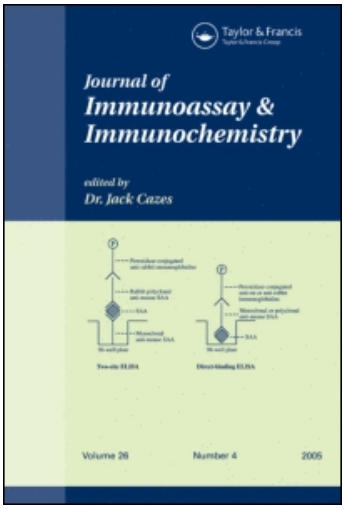

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Coated Tube Enzyme Immunoassay: Factors Affecting Sensitivity and Effects of Reversible Protein Binding to Polystyrene

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COATED TUBE ENZYME IMMUNOASSAY: FACTORS AFFECTING SENSITIVITY AND EFFECTS OF REVERSIBLE PROTEIN BINDING TO POLYSTYRENE

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ABSTRACT

Coated tube enzyme immunoassay using alkaline phosphatase conjugated to rabbit (anti-human IgG) antiserum was studied to determine conditions of maximum sensitivity. The competitive binding assay utilized showed a large increase in sensitivity with immobilized antigen levels below the levels giving rise to the maximum in the coating-antigen dilution series. The effects of reversible antigen binding to the solid phase were investigated by comparison of untreated polystyrene tubes, polystyrene tubes treated with glutaraldehyde and glass tubes activated with an aminosilane. The use of glutaraldehyde treated tubes reduced, and the use of activated glass tubes prevented the time dependent release of immobilized antigen seen with the untreated polystyrene tubes. By comparison of these solid phases, it is shown that reversible antigen immobilized in a competitive binding assay gives rise to poorer conjugate binding (three-fold), and poorer sensitivity (six-fold). A noncompetitive response was found to occur at high free antibody levels and low competing antigen concentrations. This binding behavior is moderated by the minimization of the reversible antigen immobilization.

INTRODUCTION

The coated tube method for immunoassays (1,2) offers several advantages in speed and simplicity. One of the major problems known to exist for this method, however, is the reversibility of

protein immobilization on the solid phase (3-5). Little has been done to determine the effects of this problem on the assay parameters. Both sensitivity and response effects might be expected. Also, based on computer simulations (6), the 'high dose hook effect' of some sandwich assays has been correlated with, in part, soluble antigen arising from insufficient washing of the solid phase. A similar explanation may give rise to the 'hook effect' found in the antigen dilution series of competitive assays.

Two approaches to making the protein coating irreversible are glutaraldehyde polymerization on the plastic tubes (5) and use of activated glass (7,8). Both result in a covalent bond between the protein and the solid phase.

This study reports the result of the control of this reversible protein binding in a competitive binding assay termed immunoenzymometric and classified A2i by Schuurs and Van Weemen (9). Also the effects on the sensitivity due to purification of the conjugate, change of concentration of immobilized antigen, and an unusual noncompetitive effect of soluble competing antibody at low antigen levels are presented.

MATERIALS AND METHODS

Rabbit (anti-human IgG)antiserum (catalogue #517305) was obtained from Calbiochem-Behring Corporation and human IgG from Miles Biochemicals. Alkaline phosphatase (EC 3.1.3.1) from calf intestine was supplied by Sigma Chemical Company, polystyrene tubes by Markson Science, Inc., and Ultrogel AcA-34 by LKB Instruments,

Inc. All other chemicals were obtained commercially and were of reagent grade.

Conjugation of rabbit (anti-human IgG) IgG (serum fraction) to alkaline phosphatase was performed as previously described (3). Glutaraldehyde (0.15% final concentration) was mixed with 5 mg each of antibody (as defined by titer) and enzyme in 0.05 M KH_2PO_4 - 0.9% NaCl - 0.1% NaN_3 buffer (pH 7.4) (PBS) for 2 hours. The reaction was quenched by addition of glycine and the reaction mixture dialyzed versus PBS overnight.

The conjugation solution was purified on an Ultrogel AcA34 column (1 x 63 cm) by eluting with PBS. Separation of the conjugate peak from the enzyme peak was readily achieved. Sucrose gradient purification was also performed. A 10-30% gradient, spun for 16 hours in a Beckman SW 41 rotor at 40,000 rpm also separated the conjugate from any unreacted enzyme and antibody.

