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## Sensitivity of Steroid Enzyme Immunoassays. Use of 3,3',5,5'-Tetramethylbenzidine as a Chromogen in the Assay System with Glucose Oxidase Label<sup>1)</sup>

HIROSHI HOSODA, REIKO TSUKAMOTO, KOHICHI SHORIKEN,  
WATARU TAKASAKI and TOSHIO NAMBARA\*

*Pharmaceutical Institute, Tohoku University,  
Aobayama, Sendai 980, Japan*

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The use of 3,3',5,5'-tetramethylbenzidine (TMB) as a chromogen in a testosterone enzyme immunoassay system with a glucose oxidase label is described. Glucose oxidase activity was measured with glucose, peroxidase and TMB in acetate-citric acid buffers; the optimum pH value was found to be 4.2. The glucose oxidase-labeled antigen was prepared by the *N*-succinimidyl ester method. In the competitive enzyme immunoassay, the bound and free enzyme-labeled antigens were separated by a double antibody method. A dose-response curve with a high sensitivity was obtained by the use of a minimum amount of the label at an appropriate dilution of anti-testosterone antiserum ( $K_a = 2 \times 10^{10} \text{ M}^{-1}$ ). The amount of testosterone needed to displace 50% of the bound label was 24 pg. The sensitivity was higher than that of the assay system using 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid), ABTS.

**Keywords**—enzyme immunoassay; testosterone; steroid enzyme labeling; *N*-succinimidyl ester method; glucose oxidase; 3,3',5,5'-tetramethylbenzidine; 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)

In recent years, numerous enzyme immunoassays of hormones and drugs have been developed. Enzymes currently used as labels in heterogeneous steroid enzyme immunoassays are alkaline phosphatase (AP), horseradish peroxidase (HRP),  $\beta$ -galactosidase ( $\beta$ -GAL), glucose oxidase (GOD), glucose-6-phosphate dehydrogenase, glucose dehydrogenase, glucosylase,  $\beta$ -lactamase, urease and invertase. The choice of enzyme is based on various criteria such as activity and stability.<sup>2)</sup> In order to obtain a practical basis for selecting the enzyme, we have previously compared the former three enzymes, in a colorimetric immunoassay system, with regard to the sensitivity.<sup>3)</sup>

GOD is also frequently used as an enzyme label. This enzyme catalyzes the oxidation of  $\beta$ -D-glucose to D-glucono- $\delta$ -lactone in the presence of molecular oxygen, yielding hydrogen peroxide; its activity can be measured by a colorimetric method in combination with HRP. Various chromogens are available, but some of these compounds have been shown to be carcinogenic or mutagenic in test systems<sup>4)</sup>; 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), which is mutagenic, has mainly been used in immunoassay systems.<sup>5)</sup> It has been reported that 3,3',5,5'-tetramethylbenzidine (TMB) is a safe and sensitive substrate for HRP<sup>6)</sup>; we have compared six chromogenic substrates including TMB, ABTS and *o*-phenylenediamine.<sup>7)</sup> Little information, however, is available concerning application of the chromogen TMB to GOD assay systems. This paper deals with the sensitivity obtainable with a testosterone enzyme immunoassay using the GOD-TMB system, in comparison with that of the assay using ABTS.

### Materials and Methods

**Materials**—GOD (EC 1.1.3.4) from *Aspergillus niger* (grade I, 281 U/mg) was obtained from Boehringer-

Mannheim Yamanouchi Co. (Tokyo); HRP (EC 1.11.1.7) (grade I-C, 261 units/mg) was from Toyobo Co., Ltd. (Osaka). TMB and ABTS diammonium salt were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo) and Nakarai Chemicals, Ltd. (Kyoto), respectively. The *N*-succinimidyl ester of 4-hydroxytestosterone 4-hemiglutarate was prepared by the method previously established in these laboratories.<sup>8)</sup> Anti-testosterone antiserum used was that reported in the previous paper.<sup>9)</sup> Goat anti-rabbit immunoglobulin G (IgG) antiserum and normal rabbit serum were purchased from Daiichi Radioisotope Labs., Ltd. (Tokyo).

