Immunoassays. Comparison of Four Label Enzymes in an Assay System Using a Monoclonal Anti-steroid Antibody[1)]

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The sensitivities of monoclonal antibody-based enzyme immunoassays for 11-deoxycortisol using alkaline phosphatase (AP), horseradish peroxidase (HRP), β-galactosidase (β-GAL) and glucose oxidase (GOD) as labels were compared. The anti-11-deoxycortisol antibody used was that produced in ascites by inoculating antibody-secreting hybridoma cells into mice. Enzyme labeling of 11-deoxycortisol was carried out by the *N*-succinimidyl ester method. The activated ester of 4-(2-carboxyethylthio)-11-deoxycortisol was treated with each enzyme to give a homologous enzyme-labeled antigen. In the competitive immunoassay, the bound and free enzyme-labeled antigens were separated by a double antibody method and the enzymic activity of the immune precipitate was determined by colorimetric and fluorimetric methods. The AP activity was measured in three ways, using *p*-nitrophenyl phosphate, nicotinamide adenine dinucleotide phosphate (NADP), and 4-methylumbelliferyl phosphate as substrates. *o*-Nitrophenyl β-D-galactopyranoside and 4-methylumbelliferyl β-D-galactopyranoside were used for β-GAL, and 3,3′,5,5′-tetramethylbenzidine (TMB) and 3-(*p*-hydroxyphenyl)propionic acid (HPPA) for HRP. In the case of GOD, TMB and HPPA were used in combination with HRP. A dose–response curve with a high sensitivity was obtained in each 11-deoxycortisol assay system by the use of a minimum amount of the enzyme-labeled antigen at an appropriate dilution of monoclonal anti-11-deoxycortisol antibody ($K_a = 2 \times 10^{10}$ M^{-1}). The amounts of 11-deoxycortisol needed to displace 50% of the bound label ranged from 5 to 15 pg in the colorimetric methods, and 4—9 pg in the fluorimetric methods. It was found that the assay using the HRP label was the most sensitive, and the AP, GOD and β-GAL labels gave nearly equal sensitivities. An interesting finding was obtained on the so-called bridging phenomenon, which is a factor influencing the sensitivity of hapten enzyme immunoassays.

Keywords enzyme immunoassay; monoclonal antibody; 11-deoxycortisol; steroid–enzyme labeling; immunoassay sensitivity; alkaline phosphatase; horseradish peroxidase; β-galactosidase; glucose oxidase

Enzyme immunoassays of hormones and drugs have been developed using various enzymes as labels. The sensitivity of the assay depends on the enzyme employed. Enzymes currently used in the heterogeneous enzyme immunoassay of steroid hormones are alkaline phosphatase (AP), horseradish peroxidase (HRP), β-galactosidase (β-GAL), glucose oxidase (GOD), glucose-6-phosphate dehydrogenase, glucose dehydrogenase, glucoamylase, β-lactamase, urease and invertase; the former three enzymes are most commonly used. The choice of enzyme is based on various criteria such as activity and stability.[2)] In order to obtain a practical basis for selecting the enzyme, we have previously compared AP, HRP, β-GAL, and GOD in a testosterone assay system, with regard to the effect of steroid/enzyme molar ratio in the labeling reaction on the immunoreactivity of the labeled antigen with an anti-steroid antiserum and to the assay sensitivity.[3)] There is the possibility that the heterogeneity of polyclonal antibodies influences the results on assay sesitivity. The use of monoclonal antibody eliminates this problem, simplifying the analysis of immune reactions. This paper deals with the sensitivities of monoclonal-based enzyme immunoassays for 11-deoxycortisol using AP, HRP, β-GAL, and GOD labels.

