Enzyme Labeling in Steroid Enzyme Immunoassays. Comparison of the *p*-Nitrophenyl Ester and *N*-Succinimidyl Ester Methods

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Enzyme labeling of steroids by the *p*-nitrophenyl ester method was investigated in comparison with the *N*-succinimidyl ester method. The active ester of a testosterone or 11-deoxycortisol derivative was treated with β-galactosidase and horseradish peroxidase to give labeled antigens. Various molar ratios of steroid to enzyme and pH conditions were tested. Satisfactory immunoreactivities with an anti-steroid antibody in each enzyme immunoassay system were obtained with the labeled antigens prepared at pH 8.5 by the use of molar ratios higher than 30. The enzyme labeling method should be useful in the case of polar steroids or drugs, since the *p*-nitrophenyl ester is relatively stable when compared with the *N*-succinimidyl ester.

Keywords enzyme immunoassay; steroid enzyme labeling; *p*-nitrophenyl ester methods; *N*-succinimidyl ester method; β-galactosidase; horseradish peroxidase; testosterone; 11- deoxycortisol

Enzyme-labeled antigens for use in the enzyme immunoassay of steroid hormones have usually been prepared by condensation of the carboxyl groups of a steroid hapten with the amino groups of lysine residues in an enzyme. The sensitivity and reproducibility of enzyme immunoassays are influenced by the coupling method. We have previously shown that the *N*-succinimidyl (NS) ester method is useful for alkaline phosphatase,[1] β-galactosidase (β-GAL),[2] horseradish peroxidase (HRP)[3] and glucose oxidase[4] labelings, as an alternative to the conventional methods, such as the mixed anhydride and carbodiimide methods. In general, the NS ester is stable when stored as a crystalline at 4 °C or in dioxane,[5] and hence, the active ester method is convenient for the reproducibility of enzyme immunoassay, especially, in systematic studies for obtaining a practical basis for selecting the enzyme, and on factors influencing the assay sensitivity. In the cases of some polar steroids or drugs, however, the preparation of the NS ester is troublesome because of its instability under purification and storing conditions. The activation of the carboxyl group as the *p*-nitrophenyl (*p*-NP) ester[6] may be the method of choice in such a case. This paper deals with the *p*-NP ester method in polyclonal testosterone and monoclonal 11-deoxycortisol assay systems using β-GAL and HRP as label enzymes, in comparison with the NS ester method.

Materials and Methods

Melting points were taken on a micro hot-stage apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-4 in $CHCl_3$. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were taken with a JEOL JNM-FX-100 spectrometer at 100 MHz using tetramethylsilane as an internal standard. Enzymic activity was measured with a Hitachi spectrophotometer 100-20.

Materials β-GAL (EC 3.2.1.23) from *Escherichia coli* (grade VI, 435 units/mg), and HRP (EC 1.11.1.7) (grade I-C, Reinheits-Zahl 3.15, 250 units/mg) were obtained from Sigma Chemical Co. (U.S.A.) and Toyobo Co. (Osaka), respectively. Anti-testosterone antiserum[7] and the monoclonal anti-11-deoxycortisol antibody[8] used were those reported in the previous papers. Normal rabbit serum and goat anti-rabbit immunoglobulin G (IgG) antiserum were purchased from Daiichi Radioisotope Labs., Ltd. (Tokyo); normal mouse serum and rabbit anti-mouse IgG antiserum were from MBL Co. (Nagoya). 3,3′,5,5′-Tetramethylbenzidine and *o*-nitrophenyl β-D-galactopyranoside were obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo) and Nacalai Tesque Inc. (Kyoto), respectively.

