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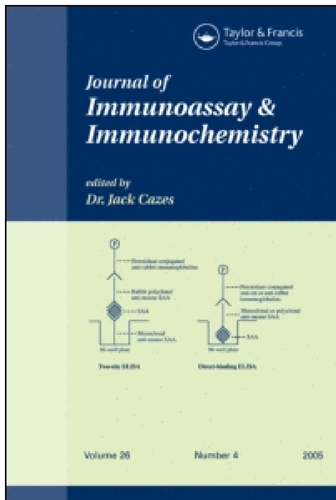

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Improvement of Estradiol Enzymoimmunoassay, Using a Monoclonal Antibody and an Avidin/Biotin Amplification System

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JOURNAL OF IMMUNOASSAY, 10(4), 339-357 (1989)

IMPROVEMENT OF ESTRADIOL ENZYMOIMMUNOASSAY, USING A MONOCLONAL ANTIBODY AND AN AVIDIN/BIOTIN AMPLIFICATION SYSTEM.

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ABSTRACT

A microtitre plate enzyme immunoassay for estradiol, using a purified monoclonal antibody covalently bound to peroxidase and a small amount of immobilized immunogen, was optimized. Decreasing the antibody concentration to $2x10^{-10}$M (Kd/5) gave optimum estradiol detectability.

The enzymatic signal was, however, very low in this assay. A 14-fold enhancement could be obtained using an avidin-biotin system in which several biotin molecules are conjugated with the antibody, providing multiple sites for binding by an avidin-enzyme complex. Further reagent concentration optimization gave an assay in which a range of 2 to 140 pg estradiol/well could be assayed simply and reproducibly.

INTRODUCTION

Competitive immunoassay sensitivity depends mainly on the affinity of the antibodies used (1). Insofar as steroids are concerned, monoclonal antibodies reported to-date have association constants which are maximally of the order of $10^{10}M^{-1}$ (2-5). This means that until a way has been found to obtain antibodies of higher affinity the improvement of assay sensitivity, whilst retaining the advantages of monoclonal

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antibody use, is limited to the optimization of experimental conditions (6) and, for enzyme immunoassays, to the introduction of an additional signal amplification step into the assay (peroxidase/antiperoxidase, avidin/biotin...). In this paper we report a comparison of ways to improve the sensitivity of a microtitre plate enzyme immunoassay for estradiol-17β using a monoclonal anti-estradiol antibody raised in this laboratory.

Firstly, the effect of optimizing reagent concentrations in an assay using peroxidase-labelled antibody was studied. These results were then compared with those obtained by introducing an avidin-biotin amplification step into the assay. Two such amplification steps have been tested: an antibody-biotin/avidin-peroxidase system and an antibody-biotin/avidin/biotin-peroxidase system. Further, we have studied whether the presence, in the biotin-antibody conjugate, of a caproic acid spacer between the two moieties improved biotin's binding by avidin.

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MATERIAL AND METHODS

Monoclonal Anti-Estradiol Antibody

Murine monoclonal antibodies were raised against 3-carboxymethyl-estradiol-bovine serum albumin (estradiol-3-CM-BSA) (38 moles steroid/mole protein) synthetized by the method of Erlanger et al (7). Ascites fluid was produced from our clones by the Société des Systèmes Biologiques (Rives, France) and monoclonal antibodies were purified as previously described for a monoclonal anti-aldosterone antibody raised here (5). The approximate affinity ($10^{9}M^{-1}$) and specificity of the antibody were determined by radioimmunoassay, using (2,4,6,7^{3}H)-estradiol (3.89 TBq/mmole, The Radiochemical Centre, Amersham, U.K.). Of the steroids tested, the antibody only cross-reacted significantly, but still at ≤1%, with estriol, estrone and testosterone.

Antibody-Peroxidase Conjugate

The purified anti-estradiol antibody was conjugated with horseradish peroxidase (type VI, Sigma, St. Louis,MO, USA) by the method of Wilson and Nakane (8). The number of enzyme molecules bound per molecule of antibody was determined from absorbances measured at 280 and 403 nm.

