Continuous monitoring of analyte concentrations*

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Abstract: We have investigated the application of a modified, heterogeneous, competitive enzyme immunoassay for the continuous measurement of small analytes in a medium stream. The analytical system contains two antibodies that are immobilized on spatially separated areas, one binding the analyte (Ab1) and the other binding the enzyme (Ab2). An analyte-enzyme conjugate serves as signal generator. The analyte-enzyme conjugate functions as a heterobifunctional shuttle that can bind to either antibody. A semipermeable membrane retains the enzyme shuttle in the internal volume of the sensor but permits the passage of small analytes from the medium stream. The amount of enzyme bound to Ab1 is inversely proportional and the amount of enzyme bound to Ab2 is directly proportional to the analyte concentration. We have demonstrated that this analytical system (1) can provide a larger total signal; (2) has a sensitivity comparable with conventional competitive immunoassays; (3) does not require the separation of bound from free antigens; and (4) is therefore suitable for the continuous measurement of analytes in a medium stream. With a model system, an increase from 0 ng ml⁻¹ to 20 ng ml⁻¹ of the steroid hormone progesterone and the subsequent fall to 0 ng ml⁻¹ could be monitored.

Keywords: immunoassay, continuous monitoring, analyte-enzyme conjugate.

INTRODUCTION

Analytical products utilizing immunoglobulins are almost exclusively disposable and use the antibodies in an irreversible mode. However, antibody-antigen binding is a dynamic equilibrium process and can lend itself to the reversible measurement of antigen concentrations. Therefore, antibodies can be used, in principle, for the continuous (i.e. reversible) measurement of analytes. Although slow in response time, such sensors become increasingly important for bioprocess control, environmental monitoring, waste and fresh water processing, aquaculture, and other applications where product fluctuations are not expected within short periods of time but which require early warning systems for exceeding limits of analyte concentrations over a longer time span.

We have investigated a new configuration of an enzyme immunoassay that can be used for the continuous measurement of low molecular weight analytes. Sensors based on the described technology will be applied where continuous records for analyte concentrations in a medium

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stream are desirable; where analyte concentrations need to be monitored over several hours; where changes in concentrations do not occur in minutes but rather in hours; and where frequent access to a medium stream is difficult.

MATERIALS AND METHODS

Materials

Horseradish peroxidase (HRP, type VI, 300 units mg\(^{-1}\) solid), cadaverine, poly-L-lysine hydrobromide (MW 421 000 by viscosity test), and 3,3',5,5'-tetramethylbenzidine (TMB) were purchased from Sigma (St Louis, MO). Monoclonal antibody specific to HRP (E47) was obtained from Zyrmed Laboratories Inc. (San Francisco, CA). Disuccinimidyl suberate (DSS) and a microdialyzer (System 100) were from Pierce (Rockford, IL). CNBr-activated Sepharose 4B, Bio Gel P-30, polypropylene monofilament cloth (113 mesh counts), and semipermeable cellulose membrane (molecular weight cutoff, 12 000 to 14 000; flat sheets) were purchased from Pharmacia Fine Chemicals, Inc. (Piscataway, NJ), Bio-Rad (Richmond, CA), Small Parts Inc. (Miami, FL), and Enka (Wuppertal, Germany), respectively. Progesterone was obtained from Steraloids (Wilton, NH), and the following progesterone derivatives were synthesized and characterized by HPLC (Schramm et al., 1987): progesterone-11α-hydroxy succinimide (P-NHS); and progesterone-11α-hemisuccinyl-1,5 diaminopentane (P-CAD) by reacting P-NHS with cadaverine. The substrate for HRP was prepared as described elsewhere (Paek et al., 1991).

