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NONSPECIFIC INTERACTION OF STREPTAVIDIN WITH UREASE-CONJUGATED ANTIBODIES

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H. Gail Thompson and William E. Lee

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Nonspecific Interaction of Streptavidin with Urease-Conjugated Antibodies

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ABSTRACT

Streptavidin (SA) biotin interactions are used in many aspects of bioanalysis and molecular biology. The biosensor of the Biochemical Detector (BCD) employs SA biotin-mediated filtration-capture onto nitrocellulose membranes in its immunoassay procedures. During the development of immunoassays for the BCD biosensor, systematic variations in the background and the sensitivity were observed. The results presented in this work indicated that the cause of these variations was nonspecific interaction of SA with one of the components of the reagent mixture, a urease-conjugated antibody. The variations could be diminished by allowing the reagents to stand at room temperature for two to three hours prior to use.

RÉSUMÉ

Les analyses biologiques et la biologie moléculaire font souvent appel aux interactions streptavidine-biotine. Les méthodes d'immunodosage sur lesquelles repose le biocapteur du Détecteur biochimique (DBC) font appel au phénomène de capture-filtration sur membrane de nitrocellulose, phénomène qui s'opère par la médiation du système streptavidine-biotine. Au cours de la mise au point des immunodosages pour le biocapteur du DBC, on a observé des variations systématiques en ce qui a trait aux valeurs de fond et à la sensibilité. Les résultats présentés dans le présent travail indiquent que ces variations étaient attribuables à un interaction non spécifique entre la streptavidine et l'un des constituants du mélange de réactifs, un anticorps conjugué à l'uréase. On peut réduire les variations en laissant les réactifs séjourner à la température de la pièce pendant deux à trois heures avant de les utiliser.

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INTRODUCTION

The streptavidin-biotin complex plays an important role in bioanalytical systems and molecular biology (1). This is due to the high affinity ($K_a = 10^{15}$ M^{-1}) of streptavidin (SA) toward biotin and to the stability of the noncovalent complex. SA is a tetrameric protein of molecular weight approximately 60,000 with each subunit capable of binding one molecule of biotin (2).

The immunoassay procedures of the Biochemical Detector (BCD) utilize SA to mediate the filtration-capture of immunocomplexes onto nitrocellulose membranes. In the reaction scheme (Figure 1) SA formed a bridge between the biotin coated membrane and the biotinylated capture antibody. The presence of antigen on the membrane was determined by a light-addressable potentiometric (LAP) sensor. The pH sensing capability of the LAP sensor was used to detect the urease-conjugated antibodies of the immobilized immunocomplex. LAP sensor assays using streptavidin-biotin mediated filtration capture have been developed for mouse imunoglobulin G (mIgG) (3) and Newcastle Disease Virus (NDV) (4).

During the initial course of the development of the assays for mIgG (3) and NDV (4), there were difficulties in obtaining reproducible results. In particular, the background of the assays would consistently increase from one assay to the next. Under these conditions it was not feasible to average replicate data, or obtain reliable benchmarks of immunoassays such as lower limits of detection (LOD).

The work presented here was undertaken to determine the cause of rising backgrounds in LAP sensor immunoassays. In this report the source of the systematic variations was identified and a method of enhancing the stability of the backgrounds of immunoassays employing SA-mediated filtration capture is presented.

MATERIAL AND METHODS

Reagents

Mouse immunoglobulin G, biotinylated anti-mIgG (goat), urease-conjugated anti-mIgG (goat), bovine serum albumin (BSA), sodium chloride, sodium dihydrogen phosphate, Tween 20, Triton X-100, phosphate-buffered saline, urease (jack bean) and urea were obtained from Sigma Chemical Co. (St Louis, MO) and used without any further purification. Monoclonal antibody to NDV was provided by R. E. Fulton, DRES and the conjugation of anti-NDV to urease was carried out by J. D. Biologicals (Scarborough, ON). Streptavidin was obtained from Scripps Laboratories (San Diego, CA) and was reconstituted in distilled water to yield a stock concentration of 10 mg/mL and stored frozen at -20°C in sealed vials.

Wash solution was prepared from 150 mM NaCl, 10 mM phosphate buffer pH 6.5 plus 0.2% Tween 20 detergent. The dilution buffer was the wash solution titrated to pH 7.0, containing 1% (w/v) BSA and 0.25% (w/v) Triton X-100. The substrate solution for the enzyme assay was wash solution containing 100 mM urea.

Apparatus

The apparatus for the immunoassays was a commercially available LAP sensor (Molecular Devices Corp., Menlo Park, CA) marketed under the name Threshold Unit™. The instrument was controlled by a microcomputer (IBM PS/2 model 30) and custom designed software (Molecular Devices Corp.). The design of the LAP sensor allowed eight samples to be tested simultaneously. Nitrocellulose membranes, coated with biotinylated BSA, were purchased from Molecular Devices Corp.