The competitive binding assay for human IgG was performed as described previously and done in triplicate (3). Incubation of 0.5 mls human IgG dilutions (range 1.6 mg/ml to 98 ng/ml) in PBS in polystyrene tubes for 3 hours at 37°C was followed by three rinses with 0.05% Tween 20-0.9% NaCl. Tubes were used on the day of preparation. A second incubation (0.4 ml total volume) containing conjugated rabbit (anti-human IgG) IgG - alkaline phosphatase plus competing soluble antigen standards (range 0.16 ng human IgG/ml to 20 μg/ml) or unknowns in a 0.6% bovine serum albumin - 0.1% Tween 20 - PBS buffer solution for 18 hours at room temperature followed. Four rinses with distilled H_2O followed and finally 0.4

ml of 3.8 mM p-nitrophenyl phosphate, substrate for alkaline phosphatase, in 0.0125 M Na_2CO_3 - 0.25 mM $MgCl_2$ buffer (pH 9.8) was added. The reaction was quenched, typically at one hour, by addition of 0.05 mls 1M NaOH, and the absorbance read at 400 nm. The above assay measures antigen (human IgG)concentration, but can easily be adapted to measure antibody (rabbit (anti-human IgG) IgG) concentration. In the latter, conjugated antibody-enzyme competes with soluble antibody for immobilized antigen.

Glutaraldehyde-treated tubes were prepared by incubation of the polystyrene tubes with 0.1% glutaraldehyde in 0.1 M Na_2CO_3 (pH 9.0) buffer at 56°C for 3 hours followed by 4 rinses with distilled water (5). Human IgG dilutions were added and incubated for 24 hours at 4°C. After coating the tubes, the above assay procedure was followed.

Activated glass tubes were prepared by an initial wash in a boiling 5% nitric acid bath for 45 minutes, followed by an incubation with a 10% α-aminopropyltriethoxysilane solution (pH 3.45) for 2.75 hours at 75°C (8). Rinses with distilled water were followed by addition of 2.5% glutaraldehyde in 0.1 M NaH_2PO_4 (pH 7.0) for 1 hour at room temperature (7). The coating with human IgG was done at 37°C for 3 hours.

RESULTS AND DISCUSSION

Effects on Sensitivity

The effect of varying the concentration of bound antigen on the enzyme linked immunosorbent assay (ELISA) response was determined. This antigen dilution series for rabbit (anti-human IgG)

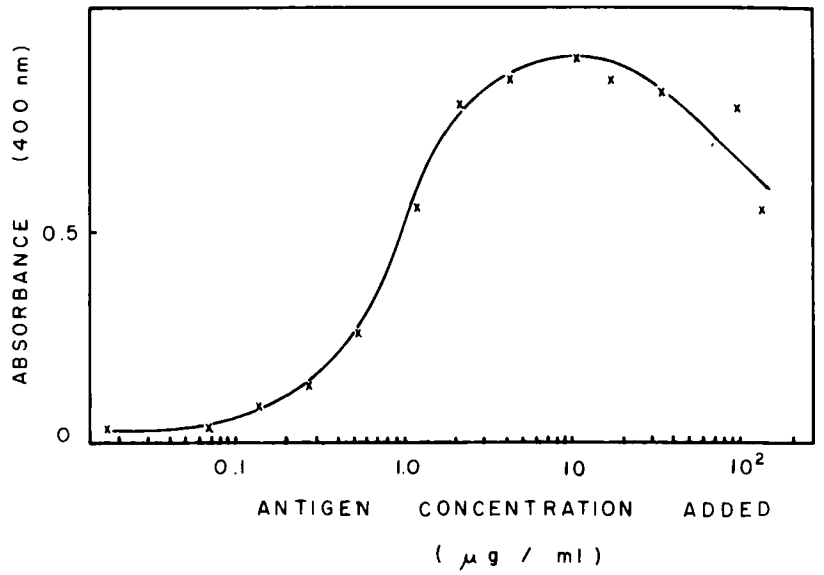

FIGURE 1. The effect of varying dilutions of the antigen (1.6 mg/ml human IgG) solution used to coat the walls of the polystyrene tubes on the response (B_o). Assay conditions are described in the Methods Section.

IgG - alkaline phosphatase binding to human IgG (antigen) adsorbed to polystyrene tubes is shown in Figure 1. This binding of enzyme labelled antibody conjugate to immobilized antigen in the absence of added soluble antigen is referred to through the text as B_o. Maximal B_o occurs at 0.01 mg/ml of added coating antigen. The maximal value is commonly used in competitive binding and sandwich assays using this technique (2,10-14). The decrease in conjugate bound at high immobilized antigen levels is analogous to the 'high dose hook effect' (6).