Materials and Methods

Materials AP (EC 3.1.3.1) from calf intestine (enzyme label for enzyme immunoassay, 2500 U/mg) and GOD (EC 1.1.3.4) from *Aspergillus niger* (grade I, 287 U/mg) were obtained from Boehringer-Mannheim Yamanouchi Co. (Tokyo); β-GAL (EC 3.2.1.23) from *Escherichia coli* (grade VIII, 580 units/mg), and HRP (EC 1.11.1.7) (grade I-C, Reinheits-Zahl 3.48, 266 units/mg) were from Sigma Chemical Co. (U.S.A.) and Toyobo Co. (Osaka), respectively. The *N*-succinimidyl ester of 4-(2-carboxyethylthio)-11-deoxycortisol (CET) was prepared by the method previously established in these laboratories.[4)] The monoclonal anti-11-deoxycortisol antibody (CET-M8) used was that produced in ascites by inoculating the hybridoma, S.CET.M8.1.1, intraperitoneally into pristane-treated BALB/c mice.[5)] Normal mouse serum (NMS) and rabbit anti-mouse immunoglobulin G (IgG) antiserum were obtained from MBL Co. (Nagoya); these sera were dialyzed against cold 0.05 M phosphate buffer (PB), pH 7.3. Nicotinamide adenine dinucleotide phosphate (NADP, sodium salt) and 3,3′,5,5′-tetramethylbenzidine (TMB) were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo). Alcohol dehydrogenase (EC 1.1.1.1.) from baker's yeast (185 units/mg), diaphorase (EC 1.6.4.3) from *Clostridium kluyveri* (type II-L, 10 units/mg) and *p*-iodonitrotetrazolium violet were obtained from Sigma Chemical Co. *p*-Nitrophenyl phosphate (*p*-NP), *o*-nitrophenyl β-D-galactopyranoside (*o*-NPG) and 4-methylumbelliferyl β-D-galactopyranoside (MUG) were purchased from Nakarai Chemicals, Ltd. (Kyoto); 3-(*p*-hydroxyphenyl)propionic acid (HPPA) and 4-methylumbelliferyl phosphate (MUP) were from Fluka AG (Switzerland) and Koch-Light Laboratories (England), respectively.

Assay Buffer PB containing 0.1% gelatin and 0.9% NaCl (gel–PBS) was used in the immunoassays with HRP and GOD as labels. In the systems with AP and β-GAL, gel–PBS containing 0.1% NaN_3 was used.

Preparation of 11-Deoxycortisol–Enzyme Conjugates AP Labeling: Dioxane solutions (0.6 ml) containing calculated amounts of CET *N*-succinimidyl ester (I) corresponding to steroid/enzyme molar ratios of 15, 20 and 30 (molecular weight (M.W.) of AP, 116500) were each added to a solution of AP (600 μg) in PB (1.2 ml) at 0 °C, and the mixture was gently stirred at 4 °C for 4 h. After addition of PB (1.2 ml), the resulting solution was dialyzed against cold PB (3 l) for 2 d. A 0.5 ml aliquot of the dialyzed conjugate solution was transferred to a test tube; the solution was stored at 4 °C at a concentration of 100 μg/ml, adjusted with assay buffer. The remaining conjugate solution was used for determination of the number of steroid molecules incorporated per enzyme molecule.

HRP, β-GAL, and GOD Labelings: A solution of I (steroid/enzyme molar ratios; 20, 30, 40 in HRP labeling, 5, 10, 15 for β-GAL, and 4, 6, 8 for GOD) in dioxane (0.15 ml) was added to a solution of an enzyme (1.5 mg) (M. W. of HRP, 42500; β-GAL, 465000; GOD, 160000) in PB (0.3 ml) at 0 °C, and the mixture was gently stirred at 4 °C for 4 h. After addition of PB (1.1 ml), the resulting solution was dialyzed against cold PB (3 l) for 2 d. A 0.5 ml aliquot of the dialyzed conjugate solution was transferred to a test tube; the solution was stored at 4 °C at a concentration of 500 μg/ml, adjusted with assay buffer.

