Electrochemical Investigation of Hapten–Antibody Interactions by Differential Pulse Polarography

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The binding of electroactively labeled estriol with estrogen-specific antibody and its subsequent displacement by unlabeled estriol have been monitored by differential pulse polarography. Estriol was found to be electro-inactive in the potential range −200 mV to −1000 mV vs a silver/silver chloride electrode. Estriol labeled in the 2 and 4 position with nitro groups was electroactive, giving two reduction waves at −422 mV and −481 mV vs a silver/silver chloride electrode. The peak current was linear with concentration over the range 60 μg/L to 3.7 mg/L. The addition of aliquots of estrogen-specific antibody reduced the peak current proportionately, indicating the binding of ligand to specific antibody. Subsequent addition of unlabeled estriol produced incremental increases in peak reduction current, indicating competitive and reversible binding of the two ligands for the antibody. Separation of bound from free labeled hapten was not necessary because reduction of the antibody-bound labeled estriol is attenuated at the electrode.

Additional Keyphrases: electrochemical techniques · estriol · immunoassay

The predominant immunoassay technique for analysis is radioimmunoassay (RIA), in which a radioactive label is attached to the antigen. This powerful method combines the selectivity of the antigen–antibody reaction with the very low detection limits of radioactivity-counting techniques. The use of radiolabeled compounds, however, entails several distinct disadvantages. The spontaneous decay of the radioactive labels means they have a finite useful shelf life and necessitates periodic recalibration of standard curves. The handling and disposal of radioactive materials involve certain legal problems and special safety precautions (1). Also, bound labeled antigen must be separated from free labeled antigen before detection by scintillation methods. Prompted by these disadvantages, investigators have studied other types of labels, including radicals detected by electron-spin resonance (2), enzymes detected by subsequent enzymic reaction (3–5), and fluorescent labels detected by fluorometry (6).

Electrochemical methods have also been examined for possible utility in measuring antibody–antigen reactions. Breyer and Radcliffe (7, 8) used polarographic methods to measure the binding of an electroactive azo-protein with specific antibody. The binding of the azo-protein by the antibody resulted in a decrease in the current for the reduction of the azo group. Similarly, Zikán and Kotýnek (9, 10) monitored the binding of dinitrophenyl-L-lysine with specific antiserum via polarography.

Meyerhoff and Rechnitz (11, 12) developed a competitive-type enzyme assay for bovine serum albumin, in which the product of urease-labeled albumin was detected potentiometrically. D'Orazio and Rechnitz (13) have also used potentiometric methods involving an ion-selective membrane electrode to monitor concentrations of complement and (or) antibody. Aizawa et al. (14) studied antigen–antibody interactions with an antigen-bound membrane for the potentiometric detection of syphilis.

Weber and Purdy (15) investigated an amperometric technique in which a solution flowing past the electrode was made up from a homogeneous voltammetric immunoassay; they determined electroactive codeine by a competitive reaction with electroactive morphine or with morphine tagged with electroactive ferrocene and morphine antibody. Yuan et al. (16) devised an amperometric method for an enzyme immunoassay of creatine kinase, in which the product of the enzyme reaction (ferrocyanide) was detected by oxidation at a platinum working electrode in a stirred solution. Similarly, Aizawa et al. (17) have also used an enzyme assay with amperometric detection of the enzyme product; in their method the antibody was chemically attached to a membrane that was placed over a Clark-type oxygen probe.

The wide dynamic range and low detection limits of modern electroanalytical techniques such as differential pulse polarography (DPP)2 and stripping voltammetry (18, 19) make labeling the antigen with an electroactive group a potentially useful approach for immunoassay methodology. The objective of this research was to explore the applicability of DPP as a technique for monitoring binding of an antibody with a hapten labeled by an electroactive group. DPP was selected for this initial research because it involves the dropping mercury electrode. This electrode, which forms a new electrode surface every 2 to 6 s, should be relatively immune to the adsorption–inhibition phenomena that can be a problem at solid electrodes with solutions containing proteins.

We had several reasons for choosing to use the estrogen estriol in a model system for evaluating DPP: Specific undiluted antiserum was readily available for estrogens. Estrogens generally show little electrochemical reduction behavior, thereby providing a “potential window” in which an electroactive label would impart electrochemical behavior to the labeled compound distinct from the unlabeled compound, and the numerous synthetic procedures for the derivatization of estriol allow easy attachment of a wide variety of possible electroactive labels. Because nitrated phenyl and phenolic compounds give well-defined waves for the reduction of the

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2 Nonstandard abbreviations: DPP, differential pulse polarography; IgG, immunoglobulin; Ab, antibody; DNE, 2,4-dinitroestriol.

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nitro group in the potential range $-300 \, \text{mV}$ to $-800 \, \text{mV}$ vs a saturated calomel electrode (20, 21), we synthesized 2,4-di-nitroestriol (DNE) for use in this study as the electroactively labeled hapten.