**Buffer Solution**—GOD activity was measured in 0.05 M acetate-citric acid, citric acid-phosphate, acetate, citrate or phosphate buffers. A 0.05 M phosphate buffer (PB), pH 7.3, was used in the enzyme labeling. In the immunoassay procedure, PB containing 0.1% gelatin and 0.9% NaCl (gel-PB) was used.

**Measurement of GOD Activity**—Determination of optimal conditions was carried out as follows: a buffer solution (2 ml) containing chromogen (0.42 mM TMB or 0.91 mM ABTS), D-glucose (0.25–1 M) and HRP (15–50 nM) was added to GOD solution (2 ng, 0.1 ml). In the case of TMB, dimethyl sulfoxide (3%) was used as a solvent. The mixture was incubated at 37 °C for 40–60 min. The reaction was terminated by addition of a stopping reagent (2 ml). The absorbance was then measured at 450 nm for TMB or 418 nm for ABTS.

**Preparation of GOD-Labeled Antigen**—This was carried out in the manner described previously.<sup>10)</sup> In short, a solution of the testosterone *N*-succinimidyl ester (29 µg) in dioxane (0.15 ml) was added to a solution of GOD (1.5 mg, M.W. 160000) in PB (0.3 ml) at 0 °C, and the mixture was gently stirred at 4 °C for 4 h. After dialysis against cold PB, the solution was stored at a concentration of 500 µg/ml, adjusted with gel-PB.

**Enzyme Immunoassay Procedure**—This was carried out in duplicate or triplicate in a glass test tube (10 ml). A solution of testosterone (0–500 pg) in gel-PB (0.1 ml) and GOD-labeled testosterone (20–100 ng) in the buffer (0.1 ml) containing 0.5% normal rabbit serum were added to diluted anti-testosterone antiserum (0.1 ml), and the mixture was incubated at 4 °C for 4 h. Goat anti-rabbit IgG antiserum (0.1 ml) diluted 1:30 with gel-PB containing 0.3% ethylenediaminetetraacetic acid was added to the incubation mixture, and the solution was vortex-mixed, then allowed to stand at 4 °C for 16 h. After addition of gel-PB (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 gel-PB (1.5 ml), and used for measurement of the enzymic activity ( $B_0$  or  $B$ ). At the same time, the procedure without addition of the first antibody was carried out to provide a non-specific binding (background) value.

**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 1–1.5 h. The reaction was terminated by addition of 0.5 M H<sub>2</sub>SO<sub>4</sub> (2 ml). The absorbance was measured at 450 nm. The background was estimated as a percentage of the intensity for  $B_0$ , using distilled water as the zero reference.

**ABTS Method:** The immune precipitate was diluted with 0.05 M citric acid-phosphate buffer, pH 5.5 (2 ml), containing 0.91 mM ABTS, 0.5 M glucose, and 25 nM HRP, vortex-mixed, and incubated at 37 °C for 1–1.5 h. The reaction was terminated by addition of 0.01% NaN<sub>3</sub>–0.5% CuSO<sub>4</sub> (2 ml). The absorbance was measured at 418 nm; the zero reference used consisted of the stopping reagent diluted 1:1 with the buffer.

## Results and Discussion

The purpose of this work was to assess TMB as a chromogen in the testosterone enzyme immunoassay system using the GOD label. Optimal assay conditions for GOD determination were first examined, and then applied in the enzyme immunoassay method. For comparison, the assay with ABTS was also carried out.

### Optimization of GOD Determination

The measurement of enzymic activity was carried out using 6 pM GOD and 0.4 mM TMB. No significant difference in intensity of the color at 450 nm was observed when D-glucose concentrations ranging from 0.25 to 1 M were used in combination with 15–50 nM HRP. GOD was thus incubated at pH 3.5–7.0 with 0.4 mM TMB, 0.5 M glucose and 25 nM HRP in 0.05 M acetate-citric acid buffer, containing 3% dimethyl sulfoxide. The effect of pH on the color intensity is shown in Fig. 1. The maximum intensity was obtained at pH 4.2. Incubations in 0.05 M acetate, citrate and citric acid-phosphate buffers gave similar results (data not shown). Stopping of the enzyme reaction was done with sulfuric acid (Table I).