Preparation of a defined progesterone-HP conjugate

The progesterone derivative, P-CAD, was chemically reacted with the enzyme, HRP, via DSS as a cross-linking reagent (Paek et al., 1991). The reaction mixture of the progesterone-HP conjugate was dialyzed, and then partially purified by exclusion chromatography on Bio Gel P-30. The conjugation mixture was finally purified on an immunoadfinity column with the IgG to progesterone, 4C10, immobilized on CNBr-activated Sepharose 4B gel (Paek et al., 1991). The purified conjugate with one progesterone molecule bound to one HRP molecule (P-HP) was selected, diluted with the same volume of gel-PBS-thimerosal, and stored at \(-4^\circ\)C. The concentration of P-HP in solution was determined by comparing its activity in a solid-phase assay with standard concentrations of free HRP (Paek et al., 1991). The enzymatic activity of the conjugate was preserved without loss for more than 1 year.
Scatchard analysis

Scatchard analyses (Scatchard, 1949) were performed to determine the concentrations of the antibodies immobilized on the discs, and the binding constants between the conjugate P-HRP and the antibodies. Known concentrations of the conjugate in gel-PBS-thimerosal (total 100 μl) were added to the antibody immobilized on the discs that were placed in microwells. After incubating for 5 h in a box maintained at 100% humidity at room temperature, the discs were washed, and then placed in separate wells. The concentrations of bound conjugate were determined by adding substrate containing TMB as chromogen, developing the colored TMB derivative, measuring the color at the absorbance of 450 nm by a spectrophotometer, and determining the concentration of the bound conjugate by means of a standard curve (Paek et al., 1991). The non-specific binding of the conjugate was measured from NS-Ab and subtracted from the total binding. All experiments were carried out in duplicate, and the means were used for the Scatchard plots. The determined binding constants of the antibodies to P-HRP are (for the concentrations of the antibodies, see below): 5.8 × 10¹⁰ l/mol for P-Ab (BQ.1), 1.3 × 10¹⁰ l/mol⁻¹ for HRP-Ab (E47), and 1.2 × 10⁹ l/mol for HRP-Ab (9G9).

Dose–response curves from the irreversible system

The performance of the immunoassays in the irreversible mode was evaluated by comparing dose–response curves with a conventional enzyme immunoassay as the control system with one antibody (P-Ab, 9.6 fmol disc⁻¹) on the disc (6 mm diameter). For the immunoassays under investigation, optimal concentrations of the two antibodies (P-Ab and HRP-Ab) in a microwell (total volume 40 μl) were determined with respect to the signal yield as follows (Paek & Schramm, 1991a): for one combination of antibodies, one disc of P-Ab and one disc of HRP-Ab (E47, 10.3 fmol disc⁻¹); for the other combination, one disc of P-Ab and two discs of HRP-Ab (9G9, 18.1 fmol disc⁻¹). Then, 10 μl of a P-HRP solution (0.75 pmol ml⁻¹), 20 μl of a progesterone standard solution, and 10 μl of gel-PBS-thimerosal were added to the microwells. After incubating for 15 h, the discs were washed, and the concentrations of the conjugate specifically bound to each antibody were measured as described above. The dose–response curves were obtained by plotting the concentration of the bound conjugate against the concentration of progesterone.

Time–response curves from the reversible system

For experiments in the reversible mode, a microdialyzer that contained wells similar to those in microplates was used. The wells were separated from the bypassing buffer by a semipermeable cellulose membrane. Varying analyte concentration over time in the external buffer was measured in the wells. The wells contained one disc (4.8 mm diameter) with immobilized P-Ab (BQ.1, 8.2 fmol disc⁻¹) and 3 discs with HRP-Ab (9G9, 13.4 fmol disc⁻¹) and 10 μl of P-HRP (0.75 pmol ml⁻¹) in gel-PBS-thimerosal in a total volume of 25 μl. The buffer chamber was filled with 100 ml degassed gel-PBS-thimerosal prior to the use. The well openings were capped, and the microdialyzer was placed in a box maintained at 100% humidity. The external medium was agitated by a magnetic stirrer, and the whole system was agitated on an orbital shaker to facilitate convectional diffusion in the sample wells. After an initial period of 2 h to reach equilibrium binding of the conjugate between the two antibodies, 2.5 ml of a 0.8 μg ml⁻¹ progesterone solution was added to the external buffer chamber to obtain a total concentration of 20 ng ml⁻¹ (time = 0). Starting from this time, P-HRP bound to each antibody on the different discs was measured in intervals by colorimetric detection as described above. After incubating for 3 h, gel-PBS-thimerosal was supplied to the buffer chamber by a peristaltic pump at a high flow rate (49 ml min⁻¹) to decrease the progesterone concentration to lower than 0.2 ng ml⁻¹ in 10 min. The flow was then maintained constant at a decreased rate (17 ml min⁻¹).