Antibody Biotinylation

Two biotin derivatives, N-hydroxysuccinimidobiotin (biotin-NHS) and biotinamidocaproate-N-hydroxysuccinimide ester (biotin-X-NHS) (Sigma chemicals) were used for biotinylation. The purified antibody was thoroughly dialyzed against 0.1M $NaHCO_3$, pH 8-8.3 and its concentration adjusted to 1 mg/ml. To this was added 50µl biotin-NHS or biotin-X-NHS in anhydrous dimethyl sulfoxide at concentrations yielding biotin/protein reagent molar ratios of 50/1 for biotin-NHS or 5, 15, 25, 35 or 50/1 for biotin-X-NHS. The reaction mixture was stirred for 2h30 at room temperature and then dialysed against 20mM Tris buffer, pH 7.5, with 0.5M NaCl (TBS) and filtered on columns (10 x 220mm) of Bio-gel P2 (Bio-Rad, CA, USA) equilibrated with the same buffer. The eluted biotinylated antibody fractions were stored in liquid nitrogen.

The degree of biotinylation was studied in microtitre plates using streptavidin-biotinylated alkaline phosphatase (Amersham Int.) Progressive dilutions of biotinylated antibody were coated in microtitre plates (Nunc, Roskilde, Denmark) and then incubated with 100µl/well streptavidin-enzyme at the dilution of 1/500 recommended by the manufacturer. Following removal of excess streptavidin-enzyme and thorough washings of the plate the alkaline phosphatase was assayed with the substrate 3mM p-nitro-phenol phosphate (Sigma chemicals) in 0.1M diethanolamine/HCl

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buffer containing 1mM $MgCl_2$. The reaction was stopped with 10mM EDTA and the optical density determined at 405nm.

Avidin-Peroxidase

Peroxidase (5mg/320µl distilled water) was activated with sodium periodate (Merck) (5mg/265µl water) for 30min at 10°C with stirring. The mixture was dialyzed against 1mM acetate buffer pH 4.4 (4 x 250ml) for 24h, dialyzed against 10mM sodium phosphate buffer pH 7.2 containing 0.15M NaCl (PBS) (2 x 250ml) for 5.5 h and filtered on an ACA 44 ultrogel (IBF, France) column (15 x 355mm) in the same buffer. The activated peroxidase was then concentrated in a Centricon-30 microconcentrator (Amicon Corp., USA).

Avidin (Sigma Chemicals, 11.3 units/mg) (2mg) in 800µl Na_2CO_3, pH 9.0, was added to the activated peroxidase (2.15mg in 190µl PBS). The mixture was stirred for 2h at 4°C, dialyzed for 3h against PBS (2 x 500ml) and filtered on a Biogel P-100 column (15 x 355mm) with the same buffer. The peroxidase/avidin molar ratio in the conjugate was determined from the absorbances at 280 and 403nm. The conjugate was stored at -20°C or in liquid nitrogen.

Peroxidase Biotinylation and Complexation with Avidin

20µl Biotin-X-NHS (4mg/ml) in 0.1M Na_2CO_3, pH 8.2, was added to 3mg peroxidase in 2ml of the same solution - a biotin/enzyme molar ratio of 2/1. The mixture was stirred for 1h at 4°C and 3h at room temperature, filtered on a Biogel P2 column (15 x 140mm) equilibrated and eluted with TBS and then concentrated in a Centricon-10 microconcentrator. 1mg (560µl, 2.25 x 10^{-8} mole) biotinylated peroxidase was reacted with an equimolar quantity (1.55mg) of avidin in 0.2ml TBS for 1h at room temperature and

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30min at 4°C and then filtered on a Biogel P-100 column (15 x 355mm) with TBS. The avidin/biotinylated peroxidase complex (A/B-HRP) was stored at 4°C or in liquid nitrogen.

Estradiol Assay

Unless otherwise specified, microtitre plate wells were coated with 25ng/well estradiol-3-CM-BSA in 50µl TBS overnight at 4°C. Remaining active sites in wells were saturated with 200µl/well acid-washed casein (30g/l in TBS) for 15min at room temperature. Wells were washed 3 times with 250µl TBS containing 1g/l acid-washed casein (the assay buffer) between each incubation step.