Figure 2 shows the dose-response curves of a series of competitive binding assays using the range of antigen coating levels of

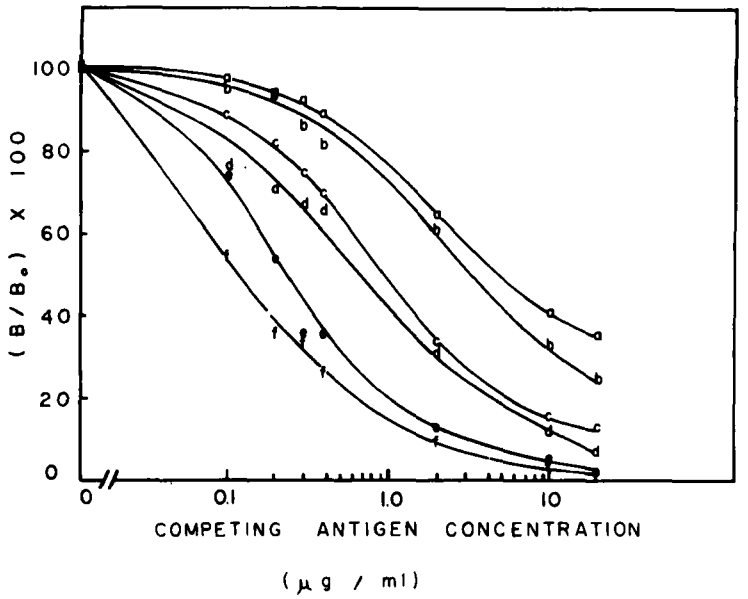

FIGURE 2. A series of competitive binding assays at different levels of immobilized antigen. The plot depicts the competition between soluble antigen and immobilized antigen for the antibody-enzyme conjugate. The relationship between the points describing each assay and the concentration of the antigen coating solution are:

Points	Coating Antigen Added
(a)	40 μg human IgG/ml
(b)	10 μg/ml
(c)	2.5 μg/ml
(d)	1.4 μg/ml
(e)	310 ng/ml
(f)	78 ng/ml

Further assay conditions are described in the Methods Section.

Figure 1. Response is plotted as B/B_o to facilitate comparisons of sensitivity. This family of curves clearly shows an increasing sensitivity to competing antigen with decreasing levels of coating antigen. Thus the sensitivity of ELISA may be enhanced significantly (in this sample ca. 40-fold with sensitivity defined by the

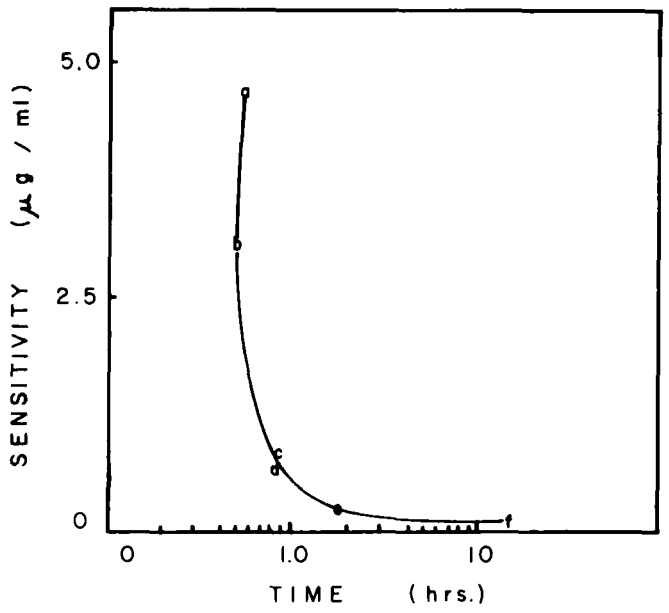

FIGURE 3. A plot illustrating the relationship between sensitivity (defined as the solution antigen concentration giving a value of B/B_o=0.5) of the competitive binding assays of Figure 2 versus the time required to obtain an equal response for the different solid-phase immobilized antigen concentration. The time of response (B_o) of each point (a-f) of Figure 2 is the time required to obtain an equal amount of product from the solid-phase bound conjugate.

midpoint of the response) by using antigen coating levels of less than maximal B_o.

A practical limitation in using the lower antigen coating levels is the time required to get a reasonable response. Even this, however, is not directly proportional to the increase in sensitivity as shown in Figure 3. This is a replot of Figure 2 sensitivities versus response (expressed as a time required to obtain an equal response). A considerable sensitivity increase

