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Bound/Free Separation Methods in Steroid Enzyme Immunoassay with Monoclonal Antibody[1)]

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Methods for separating bound and free fractions in steroid enzyme immunoassay with a monoclonal antibody are described. Three liquid-phase separation techniques and related preincubation methods were studied using β-galactosidase and horseradish peroxidase as labels. In the double- and triple-antibody systems, the effects of concentrations of first, second and third antibodies, including anti-immunoglobulin G Fc fragment antisera, and incubation times on immune precipitation were examined. Differences between the two enzyme labels in immunoreactivity were observed in some cases; this can be explained in terms of a steric interaction. Under optimal conditions, a dose–response curve with a high sensitivity was obtained in each 11-deoxycortisol assay system.

Keywords——immunoassay separation method; liquid-phase separation method; double antibody method; triple antibody method; enzyme immunoassay; monoclonal anti-11-deoxycortisol antibody; 11-deoxycortisol

Immunoassays of steroid hormones are frequently required in biochemistry and clinical chemistry. The hybridoma technique developed by Köhler and Milstein[2)] has been shown to be useful for the preparation of monoclonal antibodies to steroid hormones.[3)] An important advantage of monoclonal antibodies in clinical-chemical analysis is their availability in unlimited supply as reagents with constant binding properties. The analysis of immune reactions is also simplified by the use of monoclonal antibodies, compared to conventional polyclonal antisera.

In general, separation of bound and free fractions (B/F separation) in immunoassay procedures[4)] has been done by solid-phase methods using insoluble materials, such as beads, discs, tubes or microtiter plates; in steroid radioimmunoassays, the charcoal method is mainly employed. In some cases, however, liquid-phase separation methods are rather useful as a standard or practical means, because of their simplicity and flexibility. Little work has been done on hapten enzyme immunoassay using monoclonal antibodies, and therefore, there is limited information concerning methods of B/F separation.

We have previously reported immunoassay systems with a monoclonal antibody for the determination of 11-deoxycortisol.[3,5)] This paper deals with three liquid-phase separation techniques and related preincubation methods in an enzyme immunoassay system with the monoclonal anti-11-deoxycortisol antibody. The latter methods were designed for the purpose of giving a short practical assay time.

Materials and Methods

Materials——The monoclonal anti-11-deoxycortisol antibody (CET-M8) used was that produced in ascites by inoculating the hybridoma, S.CET.M8.1.1, which was derived from fusion of P3-NSl/l-Ag4-l myeloma cells with

spleen cells of BALB/c mice immunized with 4-(2-carboxyethylthio)-11-deoxycortisol (CET) linked to bovine serum albumin.[3,5] Normal mouse serum (NMS), rabbit anti-mouse immunoglobulin G (IgG) antiserum (Ab-2), goat anti-rabbit IgG antiserum (Ab-3), rabbit anti-mouse IgG Fc fragment antiserum (Fc-2) and goat anti-rabbit IgG Fc fragment antiserum (Fc-3) were obtained from MBL Co. (Nagoya); these sera were dialyzed against cold 0.05 M phosphate buffer, pH 7.3 (PB). β-Galactosidase (β-GAL, EC 3.2.1.23) from *Escherichia coli* (grade VI, 455 units/mg) and horseradish peroxidase (HRP, EC 1.11.1.7) (grade I-C, 261 units/mg) were purchased from Sigma Chemical Co. (U.S.A.) and Toyobo Co., Ltd. (Osaka), respectively. The *N*-succinimidyl ester of CET was prepared by the method previously established in these laboratories.[6] The assay buffer used in the immunoassay was PB containing 0.1% gelatin and 0.9% NaCl.

Preparation of Enzyme-Labeled Antigens——This was carried out in the manner described previously.[5] In short, a solution of CET *N*-succinimidyl ester (24 μg for β-GAL and 773 μg for HRP) in dioxane (0.1 ml) was added to a solution of the enzyme (1 mg) in PB (0.2 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 4 °C at a concentration of 500 μg/ml, adjusted with the assay buffer.