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a. Assays with Peroxidase-Labelled Antibody. The labelled antibody (in 50µl assay buffer) was incubated for 2h at room temperature in wells with 50µl estradiol. Following emptying and washing of wells the peroxidase activity was determined with 2mM 2,2'azino-di(3-ethylbenzothiazolin sulfonate) (ABTS) (Sigma Chemicals) and 0.7mM hydrogen peroxide in 100µl 0.1M sodium acetate buffer pH 4.5 (9). The reaction was stopped with 100µl 15mM sodium azide solution and absorbances read at 405nm with a Dynatech MR 700 microplate reader.

b. Assays with Biotinylated Antibody. The first incubation with biotinylated antibody and washing steps above were followed by incubation for 3h at room temperature with 100µl avidin-peroxidase or avidin/biotin-peroxidase (both at a final peroxidase concentration of 1.10^{-8}M) in assay buffer. After emptying and washing the wells, the peroxidase activity was determined as above.

RESULTS

Reagent Characteristics

1) The Immunoglobulin-Peroxidase Conjugate. The conjugate contained 0.8 moles peroxidase/mole immunoglobulin. The specific

activity was 80% of that of native peroxidase. The binding activity of the immunoglobulin moiety, determined by radioimmunoassay, was not modified by the covalent attachment of peroxidase. The conjugate could be stored at -20°C for at least 8 months without loss of immunological or enzymatic activity.

2) The Biotinylated Antibody.

a. The two biotin derivatives (biotin-NHS and biotin-X-NHS) were reacted with anti-estradiol antibody at the same reagent molar ratio (biotin/antibody: 50/1). The avidin-binding activity of the two conjugates, as estimated by their binding of streptavidin-biotinylated alkaline phosphatase following plate well-coating with different quantities of each conjugate, is shown in Fig. 1A. Assuming the immobilization of equal quantities of each conjugate when coating was carried out with equal concentrations, the conjugate with biotin-X-NHS had more bindable biotin. This derivative, with the spacer arm, was thus used henceforth.

b. The conjugates resulting from antibody biotinylation with biotin-X-NHS at different biotin/antibody molar ratios (5 to 50) were tested for avidin-binding as above. The results are shown in fig. 1B, in which it can be seen that increasing avidin-binding activity was obtained with increasing biotin reagent molar ratio up to a maximum. The conjugate formed using a 50/1 molar ratio was used for the remainder of the work. The binding activity of the antibody was the same in this conjugate as that of the native antibody and the conjugate could be stored in liquid nitrogen for at least 6 months without loss of activity.

3) Peroxidase-Avidin Compounds. Peroxidase was labelled with avidin either covalently (A-HRP) or indirectly, via binding between avidin and the biotinylated enzyme (A/B-HRP). The avidin/peroxidase molar ratios in the two compounds, as determined by their absorbances at 280 and 403nm, were 1/1 for

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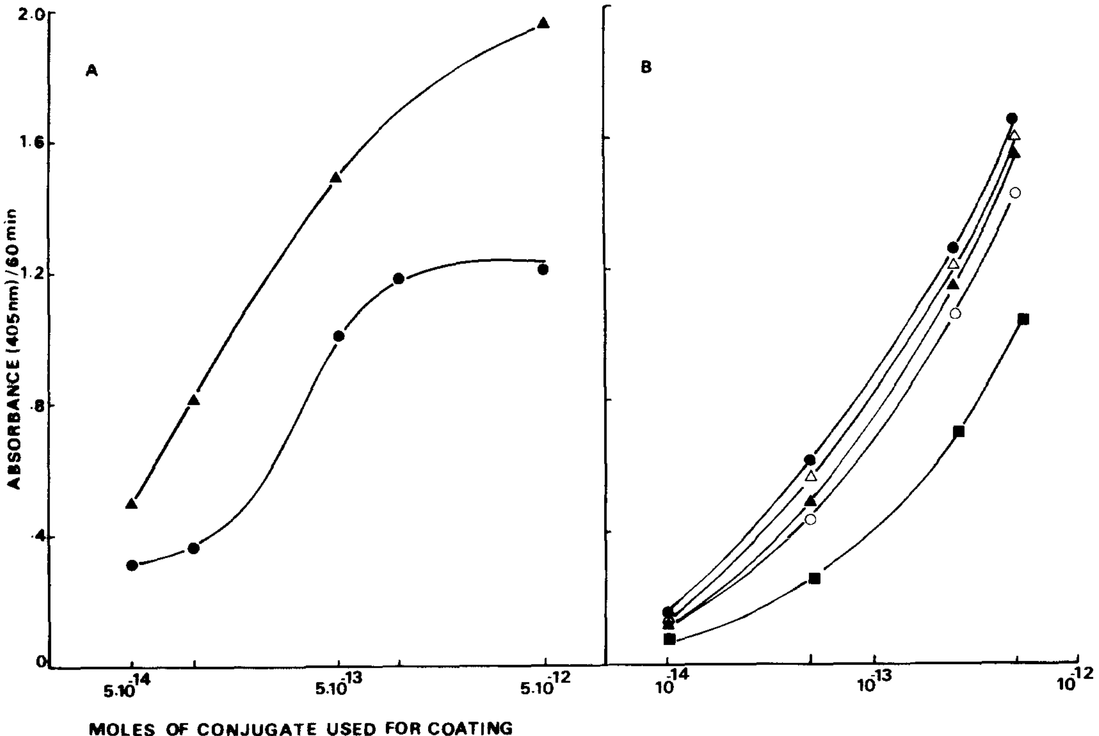