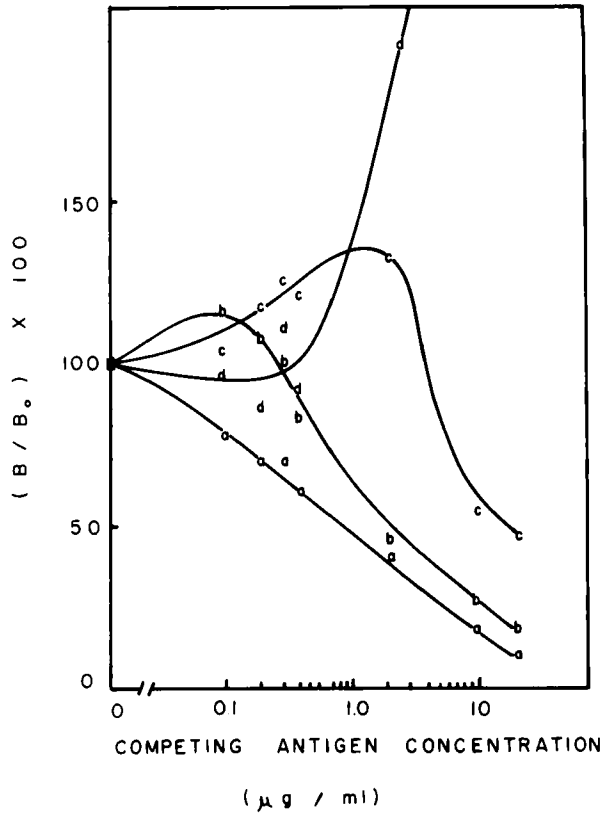

FIGURE 4. A series of competitive binding assays with added soluble antibody (rabbit (anti-human IgG) IgG). The competition is between immobilized and soluble antigen for antibody-enzyme conjugate and nonconjugated antibody. Figure 4i depicts the changing sensitivity between these curves and 4ii depicts the difference in response, B_0, of these curves when assayed at identical times. The relationship between the points describing each assay and the amount of added antibody are:

Points	Antibody Added
(a)	0
(b)	0.45 µg/ml
(c)	4.5 µg/ml
(d)	67.5 µg/ml

These assays were all done with antigen coating levels of 2.5 µg/ml. Further assay conditions are described in the Methods Section.

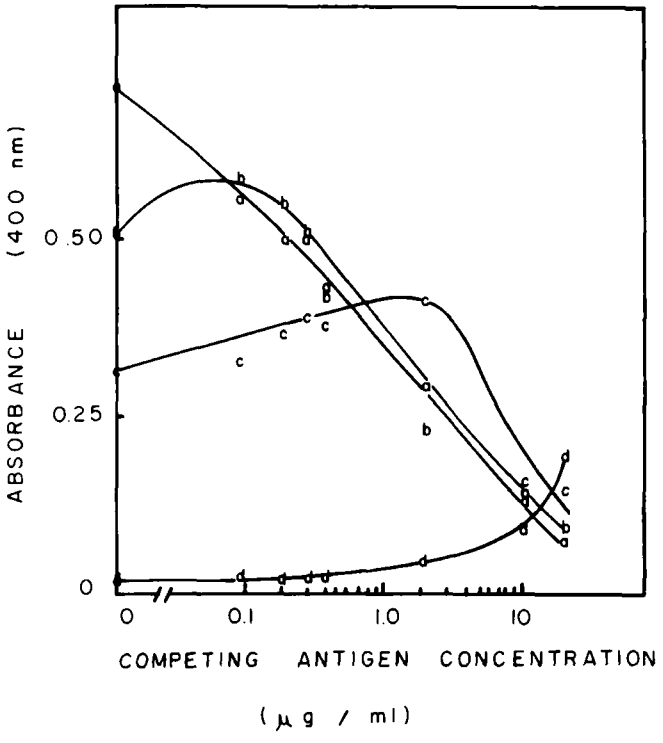

FIG. 4ii

occurs before decreased response (B_o) becomes significant and longer assay times become required to compensate for this decreased response. For example, a 7-fold sensitivity increase requires only a 1.5-fold assay time increase, and a 21-fold sensitivity increase requires only a 3.2-fold assay time increase. Variation of conjugate concentration offers no improvement in sensitivity, though it has a directly proportional effect on response (B_o).

These results agree with theoretical computer-derived models which predict optimal sensitivity where the ratio of the immo-

bilized protein concentration to the equilibrium constant ranges between 0.40 and 3.0 (15). These different values arise from different optimizing criteria in the computer models. Increasing error (for constant enzyme assay time) at lower immobilized antigen levels limits the sensitivity gain. Also, in a recent study using a nonequilibrium inhibition ELISA, minimization of the immobilized antigen increased the sensitivity, but concurrently increased the variability between assays (16).