Immunoassay Procedures

A stock solution of mIgG (1.0 mg/mL) was prepared in wash solution and stored frozen at -20°C in sealed vials. The stock solution was diluted with dilution buffer to produce the required standards for the calibration curves and the titration experiments. A reagent solution was prepared from 8.8 μg urease-conjugated anti-mIgG, 7.7 μg biotinylated anti-mIgG and 90 μg streptavidin (10 mg/mL) in 10 mL of dilution buffer. The reagent solutions were allowed to age, i.e., to stand at room temperature for three hours prior to use, except in the case of the data shown in Figure 2 where the lower curve was generated with unaged (i.e., freshly prepared) reagents.

Figure 1 provides a schematic representation of the sandwich immunoassay for mIgG. A volume of 150 μL of reagent solution was added to 100 μL of mIgG sample. The reagents and sample were mixed thoroughly for 20 sec and incubated for five minutes. At the end of the incubation period, a portion of the incubated sample-reagent mixture, 150 μL, was delivered to a well of the filter assembly of the LAP sensor. This aliquot of 150 μL contained the equivalent of 60 μL of mIgG sample and 90 μL of reagent solution.

Titrations of the urease-conjugated antibodies versus SA concentration were performed in a manner similar to above. Each reagent solution contained either urease-conjugated anti-NDV (0.4 μg per well) or urease-conjugated anti-mIgG (0.08 μg per well) and varying amounts of SA ranging from 0 to 18 μg per well. The reagent mixtures were aged for three hours prior to use. No antigen or biotinylated antibodies were used in these titrations.

Filtration Capture and Potentiometric Sensing

The sample-reagent mixture was filtered through the biotinylated nitrocellulose membrane at 250 µL per min. The streptavidin in the reagent solution acted as the bridge between the antigen-biotinylated antibody complexes and the immobilized biotin on the nitrocellulose membrane. The membrane was then washed with 0.5 mL of wash solution and filtration rate was increased to 750 µL per min.

The membrane stick containing immobilized immunocomplexes was removed from the filter assembly and inserted into the reader compartment of the Threshold Unit which contained the LAP sensor and the urea substrate solution. A plunger pressed the membrane against the surface of the silicon sensor. The instrument was designed so that the spots on the surface of the membrane, which contained immobilized immunocomplexes, aligned with the pH sensitive measurement sites on the surface of LAP sensor. At the surface of the sensor, the hydrolysis of urea to carbon dioxide and ammonia caused an increase in the pH which was detected as a change in the surface electropotential. The rate of change of pH at the surface of the silicon sensor was monitored by the rate of change with respect to time of the surface potential as µv/sec. The data was recorded and stored on the microcomputer using the custom designed software.

RESULTS

Standard Curves and Backgrounds of the mIgG Assay

Two standard curves of mIgG, carried out according to the scheme depicted in Figure 1, are shown in Figure 2; the same antigen and reagent solutions were used in each assay. The assay represented by the lower curve was performed within 5 min of the preparation of the reagent solution whereas the assay represented by the upper curve was performed after the reagent solution had been allowed to age (stand at room temperature) for 120 min. There was a marked difference in the two standard curves. The background (zero antigen) and the slope (for a given value of antigen) increased by factors of about 1.5 and 2.5, respectively, when aged reagents were used.

Backgrounds for the assay of mIgG were determined using reagent solutions that had been aged for periods of time ranging from 5 min to 48 h (Figure 3). There was a continuous rise in the output signal of the background, although the rate of increase diminished with time. The major portion of the rise in the background occurred within the first two hours and it was found that after the reagent solutions had aged for about 2 - 3 h, the rate of change of the background with respect to time was low enough that replicate assays could be undertaken. This is demonstrated by the standard curve of mIgG derived from a reagent solution aged for 3 h (Figure 4). The data points displayed represent the mean and standard deviations (SD) for three consecutive runs carried out within 90 min after the initial three hour aging. The coefficients of variation (CV, defined as SD/mean) were about 10% for each of the points on the curve, including the background. From this data, we estimated the LOD to be about 100 pg (3). Using unaged reagents comparable CVs were about 50% (data not shown).

The effects of aging of the immunoreagents were not limited to the mIgG assay. Similar results were observed in the LAP sensor assay of NDV and aging the reagents for about 3 h prior to use provided sufficient stability to permit replicate runs (analogous to that shown in Figure 4) to be carried out (4).

Titration of Urease-Conjugated Antibodies with SA

In order to identify the cause of the rising backgrounds and slopes, a titration was carried out with urease-conjugated anti-mIgG versus SA. A fixed amount (0.08 μg per well) of urease-conjugated antibody was aged for 2 h with increasing amounts of added SA (Figure 5). There was a sharp rise in the signal, and hence the amount of urease-conjugated antibody immobilized on the nitrocellulose membrane, in the range of 0 - 4 μg per well SA. These results suggest that SA undergoes a nonspecific interaction with the urease conjugated antibody and that the increase in the background and slope of the standard curve (120 min) shown in Figure 2 was also a consequence of these interactions.