Enzyme Immunoassay Procedure——This was carried out in duplicate or triplicate in a glass test tube (10 ml). The standard procedure is as follows: solutions of 11-deoxycortisol (0—500 pg) in the assay buffer (0.1 ml) and β-GAL- (100 ng) or HRP-labeled 11-deoxycortisol (10 ng) in the buffer (0.1 ml) were added to diluted CET-M8 (1 : 100000 for the β-GAL label and 1 : 60000 for the HRP label, 0.1 ml), and the mixture was incubated at 4 °C for 4 h (first incubation). B/F separation was then carried out in the manner described below (methods I, III and IV). The resulting mixture was diluted with the 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 the assay buffer (1.5 ml), and used for measurement of the enzymic activity (B_0 or B). In each system, the procedure without addition of the first antibody CET-M8 was carried out to provide a non-specific binding value. An experiment using only the enzyme label was also carried out to obtain 100% enzymic activity (T).

B/F Separation Methods——Method I: The second antibody Ab-2 (0.1 ml) diluted 1 : 10—1 : 500 with the assay buffer containing 3% ethylenediaminetetraacetic acid (buffer A) and 0.1—2% NMS (0.1 ml) in the assay buffer were added to the incubation mixture, and the solution was vortex-mixed, then allowed to stand at 4 °C for 0.5—16 h (second incubation).

Method II: A mixture of CET-M8, Fc-2 and NMS in buffer A was incubated at 4 °C for 16 h. This pre-precipitated reagent (0.3 ml) was added to a solution of 11-deoxycortisol and enzyme-labeled 11-deoxycortisol in the assay buffer (0.2 ml), and the mixture was incubated at 4 °C for 4 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.

Method III: A solution of Ab-2 (0.1 ml, 1 : 1000—1 : 10000) in buffer A was added to the incubation mixture, and the solution was vortex-mixed, then allowed to stand at 4 °C for 0—6 h (second incubation). The third antibody Ab-3 (0.1 ml) diluted 1 : 10—1 : 150 with buffer A was added to the resulting solution, and the mixture was vortex-mixed, then allowed to stand at 4 °C for 1—16 h (third incubation).

Method IV: The second antibody Ab-2 (0.1 ml, 1 : 2000—1 : 8000) and the third antibody Fc-3 (0.1 ml) diluted 1 : 10 with buffer A were added to the incubation mixture, then allowed to stand at 4 °C for 1—16 h (second incubation). A preincubation method was also examined: a solution of Ab-2 and Fc-3 was allowed to stand at 4 °C for 16 h, and used as a separating reagent.

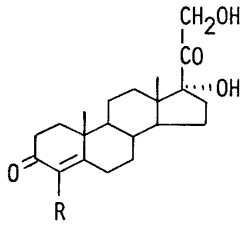
Measurement of Enzymic Activity——β-GAL: The immune precipitate was diluted with the assay buffer (1 ml) containing 0.1% $MgCl_2$ and 10% ethylene glycol, vortex-mixed, and preincubated at 37 °C for 3 min. *o*-Nitrophenyl β-D-galactopyranoside (0.06%, 1 ml) in assay buffer was added to the resulting solution and the mixture was incubated for 40—60 min. The reaction was terminated by addition of 1 M Na_2CO_3 (2 ml) and the absorbance was measured at 420 nm.

HRP: The immune precipitate was diluted with 0.1% 5-aminosalicylic acid in 0.05 M acetate buffer, pH 6.0 (1.8 ml), vortex-mixed, and preincubated at 37 °C for 3 min. Hydrogen peroxide (0.05%, 0.2 ml) was added to the resulting solution, and the mixture was incubated for 40—60 min. The reaction was terminated by addition of 0.5 M NaOH (2 ml) and the absorbance was measured at 500 nm.

In the case of the estimation of T values as described above, these procedures were applied to the enzyme solution.

Results and Discussion

The purpose of this work was to examine liquid-phase methods for B/F separation in the monoclonal antibody-based enzyme immunoassay as a model system. The monoclonal antibody CET-M8 used has been shown to be IgG_1 with kappa light chains, having an affinity constant of $2 \times 10^{10}\ M^{-1}$ for 11-deoxycortisol in the radioimmunoassay procedure.[3] Two