Determination of kinetic variables

To calculate theoretical time–response curves in the reversible system, the following kinetic variables were experimentally determined (Table 1) as described elsewhere (Schramm & Paek, 1991): the on/off-rate constants of progesterone (P) and P-HRP, the penetration layer thickness a that forms around solid surfaces and
TABLE 1 Experimentally determined kinetic variables

<table>
<thead>
<tr>
<th>Variable</th>
<th>Experimentally determined</th>
<th>Dimension</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thickness of penetration (hydrodynamic) layer</td>
<td>$8.1 \times 10^{-2}$</td>
<td>mm</td>
</tr>
<tr>
<td>Convective mass transfer coefficient of P-HRP</td>
<td>$3.7 \times 10^{-4}$</td>
<td>mm s$^{-1}$</td>
</tr>
<tr>
<td>Mass transfer coefficient for permeation of P across semipermeable membrane</td>
<td>$1.6 \times 10^{-3}$</td>
<td>mm s$^{-1}$</td>
</tr>
<tr>
<td>On-rate constant of P-HRP/P-Ab (BQ.1)</td>
<td>$6.3 \times 10^{9}$</td>
<td>litres (mol s)$^{-1}$</td>
</tr>
<tr>
<td>Off-rate constant of P-HRP/P-Ab (BQ.1)</td>
<td>$3.5 \times 10^{-4}$</td>
<td>s$^{-1}$</td>
</tr>
<tr>
<td>On-rate constant of P/P-Ab (BQ.1)</td>
<td>$4.2 \times 10^{6}$</td>
<td>litres (mol s)$^{-1}$</td>
</tr>
<tr>
<td>Off-rate constant of P/P-Ab (BQ.1)</td>
<td>$1.4 \times 10^{-3}$</td>
<td>s$^{-1}$</td>
</tr>
<tr>
<td>On-rate constant of P-HRP/HRP-Ab (9G9)</td>
<td>$4.3 \times 10^{5}$</td>
<td>litres (mol s)$^{-1}$</td>
</tr>
<tr>
<td>Off-rate constant of P-HRP/HRP-Ab (9G9)</td>
<td>$2.6 \times 10^{-4}$</td>
<td>s$^{-1}$</td>
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where agitation can no longer accelerate three-dimensional diffusion (Trurnit, 1954); and the effective diffusion coefficient of P-HRP, $D$. The convective mass transfer coefficient of P-HRP was calculated from $D/a$.

The mass transfer coefficient for the permeation of P across a semipermeable membrane was determined by using the microdialyzer. The buffer chamber of the microdialyzer was filled with PBS-thimerosal, and then 100 µl of 10 ng ml$^{-1}$ of P in PBS-thimerosal was added to the sample cells. The same operational conditions as described above were used. The concentrations of P in the wells were determined over time by colorimetric enzyme immunoassays. The concentration ratio $R$ of P at time $t$ over P at the initial time was plotted on a logarithmic scale against time. For the determination of the mass transfer coefficient for permeation $k_p$, the following equation was derived for P from the law of mass action:

$$V(dC_p, in/dt) = k_p A (C_p, out - C_p, in)$$

where $V$ is the volume of the solution in the well and $A$ is the surface area of the membrane. $C_p, in$ and $C_p, out$ are the concentrations of P in the internal and the external buffer, respectively. After integration of the equation at $C_p, out \approx 0$, the following expression was obtained:

$$\ln R = -k_p (A/V) t$$

The experimental data from the plot of $\ln R$ vs $t$ were fitted to a linear regression, and $k_p$ was calculated from the slope (Table 1).

