

**A RAPID, SIMPLE SOLID-PHASE IMMUNOFLUOROMETRIC ASSAY:
DEVELOPMENT AND CHARACTERIZATION**

by

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ABSTRACT

Two-site immunometric assays (sandwich type) give reproducible, quantitative results, but the time required for assay completion is quite long, and the steps involved is laborious. For example, a wide range of sample dilution is required because most assays have narrow, fixed ranges of sensitivity. In addition, the assay involves a sequential addition of reagents followed by a series of washing steps. In certain formats, long reagent incubation times are also required because of mass transfer limitations introduced by the size of the solid support, and because of low reagent concentrations.

In this work, we developed a novel format of two-site immunofluorometric assay which includes only one incubation step and one separation step. The assay is based on using small-sized nonporous beads (0.5 μm diameter) as a solid support and measuring the unbound fraction of labeled antibody in the liquid. Two assays were developed: one using anti-IgG polyclonal antibodies and the other using anti-bovine serum albumin monoclonal antibodies. The detection range of the polyclonal antibody assay for human IgG or mouse IgG with a 30-minute incubation was 0 - 40 $\mu\text{g}/\text{mL}$ for IgG standard. The detection range of the monoclonal antibody assay was 0 - 14 $\mu\text{g}/\text{mL}$ for bovine serum albumin (BSA) standard with 2 minutes required for the incubation. The inter-assay variability for the BSA measurement was 1.9% at 4.0 $\mu\text{g}/\text{mL}$ of BSA and intra-assay variability was 2.3% at 3.2 $\mu\text{g}/\text{mL}$ of BSA. The environmental factors such as pH, ionic strength, protein concentration and serum do not affect the binding reaction of the polyclonal antibody assay. The reaction mixture of antibody in a freeze-dried form offered reproducible, quantitative results as did the non freeze-dried form.

Intrinsic binding kinetics of ^{125}I -bovine serum albumin (^{125}I -BSA) and monoclonal anti-BSA (MAb 9.1) immobilized on non-porous polystyrene beads (0.5 μm diameter) were studied. The small sized beads minimize the mass transfer interferences on rate measurements. We demonstrated both theoretically and experimentally that the binding reaction is kinetically controlled. Rate measurements show that the association reaction is of second order and the dissociation reaction is of first order. Between 4 and 37 $^{\circ}\text{C}$ the measured equilibrium constant agrees well with the equilibrium constant calculated from the rate measurements. The temperature effects on association are much greater than on dissociation; the activation energy for association is about 9 Kcal/mole, as compared to 2 Kcal/mole for dissociation.

A model which describes an assay's dose-response was developed. The model employs a series of binding relations between the solid-phase antibody, labeled antibody and antigen to describe the multiple equilibrium reactions. Simulation results show that a dose-response curve of the assay with two monoclonal antibodies depends on a variety of factors, including the

intrinsic association constant for the binding, the cyclization factor, and the relative ratio of the cyclization factor to the equilibrium constant. The dynamic range of assay at a given condition mainly depends on the amount of antibody used, and the lowest antigen detection depends on the error in the fluorescence measurement.

The knowledge generated in this thesis should be useful in two major areas: (1) the application of the assay principle to determine protein's concentration simply and rapidly, (2) the application of the fundamental understanding for the antigen-antibody binding on a solid surface to other antibody binding systems. The rapid and simple protein immunoassay facilitates the automation of assay procedures, resulting in greater number of assays per given time. In addition, with such an assay one can monitor of a protein whose concentration changes over time. The use of small non-porous beads as a solid support in binding studies essentially avoids mass transfer limitations; such a system makes it possible to determine the intrinsic binding characteristics of any immobilized antibody on a solid surface, particularly the association and dissociation constants and equilibrium constant.

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I. INTRODUCTION

Immunoassay is an analytical method based on the use of antibodies. In protein measurements it has been shown that the immunoassay has the ability to determine the presence of a specific protein at a low concentration and in the presence of other proteins. In many areas of clinical and biological research, immunoassays have become a commonly chosen method. In clinical laboratories, enzymes, hormones, antibodies and other biologically active proteins in blood or urine are routinely measured by immunoassay for the screening or monitoring diseases. Increasingly, the immunoassays for protein quantification have also been used in biomedical research, and in the food and pharmaceutical industries for research and production processes related to the production of biologics.