11-deoxycortisol: R=H
CET: R=S(CH$_2$)$_2$COOH

Chart 1

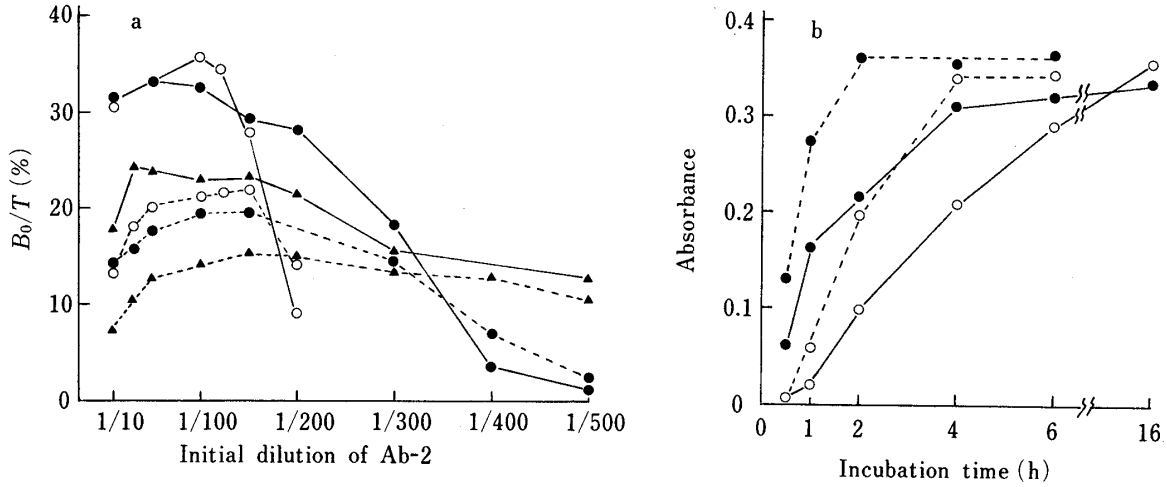


Fig. 1. Immune Precipitation in Method I with the β-GAL (——) or HRP Label (----)

a) Effects of Ab-2 and NMS concentrations. Second incubation was carried out for 16 h, using 0.5 (○), 0.25 (●) or 0.1% NMS (▲). b) Effect of second incubation time. A 1 : 30 (●) or 1 : 120 (○) dilution of Ab-2 was used in combination with 0.5% NMS.

enzymes having a relatively high or low molecular weight, β-GAL (M.W. 465000) and HRP (M.W. 42500), were employed as labels, since the molecular size of the enzyme is a possible factor influencing immune reactions. The steroid/enzyme molar ratios employed in the coupling reactions were 20 for β-GAL and 60 for HRP (the degrees of hapten substitution may be *ca.* 6 and 2, respectively).[7]

The B/F separation methods examined were double-antibody and triple-antibody systems. Previously, using a solid-phase protein A method, we have shown that the reaction of CET-M8 with an enzyme-labeled antigen was relatively rapid.[5] In this work, the first antigen–antibody reaction was carried out for 4 h at 4 °C. The initial dilutions of CET-M8 were 1 : 100000 for the β-GAL label and 1 : 60000 for the HRP label (methods I, III and IV); these were based on preliminary experiments on antibody dilution curves using method I (data not shown). The assays were assessed in terms of the absorbance of B_0 obtained upon 1 h enzymic reaction; the criterion that the absorbance of B_0 should be over 0.3 was employed.

First, the double-antibody systems were studied. There are several ways in which a second antibody can be used for B/F separation in immunoassays. The most standard approach is to use the binding of the second antibody with the first antibody in the presence of carrier globulin.

In method I, the effects of concentrations of Ab-2 and NMS, and incubation time on immune precipitation, *i.e.* absorbance or B_0/T, were examined. The results are shown in Fig. 1. With both β-GAL and HRP labels, similar results with zone phenomena were obtained. It can be seen that appropriate initial concentrations of NMS or dilutions of Ab-2 are 0.25—0.5% and 1 : 10—1 : 150 (Fig. 1a). It should be noted that a higher concentration of NMS was useful only in a narrow range of Ab-2 dilutions; for example, a significant decrease in

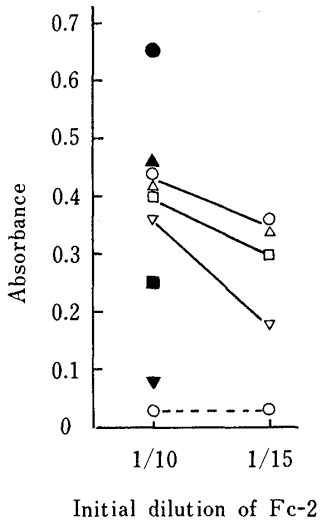


Fig. 2. Effects of CET-M8, Fc-2 and NMS Concentrations on Immunoprecipitation in Method II