An unusual noncompetitive effect obtained by adding nonconjugated antibody to a competitive binding assay was observed as shown in Figure 4. Soluble competing antigen initially results in increased conjugated antibody binding followed by the usual decreased binding at higher antigen concentrations. Figure 4i illustrates the noncompetitive nature of the response (B/B_o). Figure 4ii is the same data expressed as B_o in absorbance, and illustrates the depressing effect of nonconjugated antibody on the amount of conjugated antibody bound. The extent of this noncompetitive behavior, and the concentration at which competitive behavior re-assets dominance, is clearly a function of the nonconjugated antibody concentration. Similar results are obtained as a simple function of conjugated antibody concentration. This noncompetitive behavior thus appears to be a function of total antibody concentration rather than antibody/conjugated antibody ratio. Clearly such behavior limits the concentration of conjugated antibody and nonconjugated antibody contamination that may be utilized or present in these assays. Such behavior is not un-

precedented. Similar behavior in which the noncompetitive phase was used as the assay region has been reported in an RIA system (17) and was ascribed to being dependent on the low avidity of the antibodies used. A very moderate form was shown but not commented on in a competitive sandwich coated tube EIA (10). Preliminary results suggest that this phenomenon is due to an $(\text{antigen:antibody})_n$ aggregate bound to the walls. Although this phenomenon reduces sensitivity, the precision, λ, (9,18) defined as the standard deviation of the data divided by the slope of the log dose-response curve, is considerably increased.

This assay system shows little or no improvement in sensitivity with purification of the enzyme-antibody conjugate. Competitive binding assays comparing unpurified conjugate and conjugate fractions purified by Ultrogel chromatography or sucrose gradient centrifugation showed no difference in sensitivity. The purified fractions used were free of unconjugated antibody and enzyme. To date, EIA procedures have generally used conjugates with only preliminary purification with little investigation of purification effects. Studies using the sandwich-type assays (19) have indicated ca 5-fold sensitivity improvement upon purification. A competitive binding assay measuring the unbound activity showed a ca 5-fold sensitivity increase in contrast to these results (20). A dependence of B_o on the removal of nonconjugated antibody was noted as would be predicted from the results shown in Figure 4ii.

Reversible Protein Binding to Polystyrene

The design of the next series of experiments is illustrated in Figure 5i. The rate of desorption of human IgG immobilized on

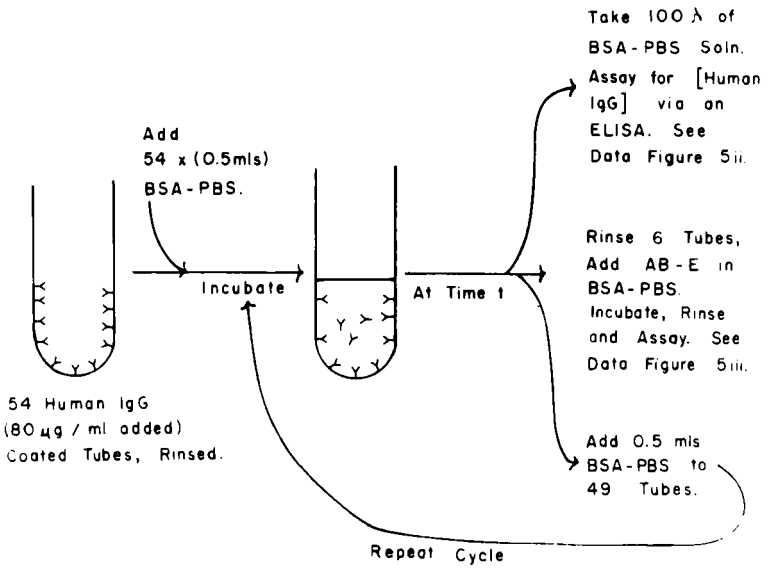

FIGURE 5. The effects of reversible antigen immobilization to the solid phase. (i). A scheme depicting the experiment performed. AB-E refers to antibody enzyme conjugate. (ii). The amount of immobilized antigen (human IgG) released as a function of time during successive incubations of 0.6% bovine serum albumin - 0.1% Tween 20 - PBS (BSA-PBS). Quantitation of the antigen desorbed in each successive incubation was performed in sextuplicate via a competitive binding assay (immobilized antigen level of 2.5 μg/ml) as described in the Methods Section. The curve for (a) points describes data for polystyrene tubes; (b), glutaraldehyde-treated polystyrene tubes; and (c) activated glass tubes. (iii). The response (B_o) of the coated tubes as affected by successive incubations of BSA-PBS. The curve for (a) points describes data for polystyrene tubes; (b) glutaraldehyde treated polystyrene tubes; and (c), activated glass tubes.

three solid phases is shown in Figure 5ii. The solid phases are untreated polystyrene, polystyrene coated with polymerized glutaraldehyde and glass activated with aminosilane and glutaraldehyde. The desorbed human IgG is measured by a competitive EIA which measures antigenically active protein. There is a rapid desorption from untreated polystyrene over the first forty-eight