In LAP sensor assays the amount of SA present is typically between 1 - 4 μg per well, the same range that shows the sharp rise in signal. Above 5 μg SA per well on the titration curve (Fig. 5) there was a slow drop in signal which corresponded to decreasing amounts of urease-conjugated antibody immobilized on the membrane. The capacity of the membrane filters to bind SA is related to the amount of biotin on the surface of the nitrocellulose matrix. For the membranes used in this work, the capacity for SA was about 5 μg per well (5). Above this amount in the titration, the SA-bound antibody conjugate was partitioned between SA which was immobilized on the membane and that which passed through. In titrations of the urease-conjugated antibody against the other component of the reagent solution, the biotinylated anti-mIgG, there was no evidence of interaction (data not shown).

A titration of urease-conjugated monoclonal anti-NDV with SA (Figure 6) yielded a result similar to Figure 5, indicating that the nonspecific interactions of SA are not limited to the polyclonal (goat) anti-mIgG system. In Figure 6 the concentration of SA which corresponded to maximum signal was approximately equal to that of the anti-mIgG titration and this provided additional evidence that the slowly decreasing portion of the curve was real and that it was due to the presence of excess SA (with respect to the capacity of the membrane) in the reaction mixture. Addition of SA to free unconjugated urease produced results that were consistent with those obtained with the urease-conjugated antibodies (Table I). The amount of SA added to the free urease was equivalent to that which produced the peak signals in Figures 5 & 6, viz., 4.5 μg per well and the resulting signals of the LAP sensor were enhanced by about 4-5 times.

The levels of output signal in these titrations (i.e., the y-axis of Figs 5&6 and Table I) displayed some differences and it is not clear at this point whether these differences were due to the extent of binding of urease to SA or to variations in the enzyme activity of the urease since each can affect the output signal.

DISCUSSION

Nonspecific interactions of SA and antibody conjugates can decrease the quality of immunoassays employing SA-biotin interactions. Both the background and the sensitivity (i.e., the slope of the standard curve, ref. 6) of the mIgG and NDV assays are subject to the effects of nonspecific interactions of SA. The results of this work indicate that, for SA-biotin mediated filtration capture immunoassays, aging the reagent solution for two to three hours prior to use stabilizes the background sufficiently to allow replicate assays to be carried out. With this technique, the LOD of a LAP sensor assay for mIgG was determined to 100 pg (Figure 4).

The similarity of the titrations of urease-conjugated polyclonal (goat) anti-mIgG and monoclonal anti-NDV suggests that these results with SA were not due to immunochemical cross-reactivity. The increase in the background in response to the addition of SA to free urease (Table I) further suggests that direct SA-urease interactions contribute to the effects shown in Figures 2, 3, 5 & 6. Urease is a globular protein of MW 480,000 (7) having as many as 12 surface exposed sulfhydryl residues. These residues can lead to aggregation of urease in solution and may play a role in urease-SA nonspecific interactions.

Another approach to enhancing the background stability of the LAP sensor assays is to remove free SA from the incubation mixture of antibodies and antigens. Rather, immediately prior to the filtration step, SA is added to the reaction as depicted in Figure 7. The effects of the slow nonspecific interactions of SA and urease can be diminished by decreasing the amount of time solubilized SA and urease are present together in the liquid phase of the assay. This method has been successful in essentially eliminating the problems associated these nonspecific interactions in LAP sensor assays for a number of antigens including mouse immunoglobulin G, Newcastle Disease Virus, *Francisella tularenis* and *Brucella abortus* (9).

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Table I. The Effect of SA on the LAP Sensor Output Signal from Unconjugated Urease

urease (μg/well)	SA (μg/well)	Signal (SD) (μv/sec)
0.08	-	9.7 (1.1)
0.08	4.5	39.5 (1.8)
0.8	-	56.1 (9.4)
0.8	4.5	270.2 (16.1)

