

An Electrochemical Enzyme Immunoassay for Chicken Luteinizing Hormone: Extension of the Detection Limit by Adequate Control of the Nonspecific Adsorption

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A noncompetitive heterogeneous enzyme immunoassay for the determination of chicken luteinizing hormone (LH) was equipped with an electrochemical endpoint in order to further enhance its sensitivity. The immunological principle of the original ELISA remained essentially unchanged, except for the fact that the peroxidase label was replaced by alkaline phosphatase, since in the upgraded version of the assay, *p*-aminophenyl phosphate was to be used as the substrate of alkaline phosphatase. Enzyme-generated *p*-aminophenol was injected into a flow-injection system and detected amperometrically in a thin-layer flow cell with a glassy carbon electrode at 0.325 V vs Ag/AgCl. A classical problem associated with this type of solid-phase immunoassay is the adsorption of proteins other than the capture antibody to the solid phase. The detection sensitivity is therefore often limited by a large background signal observed in the absence of antigen. In the present study, an experiment was designed to examine in each step of the assay the contribution of each of the potential sources of background current. It was shown that the major contribution to the background current was caused by the nonspecific adsorption of biotinylated secondary antibody. Adsorption of the secondary antibody (biotinylated goat anti-rabbit IgG) to the capture antibody (mouse anti-chicken LH β) was clearly a case of specific aspecificity, whereas adsorption to the solid phase itself had to be treated as a nonspecific aspecificity. Addition of 0.25% mouse serum to the secondary antibody as a

source of mouse immunoglobulin could overcome the cross-reaction and markedly reduced adsorption to capture antibody. The second part of nonspecific adsorption was eliminated by using combinations of Tween 20 and bovine serum albumin as blocking agents. Controlling the adsorption of the biotinylated secondary antibody in this way decreased the detection limit from 39 pg/ml in the original assay to 2.5 pg/ml in the electrochemical version. This way, the plasma volume of samples containing on the order of 1 ng/ml LH was reduced to less than 10 μ l. The linear range was 2.5–625 pg/ml. The method allowed us to measure LH in buffer and in adult and juvenile chicken plasma. © 1998 Academic Press

Key Words: enzyme immunoassay; electrochemical immunoassay; flow-injection assay; solid-phase immunoassay; luteinizing hormone; biotin-streptavidin system; nonspecific adsorption; ELISA.

Luteinizing hormone (LH)² belongs to the family of the pituitary glycoprotein hormones. The molecule is made up of two noncovalently linked subunits (α and β) and plays an important role in ovulation and luteotrophy in females and in androgenesis in males. To mea-

² Abbreviations used: LH, luteinizing hormone; RIA, radioimmunoassays; ELISA, enzyme-linked immunosorbent assay; EEIA, electrochemical enzyme immunoassay; PAPP, *p*-aminophenyl phosphate; PNPP, 4-nitrophenyl phosphate; SA-AP, streptavidin-alkaline phosphatase conjugate; PAP, *p*-aminophenol; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FIA-ED, flow-injection analysis-electrochemical detector system; IgG, immunoglobulin; TEA, triethylamine.

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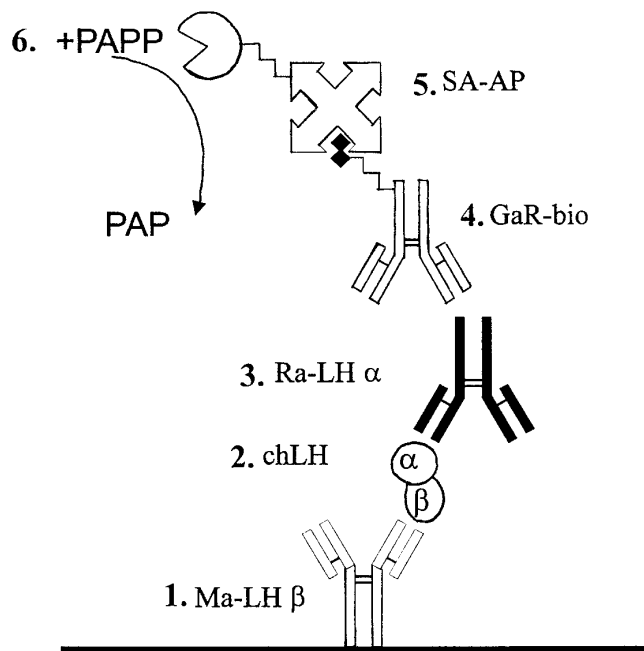


FIG. 1. Immunosassay procedure. (1) Coating of capture Ma-LH β to the polystyrene plate, (2) incubation with LH samples and standards, (3) addition of detection antibody Ra-LH α , (4) incubation with secondary antibody GaR-bio, (5) addition of the enzyme conjugate SA-AP, and (6) enzymatic reaction of PAPP.

sure this hormone in plasma or other body fluids, sensitive radioimmunoassays (RIA) have been introduced (1–5). Various RIAs using mainly ^{125}I as a label still remain the methods of choice for assaying LH to date as was done 20 years ago. Although these methods are accurate and reliable, they suffer from problems associated with the use of radioisotopes. The short half-life of the ^{125}I label further restricts their use. As an alternative to RIA, several nonisotopic immunoassays for LH using enzyme labels have been described (6, 7), but

low sensitivity and sample matrix effects seem to be the predominant problem in ELISA.