CONVENTIONAL ENZYME IMMUNOASSAYS

The majority of enzyme immunoassays for small analytes are competitive assays. Three major components participate in the analytical process: (1) a binding protein (e.g. an antibody which is usually immobilized on a solid matrix); (2) a conjugate between the analyte and an enzyme; and (3) the analyte to be measured. In the absence of the analyte in the sample, a comparatively large amount of analyte–enzyme conjugate is bound and a large signal from the enzyme is obtained on the solid matrix after the unbound components are removed. When the analyte is added, it competes with the analyte-enzyme conjugate for available binding sites on the binding protein and displaces the conjugate from the binding protein on the solid surface. Hence, a smaller signal is obtained on the solid matrix. A typical dose-response curve of an enzyme immunoassay of this type is shown in Fig. 1.

ENZYME-SHUTTLE IMMUNOASSAY

We have extended this concept by introducing into the system an additional binding protein, e.g. an antibody to the enzyme of the analyte-enzyme conjugate (Fig. 2(1)). This conjugate now has a dual function: it can either bind to the anti-analyte antibody (Fig. 2(3)) or to the anti-enzyme antibody (Fig. 2(4)). Therefore, we refer to it as a heterobifunctional conjugate (Fig. 2(2)).

For the investigation of the model system, we
Fig. 1. Dose-response curve in a competitive enzyme immunoassay with a monoclonal antibody to the steroid hormone progesterone immobilized on polypropylene discs. The amounts of analyte-enzyme conjugate (progesterone-horseradish peroxidase) and immobilized antibody to progesterone were the same as shown in Figs 6 and 7.

We used discs made of polypropylene mesh (113 mesh count) as a matrix for the immobilization of the antibodies. These discs fit into microwells as containers for the reaction. In this arrangement, the discs are removed after the reaction and separately tested for the development of a colored product (oxidation of tetramethylbenzidine). For future applications, we consider the utilization of a Clark electrode for the direct measurement of a signal. The antibodies can be immobilized on an electrode and the product generated by the enzyme can be directly measured. Preliminary experiments in our laboratory with glucose oxidase as the enzyme in the heterobifunctional conjugate have shown very similar results to those presented in this communication for horseradish peroxidase.

The amount of immobilized binding proteins, and the binding constants to the respective binding site on the heterobifunctional conjugate need to be adjusted such that most of the conjugate is bound at sensor 1 in the absence of the analyte (Fig. 3, A). This can be achieved either by using an antibody on sensor 1 with a higher affinity constant to the antigen than that on sensor 2, or by adjusting the respective amounts

Fig. 2. Enzyme immunoassay using two binding proteins and a heterobifunctional conjugate, able to bind to each of the binding proteins, as signal generator. The antibodies are immobilized at spatially distinct, separated locations.
Fig. 3. Binding of heterobifunctional conjugate on sensor 1 and sensor 2 in response to increasing analyte concentration. The sensor is constructed such that most of the conjugate molecules are bound on sensor 1 in the absence of the analyte (A). With increasing analyte concentration, an increasing number of analyte-enzyme molecules shift from sensor 1 to sensor 2 (B and C).

of antibodies immobilized on the two sensors. If the sample contains the analyte, this competes for binding sites of the binding protein on sensor 1 and the heterobifunctional conjugate is displaced from this sensor (Fig. 3, B). The displaced conjugate diffuses to sensor 2 where it is bound by the binding protein which recognizes specifically an antigenic determinant on the enzyme. At increasing analyte concentrations, the amount of conjugate remaining on sensor 1 is inversely proportional to the analyte concentration, while the number of molecules bound to the binding protein on sensor 2 is directly proportional to the amount of analyte (Fig. 3, C).

IRREVERSIBLE MODE

In this arrangement a dose-response curve can be constructed from both sensor 1 and sensor 2 (Fig. 4). While the curve for sensor 1 (Figure 4, curve 1) resembles that for conventional immunoassays (cf. Fig. 1), the dose-response curve for sensor 2 is directly proportional to the analyte concentration (Fig. 4, curve 2). The shape and the origin of the curves depend on: (a) the relative concentration of the two immobilized binding proteins; (b) the relative affinity constants of the binding proteins, and (c) the concentration of the heterobifunctional conjugate. We have developed theoretical models for the prediction of these three variables, and we have applied the results to obtain optimal experimental conditions. These investigations will be reported elsewhere (Paek & Schramm, 1991a).