The improvement of immunoassays has been carried out in order to achieve two main goals: higher sensitivity and ease of use. While the sensitivity of an immunoassay has been substantially improved, the immunoassay for protein measurements is known to be difficult to perform. By the use of new labels and signal amplification procedures, it is now possible to measure an analyte on the order of 10^3 molecules/mL (Jackson and Ekins, 1986). Many techniques have also been developed to improve the ease of use. One of the examples is the homogeneous assay that is performed without any separation. But these new techniques in many cases are only practical for determining compounds with a low molecular weight, for example, drugs or non-protein hormones. In general, however, the immunoassay procedures for measuring a protein and other high molecular weight compounds are still quite complex and takes a long time to perform.

Several reasons account for the complexity of the immunoassay for proteins. First of all, sample preparation is needed. Because of the assay's narrow and fixed dynamic range of measurement and because of the presence of components that interfere the signal of immunoassay, the sample has to be diluted with an assay buffer. Secondly, the assay often requires multiple and sequential steps. Such assay procedures include addition of reagents,

incubation of samples and separation of different phases. Lastly, determining the amount of probe is an additional step. The probe like enzyme need steps for determining the activity by using substrate. The complexity of the overall protocol results in a slow rate of measurement and poor reproducibility (Rodbard and Feldman, 1978). In addition, because of the poor reproducibility, a calibration on standard has to be performed simultaneously at each measurement.

The overall goal of this research is to provide a basic understanding and to improve the ease of use of immunoassay. In particular, the goal is to reduce the number of steps and increase the speed of assay. To achieve this goal, we selected a two-site immunofluorometric assay that was already available and developed a novel format of the assay in which the number of assay steps and time were substantially reduced.

Originally developed to facilitate analytes measurements, the two-site immunofluorometric assay provides quantitative results. It employs two kinds of antibodies: an antibody bound on solid support and an antibody labeled with a fluorophore. The fluorophore-labeled materials are stable and their quantity can be determined directly by measuring the fluorescence. Consequently, this assay needs fewer number of steps than the other two-site assays employing either radioisotopes or enzymes.

The novel format of the two-site immunofluorometric assay employs four key procedures which contribute to a greater assay speed, with a minimum of operational steps - one incubation step and one separation step. These four key procedural steps are presented below.

- (1) The use of the two antibodies that do not interfere with one another will allow the simultaneous binding of the same antigen in a single incubation step.
- (2) The use of nonporous beads with a submicron diameter as a solid support would minimize the mass transfer resistance and provide a larger surface area of bead for antibody immobilization.

- (3) The fluorescence measurement of unbound labeled antibody is from the liquid and subsequent washing step is not needed.
- (4) The use of mixtures of the solid-phase antibody and labeled antibody at different concentrations allows the preparation of reaction mixtures to extend the dynamic range without sample dilution.

The specific aims which we formulated and pursued are as follows:

- (1) To test the feasibility of the novel format of two-site immunofluorometric assay.
- (2) To develop and characterize the assay with monoclonal antibodies.
- (3) To examine the binding between an antigen and an antibody immobilized on a solid surface.
- (4) To develop a mathematical model which can describe the assay performance.

For the feasibility test, we chose to develop the assay format by using polyclonal antibodies. Human immunoglobulin G (IgG) and mouse IgG as antigens were selected because of the ease of handling and the stability of the immunoglobulins as well as availability of the different antibodies. The assay provides quantitative results with a 30-minute incubation and the dynamic measurement range can be easily expanded as the concentration of antibodies increased. The environmental factors such as pH, ionic strength, protein concentration and serum do not affect the binding reaction of the assay. To facilitate the assay procedure, we prepared the reaction mixture of antibody in a freeze-dried form to be used directly in the experimental protocols (Chapter 4.1).

In order to understand the assay quantitatively, we developed the assay with monoclonal antibodies. The assay with monoclonal antibodies for BSA showed that the results are accurate and reproducible and the required incubation time can be extremely short: 2

minutes. The effects of immobilization methods (physical adsorption and covalent binding) on assay performance were also examined (Chapter 4.2).

We studied the kinetics of binding between BSA and the monoclonal antibody immobilized on the non-porous beads (0.5 μm). After confirming that the binding reaction of the antigen and the immobilized antibody is controlled kinetically, we measured association rate, dissociation rate and equilibrium at various temperatures. Based on these measurements we determined the order of the binding reaction and the intrinsic values of the kinetic constants (Chapter 4.3).