In chicken too, LH shares a common α subunit with follicle-stimulating hormone and thyroid-stimulating hormone. Isoforms with different isoelectric points and biological activities have been reported (8). A number of chicken (c)LH isoforms which have different *in vitro* biological activities and immunoactivities in a homologous radioimmunoassay have been recently purified in considerable amounts and partially characterized by the USDA (designated USDA-cLH-K-1 to K-7) (9).

We have recently reported on the production of monoclonal antibodies (Ma-LH β) (10) against the chicken pituitary glycoprotein hormones that were characterized as cLH β specific or anti- α subunit. Based on the described LH-specific Ma-LH β , a sensitive specific sandwich ELISA has been developed in our laboratory. This ELISA uses the biotin–streptavidin amplification technique which overcomes the disadvantages associated with RIA and traditional ELISA methods (7).

Another strategy for combining high sensitivity and a nonisotopic detection system is to exploit the potential of electrochemical detection. The combination of the sensitivity of modern electrochemical detection with the selectivity and specificity of a heterogeneous enzyme immunoassay should theoretically result in extremely sensitive assays for macromolecules. Recently, the application of electrochemical techniques to immunoassay methodology, also known as electrochemical enzyme immunoassay (EEIA), has been investigated (11–16).

In the present study, we developed an EEIA method for chicken luteinizing hormone by adapting the existing colorimetric sandwich ELISA to EEIA. The adaptation consisted mainly of the optimization of the electrochemical endpoint and the efficient control of the nonspecific adsorption of detection reagents.

TABLE 1
Experimental Design and Results for Checking the Origin of the Background Current^a

Test	Mouse anti-cLH β (Ma-LH β)	Standard of cLH	Rabbit anti-cLH α (Ra-LH α)	Biotinylated goat anti-rabbit IgG (GaR-bio)	Alkaline phosphatase- streptavidin conjugate (SA-AP)	PAPP	Background current (nA)
1	–	–	–	–	–	+ ^b	4.50
2	+	–	+	+	–	+	6.94
3	+	–	+	–	+	+	8.13
4	+	–	–	+	+	+	95.50
5	+	–	–	+ ^c	+	+	17.88

^a +, present, –, absent.

^b Direct injection of 1 mM PAPP into FIA-ED system.

^c Addition of 1% mouse serum to GaR-bio.

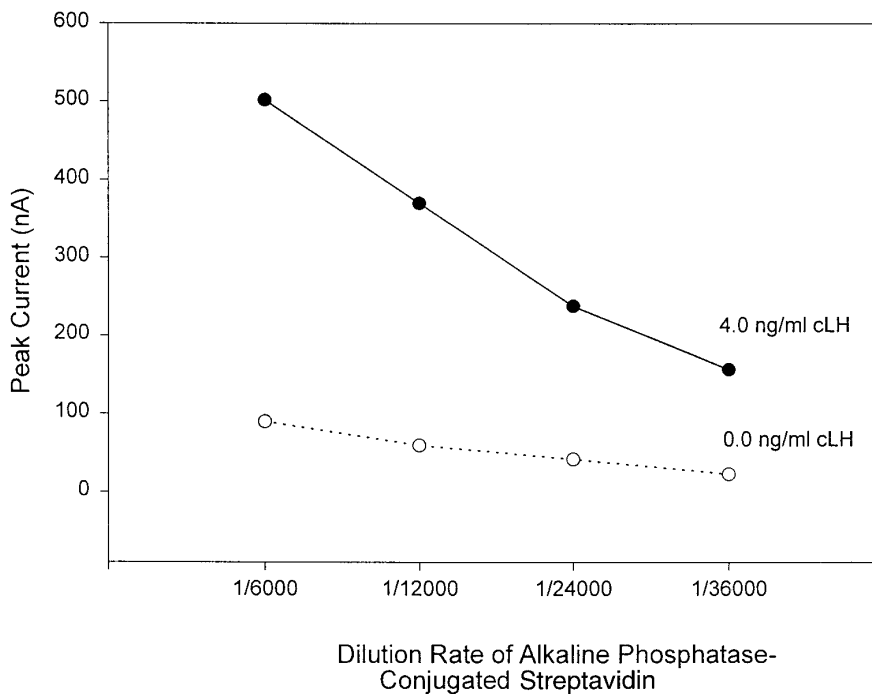


FIG. 2. Peak current as a function of enzyme SA-AP dilution.