Fig. 4. Dose-response curves on sensor 1 and sensor 2. At increasing analyte concentration the conjugate on sensor 1 dissociates proportionally (curve 1), therefore accounting for a reduced signal. The amount of conjugate bound to sensor 2 increases simultaneously (curve 2).

Briefly, the modeling is based on the following steps:

- Calculate the optimal concentration of the conjugate to obtain a maximum differential signal (for definitions see below) at the initial state (absence of analyte) for a given set of two antibody pairs. Only the concentration of the conjugate is a variable while the other factors (a) and (b) mentioned above) are constant.
- Calculate the optimal concentrations of the immobilized antibodies using the determined conjugate concentration to obtain a maximal signal difference between the initial state
(absence of analyte) and the final state (excess of analyte).

The enzyme-shuttle immunoassay requires a highly purified analyte enzyme conjugate as the signal generator. The synthesized conjugate needs to be separated from unreacted excess enzyme that does not contain an analyte ligand. In a conventional immunoassay, enzyme molecules that do not contain an analyte ligand are not part of the analytical procedure (except for potential non-specific binding). That is not so in the described system. For these studies we have used analyte–enzyme conjugates that were purified by affinity chromatography on a matrix which contained a monoclonal antibody with a low affinity constant to progesterone (Paek et al., 1990).

The signals from dose–response curves 1 and 2 can be combined to form a single standard curve by subtracting curve 2 from curve 1 (3 in Fig. 5). The maximal signal obtained from the combined curves (3) exceeds the maximum signal obtained in a conventional immunoassay (1). Theoretically, the maximal signal in a dual-antibody system from the combined dose–response curves can be twice as high compared with a conventional assay.

Two examples of binding curves are shown in Figs 6 and 7. The reagents and conditions for the measurements were the same except for the affinity constants and the concentrations of the immobilized antibody to horseradish peroxidase (HRP). The affinity constants of the HRP antibodies used in the two systems differed by about one order of magnitude. This results in a different shape and in a different relative position of dose–response curves 1 and 2. For the construction of a reversible sensor, the option to select between binding proteins of different binding constants to their respective antigens allows us to optimize the system either for a desirable reaction time, adequate sensitivity, or appropriate signal yield.

What effect does the dual-antibody system have on the detection limit and the inhibition at 50% of the labeled tracer (ED50)? As in conventional assays, the major component contributing to the sensitivity is the affinity constant of the antibody to the antigen (Jackson & Ekins, 1986). Another factor is the quality of the labeled antigen. The higher the binding constant of the tracer, the lower is the sensitivity (Bachas & Meyerhoff, 1986). This can be a problem particularly for enzyme immunoassays for small ligands because multiple ligands on the enzyme can substantially increase the binding constant.
Therefore we have used an affinity purified conjugate with a single progesterone molecule bound to each horseradish peroxidase molecule (Paek & Schramm, 1991a). The amount of antibody used in an immunoassay and the amount of tracer relative to the antibody concentration are additional variables that determine the sensitivity (Chard, 1987). While all the other factors are basically not different in a single-antibody enzyme immunoassay vs the dual-antibody system, the amount of labeled analyte participating in the competitive binding reaction is not the same.

The concentration of free labeled tracer in solution is at all concentrations of analyte lower in the dual antibody system than in conventional immunoassays because it is captured by the antibody recognizing the enzyme, i.e. it is removed from equilibrium. Therefore very little free conjugate competes with the analyte for binding sites at the anti-analyte antibody and, according to the law of mass action, a smaller amount of the analyte is required to dissociate the conjugate from this antibody. Consequently, in the presence of the same amount of antibody to the analyte and an equal concentration of the conjugate, the dual antibody system has a lower limit of detection and less external analyte is required to dissociate 50% of the tracer (ED₅₀, Table 2).