The theoretical prediction on the assay performance was performed in order to provide predictive conditions for assays with other proteins. A dose-response curve of the assay with two monoclonal antibodies depends on a variety of factors, including the intrinsic association constant for the binding, the cyclization factor, and the relative ratio of the cyclization factor to the equilibrium constant (Chapter 4.4).

The knowledge generated in this thesis should be useful in two major areas: (1) application of the assay principle to determine protein's concentration with a rapid and simple way, (2) application of the fundamental understanding for the antigen-antibody binding on a solid surface to other antibody binding system.

The potential of this rapid and simple protein immunoassay is numerous. Such an assay in general facilitates the automation of assay procedures, resulting in a greater number of assays per given time. Another major attribute of such an immunoassay is the monitoring of a protein whose concentration changes over time. This behavior, for example, is encountered in many therapeutic proteins when produced by cultured cells in bioreactors or during purification processes following production. Monitoring of these processes demands measuring the concentration of protein when their concentrations vary with time rapidly.

Many immobilized antibodies are currently used in purification and quantitation of proteins. In selection of an antibody, the key factors to consider are the characteristics of the binding between the antigen and its immobilized antibody; in particular the intrinsic value of

kinetic parameters. The use of small non-porous beads as a solid support in binding studies essentially avoids mass transfer limitations; such a system makes it possible to determine the intrinsic binding characteristics of any immobilized antibody on a solid surface.

II. LITERATURE SURVEY

2.1. Immunoassay for protein measurement

Immunoassays have been used for measurement of many biologics at low concentrations. One of the frequently measured biologics is proteins. In clinical purposes proteins in body fluids are measured for test and diagnosis: among them are calcinoembriogenic antigen (CEA) for cancer monitoring, immunoglobulin E (IgE) for allergy testing, human gonadotropin (hCG) for pregnancy tests, and rheumatoid factor (RF) for autoimmune disease diagnosis. In addition to clinical diagnosis, the measurement of proteins has become a key procedure in research and production of genetically engineered proteins. Proteins including interleukines, interferons, and growth factors have become commercially valuable products, and the number of such proteins will keep increasing.

The selection of an immunoassay for protein measurement is not a simple task due to the diversity of immunoassays. Many immunoassays differ in sensitivity, accuracy and ease of use. Some assays have sensitivity to measure a nanomole or even picomole of protein. These assays usually employ signal amplification steps such as the use of biotin-avidin complex (Kohen *et al.*, 1986). Because of multiple steps, performing the assay is laborious and time consuming. On the other hand, some assays such as latex agglutination, require only one incubation step before visual detection of changes in turbidity. The assay procedure is simple and easy to perform, but the assay only gives qualitative results because of lack of sensitivity (Robbins *et al.*, 1962).

The intension of this section is to provide an overview on immunoassays for proteins, particularly on the development and application of immunoassays for a rapid measurement. To provide background information we first describe the key assay components: proteins, antibodies, and their binding reactions. Immunoassays are then classified into three assay types by the binding reaction. The fundamentals of the three types and their common characteristics are discussed. Because the multiple, complex procedures

limit the assay speed, the effects of individual steps on overall assay speed are discussed. Through a literature survey the advantages and disadvantages of currently available assays for a rapid measurement are reported.

2.1.1. Proteins, antibodies and their binding

2.1.1.1. Proteins

In an immunoassay for proteins, the key assay procedure is binding reactions between proteins and their antibodies. A protein molecule has multiple binding sites on its surface. The domains responsible for the binding to antibodies are known as epitopes (Sela, 1969). Epitopes are a portion of amino acid chains which may be distant in sequence but close in space. The effective size of epitope may correspond in size to three to four glucose molecules, having no relationship to the overall size of the protein (Schechter *et al.*, 1970). On a protein molecule each epitope has its own unique binding specificity to antibodies. However, if a protein has a repeated sequence or subunits of identical structure, the protein can have the same epitopes on a single molecule.

2.1.1.2. Antibodies

In immunoassays, antibodies are a binding reagent to an analyte. For an assay development, it is desirable to select an antibody with high affinity and defined specificity to the antigen. Generally, antibodies with a higher affinity gives better assay performance in sensitivity and accuracy (Berson and Yalow, 1973). The use of antibodies with defined specificity avoids an interference by the presence of a compound with a similar binding specificity to the antigen (Berson and Yalow, 1963).