MATERIALS AND METHODS

Anti-gonadotropins. The immunological reagents in the present EEIA of the chicken LH were the same as described before (7). Briefly, three monoclonal anti-chick-

en-LH β antibodies (designated IIC7E10, IVB10F10, and IVE7C9) that could be easily produced in high-titer ascites were mixed in equal quantities after purification and further used as a coating cocktail Ma-LH β at a total IgG working concentration of 1 μ g/ml coating buffer (0.1

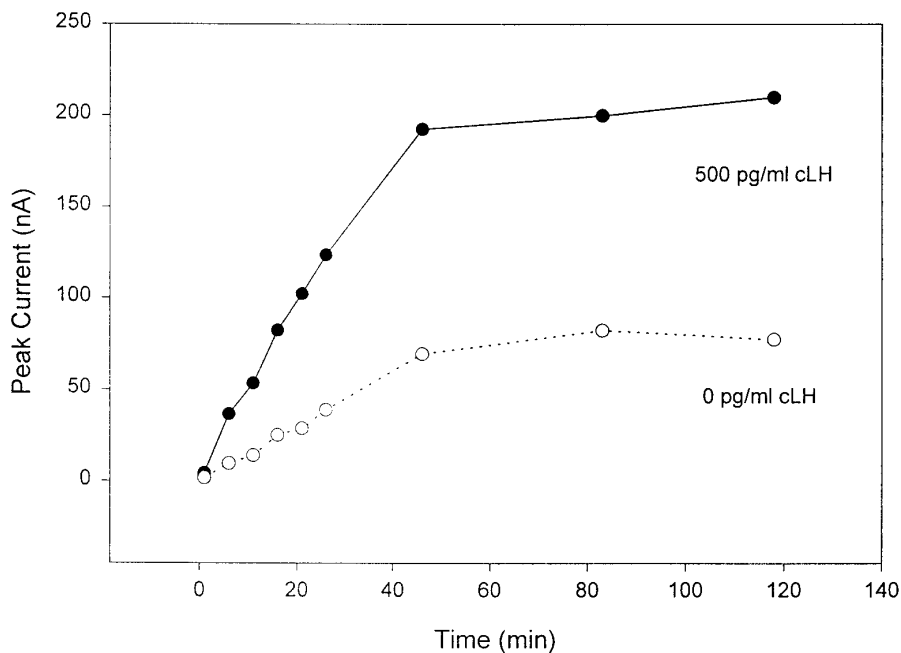


FIG. 3. Peak current as a function of enzymatic reaction time.

M NaHCO₃, pH 9.3). The detection antibody was a polyclonal rabbit anti-chicken LH α (Ra-LH α) which was also produced in our laboratory (7), used in a 1/10,000 working dilution (starting from neat serum).

Detection reagents. The secondary antibody was biotinylated goat anti-rabbit IgG (GaR-bio, E432; DAKO A/S, Glostrup, Denmark), used at a dilution of 1/5000 (stock solution, 1.0 g/L). Later in the procedure, 0.25% mouse serum was added. As an enzyme label, the alkaline phosphatase-streptavidin conjugate (SA-AP, P396; DAKO A/S) was used at a 1/6000 dilution (stock solution, 0.3 g/L) instead of the peroxidase used in the colorimetric version of the assay. The substrate *p*-aminophenyl phosphate (PAPP) was unavailable commercially and was synthesized from 4-nitrophenyl phosphate (PNPP) (Sigma, No. 3254) by hydrogenation (17). PNPP (100 mg) was dissolved in 25 ml of degassed water and the pH was adjusted to 6.3 with 3.0 μ l 6 N HCl, and then 30 mg 10% Pd/C catalyst (Aldrich, No. 205699) was added. The reductive reaction was allowed to proceed under 1 bar of hydrogen at room temperature for 90 min. In order to assess the purity and the yield of the synthesized product, ion-pair chromatography was used. The column was Bioanalytical Systems Phase II C₁₈ column (100 \times 3.2 mm, 3- μ m particles) and the mobile phase was 0.4% triethylamine (TEA) aqueous solution (2 ml TEA + 500 ml Milli-Q water); pH was adjusted to 4.5 by acetic acid. The detection was achieved at 254 nm by UV absorbance detection (LKB Bromma 2141 Variable). The molar yield of PAPP was over 99%. A 1 mM PAPP working solution was diluted from 10 mM stock solution (stored at -70°C) with carrier fluid. *p*-Aminophenol (PAP) was purchased from Sigma.