Synthesis of Steroid NS Esters The NS esters of 4-hydroxytestosterone 4-hemiglutarate (T·HG),[7] 4-(carboxymethylthio)testosterone (T·CMT), 4-(2-carboxyethylthio)testosterone (T·CET),[9] and 4-(2-carboxyethylthio)-11-deoxycortisol (S·CET)[10] were prepared by the method described previously.[9] In short, a solution of the carboxylated steroids (0.5 mmol), *N*-hydroxysuccinimide (0.7 mmol), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl (0.7 mmol) in 95% dioxane (2 ml) was stirred at room temperature for 2 h. The resulting solution was diluted with AcOEt, washed with H_2O, dried over anhydrous Na_2SO_4, and passed through an Al_2O_3 layer. The filtrate was evaporated down to give the active ester.

General Procedure for the Preparation of Steroid *p*-NP Esters A solution of the carboxylated steroids (T·HG, T·CMT, T·CET, S·CET) (1 mmol), *p*-nitrophenol (1.3 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl (1.8 mmol) in 95% dioxane (5 ml) was stirred overnight at room temperature. The resulting solution was diluted with AcOEt, washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated down. The product was purified by chromatography on silica gel using hexane–AcOEt as an eluent, and recrystallized from an appropriate solvent.

T·HG *p*-NP Ester Colorless semi-crystals. ^{1}H-NMR ($CDCl_3$) δ: 0.80 (3H, s, 18-CH_3), 1.27 (3H, s, 19-CH_3), 2.4—2.9 (4H, 4-$COCH_2CH_2\underline{CH_2}CO$), 3.65 (1H, t, J=8 Hz, 17α-H), 7.30, 8.30 (each 2H, d, J=9 Hz, aromatic H).

T·CMT *p*-NP Ester Colorless needles from acetone–hexane. mp 135—137 °C. $[\alpha]_D^{20}$ +86° (c=0.4). *Anal.* Calcd for $C_{27}H_{33}NO_6S$: C, 64.91; H, 6.66; N, 2.80. Found: C, 64.88; H, 6.84; N, 2.74.

T·CET *p*-NP Ester Colorless needles from acetone–hexane. mp 112—114 °C. $[\alpha]_D^{20}$ +96° (c=0.4). *Anal.* Calcd for $C_{28}H_{35}NO_6S$: C, 65.48; H, 6.87; N, 2.73. Found: C, 65.28; H, 6.86; N, 2.64.

S·CET *p*-NP Ester Colorless semi-crystals. ^{1}H-NMR ($CDCl_3$) δ: 0.71 (3H, s, 18-CH_3), 1.23 (3H, s, 19-CH_3), 2.6—3.2 (4H, 4-SCH_2CH_2CO), 3.70 (1H, m, 6α-H), 4.30, 4.68 (each 1H, d, J=20 Hz, 21-H), 7.31, 8.28 (each 2H, d, J=9 Hz, aromatic H).

Buffer Solution 0.05 M Phosphate buffers (PBs), pH 5.4—8.0, were used in the study on stability and reactivity with glycine of the active ester, and in the enzyme labeling. 0.05 M Borate buffers (pH 8.5—9.0) were also employed in the labeling reaction. In the immunoassay, PB (pH 7.3) containing 0.1% gelatin and 0.9% NaCl was used.

Determination of Hydrolysis Rate of the Steroid NS and *p*-NP Esters PBs, pH 5.4—8.0 (0.2 ml), were each added to a solution of the CET NS or *p*-NP ester (0.05 mmol) in dioxane (0.1 ml), and the solution was allowed to stand at 26 °C. An aliquot of the reaction mixture was subjected to thin-layer chromatography (TLC) on silica gel using AcOEt–MeOH (4:1) as a developing solvent. The ratio of the remaining ester and hydrolyzate was determined with a Shimadzu dual-wavelength TLC scanner (240 nm); the rate of hydrolysis was calculated on the basis of the ratio values, since no side reaction occurred under the conditions tested.