Determination of the Number of 11-Deoxycortisol Molecules Incorporated per Enzyme Molecule Spectrometric analysis was carried out by comparing the absorbances at 250 and 280 nm (403 nm in the case of HRP) of the conjugate with those of CET and the enzymes as controls in PB (ε value for the steroid was 11000 at 250 nm).

Enzyme Immunoassay Procedure This was carried out in duplicate or triplicate in glass test tubes (10 ml). The standard procedure with the AP label is as follows: solutions of 11-deoxycortisol (0—500 pg) in assay buffer (0.1 ml) and AP-labeled 11-deoxycortisol (5—10 ng) in the buffer (0.1 ml) were added to diluted CET-M8 (0.1 ml), and the mixture was incubated at 4 °C for 4 h (first incubation). Rabbit anti-mouse IgG antiserum (0.1 ml) diluted 1 : 120 with assay buffer and 0.5% NMS (0.1 ml) in the buffer were added to the incubation mixture, and the solution was vortex-mixed, then allowed to stand at 4 °C for 16 h. After addition of 0.05 M carbonate buffer (pH 10.0) containing 0.1% gelatin, 0.9% NaCl, and 0.1% NaN_3 (1.5 ml), the resulting solution was centrifuged at 3000 rpm for 10 min, and the supernatant was removed by aspiration. The immune precipitate was washed once with the carbonate buffer (1.5 ml), and used for measurement of the enzymic activity (B_0 or B).

In the enzyme immunoassays using HRP, β-GAL and GOD as labels, the first incubation procedure was carried out in a manner similar to that described above; the amounts of the labeled antigen used were 1—2 ng, 10—50 ng, and 5—50 ng with the HRP, β-GAL, and GOD labels, respectively. B/F separation was then carried out as follows: rabbit anti-mouse IgG antiserum (0.1 ml) diluted 1 : 120 with assay buffer containing 0.3% ethylenediaminetetraacetic acid and 0.5% NMS (0.1 ml) in the buffer were added to the incubation mixture, and the solution was vortex-mixed, then allowed to stand at 4 °C for 16 h. The resulting mixture was diluted with assay buffer (1.5 ml) and centrifuged at 3000 rpm for 10 min, and the supernatant was removed by aspiration. The immune precipitate was washed once with assay buffer (1.5 ml), and used for measurement of the enzymic activity. In each system, the procedure without addition of the first antibody CET-M8 was carried out to provide non-specific binding values; the background was estimated as a percentage of the absorbance or the fluorescence intensity for B_0, using distilled water in colorimetry or a blank solution in fluorimetry as the zero reference.

Measurement of AP Activity NADP Method: The immune precipitate was diluted with 0.05 M diethanolamine buffer, pH 10.0 (DEA, 1 ml), containing NADP (20 μM) and 0.01% $MgCl_2$, vortex-mixed, and incubated at 25 °C for 40 min. A PB solution (1 ml) containing alcohol dehydrogenase (100 μg), diaphorase (100 μg), 0.8 mM *p*-iodonitrotetrazolium violet, and 2% ethanol was added to the incubation mixture, and the whole was incubated at 25 °C for 20 min. The reaction was terminated by addition of 0.1 M HCl (2 ml) and the absorbance was measured at 492 nm.

p-NP Method: A solution of *p*-NP (1 mM) in 1 M DEA (2 ml) containing 0.01% $MgCl_2$ was added to the assay tube, and the mixture was incubated at 37 °C for 1 h. The reaction was terminated by addition of 0.1 M NaOH (2 ml) and the absorbance was measured at 405 nm.

MUP Method: A solution of MUP (0.005%) in 0.05 M DEA (2 ml) containing 0.01% $MgCl_2$ was added to the assay tube, and the mixture was incubated at 37 °C for 30 min. The reaction was terminated by addition of a 0.5 M phosphate buffer, pH 10.4 (2 ml). The fluorescence intensity was then measured at 450 nm with excitation at 360 nm; the zero reference used consisted of these solutions except the substrate solution.