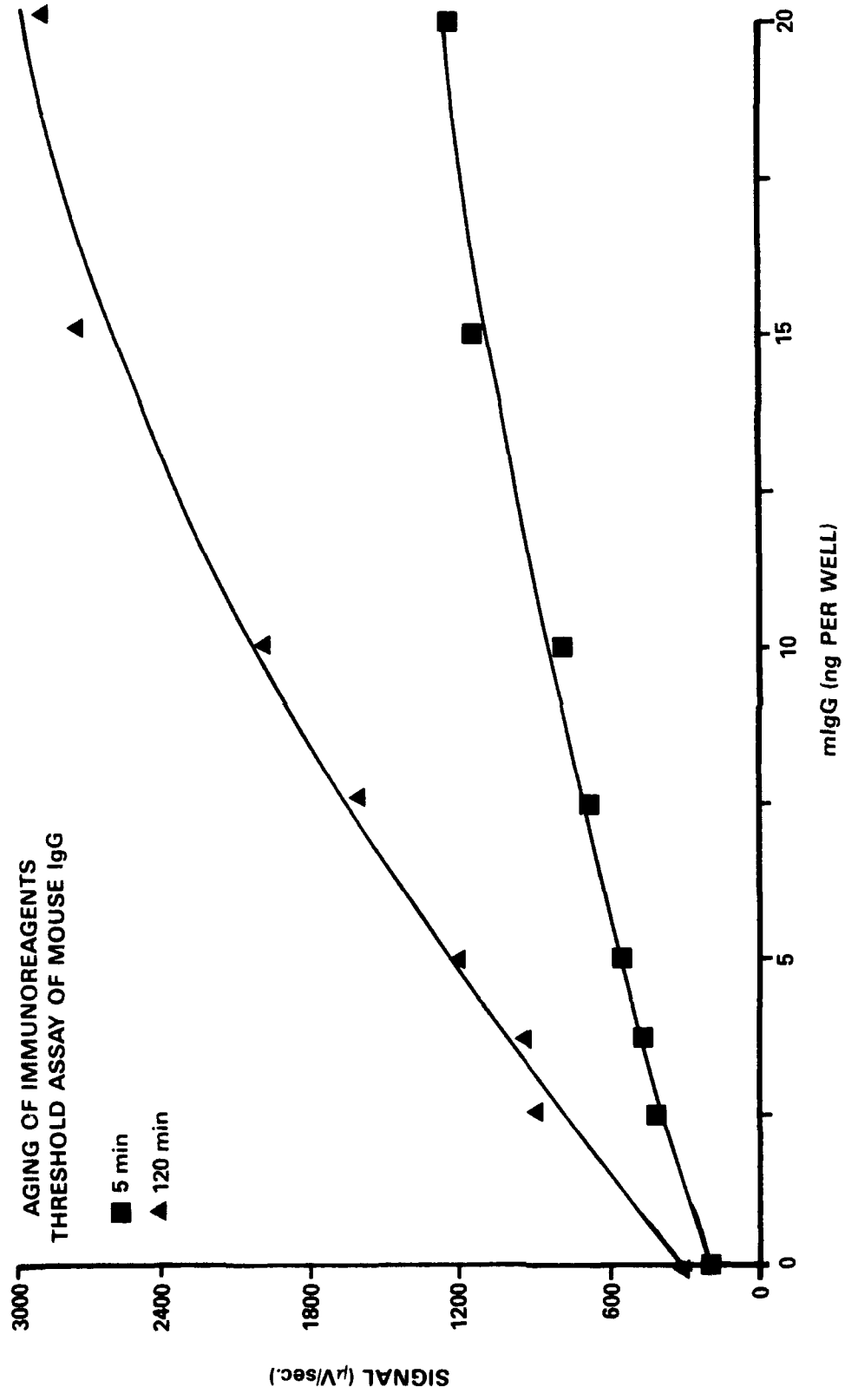

Figure 2

Standard calibration curves of mIgG obtained from reagent solutions aged for 5 and 120 min.