Reaction of the Steroid NS and *p*-NP Esters with Glycine Glycine (0.2 mmol) in dioxane (0.1 ml) was added to a solution of T·CET NS or *p*-NP ester (0.05 mmol) in PBs, pH 5.4—8.0 (0.2 ml), and the solution was allowed to stand at 4 °C. An aliquot of the reaction mixture was subjected to TLC on silica gel using $CHCl_3$–MeOH–AcOH (40:5:0.4) as a developing solvent. The yield of the product was determined with the TLC scanner described above.

Enzyme Labeling of Steroid by the Active Ester Methods Dioxane solutions (0.1 ml) containing calculated amounts of the NS or *p*-NP ester

corresponding to steroid/enzyme molar ratios of 10—100 (molecular weight of HRP,[11] 44000; β-GAl,[12] 465000) were each added to a solution of HRP or β-GAL (200 μg) in PB (pH 5.4—8.0, 0.2 ml) at 0 °C, and the mixture was gently stirred at 4 °C for 4 h. After addition of the corresponding PB (1.2 ml), the resulting solution was dialyzed against the cold buffer (2 l) for 2 d, then against PB, pH 7.3 (1 l), for 12 h. A 1 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 the assay buffer.

In the practical *p*-NP and NS ester methods, the β-GAL or HRP labeling was carried out with 1 mg of enzyme at pH 7.3—9.0 in dioxane (0.1 ml)–buffer (0.2 ml). The steroid/enzyme molar ratios examined were 10—100 in both labeliongs. The buffer used for dialysis was PB, pH 7.3.

Enzyme Immunoassay Procedure This was carried out in triplicate in glass test tubes (10 ml). The standard procedure for immunoreactivity of the testosterone labels is as follows: HRP- (2 ng) or β-GAL-labeled antigen (100 ng) in the assay buffer (0.1 ml) containing 0.5% normal rabbit serum was added to the diluted anti-testosterone antiserum (0.1 ml), and the mixture was incubated at 4 °C for 4 h (first incubation). Goat anti-rabbit IgG antiserum (0.1 ml) diluted 1 : 30 with the assay buffer 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 the assay buffer (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 assay buffer (1.5 ml), and used for measurement of the enzymic activity (B_0).

In the monoclonal 11-deoxycortisol assay system,[8b] the first incubation procedure using monoclonal anti-11-deoxycortisol antibody and enzyme-labeled 11-deoxycortisol was carried out in a manner similar to that described above. *B*/*F* separation was then carried out, using rabbit anti-mouse IgG antiserum diluted 1 : 120 with the assay buffer and 0.5% normal mouse serum.[13]

In each system, the procedure without addition of the first antibody was carried out to provide non-specific binding values. An experiment using only the enzyme label was also carried out to obtain 100% enzymic activity (*T*).

Measurement of HRP Activity The immune precipitate or enzyme solution was diluted with 0.05 M acetate–citric acid buffer, pH 4.2 (1.8 ml), containing 0.42 mM 3,3′,5,5′-tetramethylbenzidine and 3% dimethylsulfoxide, 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 the addition of 0.5 M H_2SO_4 (2 ml) and the absorbance was measured at 450 nm.

Measurement of β-GAL Activity The immune precipitate or enzyme solution was diluted with the assay buffer (1 ml) containing 0.1% $MgCl_2$ and 10% ethylene glycol, vortex-mixed, and preincubated at 37 °C for 3 min. *o*-Nitrophenyl β-D-galactopyranoside (0.06%, 1 ml) in the assay buffer was added to the resulting solution, and the mixture was incubated for 1 h. The reaction was terminated by the addition of 1 M Na_2CO_3 (2 ml) and the absorbance was measured at 420 nm.