Measurement of HRP Activity TMB Method: The immune precipitate was diluted with 0.05 M acetate–citric acid buffer, pH 4.2 (1.8 ml), containing 0.42 mM TMB and 3% dimethyl sulfoxide, vortex-mixed, and preincubated at 37 °C for 3 min. Hydrogen peroxide (0.02%, 0.2 ml) was added to the resulting solution, and the mixture was incubated for 1 h. The reaction was terminated by addition of 0.5 M H_2SO_4 (2 ml) and the absorbance was measured at 450 nm.

HPPA Method: A solution of HPPA (0.08%) in PB (1.2 ml) was added to the assay tube, and the mixture was preincubated at 25 °C for 3 min. Hydrogen peroxide (0.01%, 0.2 ml) was added to the resulting solution, and the mixture was incubated for 30 min. The reaction was terminated by addition of 3% NaN_3–0.5 M NaOH (1 : 1, 2 ml). The fluorescence intensity was then measured at 405 nm with excitation at 320 nm; the zero reference used was the NaN_3–NaOH solution diluted 1 : 1.7 with H_2O.

Measurement of β-GAL Activity *o*-NPG Method: The immune precipitate was diluted with assay buffer (1 ml) containing 0.1% $MgCl_2$ and 10% ethylene glycol, vortex-mixed, and preincubated at 37 °C for 3 min. *o*-NPG (0.12%, 1 ml) in assay buffer was added to the resulting solution, and the mixture was incubated for 1 h. The reaction was terminated by addition of 1 M Na_2CO_3 (2 ml) and the absorbance was measured at 420 nm.

MUG Method: Assay buffer (1 ml) containing 0.1% $MgCl_2$ and 10% ethylene glycol was added to the assay tube, and the mixture was preincubated at 37 °C for 3 min. MUG (0.007%, 1 ml) in assay buffer was added to the resulting solution, and the mixture was incubated for 30 min. The reaction was terminated by addition of 1 M Na_2CO_3 (2 ml) and the fluorescence intensity was measured at 450 nm with excitation at 360 nm; the zero reference used consisted of these solutions except the substrate solution.

Measurement of GOD Activity TMB Method: The immune precipitate was diluted with 0.05 M acetate–citric acid buffer, pH 4.2 (2 ml), containing 0.42 mM TMB, 3% dimethyl sulfoxide, 0.5 M glucose, and 25 nM HRP, vortex-mixed, and incubated at 37 °C for 1 h. The reaction was terminated by addition of 0.5 M H_2SO_4 (2 ml). The absorbance was measured at 450 nm.

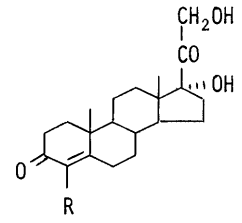
HPPA Method: A 0.05 M acetate buffer, pH 5.0 (1.2 ml), containing 0.08% HPPA, 0.5 M glucose, and 25 nM HRP was added to the assay tube, and the mixture was incubated at 25 °C for 30 min. The reaction was terminated by addition of 3% NaN_3–0.5 M NaOH (1 : 1, 2 ml). The fluorescence intensity was measured at 405 nm with excitation at 320 nm; the zero reference used consisted of these solutions.

Immunoreactivity Studies Colorimetric immunoassay procedures for B_0 values were carried out in the manner described above, using a 1 : 5000 dilution of CET-M8 (in the case of the HRP label, incubation time was 30 min). Simultaneously, an experiment using only the enzyme was carried out to obtain 100% enzymic activity (T); the absorbance obtained was corrected for incubation time. The amounts of the label used and the incubation times for T were as follows: AP (*p*-NP): 10 ng, 30 min; HRP: 2 ng, 10 min; β-GAL and GOD: 50 ng, 30 min.