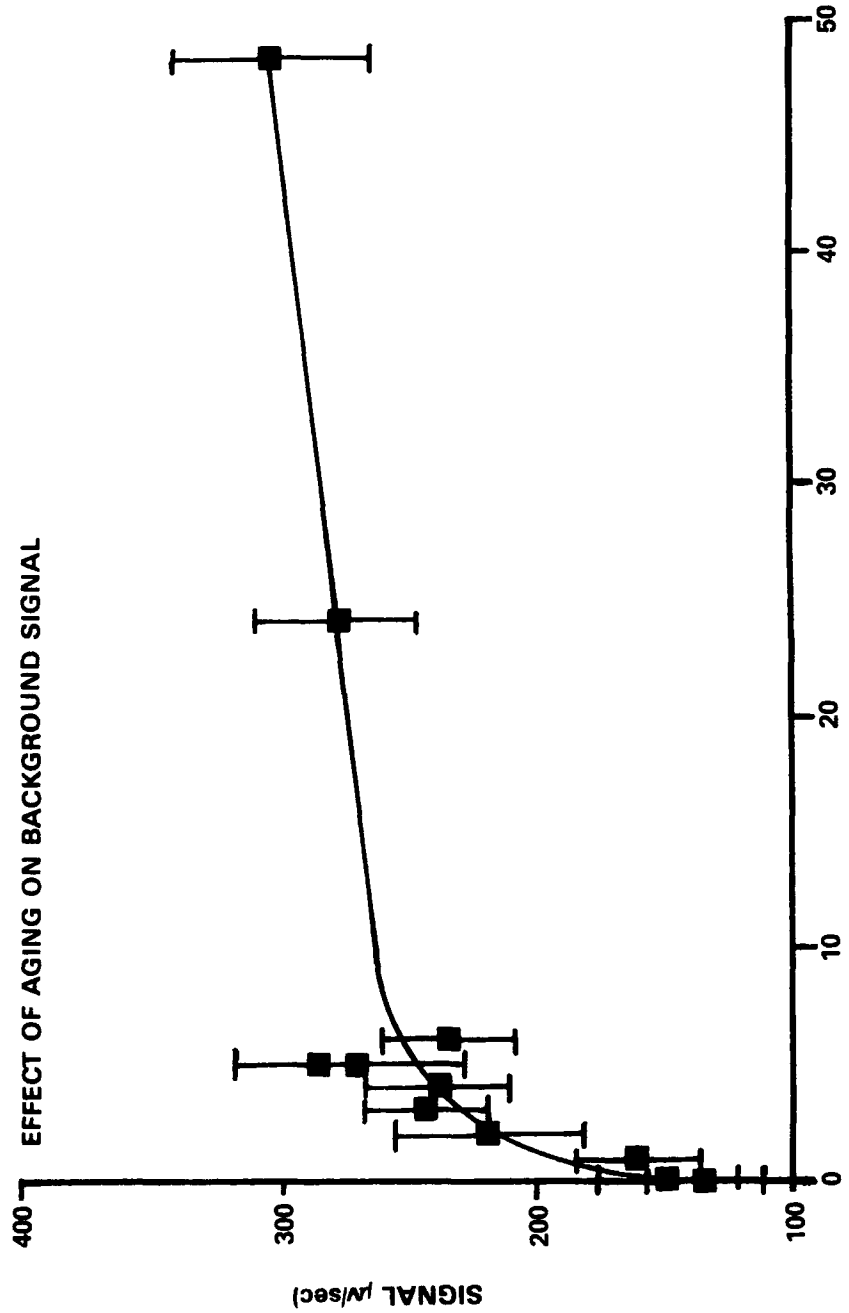

Figure 3

The background (zero antigen) for the mIgG assay obtained from reagent solutions aged for periods of time up to 48 h.