In the assay using the HRP label, 0.5% NMS and CET-M8 diluted 1 : 10000 (●), 1 : 20000 (▲), 1 : 30000 (■) or 1 : 60000 (▼) were used. With the β-GAL label, 0.1 (□), 0.25 (○), 0.5 (△) or 1% NMS (▽) was tested at 1 : 10000 (——) or 1 : 100000 (- - - -) dilution of CET-M8.

reactivity was observed with over 1 : 150 dilution of Ab-2 in the assay using 0.5% NMS. We can use 0.1% NMS even at 1 : 500 dilution of Ab-2, though the activity is about one-half of the maximum value. Figure 1b shows that the reactivity in the system with the HRP label reaches a plateau at a shorter time than with the β-GAL label, and satisfactory results are obtained upon 6—16 h incubation in all cases. The use of a 1 : 30 dilution of Ab-2 is advantageous in shortening the incubation time.

Method II utilized an antiserum specific for the Fc region of the first IgG, that is, rabbit anti-mouse IgG Fc antiserum (Fc-2) as a second antibody. This enabled us to prepare the pre-precipitated first antibody reagent, which is ideally suited for obtaining rapid assay results. In the unique method, 1 : 10000—1 : 20000 dilutions of CET-M8 in combination with 0.1—0.5% NMS and a 1 : 10 dilution of Fc-2 (1 : 1 : 1) were found to give satisfactory binding values (Fig. 2). Thus, we can shorten the total assay time. The need for a higher concentration of the first antibody CET-M8, when compared with that in method I, is a future subject to be clarified. Under some conditions, it may be possible to eliminate the need for the carrier NMS.

Triple-antibody systems were the next subjects. The use of a third antibody has the advantage of obviating the need for NMS, which is difficult to obtain in large amounts for the support of routine immunoassays.[8]

In method III, the effects of concentrations of Ab-2 and the third antibody Ab-3, and incubation times on immune precipitation were examined. Figure 3a shows the relationship between the antibody concentrations and the B_0/T values obtained after 6-h second and 16-h third incubations. We can say that appropriate dilutions of Ab-2 are 1 : 5000—1 : 8000 in combination with 1 : 30—1 : 50 dilutions of Ab-3. The effects of the second incubation time on the absorbance are shown in Fig. 3b (the third incubation was 16 h). The results demonstrated that the simultaneous addition of the two antibodies (time 0) gave a low absorbance for B_0, whereas satisfactory values were obtained within 6 h, for example, even 30 min, at 1 : 5000 dilution of Ab-2 and 1 : 30 dilution of Ab-3. The results on the third incubation time are shown in Fig. 3c, where second incubation was carried out for 6 h. It is evident that the reaction is significantly influenced by the antibody concentrations, showing that the incubation time of 16 h is sufficient under the conditions tested. It is possible to obtain comparable results with a shorter third incubation period (4 h) by the use of a high Ab-2 concentration (1 : 2000) in combination with a 1 : 10 dilution of Ab-3. In Fig. 3d, antibody dilution curves with respect to the first antibody are shown. A typical zone phenomenon can be seen in each system, showing that the appropriate dilutions of CET-M8 were *ca.* 1 : 60000 for the HRP label and *ca.* 1 : 100000 for the β-GAL label.