In the case of ABTS, problems were encountered. Sulfuric acid<sup>5a)</sup> or sodium azide<sup>5b)</sup> has been used as a stopping agent in immunoassay systems, but these agents were not satisfactory, because of the instability of the developed color (418 nm). We tested the combination of

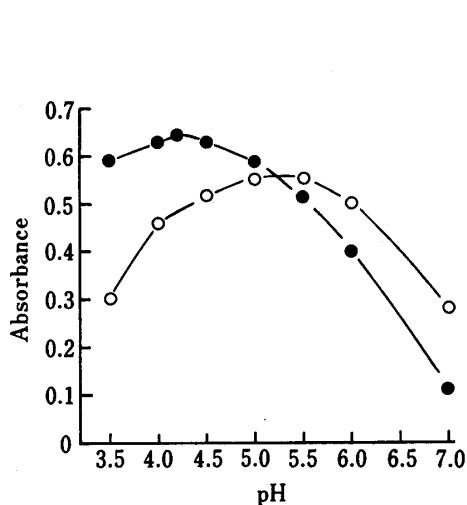


Fig. 1. Effect of pH on Absorbance in Assays Using TMB (●) and ABTS (○)

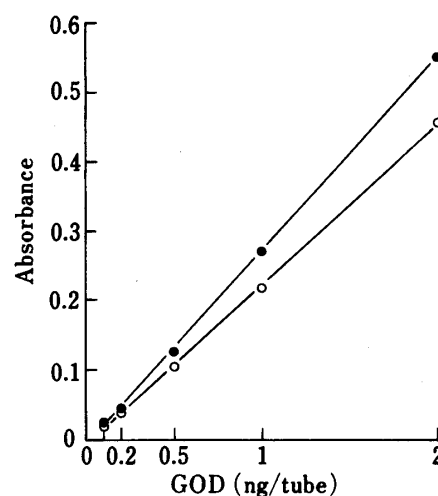


Fig. 2. Relationship between Absorbance and Amount of GOD in Assays Using TMB (●) and ABTS (○)

TABLE I. Effect of the Stopping Reagents on Stability of Absorbance<sup>a)</sup>

Time (min)	TMB		ABTS		
	0.5 M H <sub>2</sub> SO <sub>4</sub>	0.5 M H <sub>2</sub> SO <sub>4</sub>	0.5% NaN <sub>3</sub>	0.05% NaN <sub>3</sub>	0.01% NaN <sub>3</sub> - 0.5% CuSO <sub>4</sub>
0	0.511	0.441	0.436	0.444	0.434
90	0.497	0.339	0.015	0.582	0.453

a) Absorbance values (TMB 450 nm, ABTS 418 nm) were obtained 90 min and within 10 min (time 0) after addition of the stopping reagent.

sodium azide and Cu<sup>2+</sup> as inhibitors for HRP or GOD<sup>11)</sup>; suitable initial concentrations were found to be 0.01% sodium azide–0.5% cupric sulfate, as shown in Table I. GOD was incubated in 0.05 M citric acid–phosphate buffers, containing 0.9 mM ABTS, 0.5 M glucose and 25 nM HRP; the optimum pH value was 5.5 (Fig. 1). The assay in 0.05 M citrate buffers gave similar results, whereas acetate, acetate–citric acid and phosphate buffers were no good owing to the insolubility of the stopping reagent.

Relationship between absorbance and GOD concentration, under the optimal conditions with each chromogen, is shown in Fig. 2. In both cases, a linear relationship was observed, and the minimal detectable amount of GOD was *ca.* 100 pg per tube. It can be seen, however, that TMB gives a more sensitive response than ABTS.