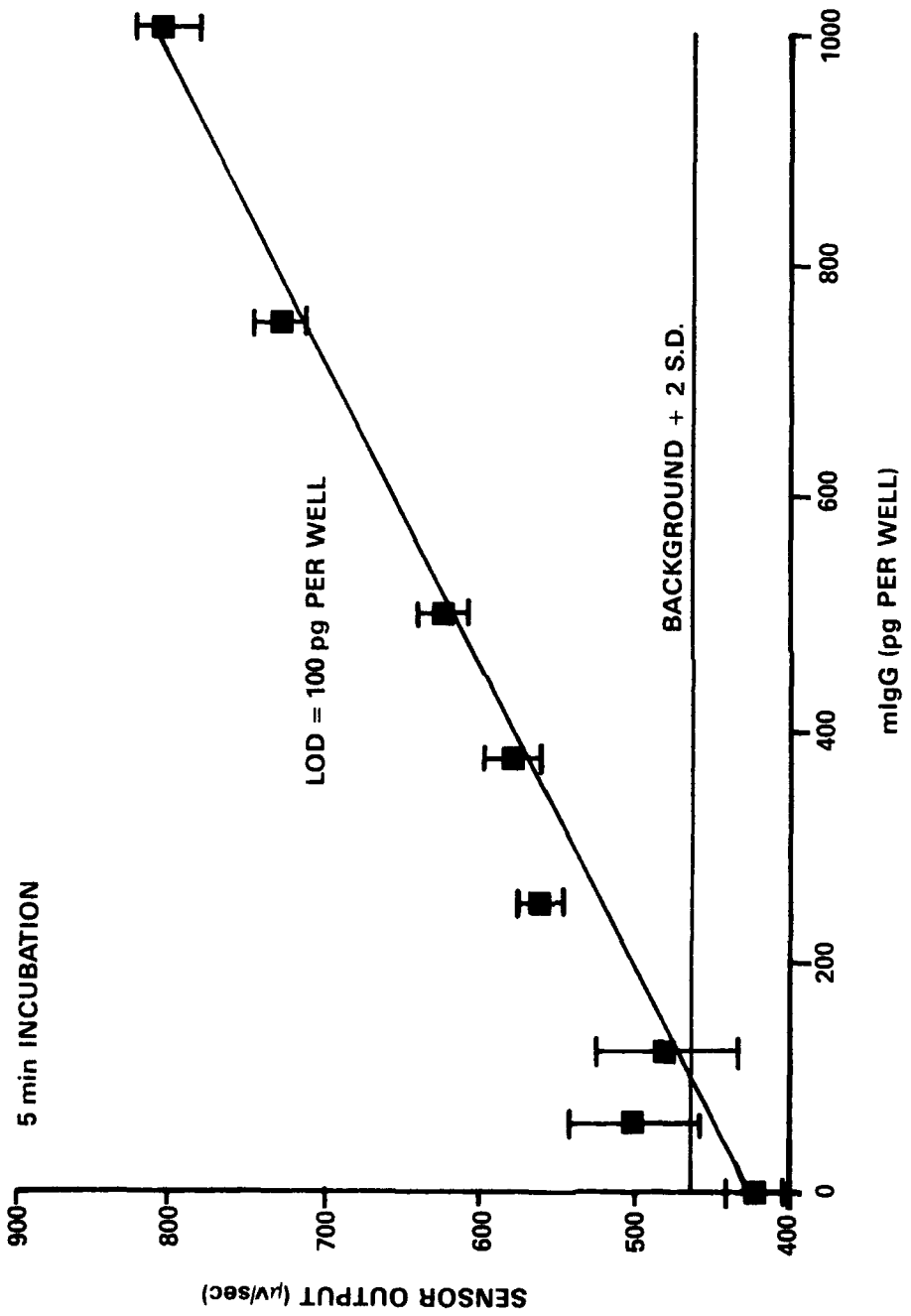

Figure 4

A standard calibration curve of mIgG obtained from reagent solutions aged for three hours. The antigen, mIgG, was incubated with reagent solution for 5 min. Each point represents the mean of three determinations and the bars represent ±1 SD. The calibration curve was obtained from a linear regression of the data points ($y = 425 + 0.360x$, $r^2 = 0.989$). The LOD was taken to be the intersection of the calibration curve with the background +2 SD (see ref. 3).