Apparatus. A Waters Model 625 LC system (Waters Assoc., U.S.A.) was used to pump the carrier fluid at a flow rate of 0.5 ml/min. A Waters 715 Ultra WISP sample processor was used for automatic injection. A BAS LC-4C amperometric detector (Bioanalytical Systems, BAS Europe, Belgium), which was equipped with a glassy carbon working electrode, was used for electrochemical quantification of PAP. The operating potential was +325 mV versus an Ag/AgCl reference electrode. The cell gasket was 16 μ m.

Carrier fluid. The carrier fluid consisted of 0.1 M tris(hydroxymethyl)aminomethane, 1 mM MgCl₂ (Merck), and 0.2 g/L NaN₃ (Merck), pH 9.0. This buffer was also used as the enzymatic reaction buffer.

Immunoassay procedures. All experiments were performed at room temperature under continuous agitation (80 rpm), unless otherwise stated. A PBST/BSA buffer (10 mM K₂HPO₄, 0.15 M NaCl, pH 7.4) containing 0.01% (w/v) NaN₃, 0.5% (v/v) Tween 20, and 1% (w/v) bovine serum albumin (BSA) (Sigma A-7030) was

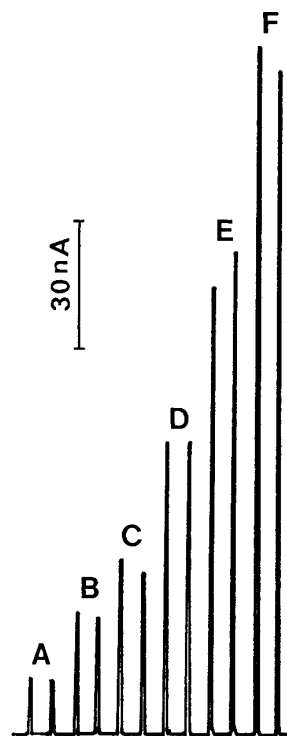


FIG. 4. Peak current as a function of cLH concentration. Each concentration was run in duplicate: (A) 0.0, (B) 39.0, (C) 78.0, (D) 156, (E) 321.0, and (F) 625.0 pg/ml. SA-AP dilution was 1/6000. Enzymatic reaction time was 15 min.

used for dilution of antibodies, standard, and enzyme label and washing of microtiter plates. In between the different steps of the protocol, plates were routinely rinsed four times with PBST/BSA buffer. Flat-bottom medium-binding polystyrene microtiter plates were from Costar (Cambridge, MA). Samples were standardized against a serial dilution of USDA-cLH-K-3 supplied by the USDA Animal Hormone Program. Working solutions were prepared from a 100 ng/ml stock solution. Blood samples were collected into heparinized tubes and immediately centrifuged. Plasma samples were stored at -70°C until the day of assay.

All steps are shown in Fig. 1. The capture Ma-LH β was passively adsorbed to polystyrene plates for 5 h (200 μ l/well); subsequently, excess protein binding sites were blocked by a 1-h incubation at 37°C with 1% (w/v) BSA buffer (10 mM K₂HPO₄, 0.15 M NaCl, 0.01% NaN₃, pH 7.6), 220 μ l/well. Serial dilutions of USDA-cLH-K-3 ranging from 0.31 pg/ml to 5 ng/ml were made in PBST/BSA buffer and incubated overnight (200 μ l/well). Plasma samples were serially diluted in PBST/BSA buffer and incubated likewise. The next day, the detection antibody Ra-LH α was added and left for 3 h. Then the secondary antibody GaR-bio was added for 1 h with or without a preincubation step of the antibody solution with 0.25%