### Enzyme Immunoassay Method

In order to control the molar ratio of steroid to enzyme in a labeled antigen, since this influences assay sensitivity, enzyme labeling was carried out by the *N*-succinimidyl ester method.<sup>10)</sup> The *N*-succinimidyl ester of 4-hydroxytestosterone 4-hemiglutarate was treated with GOD in phosphate buffer (pH 7.3)–dioxane (Fig. 3). The activated ester should react readily with free amino groups of this enzyme. The steroid/enzyme molar ratio employed in the coupling reaction was 6. Selection of the ratio was based on both immunoreactivity of the labeled antigen and sensitivity of the assay, namely, on a balance between sensitivity and precision of assay.<sup>10)</sup> The conjugate was dialyzed against the buffer to remove the unreacted steroid. No significant loss of enzymic activity was observed under the coupling conditions used. Spectrometric analysis showed that the degree of hapten substitution was *ca.* 2. With

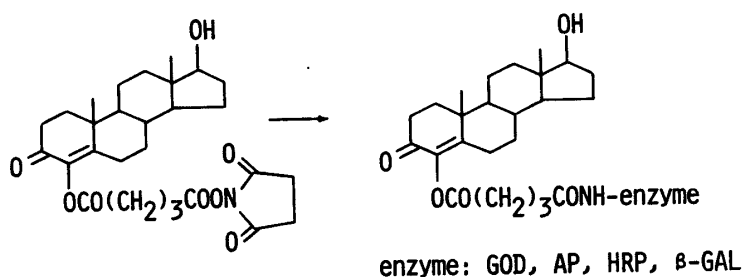


Fig. 3. Preparation of Enzyme-Labeled Antigen

TABLE II. Inhibition of Bound Enzymic Activity of the GOD-Labeled Antigen by 50 pg of Testosterone

Amount of label (ng)	Antiserum dilution	TMB		ABTS	
		Inhibition (%)	Absorbance <sup>a)</sup>	Inhibition (%)	Absorbance <sup>a)</sup>
100	1:10000	54	0.53 (13)	51	0.47 (14)
	1:15000	60	0.43 (16)	54	0.34 (20)
50	1:8000	53	0.41 (5)	44	0.37 (12)
	1:10000	65	0.35 (6) <sup>b)</sup>	57	0.31 (15) <sup>b)</sup>
	1:15000	66	0.24 (8)	62	0.22 (21)
20	1:2000	25	0.28 (5)	22	0.20 (20)
	1:5000	36	0.23 (6)	42	0.18 (23)
	1:8000	62	0.19 (7)	62	0.15 (27)

a) Absorbance value for  $B_0$  obtained after a 1 h enzyme reaction. Figures in parentheses indicate non-specific binding (background) (%). b) The dose-response curves shown in Fig. 4 were obtained with these systems.

this label, a pH effect similar to that with native GOD was observed on color intensity in assays using TMB and ABTS.

The anti-testosterone antiserum used was that elicited in a rabbit by immunization with the conjugate of 4-hydroxytestosterone 4-hemiglutarate with bovine serum albumin, that is, the combination of antibody and labeled antigen is homologous.<sup>12)</sup> The ultimate sensitivity of competitive enzyme immunoassay depends on the affinity of the antibody. The present antiserum has an affinity constant of  $2 \times 10^{10} \text{ M}^{-1}$  for testosterone in the radioimmunoassay procedure,<sup>9)</sup> and showed weak, if any, binding affinity for the bridge portion between enzyme and steroid in a labeled antigen.<sup>13)</sup> 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 the colorimetric methods described above.

Sensitivities obtainable with the assay systems were tested by examining the inhibition of enzymic activity caused by the addition of 50 pg of testosterone per tube, in which various amounts of the GOD label and dilutions of the anti-steroid antiserum were used. The assays were assessed in terms of the absorbance for  $B_0$  and non-specific binding (background). The criteria that the absorbance obtained upon 1 h enzymic reaction and the background should be over 0.2 and less than 20%, respectively, were employed in this work. The results are listed in Table II. It is clear that the immunoassay using TMB is more sensitive than that with ABTS. The former assay gave satisfactory results with respect to the background.