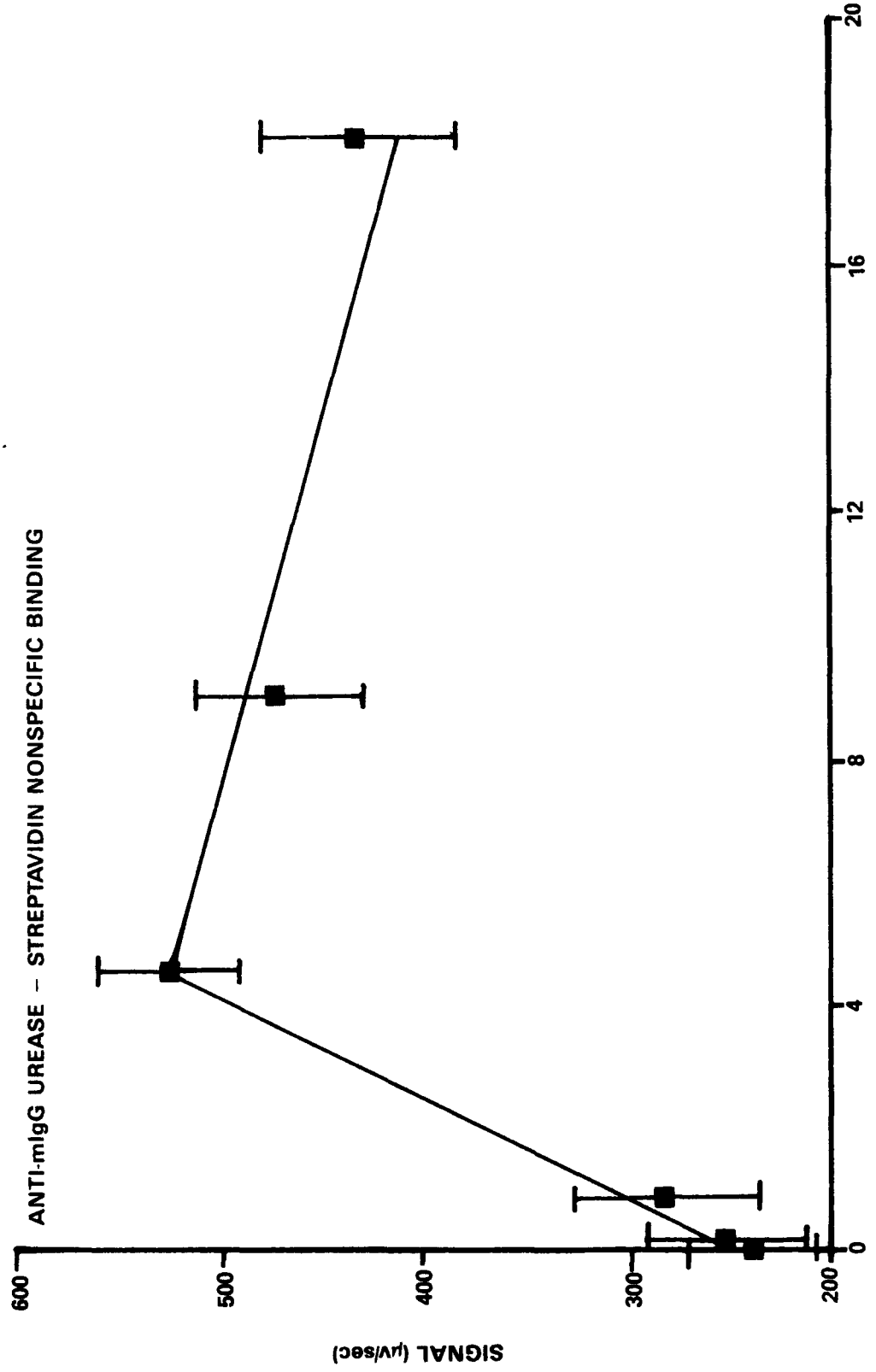

Figure 5

Titration of urease conjugated anti-mIgG (0.08 μg/determination) with streptavidin. Each point represents the mean of eight determinations and the bars represent ±1 SD.

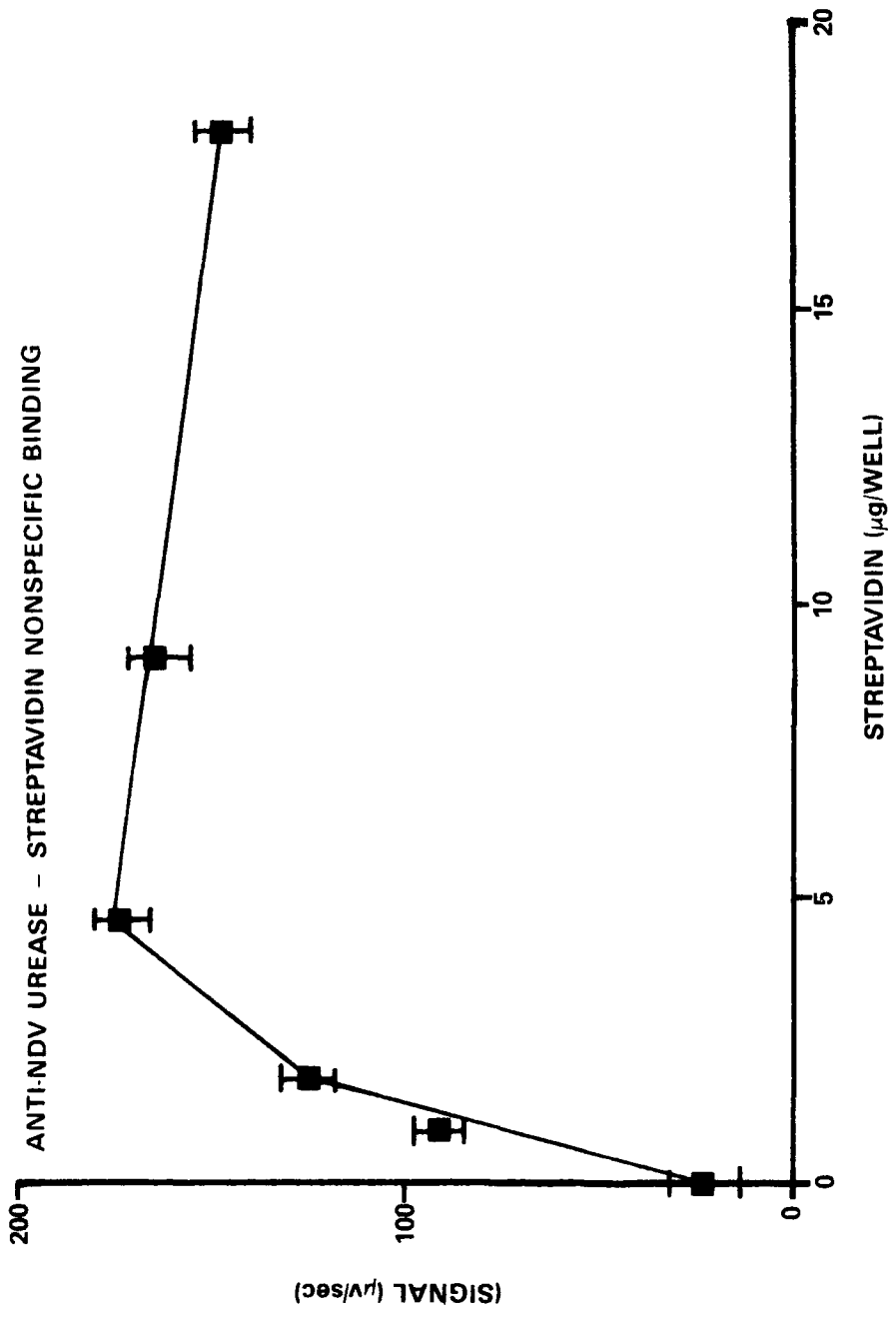

Figure 6

Titration of urease conjugated anti-NDV (0.40 μg/determination) with streptavidin. Each point represents the mean of eight determinations and the bars represent ±1 SD.