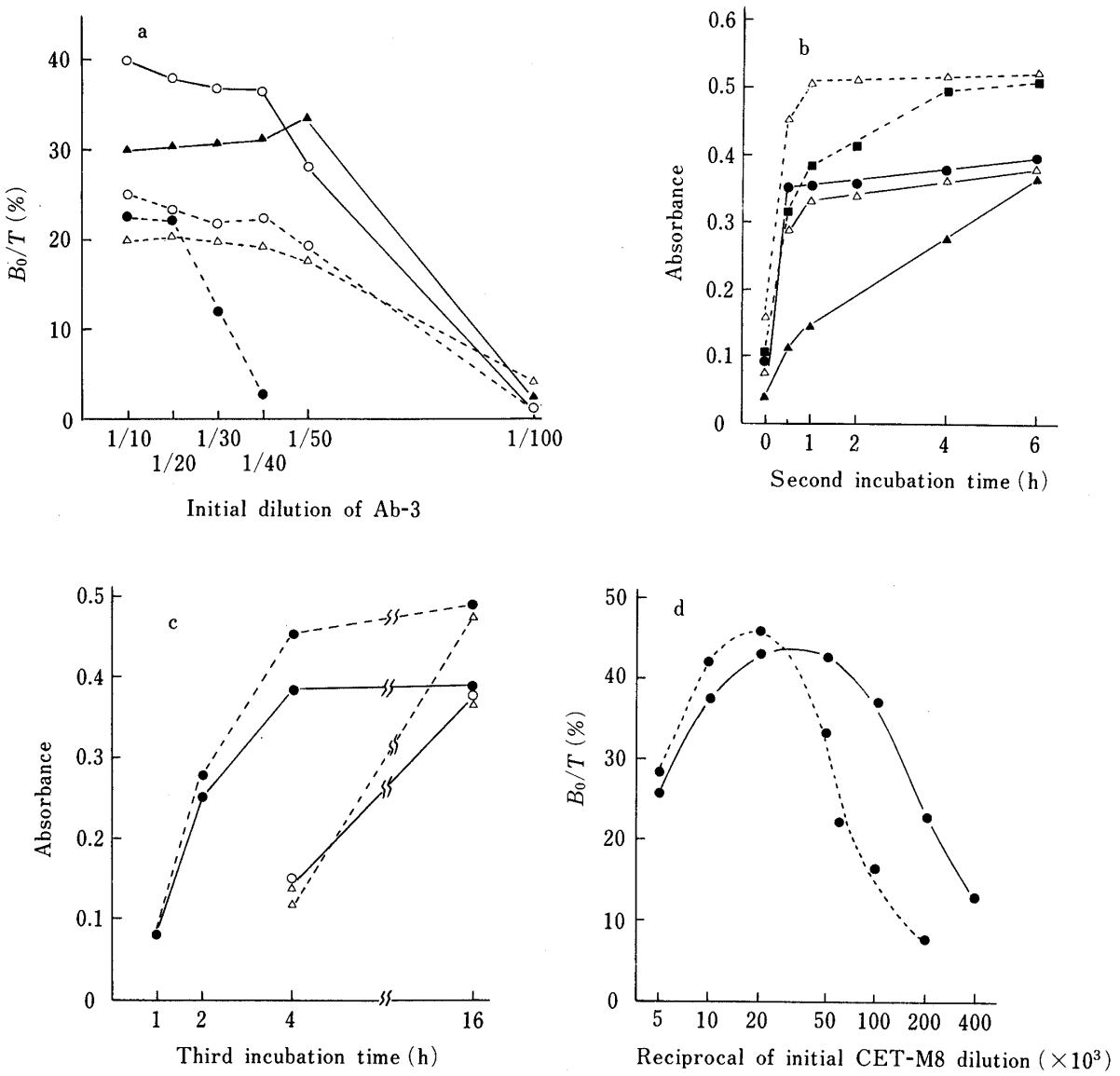


Fig. 3. Immune Precipitation in Method III with the β-GAL (——) or HRP Label (----)

a) Effects of Ab-2 and Ab-3 concentrations. The initial dilutions of Ab-2 used were 1 : 2000 (●), 1 : 5000 (○), 1 : 8000 (▲) and 1 : 10000 (△). b, c) Effect of incubation time. The antibody dilution combinations (Ab-2/Ab-3) used were 1 : 2000/1 : 10 (●), 1 : 5000/1 : 10 (○), 1 : 3000/1 : 30 (▲), 1 : 5000/1 : 30 (△) and 1 : 8000/1 : 30 (■). Time 0 in second incubation means simultaneous addition of Ab-2 and Ab-3. d) The antibody dilution curves were obtained at a dilution of 1 : 5000 Ab-2 and 1 : 30 Ab-3. The first, second, and third incubations were carried out for 4, 6 and 16 h, respectively.