Results and Discussion

The purpose of this work was to assess the *p*-NP ester method as an enzyme labeling technique, in comparison with the NS ester method. It has been suggested that the *p*-NP ester is more stable than the NS ester.[14] Therefore, we compared the two active esters in the practical enzyme immunoassay procedure for the determination of steroids. The steroid derivatives used as haptens in this study were T·HG, T·CMT, and T·CET in the testosterone assay system. The aim of the employment of the various haptens was to examine the effect of the bridge length on the immunoreactivity of the labeled steroids prepared by the labeling methods. In order to test the applicability of the enzyme labeling at an higher pH, the monoclonal assay system for alkaline-sensitive 11-deoxycortisol was also studied. The *p*-NP and NS esters were prepared from the carboxylated derivatives by condensation with *p*-nitrophenol or *N*-hydroxysuccinimide in the presence of a water-soluble carbodiimide.

First, the stability of the *p*-NP and NS esters of CET was studied. The hydrolysis of the esters was investigated at pH 5.4—8.0 in 0.05 M phosphate buffer–dioxane. The rate of the reaction could be followed by the ratio of ultraviolet absorbance of the α,β-unsaturated ketone system in the ester and hydrolyzate at 240 nm on the TLC. The effect of pH on the stability of the esters of T·CET is shown in Fig. 1. It can be seen that the *p*-NP ester is more stable than the NS ester under the conditions tested. Both the esters were relatively stable at pH 5.4. The rate of hydrolysis increased with increasing pH value, where pseudo-first-order kinetics

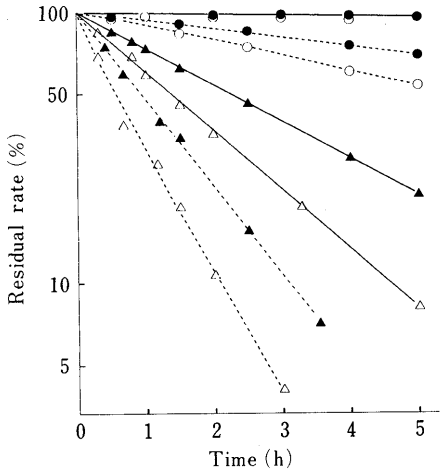
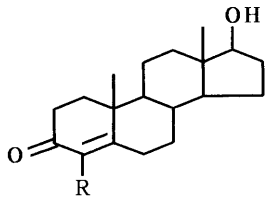
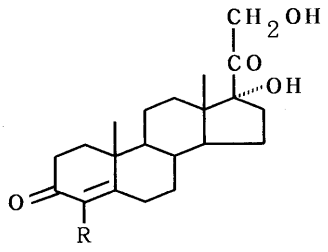


Fig. 1. Stabilities of the *p*-NP (—) and NS (---) Esters of T·CET at pH 5.4 (●), 6.2 (○), 7.3 (▲) or 8.0 (△) in Aqueous Medium



testosterone: R=H
T·CMT: R=SCH_2COOH
T·CET: R=$S(CH_2)_2COOH$
T·HG: R=$OCO(CH_2)_3COOH$



11-deoxycortisol: R=H
S·CET: R=$S(CH_2)_2COOH$

Chart 1

were observed. The reaction rate of the NS ester is 2—3 times as fast as that of the *p*-NP ester at pH 7.3—8.0. The half-time values of the hydrolysis at pH 7.3 were 55 and 135 min with the NS and *p*-NP esters, respectively. With the esters of S·CET, similar results were obtained.

Next, reactivities of the active esters of T·CET with the amino group of glycine were examined. The *p*-NP and NS esters were treated with 4 eq of glycine at various pHs in phosphate buffer–dioxane. The effect of pH on the reactivity is shown in Fig. 2. Distinct differences in reactivity can be observed. With the NS ester at pH 7.3, the formation of the product was efficient, resulting in almost 100% yield after the reaction time of 20 min. In the case of the *p*-NP ester, the yield was only 5% after 1 h at pH 7.3, despite the fact that a large amount of the starting compound still remained intact without being hydrolyzed. At pH 8.0, the reactivity of the *p*-NP ester was somewhat increased, but less than that of the NS ester at pH 6.2.