Dose-response curves for testosterone enzyme immunoassays under the optimal conditions for each system are shown in Fig. 4. It can be seen that the assay using TMB is highly sensitive; the amount of testosterone needed to displace 50% of the bound label is 24 pg. Taking account of the amount of the enzyme-labeled antigen fixed (50 ng of the label corresponds to ca. 90 pg of testosterone, if the degree of hapten substitution is 1), the sensitivity of the assay using the GOD label is rather high. A possible explanation for this is

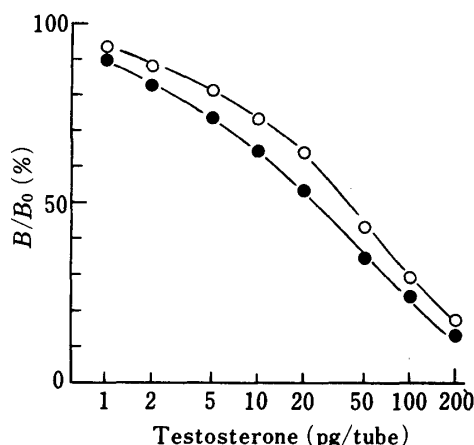


Fig. 4. Dose-Response Curves for Testosterone Enzyme Immunoassays Using TMB (●) and ABTS (○)

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

TABLE III. Sensitivity of Colorimetric Enzyme Immunoassays for Testosterone Using Various Enzyme Labels

Enzyme label	Molar ratio <sup>a)</sup>	Detection <sup>b)</sup>	Amount of label <sup>c)</sup> (ng)	Antiserum dilution	Sensitivity <sup>d)</sup> (pg)
AP	30 (1)	<i>p</i> -NP	10 (24)	1:25000	50
HRP	30 (2)	TMB	2 (14)	1:30000	9
$\beta$ -GAL	10 (3)	<i>o</i> -NPG	50 (25)	1:10000	90
GOD	6 (2)	TMB-HRP	50 (90)	1:10000	24

a) Steroid/enzyme molar ratios in the enzyme labeling by the *N*-succinimidyl ester method. Figures in parentheses indicate degrees of hapten substitution. b) Substrate or chromogen systems in the measurement of enzymic activity. c) Figures in parentheses indicate the amount of testosterone (pg), which is calculated by assuming that the degree of hapten substitution is 1. d) The amount of testosterone needed to displace 50% of the bound label in each dose-response curve. Absorbance values for  $B_0$  obtained after a 1 h enzyme reaction were over 0.2.

that the reactivity of the label with the anti-steroid antiserum is relatively low owing to steric hindrance. The sensitivity is approximately one-half of that of the fluorimetric assay using 3-(*p*-hydroxyphenyl)propionic acid.<sup>10)</sup> Direct comparison can also be done with the previous colorimetric assays using AP, HRP and  $\beta$ -GAL as labels,<sup>3)</sup> according to our criteria (Fig. 3, Table III). The present sensitivity is low when compared with that of the assay using the HRP label, but higher than those with the AP-*p*-nitrophenyl phosphate (*p*-NP) and  $\beta$ -GAL-*o*-nitrophenyl  $\beta$ -D-galactopyranoside (*o*-NPG) systems. We are interested in the result on the order of assay sensitivity; this problem is under investigation. The minimal detectable amounts of testosterone, that is, twice the standard deviation of the zero determination ( $B_0$ ,  $n=10$ ), were 2 and 3 pg in the present assays using TMB and ABTS, respectively.