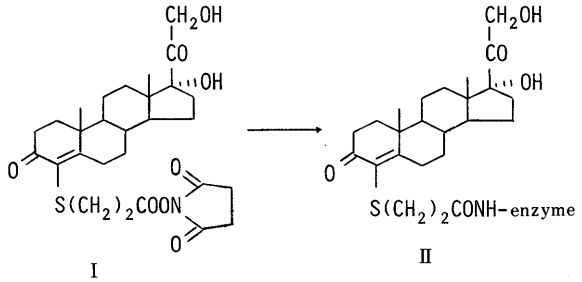
Results and Discussion

The purpose of this work was to compare the sensitivities obtainable with the 11-deoxycortisol enzyme immunoassay systems using the monoclonal antibody CET-M8, when AP, HRP, β-GAL and GOD were employed as labels in colorimetric and fluorimetric detection methods. In general, development of a specific and sensitive assay system is not always easy owing to various factors. There are several factors determining the sensitivity, such as the affinity constant (K_a) of the antibody, methods for enzyme labeling, immunoreaction and enzyme activity measurement, and the bridge binding phenomenon.

CET *N*-succinimidyl ester (I) was treated with each enzyme in PB (pH 7.3)–dioxane to give enzyme-labeled antigen (II). The activated ester should react readily with free amino groups of these enzymes. The steroid/enzyme



11-deoxycortisol : R=H
CET : R=S(CH2)2COOH



enzyme : AP, β-GAL, GOD, HRP

Chart 1

Table I. Enzyme-Labeled Antigens and Their Reactivities with the Monoclonal Antibody CET-M8

Enzyme label	Molar ratio[a]	Degree of substitution	B_0/T
AP	20	0.5	0.16[b]
HRP	30	1.8	0.24
β-GAL	5	2.0	0.28
GOD	6	1.0	0.16

a) Steroid/enzyme molar ratios in the enzyme labeling by the *N*-succinimidyl ester method. The final enzyme concentrations were 2.9, 78, 7.2 and 21 μM in the AP, HRP, β-GAL and GOD labelings, respectively. *b*) *p*-NP detection method.

molar ratios employed in the coupling reactions were 15—30, 20—40, 5—15 and 4—8 for AP, HRP, β-GAL and GOD labels, respectively. Selection of the ratio was based on the previous findings.[3a] The conjugates were dialyzed against the buffer to remove the unreacted steroid. No significant loss of enzymic activity was observed under the coupling conditions used. The number of steroid molecules incorporated per enzyme molecule (degree of hapten substitution), determined by spectrometric analysis, was listed in Table I.

The anti-steroid antibody (IgG_1) employed in this work was that secreted from the hybridoma, S.CET.M8.1.1, which was derived from fusion of P3-NS1/1-Ag4-1 myeloma cells with spleen cells of BALB/c mice immunized with CET linked to bovine serum albumin, that is, the combination of antibody and labeled antigen is homologous.[6] This monoclonal antibody showed a high affinity for 11-deoxycortisol ($K_a = 2 \times 10^{10}$ M^{-1}) in the radioimmunoassay procedure.[5a] The bound and free enzyme-labeled antigens were separated by a double antibody method.[7] The enzymic activity of the immune precipitate was determined by colorimetric or fluorimetric methods. In the former methods, the assays were assessed in terms of the absorbance for B_0 and non-specific binding (background). The criteria that the optical density obtained upon 1 h incubation and the background should be at least 0.3 and less than 20% (10% in fluorimetry), respectively, were employed. The substrates used were *p*-NP, NADP[3b,8] and MUP for the AP label, *o*-NPG and MUG for the β-GAL label, and TMB and HPPA for the HRP label; in the detection of GOD activity, TMB–[3c] and HPPA–HRP systems were employed. These reagents were chosen because they are commonly used or are the most sensitive.