Immunoreactivities obtainable with the assay systems using the enzyme-labeled antigen prepared by the active ester method were then studied (Fig. 3). Two enzymes having a relatively high or low molecular weight, β-GAL and HRP, were employed as labels, since the molecular size of enzymes is a possible factor influencing the labeling and immune reactions. The enzyme labeling was first carried out at pH 5.4—8.0 by mixing the active ester with the enzyme in phosphate buffer–dioxane at 4 °C for 4 h. The active ester should react with free amino groups of these enzymes. Various molar ratios of the steroid to enzyme, ranging from 10 to 100, were used. The reaction mixtures were dialyzed against the buffer to remove the unreacted steroid and *p*-nitrophenol or *N*-hydroxysuccinimide. The loss of enzymic activity was less than 20% under the coupling conditions used. Determination of the number of steroid molecules incorporated per enzyme molecule was not essential for the present purpose, and hence, was not carried out. The anti-testosterone antiserum used in the enzyme immunoassay was that obtained in a rabbit by immunization with the conjugate of T·HG with bovine serum albumin (BSA).[7] In the system with the 11-deoxycortisol label, the monoclonal anti-steroid antibody was employed, which was secreted from the hybridoma derived from fusion of P3-NS1/1-Ag4-1 myeloma cells with spleen cells of BALB/c mice immunized with S·CET linked with BSA.[8a] The bound and free enzyme-labeled antigens were separated by a double antibody method in both assay systems. The enzymic activity of immune precipitate was determined by colorimetric methods using 3,3′,5,5′-tetramethylbenzidine for HRP and *o*-nitrophenyl β-D-galactopyranoside for β-GAL as substrates.

The binding abilities of a fixed amount of the labeled antigens, in the testosterone system, were investigated at 1 : 500 dilution of the anti-testosterone antiserum. The results on the immunoreactivity of the enzyme-labeled T·HG, T·CMT and T·CET prepared at pH 7.3 by the active ester methods employing various steroid/enzyme molar ratios are shown in Fig. 4. In the assay systems with the β-GAL and HRP labels prepared by the NS ester method, the immunoreactivity (B_0/T) increased with increasing molar ratio in the labelings and satisfactory binding abilities were obtained with molar ratios higher than 20. On the other hand, the *p*-NP ester method gave much less reactive labels under the conditions tested in both enzyme systems. Although the difference between the two active ester methods in the effect of bridge length on the immunoreactivity was not significant in the present case, this factor may be important in some haptenic derivatives showing the steric hindrance. In Fig. 5, the results on the reactivity of the enzyme-labeled T·HG prepared at various pHs are shown. With the NS ester method, high reactivities were obtained at pH 8.0 as well as at pH 7.3 and lower reactivities were observed at pH 5.4—6.2. In contrast, the *p*-NP method did not give satisfactory results with respect to immunoreactivity, especially in the case of HRP labeling; this gave a label showing a very low reactivity even at pH 8.0. Similar results were obtained in the monoclonal 11-deoxycortisol assay system (data not shown). The results of the difference in reactivity between the HRP and β-GAL labels are reasonably related to the numbers of lysine residues in the enzyme molecules; the values have been

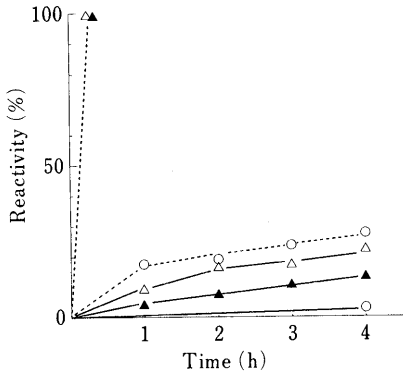


Fig. 2. Reactivities of the *p*-NP (—) and NS (---) Esters of T·CET with Glycine at pH 6.2 (○), 7.3 (▲) or 8.0 (△)