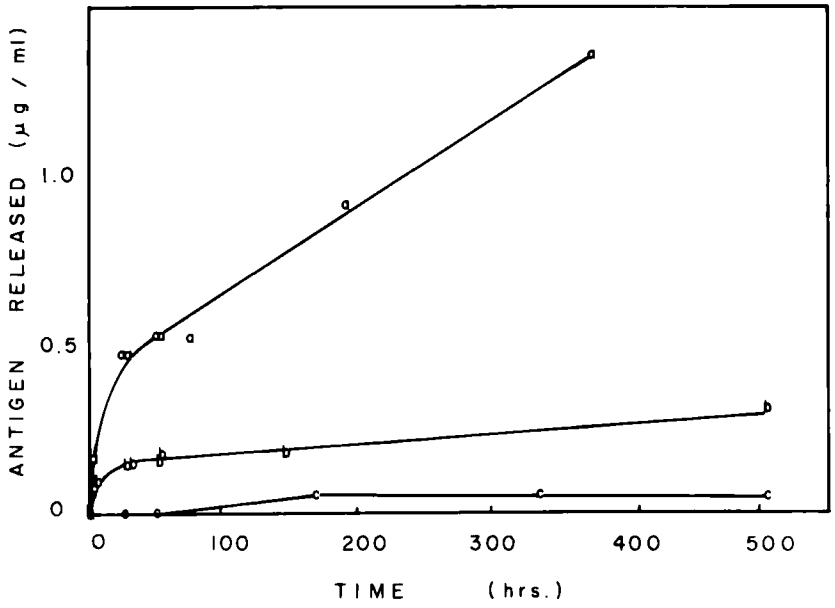

FIG. 5ii

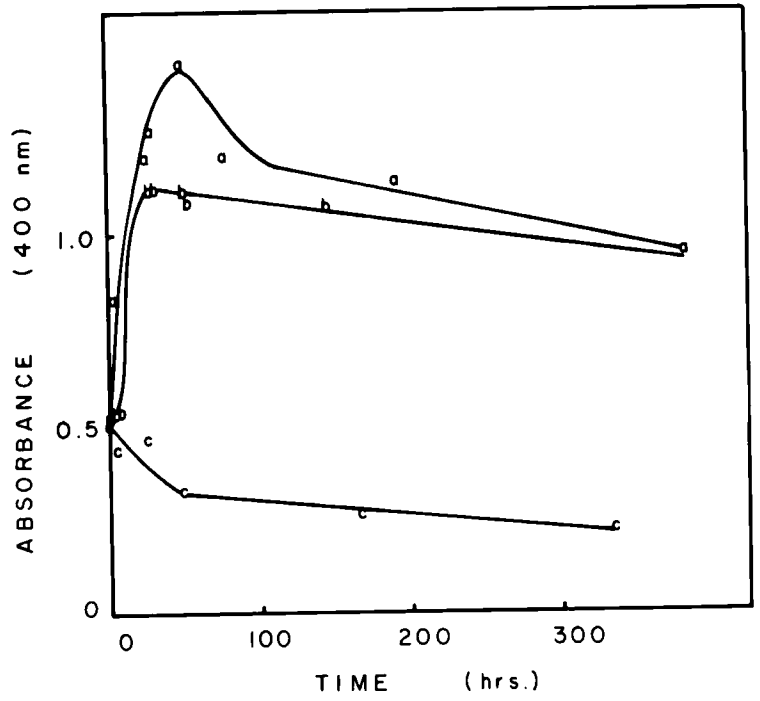

FIG. 5iii

hours followed by a much slower second phase desorption. The glutaraldehyde treatment reduces both phases with the initial rapid phase desorption being approximately 4-fold less. Immobilization on activated glass appears to be irreversible as no detectable IgG is desorbed up to one week. The coating concentration used was 80 µg human IgG/ml. Based on data of Canterero (21), 32 µg human IgG would be immobilized under these conditions. Approximately 0.5 µg is desorbed from untreated polystyrene during the 48 hour incubation for a loss of 1.5% of the immobilized antigen. This immobilized level is slightly higher than the amount corresponding to the maximal response in the antigen dilution series seen in Figure 1. A lower concentration was used initially, and the rapid 48 hour desorption using untreated polystyrene tubes was seen, but since proportionately less human IgG was being desorbed, the slower second phase desorption was not detected, most likely due to the detection limits of the assay.

These glutaraldehyde results contrast with a recent study finding that glutaraldehyde did not prevent desorption of protein from the polystyrene surface (22). The glutaraldehyde treatments differ markedly, however.

Desorption of BSA from polystyrene, nylon and cyanogen bromide activated paper has been shown to be similar to these results (23). Alternative immobilization procedures such as immunological complexation and covalent attachment of antigen to absorbed protein yield little improvement in sensitivity (24).