Some remarks are required to clarify the differences in performance of conventional enzyme immunoassays compared with the dual-antibody system. Taking into account the many factors that are contributing to an optimized assay with high sensitivity (limit of detection and ED₅₀), it is possible that the conventional immunoassay permits a higher degree of variation of components to achieve maximal sensitivity. For the dual-antibody system the binding constants of the two antibodies and their

<table>
<thead>
<tr>
<th>System</th>
<th>Kₚ-Ab/Kₜₜ-Ab</th>
<th>[HRP-Ab]/[P-Ab]</th>
<th>Detect limitᵇ</th>
<th>ED₅₀</th>
</tr>
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<tbody>
<tr>
<td>Conventional P-Ab</td>
<td>∞</td>
<td>—</td>
<td>6-0 x 10⁻⁹</td>
<td>2-3 x 10⁻⁸</td>
</tr>
<tr>
<td>Model A P-Ab: HRP-Ab (E47)</td>
<td>4:3</td>
<td>1</td>
<td>7-6 x 10⁻¹⁰</td>
<td>6-7 x 10⁻⁹</td>
</tr>
<tr>
<td>Model B P-Ab: HRP-Ab (9G9)</td>
<td>50</td>
<td>4</td>
<td>2-0 x 10⁻⁹</td>
<td>1-8 x 10⁻⁸</td>
</tr>
</tbody>
</table>

ᵃThe concentrations of P-Ab and P-HRP conjugate were the same in all systems.
ᵇDefined as two standard deviations from zero standard.
affinity constants need to be matched such that at the initial state (no external analyte present), the majority of the conjugate binds to the analyte antibody. As a result, the minimal amount of analyte antibody required for the dual-antibody system may be higher than in a conventional assay. We are now in the process of assessing the limitations of each system in comparative studies and we will report on the results elsewhere.

REVERSIBLE MODE

The analytical system using two antibodies with a heterobifunctional conjugate can be operated in the reversible mode and is therefore suitable for continuous monitoring of analytes. For this purpose the internal compartment of the sensor is separated from the medium stream by a semipermeable membrane (Fig. 8).

Similar to the system described above, if a medium stream free of the analyte passes by the sensor compartment, the majority of the heterobifunctional conjugate is bound at sensor 1 (Fig. 9, left). If the medium contains the analyte, the equilibrium is shifted towards binding of a larger portion of the conjugate at sensor 2 (Fig. 9, right).

Using two antibodies for continuous monitoring has three advantages: (1) responses can be obtained on two sensors for each measurement, thus increasing the signal yield; (2) the concentration of free (i.e. unbound) analyte-enzyme conjugate is low at all times, thus reducing the background signal; and (3) a sensitivity comparable to conventional enzyme immunoassays can be obtained even in the continuous mode because the dissociated conjugate is removed from the bulk solution and, therefore, from competition with the analyte, thus favoring binding of the analyte according to the law of mass action.

On the basis of the physical parameters of the system (Table 1), we calculated the optimal concentrations of the two antibody pairs, BQ.1 (P-Ab) and 9G9 (HRP-Ab), and the analyte-enzyme conjugate (P-HRP). In addition we estimated the diffusion coefficients for the analyte and the conjugate in the internal space of the sensor (Schramm & Paek, 1991). The time-response curve from the enzyme-shuttle system in the reversible mode was predicted from a kinetic mathematical model that accounts for the following processes: the permeation of the analyte across

Fig. 8. For continuous measurement of the analytes, the compartment containing the sensors with antibody 1 and antibody 2 is enclosed by a semipermeable membrane. This membrane allows free passage of small molecules but retains the heterobifunctional conjugate within the compartment.

Fig. 9. Continuous monitoring of the analytes in a medium stream. In the absence of the analyte, the heterobifunctional conjugate generates a signal predominantly on sensor 1 (left). At high concentrations of the analyte in the medium, the signal shifts to sensor 2 (right).
Fig. 10. Mathematical modeling of the enzyme-shuttle sensor upon exposure of elevated concentration of analyte in the external medium (top). After establishing equilibrium (A) the concentration of heterobifunctional analyte changes in response to the increased analyte concentration (B). After the external analyte concentration diminishes, the concentration of conjugate on the two sensors remains constant for a delay period (C) until it approaches the equilibrium of the initial phase (D). The sum of the two dose-response curves is shown at the bottom.