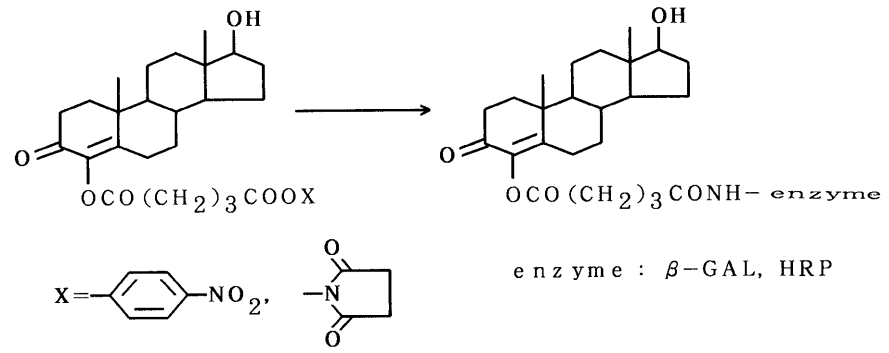


Fig. 3. Enzyme Labeling of T·HG by the *p*-NP and NS Ester Methods

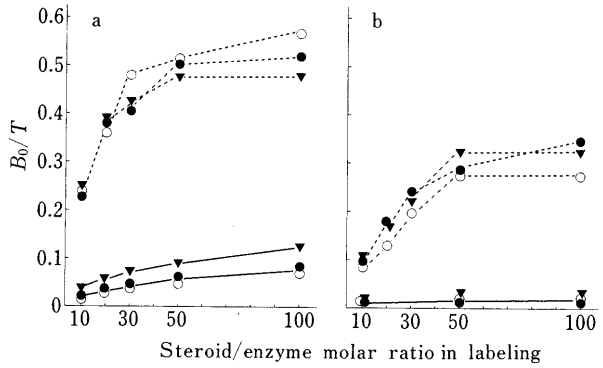


Fig. 4. Binding Abilities of the β-GAL- (a) and HRP-Labeled (b) T·HG (●), T·CMT (▼) and T·CET (○) Prepared at pH 7.3 by the *p*-NP (—) and NS (---) Ester Methods Using Various Steroid/Enzyme Molar Ratios

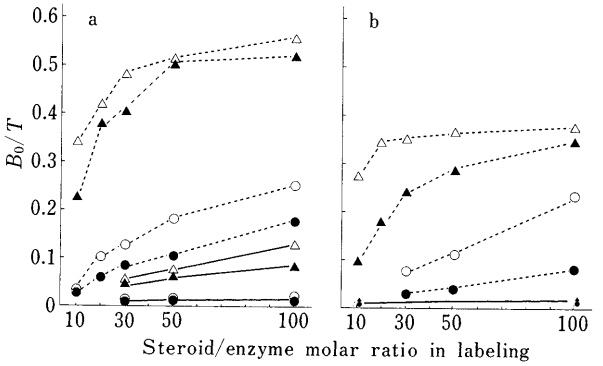


Fig. 5. Binding Abilities of the β-GAL- (a) and HRP-Labeled (b) T·HG Prepared at pH 5.4 (●), 6.2 (○), 7.3 (▲) or 8.0 (△) by the *p*-NP (—) and NS (---) Ester Methods Using Various Steroid/Enzyme Molar Ratios