The binding abilities of a fixed amount of the labeled antigens were investigated at 1 : 5000 dilution of CET-M8. The immunoreactivity (B_0/T) increased with increasing molar ratio in the labelings. The results obtained in the colorimetric assays with the labels prepared at a given molar ratio are listed in Table I. The reactivity values ranged from 0.16 to 0.28. These labels were found to give a satisfactory result with respect to the assay sensitivity, according to our criteria (the label obtained at a higher ratio gave a low sensitivity). It should be noted, with the GOD label, that a significant decrease in the immunoreactivity was observed under the present storage conditions. The reason for this unexpected result remains to be clarified.

In order to determine an appropriate dilution of CET-M8 for use in enzyme immunoassay, antibody dilution curves were constructed with each system, and the results obtained in the colorimetric methods are shown in Fig. 1. The binding ability was expressed for convenience as a percentage of that with 1 : 5000 dilution. The dilution showing 50% binding can be defined as a titer. Therefore, the use of CET-M8 diluted approximately 1 : 60000 in the assays using the HRP and GOD labels may be suitable for obtaining dose–response curves; in the cases of the AP and β-GAL labels, 1 : 130000—1 : 150000. In practice, however, various dilutions were employed in the comparative study of sensitivity.

Sensitivities obtainable with all the assay systems were

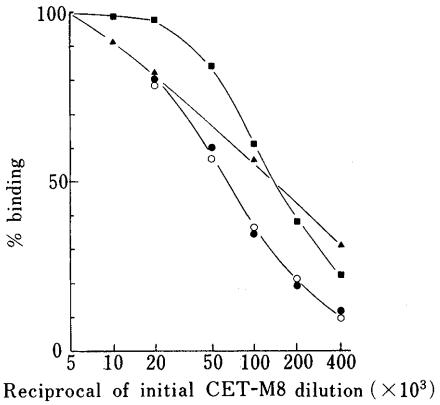


Fig. 1. Antibody Dilution Curves with the HRP (●)-, AP (▲)-, β-GAL (■)- and GOD (○)-Labeled Antigens

Assay conditions are given in Table I or in the text.

Table II. Sensitivity of 11-Deoxycortisol Enzyme Immunoassay Using the Monoclonal Antibody CET-M8

Enzyme label	Detection system	Amount of label (ng)	Antibody dilution[a] (×10⁴)	Sensitivity (pg)	Absorbance for B_0[b]	
AP	*p*-NP	10	5	52	0.89	(6)
			10	29	0.72	(7)
			20	17	0.51	(10)
			40	10	0.38	(14)[c]
	NADP	5	5	31	1.6	(7)
			10	19	1.5	(8)
			20	11	1.4	(8)
			40	9	0.70	(16)
	MUP	5	20	14	—	(8)
			40	8	—	(10)
HRP	TMB	2	5	23	1.7[d]	(3)
			10	13	1.6	(3)
			20	7	1.1	(5)
			40	5	0.52	(10)[c]
	HPPA	1	20	4	—	(5)
β-GAL	*o*-NPG	50	5	51	0.77	(6)
			10	26	0.65	(7)
			20	15	0.45	(10)[c]
			40	11	0.30	(15)
	MUG	10	20	9	—	(6)
			40	8	—	(14)
GOD	TMB–HRP	50	5	36	1.1	(3)
			10	24	0.70	(4)
			20	11	0.41	(7)[c]
			40	7	0.18	(16)
	HPPA–HRP	5	20	6	—	(7)

a) Reciprocal of CET-M8 dilution. *b*) The absorbance obtained after a 1 h enzyme reaction. Figures in parentheses indicate background (%). *c*) The dose–response curves shown in Fig. 2 were obtained with these systems. *d*) Incubation was carried out for 30 min.