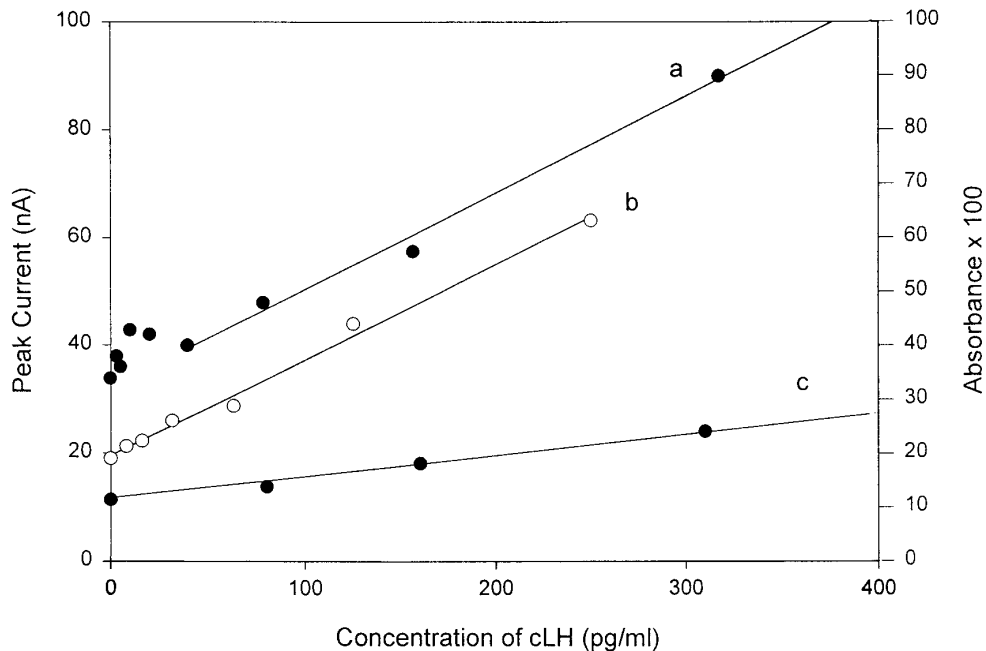


FIG. 5. Standard calibration curve of cLH (a) obtained by EEIA before reducing the background current, (b) obtained by EEIA after decreasing the background current, and (c) obtained by colorimetric ELISA.

mouse serum for 3 h. Finally, SA-AP was added for another 30 min. After four rinses with PBST/BSA buffer (2 s soaking time in between rinses) and one 5-min rinse with carrier fluid, 1 mM PAPP substrate (200 μ l/well) was added and after 15 min. 50 μ l of the reaction mixture

was aspirated for analysis. Five microliters of the sample was finally injected into the flow injection analysis-electrochemical detector (FIA-ED) system. The samples were staggered 0.2 min apart to allow sufficient time to record the signal before the next injection. The resulting

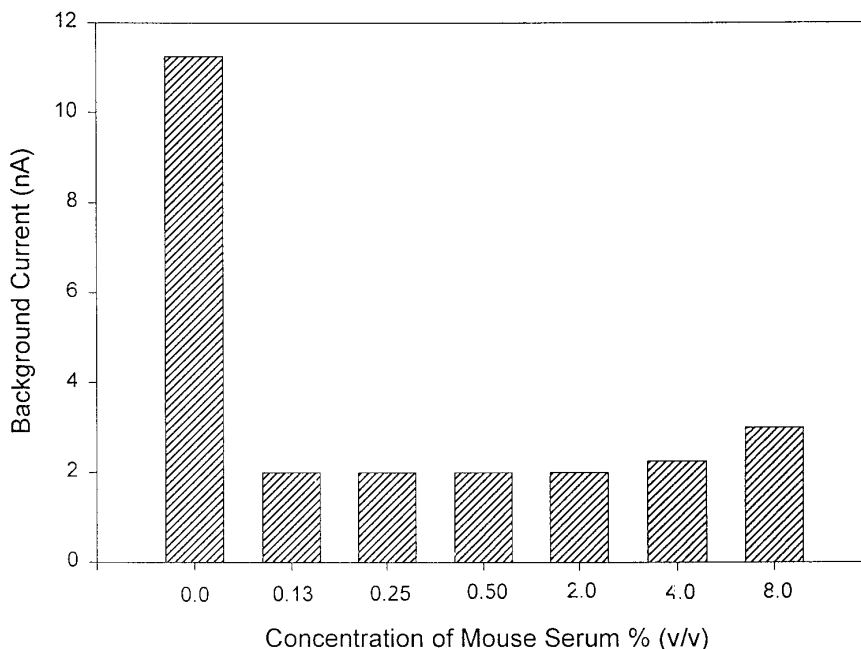


FIG. 6. Effect of mouse serum concentration on the background current.

TABLE 2
Summary of Cause and Remediation for the Background Current

Test	Origin of the background current	Remedy for decreasing background
1	Nonenzymatic hydrolysis of PAPP.	Keep PAPP in the dark and at 4°C.
2	a. Nonenzymatic hydrolysis of PAPP. b. Contaminative enzymatic hydrolysis of PAPP by endogenous enzyme.	a. Keep PAPP in the dark and at 4°C. b. Add levamisole to antisera and BSA solution.
3	a. Nonenzymatic hydrolysis of PAPP. b. Contaminative enzymatic hydrolysis of PAPP by endogenous enzyme. c. Nonspecific adsorption of enzyme (SA-AP).	a. Keep PAPP in the dark and at 4°C. b. Add levamisole to antisera and BSA solution. c. Not significant.
4	a. Nonspecific adsorption of GaR-bio to the capture antibody. b. Nonspecific adsorption of GaR-bio to the solid support.	a. Add 1% mouse serum to GaR-bio. b(1). Add 0.5% Tween 20 to wash buffer. b(2). Add 1.0% BSA to wash buffer.
5	Nonspecific adsorption of GaR-bio to the solid support.	Add 0.5% Tween 20 and 1.0% BSA to wash buffer.

peak heights were then used to construct a standard calibration curve.