FIGURE 1 - Avidin-binding activity of biotin-antiestradiol conjugates synthetized from: A - N-hydroxysuccinimidobiotin derivative (●) or from biotinamidocaproate-N-hydroxysuccinimid ester (▲) in a molar ratio biotin/antibody of 50/1. B - biotinamidocaproate-N-hydroxysuccinimid ester in various biotin/antibody molar ratios: 5/1 (■), 15/1 (○), 25/1 (▲), 35/1 (△), and 50/1 (●).
Increasing amounts of biotinylated antibody are coated in plate wells. Following saturation with casein 3% in TBS and incubation with streptavidin-alkaline phosphatase, the enzyme was assayed and optical densities determined at 405 nm.

A-HRP and 0.7/1 for A/B-HRP. The peroxidase activity in both compounds was the same as that of native peroxidase. A-HRP retained 80-90% of its activity following storage at -20°C but it was over 90% active if stored at -196°C. A/B-HRP was 84% active when stored at -196°C.

Enzyme Immunoassays

1) Immunoassay with the Antibody-Peroxidase Conjugate. The effect of antibody-peroxidase concentration ((coating with 25ng (0.36 pmol) immunogen, containing 13.7 pmol estradiol)), is shown in Fig.2. The lower limit of detection (given by the readings equal to twice the standard deviation in the absence of competing estradiol) and the amount of estradiol displacing 50% of bound antibody for each curve are shown in Table I. The lower limit of detection was greatly decreased by reducing the antibody concentration, as was the amount of estradiol required to displace 50% bound antibody. The curve slopes at the mid-point were similar, indicating similar assay precisions.

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2) Immunoassay with the Biotinylated Antibody and either Avidin-Peroxidase (A-HRP) or Avidin/Biotinylated Peroxidase (A/B-HRP). All assays, using A-HRP or A/B-HRP, were carried out with the same saturating concentration of peroxidase (>50 times the antibody concentration). It was found that 3h was necessary to attain equilibrium in the avidin/biotin reaction step when using low antibody concentrations . So this equilibration time was used for all assays. On the other hand the duration of the peroxidase assay step depended on the rate of colour development in a given assay and was in the range 6-30min (the reaction was linear for at least 35min).

A comparison between assay curves obtained with the same biotinylated antibody concentration ($2x10^{-10}$M) and identical