**Materials and Methods**

*Equipment.* For DPP we used a Model 174A polarographic analyzer (Princeton Applied Research Corp. [PARC], Princeton, NY 08540) with a Model 2000 x-y recorder (Houston Instruments, Bellaire, TX 77401), a Model 303 Static Mercury Drop Electrode (PARC), a saturated Ag/AgCl reference electrode, and a platinum auxiliary electrode. All polarography was with these scan ranges: range of scan, $-200$ to $-1700 \, \text{mV}$ vs Ag/AgCl; pulse amplitude, 26 mV; scan rate, 10 mV/s; drop time, 1.0 s. Solutions were purged for 5 to 12 min with nitrogen passed through vanadium chloride deoxygenating towers. They then were blanketed with nitrogen while recording polarograms. Polarograms were repeated three times to obtain average values. The solution volumes used in this study were about 5.0 mL.

*Materials.* Estriol, estradiol, and bovine IgG were of the highest available purity (Sigma Chemical Co., St. Louis, MO 63178). Estrogen-specific monoclonal antisera was a gift from New England Nuclear, Boston, MA 02118. Estriol-3-$\beta$-nitro was doubly distilled and passed through an ion-exchange column. All other chemicals were reagent grade or better.

The supporting electrolyte was potassium phosphate buffer, 0.1 mol/L, pH 7.4. The analysis buffer was purged with $N_2$ for 1 h and then pre-electrolyzed over a mercury pool electrode maintained at $-1.3 \, \text{V}$ vs a saturated calomel electrode for at least 48 h to remove trace-metal impurities.

*Labeling of estriol.* Estriol was nitro-labeled in the 2 and 4 positions by the procedure of Könyves and Ölsén (22). The product was separated from an impurity by column chromatography on silica gel 60 and elution with acetone/chloroform (1/1, by vol). The isolated product melted at 225 °C. The labeled estriol was dissolved in ethanol/water (1/1, by vol) solution and used as a standard solution.

*Characterization of antisera.* The estrogen-specific monoclonal antisera was raised in mice against estriol conjugated at the 16 position to the carrier, and showed the following cross reactivities: estrone 69.8%, estradiol 62.7%, and estriol-3-ace te less than 1% (private communication from New England Nuclear). The IgG fraction was isolated by use of a Sephacryl S200 column with potassium phosphate buffer (0.1 mol/L, pH 7.4) as the eluent. The isolated IgG fraction was dialyzed overnight against 10 mmol/L potassium phosphate buffer (pH 7.4), lyophilized, and reconstituted with de-ionized water before use as the antisera in this study. Immunoprecipitation of the isolated IgG fraction with goat anti-mouse IgG confirmed the identity of the antisera.

**Results and Discussion**

*Polarography.* We recorded polarograms of solutions of phosphate buffer, estriol, and specific antisera to determine the electrochemical "potential window" available for this study (Figure 1). All three solutions were electro-inactive in the potential range $-200 \, \text{mV}$ to $-1000 \, \text{mV}$ vs Ag/AgCl. Thus this relatively large "potential window" is available for the observation of the labeled material without interference from these assay components.

Figure 2 shows the polarogram of a solution of DNE, used to determine the reduction peak potential of the electroactive nitro labels. DNE has two distinct reduction waves with peak potentials of $-422 \, \text{mV}$ and $-481 \, \text{mV}$ vs Ag/AgCl. Both waves were observed at scan rates of less than 10 mV/s. At faster scan rates there was a loss of resolution, showing a single non-symmetric wave with a peak potential of $-481 \, \text{mV}$ vs Ag/AgCl. The peak current of the reduction wave was linear as a function of concentration over the range 0.1 to 3.7 mg/L. Introducing the nitro groups into the A ring of estriol thus produced an electroactive labeled hapten that is electrochemically distinguishable from the unlabeled hapten.

*Antibody–DNE Binding.* To determine the effect of the antibody on the reduction of DNE, we recorded successive polarograms of a DNE solution before and after the addition of successive aliquots of the estrogen antibody (Figure 3). The solutions were deoxygenated slowly for 12 min after each addition before the polarograms were recorded. The binding of DNE by antibody is demonstrated by the sequential decrease in the reduction peak current.