Examination of the effect of this immobilized antigen desorption on several parameters of this assay reveals a number of

important changes. The response of the assay B_o increases as shown in Figure 5iii. This increased response occurs concurrently with the desorption and removal of the immobilized antigen (Figure 5ii). In this experiment, the antigen-coated tubes, pre-incubated as in Figure 5i, for varying times, are then rinsed three times and conjugate is added, incubated, rinsed and the tubes are assayed. As can be seen, there is a rapid rise in the response as antigen is desorbed and removed from the tubes, followed by a slow increase in response with still longer times. An explanation may lie in the fact that the antigen desorbed from the walls during an incubation with conjugate would serve to compete with the immobilized antigen for conjugate. By removal of this soluble antigen, additional conjugate is available to shift the equilibrium further toward the bound conjugate. The glutaraldehyde treatment gives rise to a lesser increase, and activated glass tubes show no increase at all in accord with their respective reduced and zero-desorbed antigen shown in Figure 5ii.

The effect on B_o response of varying immobilized antigen levels for the glutaraldehyde treated polystyrene and activated glass tubes can be seen in Figure 6. The decrease in conjugate bound at high antigen levels seen in Figure 1 for untreated polystyrene tubes is still evident in Figure 6 for the treated tubes. One postulated explanation for the decreased conjugate binding at high immobilized antigen levels is a carryover of desorbed antigen to the conjugate incubation. Rodbard et al (6) suggest insufficient washing as an origin for such soluble antigen, but reversibly bound antigen would be an alternate source. The

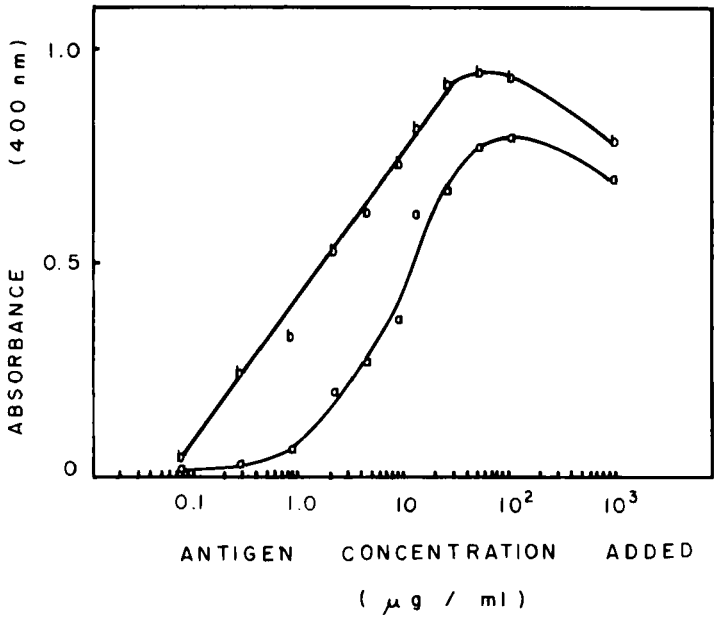

FIGURE 6. A set of antigen dilution curves based on varying concentrations of immobilized antigen. The curve for (a) points described data for glutaraldehyde treated polystyrene tubes and (b), activated glass tubes.

fact that low conjugate binding at high immobilized antigen remains for both covalent treatments indicates that antigen desorption is not the sole cause of the effect. However, both covalent methods do shift the peak of the response, B_o which may reflect an increase in conjugate binding because of the lack of desorbed antigen acting as competing soluble antigen.

The sensitivity of a competitive binding assay is also affected as shown in Figure 7. Incubation of the coated tubes, as in Figure 5ii, for forty-eight hours in BSA - Tween 20 - PBS, removes most of the reversibly immobilized antigen and results in