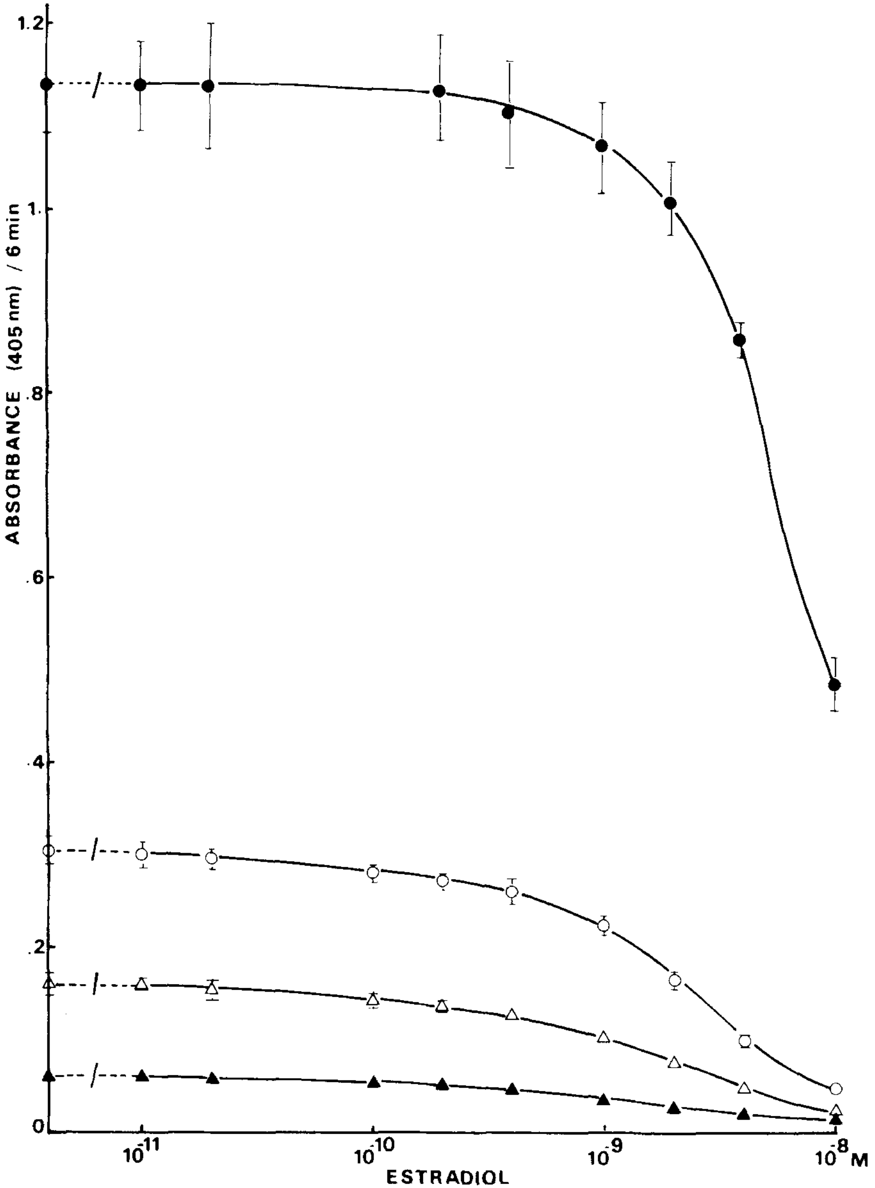

FIGURE 2 - Displacement curves obtained with decreasing concentrations of peroxidase-labelled antibody: $5x10^{-9}$M ●-● , $1x10^{-9}$M ○-○ , $5x10^{-10}$M △-△ and $2x10^{-10}$M ▲-▲ . Microtitre plate wells were coated with 25ng estradiol-3-CM-BSA and saturated with casein 3% in TBS. Following incubation with increasing amounts of estradiol and enzyme-labelled antibody, the peroxidase activity in wells was assayed and optical densities determined at 405nm.

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TABLE 1

PERFORMANCES of EIAs using DECREASING CONCENTRATIONS of ANTIBODY-PEROXIDASE CONJUGATE

Antibody concentration $M \times 10^{-10}$	Detection limit		Free estradiol displacing 50% of antibody	
	$M \times 10^{-10}$	pg/well	$M \times 10^{-10}$	pg/well
50	16.8	22	84	114
10	2.2	3	23	32
5	2.2	3	18	24
2	1.2	2	16	22

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concentrations (1×10^{-8}M) of the two peroxidase reagents A-HRP and A/B-HRP) is shown in Fig.3. This figure also shows the curve obtained with the antibody-peroxidase conjugate. The curves are shown normalized to a 6 min enzyme assay incubation time. Displacements of antibody as a function of increasing amounts of competing estradiol are plotted as the peroxidase activity at each point relative to that in the absence of competing estradiol for a given curve (Fig. 3A) and as the optical densities measured at each point (Fig. 3B). The standard deviations of the relativized points (n=8) are not shown but are all similar for a given free estradiol concentration. The signal obtained with A-HRP was 14-fold greater than that with antibody-peroxidase and 3-fold greater than with the A/B-HRP conjugate. Using this A-HRP conju-gate, a 10-fold decrease in biotinylated antibody concentration (with the same amount of immobilized steroid in the