Experiment 1. To examine the sources of background current in each immunoassay step, the following experiment was performed in 96-well polystyrene microtiter plates as shown in Table 1. In row 1, 1 mM PAPP was injected directly into the FIA-ED system. In the other rows, the Ma-LH β was adsorbed to the plastic surface for 5 h and subsequently blocked by a 1-h incubation at 37°C with 1% BSA buffer; standards or samples were omitted and instead 200 μ l PBST buffer was incubated overnight. The next day, 200 μ l PBST buffer was added in rows 4 and 5 and Ra-LH α was added in rows 2 and 3 and left for 3 h. Then GaR-bio was added either with (row 5) or without (rows 2 and 4) preincubation with 1% mouse serum. Two hundred microliters of PBST buffer was added in row 3. Incubation time was 1 h. After rinsing, 200 μ l of a 1/6000 diluted SA-AP solution was added in rows 3, 4, and 5, and PBST buffer was added in row 2 for 30 min. The remaining steps were as described in the immunoassay procedures.

Experiment 2. To test the specificity of the assay in different types of chicken plasma, juvenile and adult chicken plasma samples were serially diluted and further treated similarly to the cLH standard curve in order to assess the parallelism of the dilution curves.

RESULTS AND DISCUSSION

Flow injection analysis-electrochemical detection system. In order to obtain the highest sensitivity for this immunoassay, some adaptations of the FIA-EC system were necessary. The effective volume of the

electrochemical cell was reduced by using a 16- μ m thin-layer gasket and the injection volume was only 5 μ l. Because electrochemical detection is an interfacial rather than a bulk-solution phenomenon, a lower flow rate of carrier fluid provided a higher mass sensitivity and less carrier fluid consumption. However, the inherent sensitivity of the detection system was high enough to allow a 500 μ l/min flow rate.

The cyclic voltammograms of *p*-aminophenol show chemically reversible behavior and easy oxidation (15). The optimal potential, determined by repeatedly injecting a single concentration of PAP into the detection system at different applied potentials, was 325 mV. The magnitude of the oxidation current of PAP at this potential was directly proportional to the concentration of PAP and resulted in sufficient peak height and low baseline noise. At this potential, spontaneous oxidation of the substrate PAPP was avoided. The dynamic range of the plot was from 0.1 to 4.0 μ M and the detection limit was 9 nM PAP, as calculated by using a signal-to-noise ratio of 3. This detection limit means that only 1/100,000 parts of the PAPP present must be converted into PAP to allow detection (1 mM PAPP was added).

Optimization of immunoassay parameters. Capture antibody, detection antibody, and secondary anti-

TABLE 3
Effect of Tween 20 and BSA on the Signal to Noise Ratio

	Concentration	Signal/noise
Tween 20 (v/v)	0.5%	3.07
	0.05%	7.45
BSA (w/v)	1.0%	8.75
	0%	14.82