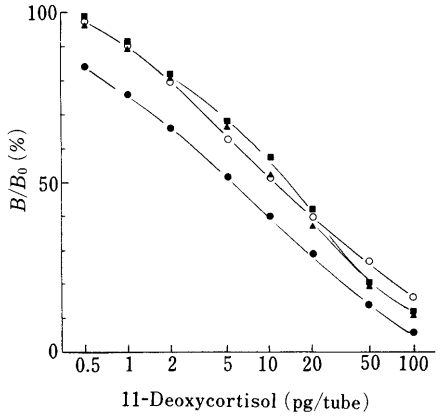


Fig. 2. Dose–Response Curves for Colorimetric 11-Deoxycortisol Enzyme Immunoassays Using HRP (●), AP (▲), β-GAL (■) and GOD (○) Labels

Assay conditions are given in Table II or in the text.

tested by obtaining the dose–response curves for 11-deoxycortisol: the sensitivity was expressed as the amount of 11-deoxycortisol needed to displace 50% of the bound label. The results are listed in Table II; 5, 2, and 50 ng of the AP, HRP, and β-GAL or GOD labels correspond to *ca.* 15, 16, 37, and 108 pg of 11-deoxycortisol, respectively, if the degree of hapten substitution is 1. With all the assay systems, satisfactory sensitivities were obtained. It is clear that a sensitivity value of less than 10 pg can be obtained in all the fluorimetric assay systems and in the colorimetric methods using the HRP and AP (NADP) labels; in the other colorimetric systems, the sensitivity values were 10—20 pg. It should be mentioned that, among the fluorimetric methods, the assay using the β-GAL label did not exceed the system with the HRP label in sensitivity, although the minimal detectable amount of the label or native β-GAL is rather small when compared with that of the latter enzyme.

Dose–response curves for colorimetric 11-deoxycortisol enzyme immunoassays under the optimal conditions for each system are shown in Fig. 2. It can be seen that the highest sensitivity was obtained by the use of HRP, and the AP, GOD and β-GAL labels gave similar results. The minimal detectable amount of 11-deoxycortisol, defined as twice the standard deviation of the zero determination (B_0, $n=10$), was 0.2 pg in the assay with the HRP label; in the cases of the AP, GOD and β-GAL labels, the values were 0.8—1 pg. In the fluorimetric assays, the detection limit values were 0.5—0.6 pg.

The present work showed that, even in the colorimetric enzyme immunoassay, excellent sensitivities can be obtained under appropriate conditions. The affinity constant of the antibody limits the ultimate sensitivity of competitive enzyme immunoassay; in the present assay, the sensitivity value may be 4—6 pg/tube. It should be noted that the steroid/enzyme molar ratio in the active ester method for enzyme labeling and, in general, the bridging phenomenon[6,9] are important factors influencing the sensitivity. The latter problem arises when the antibody shows an affinity for not only the steroid molecule but also the bridge portion in the labeled antigen. The effect of this factor on the present sensitivity must be little, if any, since a heterologous assay system using 11-deoxycortisol 3-(*O*-carboxymethyl)oxime[5b] or 4-hemisuccinyloxy-11-deoxycortisol as a labeling antigen gave a sensitivity similar to that of the assay with CET (data not shown). Interesting findings were obtained upon comparison with the results of the previous testosterone assays, [3b] in which the sensitivities obtained were 9 and 90 pg in the colorimetric assays using HRP- and β-GAL-labeled antigens, respectively. In the present assay systems, the difference in sensitivity between the two enzyme labels was not so large (5 pg *vs.* 15 pg in Fig. 2). Similar features were observed with the fluorimetric assays.[3a] Previously, using β-GAL as a label in bridge heterologous assay systems for cortisol or 11-deoxycortisol, we showed that the bridge length is an important factor influencing the sensitivity, and the use of a shorter bridge for enzyme labeling results in an increase in sensitivity.[9] Thus, the present results on the assay sensitivity, together with the previous findings,[3b,9] suggest that the bridge length effect may depend on the label enzyme. It is hoped that systematic studies will be done to elucidate this important problem.

The information obtained here should be helpful in the further development of enzyme immunoassay for steroid hormones and other haptenic compounds. Studies on the labeling of steroids with other enzymes currently used in enzyme immunoassays and on bridging phenomena are in progress.

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