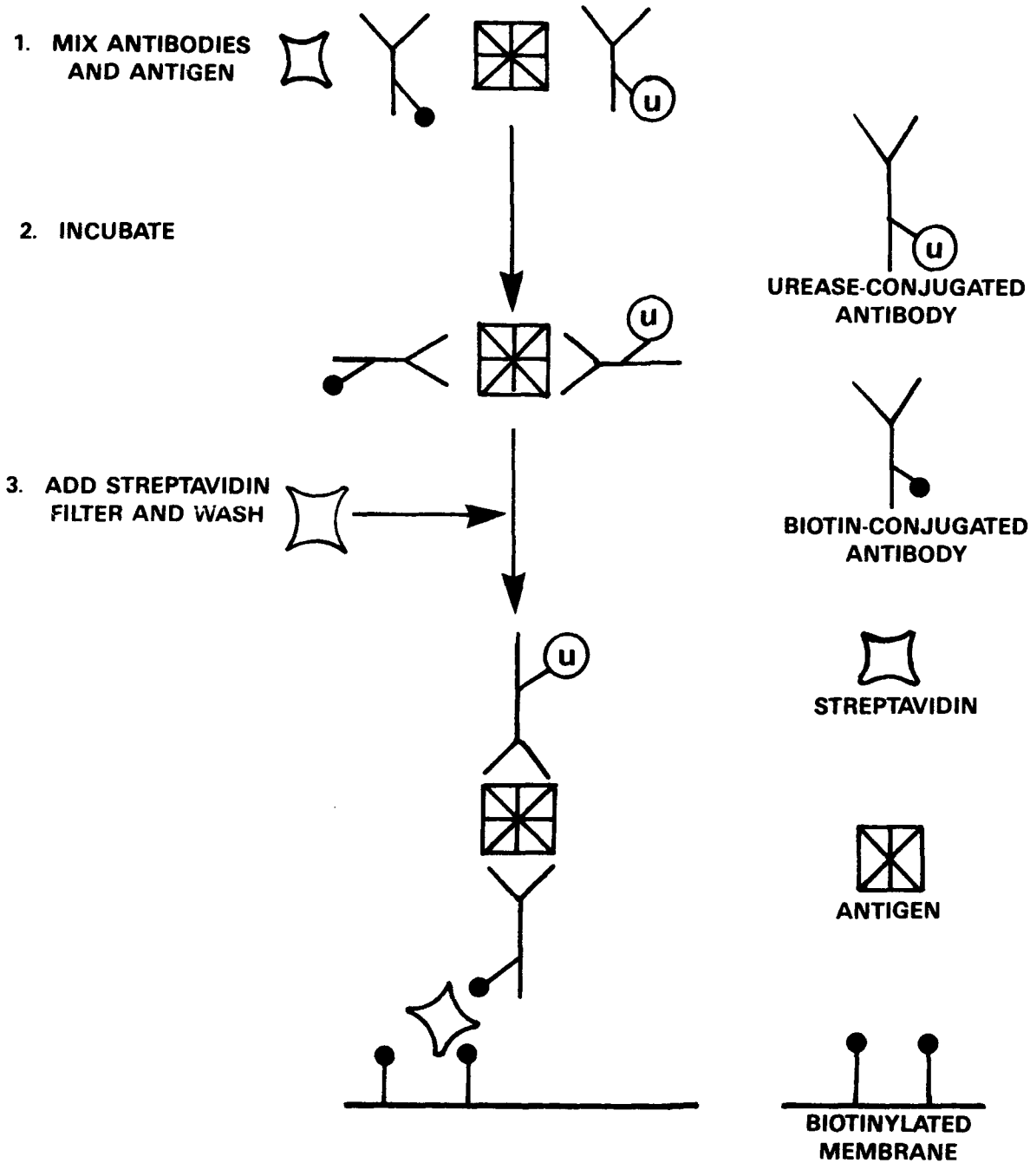

Figure 7

Modified reaction scheme for the LAP sensor.

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Streptavidin (SA) biotin interactions are used in many aspects of bioanalysis and molecular biology. The biosensor of the Bio-Chemical Detector (BCD) employs SA biotin-mediated filtraion-capture onto nitocellulose membranes in its immunoassay procedures. During the development of immunoassays for the BCD biosensor, systematic variations in the background and the sensitivity were observed. The results presented in this work indicated that the cause of these variations was nonspecific interaction of SA with one of the components of the reagent mixture, a urease-conjugated antibody. The variations could be diminished by allowing the reagents to stand at room temperature for two to three hours prior to use.

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