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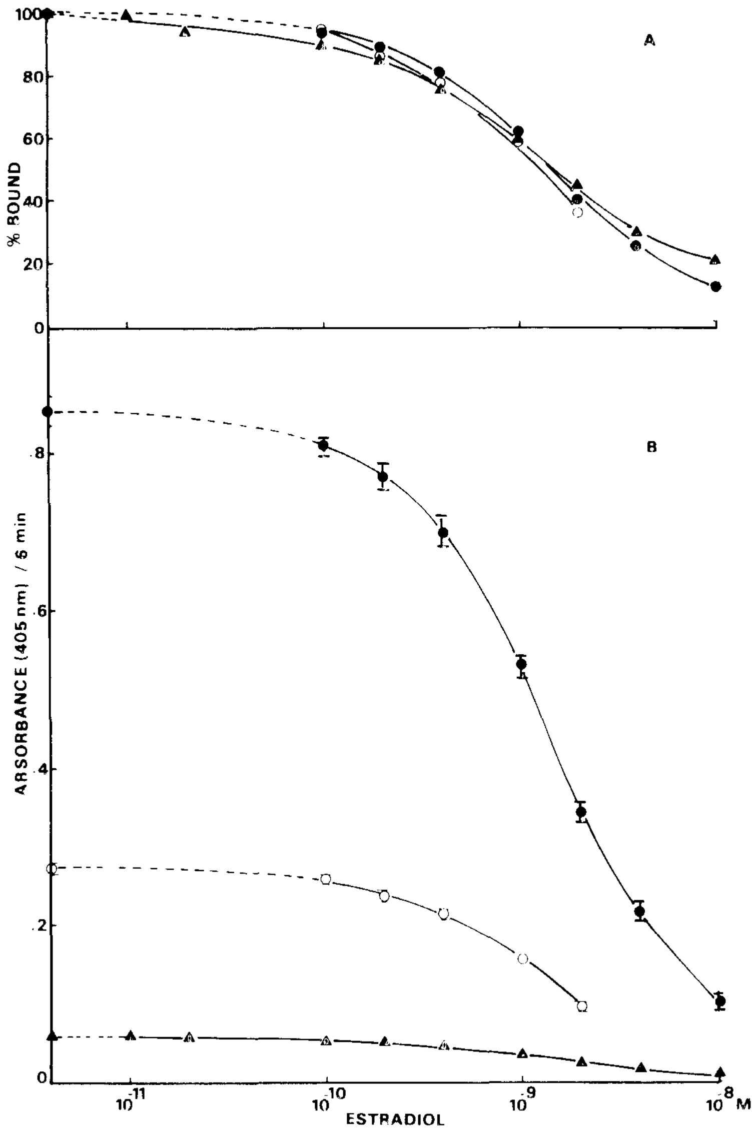

FIGURE 3 - Displacement curves obtained with three different systems:
- ▲-▲ peroxidase-antiestradiol conjugate;
- ○-○ biotinylated anti-estradiol and avidin/biotinylated peroxidase;
- ●-● biotinylated antibody and avidin-peroxidase.
Microtitre plate wells were coated with 25ng estradiol-3CM-BSA and saturated with casein 3% in TBS. The same concentration of antibody (2×10^{-10}M) was used in each system and incubated with increasing amounts of free estradiol. With biotinylated antibody this was followed by a second incubation step with one of the avidin-peroxidase conjugates. Curves A: the enzymatic signal at each point is relativized to that obtained in the absence of competing estradiol. Curves B: the enzymatic signal is plotted as optical density at 405 nm following peroxidase assay.

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TABLE 2

FREE ESTRADIOL CONCENTRATIONS DISPLACING 50% of ANTIBODY and ESTRADIOL DETECTION LIMIT using 3 different IMMUNOGEN COATINGS and 3 different ANTIBODY-BIOTIN-X-NHS CONCENTRATIONS.