Method IV utilized the third antibody Fc-3 specific for the Fc region of the second IgG. We examined the procedures with and without preincubation of Ab-2 (1 : 5000) and Fc-3 (1 : 10). The relationships between absorbance and second incubation time are shown in Fig. 4. It is clear that the preincubation is effective in increasing the apparent reactivity. The effect in the assay using the HRP label was more significant than with the β-GAL label. This, together with the results obtained in method I (Fig. 1b), seems to be ascribable to the difference in molecular size between the enzymes: the steric interaction between the label and antibody molecules, which causes interference in forming the immune complex, must be more marked in the assay using the larger β-GAL label.

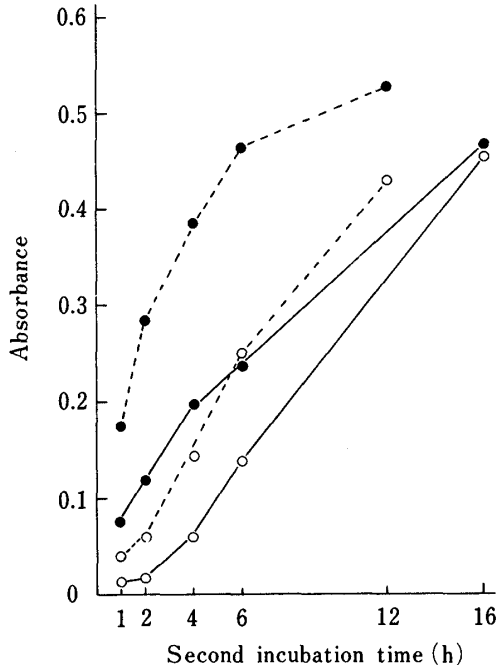


Fig. 4. Effect of Preincubation between Ab-2 and Fc-3 on Absorbance in Method IV with the β-GAL (——) or HRP Label (– – – –)

Preincubation (●) was carried out for 16 h at a dilution of 1 : 5000 Ab-2 and 1 : 10 Fc-3. ○, non-preincubation method.

TABLE I. B/F Separation Methods in 11-Deoxycortisol Enzyme Immunoassay Using the Monoclonal Antibody CET-M8[a]

Method	Second antibody	Precipitating reagent	Assay time[b] (h)
I	Ab-2 (1 : 30)	0.5% NMS	8
II	Fc-2 (1 : 10)	0.25% NMS	4
III	Ab-2 (1 : 2000)	Ab-3 (1 : 10)	10[c]
IV	Ab-2 (1 : 5000)	Fc-3 (1 : 10)	16[d]
Protein A[5]	Ab-2 (1 : 1000)	5% IgGsorb	6

a) In method I, III or IV, or in the protein A method, the first incubation between CET-M8 (1 : 100000) with the β-GAL label was carried out for 4 h. In method II, the initial dilution of CET-M8 was 1 : 10000. Absorbances for B_0 obtained after a 1 h enzymic reaction were over 0.3. Figures in parentheses indicate antiserum dilution. *b*) This includes the first incubation time (4 h), and not the preincubation and enzymic reaction times. *c*) The second and third incubation times were 2 and 4 h, respectively. *d*) The time in the preincubation method.

The standard conditions with the β-GAL label in these B/F separation methods, including the previous protein A method,[5] are listed in Table I. In the present methods, the immune precipitates obtained were soluble in the solution used for the enzyme reaction or for stopping the enzymic activity. Under the given conditions, a dose–response curve with a high sensitivity was obtained in each 11-deoxycortisol assay system (the non-specific binding was less than 15% of the B_0 value)—the amount of 11-deoxycortisol needed to displace 50% of the bound β-GAL label was *ca.* 70 pg in method III, for example. The separation methods should be applicable to assay systems with other enzymes currently used as labels (ethylenediaminetetraacetic acid[4a] is not necessarily essential for the formation of the immune precipitates). We used the commercially available second and third antibodies. At the present time, the anti-Fc antisera are very expensive and their titers are somewhat low. The incubation time required in method IV, especially with the β-GAL label, was rather long when compared with that in method III, although the use of a larger amount of the pre-precipitated antibody reagent gave a comparable result (data not shown). Ethylene glycol-assisted precipitation may be worthy of

examination.[8,9]

The information obtained here should be helpful for the further development of enzyme immunoassay or other immunoassay systems using monoclonal antibodies for steroid hormones and drugs. Studies on factors influencing the sensitivity and specificity of enzyme immunoassay by the use of the monoclonal antibody are in progress.

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