The rate equations describing the kinetic processes were simultaneously solved by a numerical method as described elsewhere (Paek & Schramm, 1991b), and we subsequently
investigated experimentally the predicted performance. For the model, we assumed raising the concentration of progesterone in the external medium from 0 ng ml\(^{-1}\) to 20 ng ml\(^{-1}\) (hour 0), maintaining this concentration for a period of 3 h and subsequently exposing the sensor to a medium without progesterone (Fig. 10, top). When newly assembled, the sensor comes to equilibrium within less than 2 h, i.e. the progesterone-enzyme conjugate is bound to sensor 1 and sensor 2 in a certain ratio within the internal compartment (Fig. 10, A). Raising the external progesterone concentration causes an increase in the conjugate on sensor 2 (gray line) and a decrease on sensor 1 (black line) (Fig. 10, B). After withdrawal of progesterone, the concentration of the conjugate on both sensors remains about the same for an additional hour and then begins to return to the initial state (C) which is reached under these conditions at hour 8, i.e. about 5 h after progesterone withdrawal.

We have experimentally investigated the theoretical model under the same conditions as mentioned above. The antibodies to progesterone and horseradish peroxidase were immobilized on discs of polypropylene mesh that fit into sample wells of a microdialyzer. The bottoms of the wells were sealed with a semipermeable cellulose membrane (thickness of 8 \(\mu\)m). The wells were exposed to a medium stream. Discs were removed at various time intervals and bound progesterone-horseradish oxidase conjugate on the discs was quantitatively measured by color development with tetramethylbenzidine (Bos et al., 1981).

The results are shown in Figs 11 and 12. We found that the dose-response curves at the two compartments of the sensor follow the theoretically determined response fairly closely during the phase of increased analyte concentration in the external medium (B in Figs 11 and 12). There is also close correlation with the predicted model for about 90 min after withdrawal of the analyte (phase C). Thereafter, the concentration of the analyte-enzyme conjugate deviates from the predicted response. As a result, the initial state (phase D) is reached later. We attribute the delayed recovery to a slower than expected diffusion of analyte-enzyme molecules from the polypropylene mesh containing the anti-enzyme antibody and, possibly, to re-association of the conjugate. This theory is currently being investigated by using different modes of agitation during the recovery period and by elevating the temperature of the by-passing external medium. Another working hypothesis is that potential degradation of the enzyme occurs over time, which will be tested by adding non-diffusible stabilizers to the reaction cell.

**CONCLUSIONS**

We have shown that a competitive, heterogeneous enzyme immunoassay can be used for the continuous measurement of analytes in a medium stream if two antibodies, binding to different sites on a heterobifunctional conjugate (the signal generator), are immobilized on spatially separated areas of the sensor. A sensor based on this principle has advantages and
limitations. The major advantage is the continuous monitoring of media to detect a threshold levels of analytes.

The limitations of this system are not yet fully established. Since binding proteins are involved in the detection of the analytes, the response time cannot be measured in minutes but in hours. In the described application, two antibodies with high binding constants \((5.8 \times 10^{10}\) and \(1.2 \times 10^9\) mol\(^{-1}\)) were used and the increase of analyte from 0 ng ml\(^{-1}\) to 20 ng ml\(^{-1}\) in the medium stream could be detected within 10 min, but maximal response was reached about 3 h after onset of the elevated external analyte concentration (Fig. 11). The decline in progesterone as the analyte could be clearly detected with a delay of 2 h but did not reach the initial state after more than 5 h. Several improvements are possible in the experimental arrangement, which will affect the performance of this sensor.

To establish the principle of the dual-antibody system, we used an off-line detection method, i.e. we had to destroy the wells containing the discs with the immobilized antibodies for measurement. In future applications, glucose oxidase can be used as an enzyme and antibodies can be directly immobilized on a Clark electrode. Many media used for bioprocessing already contain glucose, the substrate for glucose oxidase, but the addition of glucose to a medium stream for measurement is feasible. This would permit direct, on-line, real-time monitoring of the signal.

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