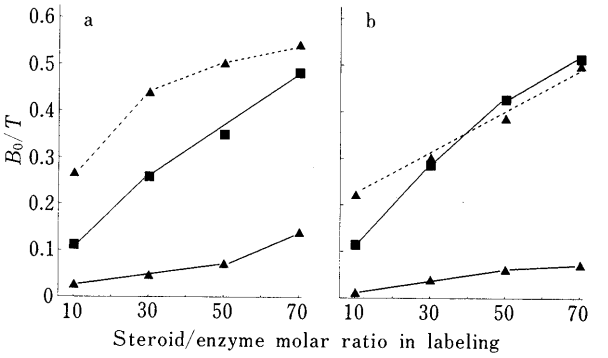


Fig. 6. Binding Abilities of the β-GAL- (a) and HRP-Labeled (b) S·CET Prepared at pH 7.3 (▲) or 8.5 (■) by the Practical *p*-NP (—) and NS (---) Ester Methods Using Various Steroid/Enzyme Molar Ratios

reported to be 6 for HRP[11] and 80 for β-GAL.[12]

Finally, based on the findings obtained above, we explored the practical enzyme labeling conditions in the *p*-NP ester method, using various alkaline conditions, steroid/enzyme molar ratios and reaction times. It was found, in both β-GAL and HRP labelings, that when the labeling was carried out at pH 8.5 for 4 h with the steroid/enzyme molar ratio of higher than 30, the *p*-NP ester method gave satisfactory results comparable to those obtained with an appropriate label prepared by the NS ester method. The results obtained with the monoclonal 11-deoxycortisol assay system are shown in Fig. 6. In testosterone and 11-deoxycortisol assay systems using the labeled antigen prepared at a molar ratio of 30, the desired dose-response curves for the steroids with a high sensitivity equal to that in the previous works[15] could be obtained (data not shown). The use of a higher pH value than 9 resulted in the loss of β-GAL activity. With the HRP labeling, a somewhat higher pH can be used, but it is not effective in increasing the immunoreactivity; with the 11-deoxycortisol assay system, a marked decrease in the reactivity was observed. This is ascribable to the instability of the dihydroxy acetone chain in the 11-deoxycortisol molecule. The standard conditions in the NS[3] and *p*-NP ester methods for β-GAL and HRP labelings are listed in Table I.

TABLE I. Enzyme Labeling Methods in Steroid Enzyme Immunoassay[a]

Method	Label enzyme	pH	Molar ratio[b]
NS	β-GAL	7.3	10—20
	HRP	7.3	10—60
p-NP	β-GAL	8.5	30—60
	HRP	8.5	30—60

a) Enzyme labeling with 1 mg of enzyme in dioxane (0.1 ml)-phosphate or borate buffer (0.2 ml) at 4 °C for 4 h. Final enzyme concentrations were 7.1 and 76 μM in the β-GAL and HRP labelings, respectively. *b*) Molar ratios of the steroid active ester to enzyme.

In the present work, the *p*-NP ester was shown to be more stable than the NS ester under the neutral and alkaline conditions. The difference in stability of these esters was also observed in the purification by column chromatography on silica gel (data not shown). Thus, the *p*-NP ester method must be useful for the enzyme labeling of carboxylated haptens, especially with highly polar derivatives, when the isolation of active intermediates is needed. It should be noted that, with this active ester method, the enzyme and hapten itself used must be stable under alkaline conditions, if not, the employment of a large excess amount of the ester or a longer reaction time may be the method of choice for the preparation of a labeled antigen suitable for enzyme immunoassay.

The findings obtained here should be useful in the further development of enzyme immunoassay for steroid hormones and other haptenic compounds. We recommend molar ratios of 30—60 in both β-GAL and HRP labelings at pH 8.5 by the *p*-NP ester method (Table I). In the case of the NS ester method, the use of the molar ratios of 10—20 in the β-GAL labeling and 10—60 in the HRP labeling at pH 7.3 gives a good result with respect to the immunoassay sensitivity.[3] In general, however, it is desirable to estimate the degree of hapten substitution, since the labeling rate is influenced by various factors, such as pH, solvent volume, and reactivity of hapten derivatives. The present information should be helpful in enzyme labeling using alkaline phosphatase, glucose oxidase or other enzymes, and in the preparation of a hapten–carrier conjugate for use as an immunogen or the coated antigen in the microtiter plate immunoassay system.

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