Two explanations for the decrease in peak current for the reduction of DNE from the addition of specific antibody are plausible. The antibody binding of DNE may sequester the electroactive nitro groups from the electrode surface by burying them in the binding pocket. The DNE–Ab complex would be electro-inactive and the decrease in peak current...
would therefore be due to a decrease in the concentration of
the reducible solution species. It is also possible, however, that
the DNE–Ab complex is still electroactive, in which case the
decrease in peak current would be due to the large decrease
in the diffusion coefficient of the DNE–Ab complex (D_{DNE-
Ab}), as compared with that of free DNE (D_{DNE}). The diffusion
coefficients of globular proteins of M, 150 000−160 000 in
aqueous media are reportedly in the 10^{−7} cm^{2}/s range (23),
whereas those of small organic compounds such as estriol are
in the 10^{−5} cm^{2}/s range (24). In polarography the peak current
is directly proportional to the square root of the diffusion
coefficient of the electroactive species and therefore the
decrease could be attributable to a change in diffusion coefficient
upon binding. In either case, the effect of binding is clearly
measurable without separation of the bound-labeled from the
free-labeled species. The increase in peak current observed
for the addition of the first aliquot of antisera is probably due
to some type of adsorption phenomenon at the electrode
surface.

Reversibility of antibody–DNE binding. For an antigen–
antibody reaction to serve as a functional competitive-type
analytical method, the bound labeled hapten must be re-
vessibly displaced from the antibody by unlabeled hapten, and
the amount of displaced labeled hapten should be propor-
tional to the concentration of unlabeled hapten in the stan-
dard or sample to be analyzed. We tested this by deoxygen-
ating a solution of DNE plus antisera slowly for 12 min and
recording the polarogram. Subsequently, we added successive
aliquots of unlabeled estriol to this DNE–Ab solution, de-
obxygenated the solution slowly for 5 min, and recorded the
polarograms. A plot of the resulting peak currents is shown
in Figure 4.

The reversible displacement of DNE from antibody-binding
sites is demonstrated by the sequential increase in peak cur-
rent with successive aliquots of unlabeled estriol. The dis-
placement of DNE from the antibody-binding sites by estriol
increases the concentration of free DNE capable of being re-
duced and correspondingly decreases the concentration of
bound DNE. The quantitative detection of this reversible
displacement would form the basis of an immunoassay based
on DPP. Estrone and estradiol were also able to displace
antibody-bound DNE, as would be expected from the reported
cross reactivity of the antisera with these steroids. Proges-
terone, however, did not displace DNE from the antibody-
binding sites, indicating that the reaction is specific for es-
trogens.

Bovine IgG–DNE–estriol interactions. Bovine IgG was
used to probe for nonspecific interactions between DNE and
the globular IgG proteins. Aliquots of a bovine IgG solution
were added successively to a solution of DNE; polarograms
were recorded after the resulting DNE solutions had deoxygen-
ated slowly for 12 min. Figures 5 shows a plot of normalized
peak current vs concentration of bovine IgG.

With the addition of successive aliquots of the IgG, the
normalized peak current decreased to a limiting value, after
which further aliquots of bovine IgG produced no further
decrease in normalized peak current. Subsequent addition of
an aliquot of an unlabeled estriol solution to the DNE–bovine
IgG solution failed to increase the normalized peak current
(Figure 5).

The small decrease in the normalized peak current upon the
addition of nonspecific bovine IgG is probably caused by
protein coating the electrode surface and thereby inhibiting
somewhat the access of the electroactive species to the elec-
trode surface. [The effect of adsorption on polarograms is well
documented (25).] However, regardless of the cause of this
decrease, the fact that the addition of unlabeled estriol does
not increase the peak current indicates that there is no specific
binding like that observed with the estrogen-specific anti-
sera.
The addition of successive aliquots of estrogen antisera to a solution of DNE containing bovine IgG caused incremental decreases in the normalized peak current, whereas further additions of a bovine IgG solution resulted in no significant decrease in normalized peak current. Also, the addition of an aliquot of estriol to a solution of DNE containing estrogen antisera plus bovine IgG increased the normalized peak current, in contrast to the addition of estriol to the DNE solution containing bovine IgG only (Figure 5). These results indicate that a specific hapten–antibody interaction is responsible for the decrease in peak current observed for DNE/estrogen antisera combination, but not for the small decrease observed with the DNE/bovine IgG combination.

Binding of DNE with antisera and its subsequent displacement by estriol is readily observable in urine, but the reduction in human serum is greatly attenuated; as a result, the binding and displacement of DNE in serum was not observable in the concentration range used for the assay. Whether the attenuation of DNE reduction is caused by severe electrode coating or a nonspecific binding of the DNE by serum proteins has yet to be evaluated.

In summary, with this model system involving estriol, we have demonstrated the feasibility of using an electrochemically labeled hapten and detection by DPP to monitor the competitive and reversible binding of labeled and unlabeled hapten by a specific antibody. Although estriol was labeled in the A ring, where the antisera should demonstrate its highest specificity, we could observe binding and release of the labeled hapten from the antibody. Therefore, this method, in principle, could be used in an immunosensor technique with the advantage of not requiring separation of bound and free labeled material. It could also be used in the titration of antibody-binding sites, as well as in measurement of binding constants.

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