Immunogen coating	25 ng/well			15 ng/well			5 ng/well		
Biotin-antibody concentration x 10^{-10}M	2.0	1.0	0.5	2.0	1.0	0.5	2.0	1.0	0.5
OD/6min without free estradiol	0.85	0.27	0.16	0.34	0.20	0.11	0.27	0.16	0.09
Detection limit x 10^{-10}M	1.0	1.4	1.4	0.7	0.5	0.5	0.5	0.4	0.3
Free estradiol displacing 50% x 10^{-10}M	15	14	12	10	8	8	8	6	5

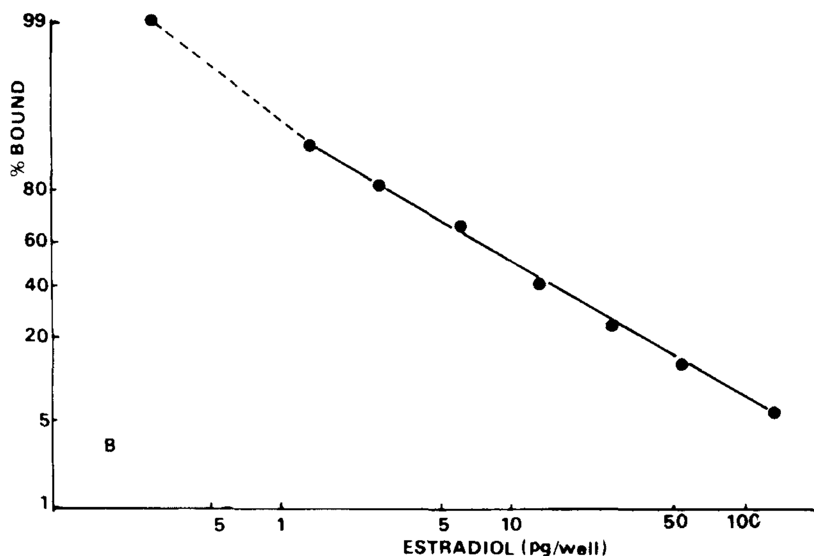

FIGURE 4 - Logit-log representation of a typical standard curve for the estradiol assay obtained with a 5 ng estradiol-3-CM-BSA coating, with a 2 x 10^{-10}M concentration of biotinylated antibody and a 1 x 10^{-9}M concentration of peroxidase-avidin conjugate.

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wells) did not lead to any improvement in the assay's lower limit of detection. On the other hand the effect of lowering both the biotinylated antibody concentration (2 to 0.5 x 10^{-10}M) and that of the amount of immunogen used for well-coating (25 to 5ng) is shown in Table II.

Finally, Fig. 4 shows a logit/log plot of the standard assay displacement curve obtained following well-coating with 5ng immunogen and using 2 x 10^{-10}M biotinylated antibody. This plot is linear up to 140 pg estradiol. Using these conditions, the lower limit of detection is 0.5 pg (2.0 fmol) estradiol (Table II).

DISCUSSION

Recent work in this laboratory (10) has shown that the sensitivity of a competitive enzyme immunoassay using a monoclonal antibody (for aldosterone) can be improved by decreasing the amount of steroid immobilised in plate wells to an optimum amount, further decreases leading to a loss of sensitivity. Further, Ekins (11) has used computer simulation of a radioimmunoassay to show that optimum assay sensitivity is obtained with very low antibody concentrations.

The first part of the work on reagent concentration optimization was carried out using an antibody-peroxidase conjugate prepared by the periodate method (8) since we have previously found it gives higher yields of active conjugate and higher enzyme/antibody molar ratios than the glutaraldehyde method (5). Optimizing the concentration of this reagent, we found a 10-fold improvement in estradiol detectability when the peroxidase-antibody concentration was decreased from 50 to 2 x 10^{-10}M. Despite the improved detectability, the low reagent concentration resulted in dramatically weak coloration. We therefore investigated methods for signal amplification and for this the avidin/biotin binding system was used.

Firstly we investigated the optimum conditions (nature and concentration of the reagent) for biotinylating the particular monoclonal antibody concerned here. Since the question at issue was the optimization of biotinylation with respect to the assay signal amplification obtainable, the biotinylation reactions were followed in terms of degree of avidin binding to the product rather than in terms of the absolute amount of biotin conjugated to the antibody. While the two reagents tested (B-NHS and B-X-NHS) may, at equal concentrations, have yielded identical biotin/antibody molar ratios in the products, the fact that one product's biotin moieties could be bound by more avidin molecules was empirically more relevant. Under identical reaction

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conditions, biotinylation with the reagent having a caproic acid spacer arm (biotin-X-NHS) yielded a product with more bindable biotins per molecule of antibody than the reagent without the spacer arm (biotin-NHS).