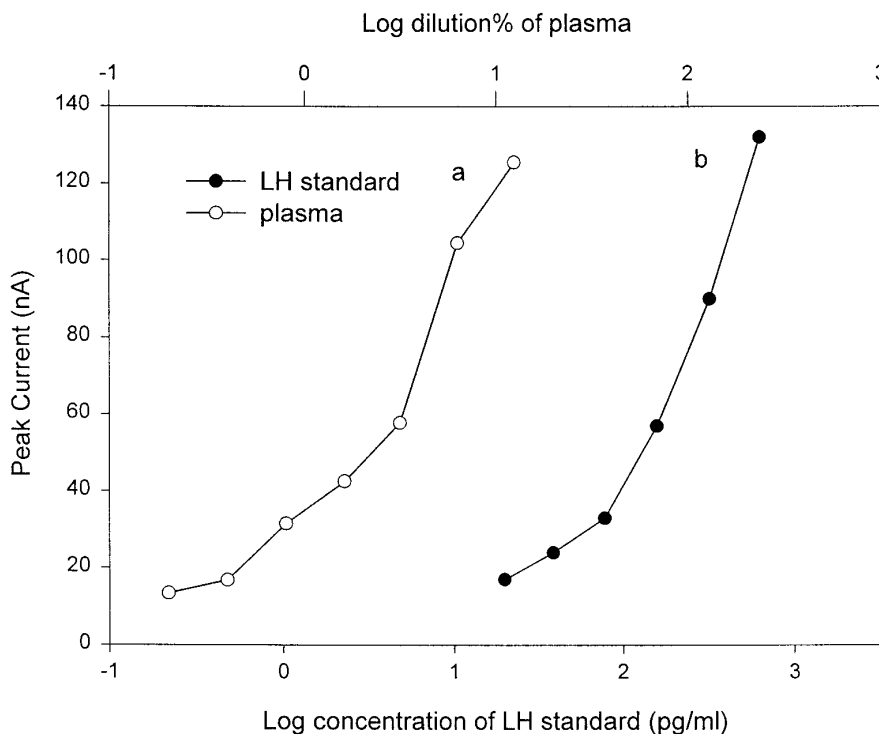


FIG. 7. Peak current as a function of the dilution of (a) cLH standards and (b) juvenile chicken plasma. The dilutions of juvenile chicken plasma were 12.5, 6.25, 3.125, 1.6, 0.8, 0.4, and 0.2%. The concentrations of cLH standards were 19.0, 39.0, 78.0, 156, 317, and 625 pg/ml.

body concentrations were adopted from the colorimetric assay as described earlier (7). To optimize the concentration of SA-AP for EEIA, different enzyme-conjugate dilutions were tested with standard LH concentrations of 4 and 0 ng/ml (Fig. 2). Both signal and background kept increasing with increasing concentration of the enzyme. The optimal dilution was 1/6000. To study the substrate incubation time, the assay was carried out with LH standard concentrations of 500 and 0 pg/ml; 1 mM PAPP was added for various periods of time at the end of the EEIA procedure (Fig. 3). The resulting current reached a plateau after 40 min, but for practical reasons, the enzymatic reaction was stopped after 15 min of incubation, since this both yielded sufficient sensitivity and did not compromise the stability of the substrate. The blank current was likely to be due to the nonspecific adsorption of the antibody-enzyme conjugate that converted PAPP to PAP as discussed below. PAPP was diluted in substrate buffer containing tris(hydroxymethyl)aminomethane, which is known to promote transphosphorylation of the substrate. Addition of magnesium ions to the reaction mixture was essential for optimal enzymatic activity (15).

Nonspecific adsorption. Under the above assay conditions, a series of LH standards was assayed and the FIA peaks are shown in Fig. 4. A standard calibration

curve is shown in Fig. 5a. It was observed that the linear range of the assay was only from 39 to 5000 pg/ml, and the oxidation current at zero antigen concentration was very high. Therefore, experiments were performed to test the sources of the background current in each step of the assay (see Table 1). Since PAPP is known to undergo nonenzymatic hydrolysis in solution, 1 mM PAPP was injected directly into the FIA-ED system (row 5 in Table 1). This revealed that a small amount of PAP was indeed present in the substrate, but this could not possibly account for the observed background current.

As a test for endogenous alkaline phosphatase activity of any of the immunological reagents used during the detection protocol, the reaction product was measured in the absence of SA-AP (Table 1, row 2). Again, only a minor portion of the background current could be attributed to this cause. Addition of levamisole blocks the endogenous alkaline phosphatase and reduces the background current; but in our work, this contamination was quite small and addition of levamisole did not substantially lower the background (results not shown).

The result of test 3 represents the background current contributed by nonspecific adsorption of the enzyme conjugate to the plate or the capture antibody in the absence of biotinylated antibody. Because the background current