It should be noted that other chromogens, such as *o*-phenylenediamine, 5-aminosalicylic acid, 3-amino-9-ethylcarbazole and 3-methyl-2-benzothiazolinone hydrazone, which gave satisfactory sensitivities when used in the enzyme immunoassay with HRP as a label,<sup>7)</sup> were less sensitive (data not shown). To our knowledge, this is the first application of TMB in heterogeneous hapten immunoassay using a GOD label, although this chromogen has been employed in a dry-reagent strip system.<sup>14)</sup> The high sensitivities of the present and other enzyme systems<sup>3,10)</sup> are ascribable, in part, to the use of an appropriate molar ratio of steroid to enzyme in the enzyme labeling by the *N*-succinimidyl ester method. We recommend molar ratios of 4–8, 20–40, 10–60, and 10–20 in the GOD, AP, HRP, and  $\beta$ -GAL labelings, respectively.

The information obtained here, together with the previous findings on sensitivity of the

assays using AP, HRP and  $\beta$ -GAL, should be helpful as a practical basis for selecting the label enzyme in the further development of hapten enzyme immunoassays.

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#### References and Notes

- 1) Part CCXXXVII of "Studies on Steroids" by T. Nambara; Part CCXXXVI: T. Ohkubo, F. Tsuchiko and T. Nambara, *Chem. Pharm. Bull.*, **36**, 419 (1988).
- 2) G. B. Wisdom, *Clin. Chem.*, **22**, 1243 (1976); A. H. W. M. Schuurs and B. K. Van Weemen, *Clin. Chim. Acta*, **81**, 1 (1977).
- 3) H. Hosoda, W. Takasaki, R. Tsukamoto and T. Nambara, *Chem. Pharm. Bull.*, **35**, 3336 (1987).
- 4) C. E. Voogd, J. J. Van der Stel and J. J. J. A. A. Jacobs, *J. Immunol. Methods*, **36**, 55 (1980); R. H. Yolken, *Rev. Infect. Diseases*, **4**, 35 (1982).
- 5) a) K. Kawamura, M. Tanaka, F. Kamiyama, K. Higashino and S. Kishimoto, *Clin. Chim. Acta*, **131**, 101 (1983); b) R. Maiolini, B. Ferrua and R. Masseyeff, *J. Immunol. Methods*, **6**, 355 (1975); c) W. H. W. Albert, G. Kleinhammer, R. Linke, P. Tanswell and F. Staehler, "Enzyme Labelled Immunoassay of Hormones and Drugs," ed. by S. B. Pal, Walter de Gruyter, Berlin, 1978, p. 153; G. Holder, *ibid.*, p. 221; R. B. Johnson, Jr., R. M. Libby and R. M. Nakamura, *J. Immunoassay*, **1**, 27 (1980).
- 6) E. S. Bos, A. A. Van der Doelen, N. Van Rooy and A. H. W. M. Schuurs, *J. Immunoassay*, **2**, 187 (1981).
- 7) H. Hosoda, W. Takasaki, T. Oe, R. Tsukamoto and T. Nambara, *Chem. Pharm. Bull.*, **34**, 4177 (1986).
- 8) H. Hosoda, Y. Sakai, H. Yoshida, S. Miyairi, K. Ishii and T. Nambara, *Chem. Pharm. Bull.*, **27**, 742 (1979).
- 9) H. Hosoda, K. Tadano, S. Miyairi and T. Nambara, *J. Steroid Biochem.*, **10**, 513 (1979).
- 10) H. Hosoda, R. Tsukamoto, M. Shishido, W. Takasaki and T. Nambara, *Chem. Pharm. Bull.*, **35**, 4856 (1987).
- 11) S. Nakamura and Y. Ogura, *J. Biochem. (Tokyo)*, **64**, 439 (1968).
- 12) B. K. Van Weemen and A. H. W. M. Schuurs, *Immunochemistry*, **12**, 667 (1975); H. Hosoda, N. Kobayashi, N. Ishii and T. Nambara, *Chem. Pharm. Bull.*, **34**, 2105 (1986).
- 13) H. Hosoda, H. Yoshida, Y. Sakai, S. Miyairi and T. Nambara, *Chem. Pharm. Bull.*, **28**, 3035 (1980).
- 14) P. Rupchock, R. Sommer, A. Greenquist, R. Tyhach, B. Walter and A. Zipp, *Clin. Chem.*, **31**, 737 (1985).