It was found that bindable biotin conjugated to the antibody increased with increasing reagent molar ratio (biotin-X-NHS/antibody) up to approximately 50/1. This optimum reagent molar ratio was higher than that of 15/1 found by Gretch et al. (12) though they did find a higher value for one of the antibodies they studied and thus the optimum may be specific to a given antibody. However undesirable effects of excessive biotinylation have been pointed out (13, 14). Indeed, we have found that biotin:antibody ratios of 100/1 lead to loss of antibody activity.

This antibody-biotin reagent could then be used in assays involving the enzyme, peroxidase, either directly covalently linked to avidin or biotinylated and non-covalently bound to avidin. For synthesizing the biotinylated peroxidase (B-HRP), given the results above, the biotin reagent with the spacer arm (biotin-X-NHS) was used. However, while a maximum number of active biotin moieties per antibody molecule is required for optimal amplification, a biotin/peroxidase molar ratio of unity is required to avoid one enzyme molecule labelling more than one antibody molecule. Thus a low (2/1) biotin-X-NHS/peroxidase molar ratio was used for biotinylating the enzyme - this yielded a mean molar ratio of 0.7 active biotins per peroxidase. Similarly, for the assay in which the enzyme is covalently linked directly to avidin (A-HRP), an equimolar ratio of avidin to antibody is required to maximize the number of enzyme molecules binding to an antibody via the biotin/avidin system. In this case a final avidin/peroxidase molar ratio of 1/1 was obtained.

Comparison of the assays using these two reagents showed the superiority of the A-HRP system over the A/B-HRP system. Thus the

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signal amplifications with the two assays were respectively 14 times and 4.5 times the signal obtained using the peroxidase-antibody conjugate. This agrees with observations of two authors reporting unsuccessful attempts to increase ELISA sensitivity with the A/B-HRP system (12,15).

It therefore remained to optimize this assay using biotinylated antibody and the avidin-peroxidase conjugate in terms of reagent concentration. It was found that further lowering the antibody concentration beyond that used with the peroxidase-antibody conjugate, while maintaining the same immunogen coating (25ng/well), did not improve assay sensitivity. This antibody concentration corresponded to approximately one fifth of the antibody's dissociation constant. Decreasing the amount of immunogen coated in wells, found to increase assay sensitivity by Hanquez et al. (10), was also tested. We were able to obtain a 2-fold improvement in the assay detection limit when wells were coated with 5ng/well immunogen and then a corresponding decrease in antibody concentration to 0.5×10^{-10}M yielded another 1.6-fold improvement in detection limit. However, under these conditions, the peroxidase/substrate incubation had to be lenghthy in order to obtain a sufficient signal. We therefore prefer to sacrifice some assay sensitivity, and use an antibody concentration of 2×10^{-10}M with immunogen coated at 5ng/well, in order to obtain an increased signal. Such a sacrifice could of course be avoided using a fluorogenic or luminogenic enzyme substrate, though the relevant signal detection apparatus would also be required.

The assay as finally developed had a detection limit of 0.5 pg (2.0 fmol) estradiol and a logit/log plot was linear over the range 1.40 to 140 pg/well (5 to 500 fmol). The precision of this assay was also high since the coefficients of variation of each point on the assay curve were all below 2.5% (n = 8). The detection limit found here is the same as that reported by Marcus

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and Durnford (16) for an estradiol assay involving peroxidase-labelled steroid and antibody coated in wells. A similar assay, but with a fluorescent signal, gave an improved detection limit of 0.3 pg/well (17). However, in such assays, the biological sample is present in wells at the same time as the enzyme label so the risks of interference with enzyme activity may be increased.

It seems that the assay described here, given the characteristics (affinity, specificity) of the antibody used, exhausts the possibilities so far available in enzyme immunoassay technology with respect to all the steps up to the final enzyme assay. The sensitivity and precision of the assay are suitable for the determination of urinary or plasma estradiol determination in menstruating women, particularly for the prediction of ovulation.

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