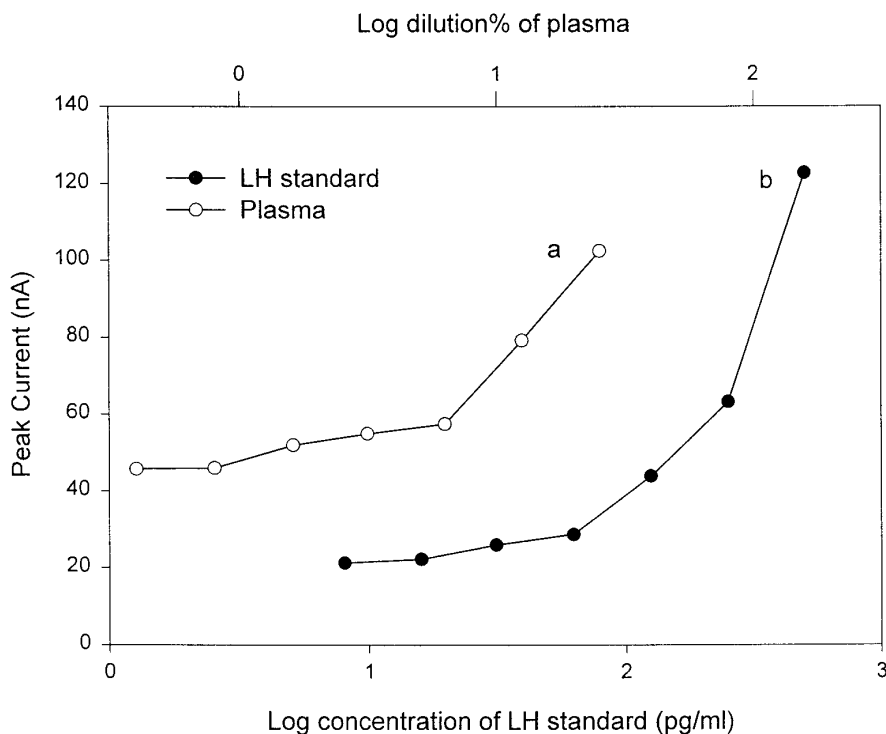


FIG. 8. Peak current as a function of the dilution of (a) cLH standards and (b) adult chicken plasma. The dilutions of adult chicken plasma were 25.0, 12.5, 6.25, 3.125, 1.6, 0.8, 0.4, and 0.2%. The concentrations of cLH standards were 2.5, 5.0, 10.0, 20.0, 78.0, 156, 317, and 625 pg/ml.

in row 3 was nearly the same as in row 2, nonspecific adsorption of enzyme to the polystyrene surface or the capture antibody appeared to be minimal.

The data from row 4 clearly show that the major source of PAP production at zero antigen concentration had to do with the binding of the secondary antibody (i.e., goat anti-rabbit IgG). A first cause can be the interaction of the secondary antibody with molecules other than the detection antibody (so-called specific nonspecificity). The magnitude of this depends upon the species specificity of the secondary antibody. Secondly, nonspecific binding might be caused by adsorption of this secondary antibody to the solid support (nonspecific nonspecificity). Addition of 1% mouse serum to the secondary antibody solution clearly shows that the former type of nonspecific adsorption takes place in this case (Table 1, row 5). The most likely explanation is that the GaR-bio cross-reacts with the capture antibody (Ma-LH β), generating a false positive response. This cross-reactivity was efficiently blocked by preincubating the secondary antibody with 1% normal mouse serum for 3 h. Figure 6 shows the effect of the addition of different amounts of mouse serum on the reduction of the background current. Concentrations from 0.125 to 4% were equally effective in reducing the background current, which was minimal under those conditions. The remaining background current in

row 5 indicates that the nonspecific binding also occurs through the interaction of the conjugate with vacant sites on the hydrophobic polystyrene surface. This type of binding is due mainly to entropically driven hydrophobic interactions of proteins with nonpolar surfaces. In the case of a plastic surface such as polystyrene, adsorption can also be due to electrostatic interactions between the surface and the protein. Tween 20 and BSA reduce hydrophobically based adsorption, but cannot eliminate the adsorption due to electrostatic interactions. Tween 20 is a nonionic detergent which is commonly employed in immunoassays to block vacant sites on the solid support which were not occupied by the capture antibody (16). So the Tween 20 concentration in PBST buffer was changed from 0.05 to 0.5% (v/v). The result in Table 3 shows the benefits of using BSA as an additional site blocker of conjugate adsorption. If BSA is present in all solutions starting from the first rinse, the adsorption is even further reduced. Accordingly, 0.5% Tween 20 and 1% BSA were added to all buffer solutions and used for dilution of antibodies, standard, and enzyme label and for washing of microtiter plates, with the exception of the coating buffer and the substrate solution. The general cause and remedy for background current are summarized in Table 2 which further explains Table 1.

Analytical performance and specificity. Using the adapted assay protocol, a new calibration curve was constructed (Fig. 5b). In comparison with the standard calibration curve obtained by ELISA (Fig. 5c), the detection limit was extended by more than 1 order of magnitude (from 39 to 2.5 pg/ml). The method gave good intra-assay precision (CV = 6.9%, $n = 4$) over its linear dynamic range of 2.5 to 625 pg/ml (0.125–313 pg/well). The limit of detection of the assay was 2.5 pg/ml (defined as the mean signal of four replicates of zero calibrator plus two times the standard deviation). Parallelism was observed between serial dilutions of standard USDA-cLH-K-3 and plasma samples from juvenile chicken and adult chicken (Figs. 7 and 8). These dilution curves illustrate the sensitivity of the assay and the small amount of plasma needed (i.e., less than 10 μ l). The concentrations of LH in juvenile chicken and adult chicken plasma were 6.35 and 1.66 ng/ml.

In conclusion, the inherent sensitivity of FIA-EC allowed the development of an ultrasensitive enzyme immunoassay for cLH, on condition that the nonspecific adsorption (which was much less important in the original colorimetric ELISA) was efficiently reduced. Moreover, the method requires a very small sample volume, which not only provides the possibility of determining LH in chicken embryo plasma samples, but which also may decrease potential interference with matrix components present in chicken plasma or pituitary extracts from juvenile and adult chickens.

Because EEIA is still in the developing stage as a technique, the instrumentation is not as convenient as the classical ELISA equipment. However, the newly developed "Endurance" autosampler (Spark, the Netherlands) would be a way to automatically inject the 96 samples from a microtiter plate.

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