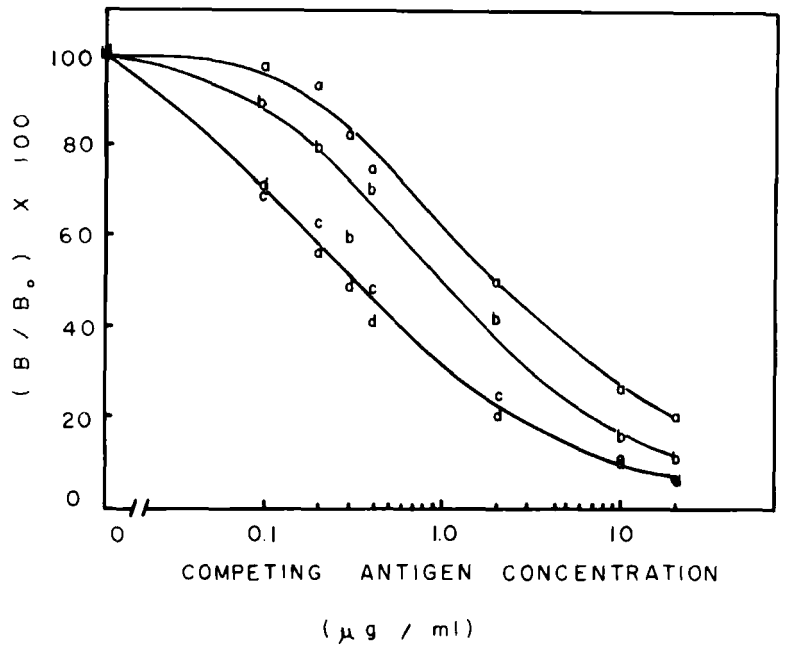

FIGURE 7. A series of competitive binding assays measuring antigen (human IgG) concentration. Human IgG added for immobilization on the solid phase was 2.5 μg/ml. The curve for (a) points describes data for polystyrene tubes; (b), 48 hour BSA-PBS preincubated polystyrene tubes (3 successive incubations for 3, 21, and 24 hours respectively); (c), glutaraldehyde treated polystyrene tubes; and (d), activated glass tubes.

a 2-fold increase in the sensitivity. The glutaraldehyde polymerization and the activated glass treatments both yield 6-fold improvements over the untreated tubes. This latter sensitivity increase may involve less immobilized antigen desorption adding to the soluble competing antigen concentration. It also may involve the effect due to shifted antigen dilution curves, as less human IgG may be immobilized at comparable added concentrations, and may result in an increased sensitivity as shown in Figure 2.

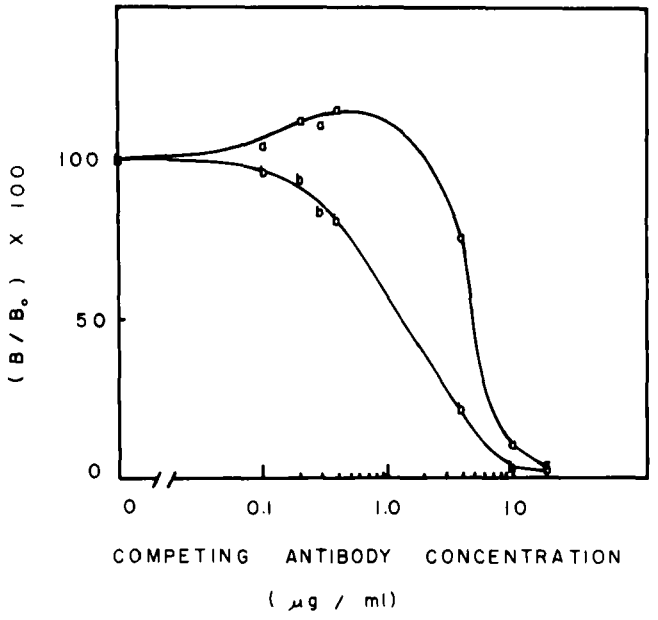

FIGURE 8. A set of competitive binding assays where the competition is between soluble, nonconjugated antibody and antibody-enzyme conjugate for bound antigen. The immobilized antigen level corresponds to 2.5 μgm human IgG/ml added. The curve for (a) points describes data for polystyrene tubes and for (b) points describes 48 hour BSA-PBS preincubated polystyrene tubes (3 successive incubations for 3, 21, and 24 hours respectively).

An additional phenomenon which affects these assays is the antibody concentration dependent noncompetitive response (vide supra). This has been postulated to be dependent on formation of immobilized antigen-conjugate aggregates and, as such, should be dependent on soluble antigen concentration. Thus a reduction in desorbed antigen concentration by prevention of the immobilized antigen desorption should reduce this noncompetitive response. This does occur as shown in Figure 8. Under conditions that produce a noncompetitive phase in the untreated coated tube procedure, the preincubated tubes produce no apparent noncompetitive phase.

Finally comparison of the error of the different solid phases shows that the covalent binding procedures are more precise, yielding coefficients of variation of 10.1 ± 6.0%, 7.3 ± 4.3% and 12.7 ± 7.8% respectively for activated glass, glutaraldehyde treated polystyrene, and untreated polystyrene.

Thus significant effects on response, sensitivity, precision, and the low competing antigen noncompetitive phase are seen as a function of antigen desorbed from the solid phase in competitive binding assays. The speed and simplicity of the coated tube method (and presumably microtiter plates as well) may be retained, while considerably improving the assay if a covalent binding method is used.

ACKNOWLEDGEMENTS

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