A conventional technique for the antibody preparation is by immunizing an animal with a protein. Purified or partially purified proteins are introduced to an animal by intradermal and subcutaneous injection (Vaitukaitis *et al.*, 1971). Immune response to the protein enables cell lines of B-lymphocyte to produce a mixture of antibodies in the sera. The

antibodies known as polyclonal antibody differ in physical, chemical and immunological properties.

The antibody molecule most abundantly found in antisera is immunoglobulin G (IgG) which has two binding sites to antigen. Figure 1 shows a structure of IgG molecule which consists of two sets of two polypeptide chains known as the light and heavy chain. By the action of papain, an IgG can be cleaved into three 50-KDa fragments: two fragments, each of which contains one binding site for antigen, are called Fab fragments, and the other fragment called Fc which does not bind antigen (Nisonoff, 1985). The antigen binding site locates on the end of Fab fragments, and the size is only few percent of the size of antibody molecule (Poljak, 1975).

During the early development of immunoassays, unpurified antisera was the common binding reagent. Later, a purified form of immunoglobulins from antisera started to replace the antisera; the use of the purified reagents have improved assay performance in sensitivity and accuracy (Shield and Turner, 1986). Furthermore, fractions of immunoglobulin such as Fab, or $F(ab')_2$ - dimeric form of Fab, have been used as a binding reagent. When an analyte in serum is measured, some compounds from the serum can interact with the Fc fragment of the antibody. The use of the antibody fragments reduces the nonspecific interaction (Moussebois *et al.*, 1983).

One major breakthrough in antibody preparation is the development of monoclonal antibody by the cell fusion technique (Köhler and Milstein, 1975). By growing a single cell line of hybridoma, it is possible to prepare a large quantity of antibody with homogeneous binding properties. In addition, an antibody with the same quality can be produced over a long period of time by growing the cell line over and over.

The monoclonal antibody preparation can be highly specific to the antigen; the high specificity avoids interference by the presence of cross-related compounds with common epitopes so that the specificity of the assay improves (Wallis *et al.*, 1982). Because of its homogeneous and highly specific binding characteristics and because of possibility of the

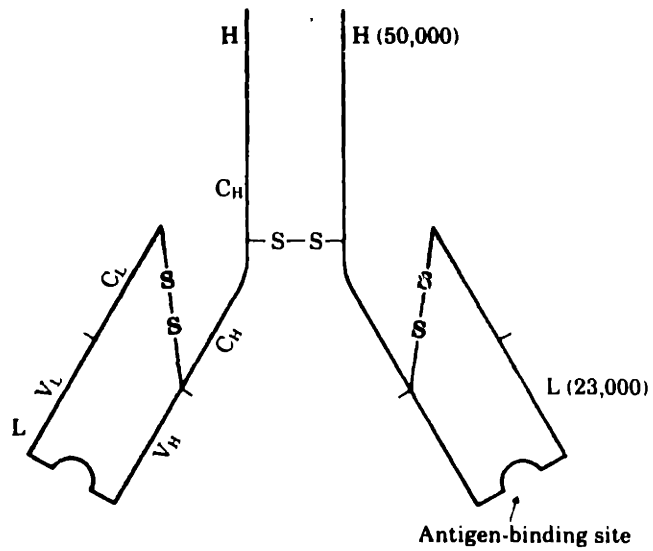


Figure 1. Diagrammatic sketch of the structure of IgG. In an individual molecule, the two H chains are identical to one another, as are the two L chains. Note that the variable regions of the L and H chains (V_L and V_H) are approximately equal in size, whereas the constant region of the H chain (C_H) is about three times as large as C_L (Nisonoff, 1985).

consistent production of antibody, the use of monoclonal antibody in immunodiagnostics increases.

2.1.1.3. Binding Reaction

For the development of immunoassays for proteins, three binding characteristics have been used: a specific binding between an antibody and antigen, a multiple binding reaction of antibodies on a single protein, and a formation of an immune complex of proteins-antibodies mixture.

The binding between an antigen and an antibody is known to be one of the most highly specific reactions in nature. The antigen-antibody binding reaction in solution is a bimolecular reaction (the association reaction is of first order, the dissociation reaction is of second order, and the reactions are reversible). The value of the equilibrium constant represents the extent of affinity for the binding, and for monoclonal antibodies-protein antigen binding the equilibrium association constant ranges between $10^5 - 10^{11}$ L/mole (Morgan *et al.*, 1984; Soos *et al.*, 1984).

A multiple binding of antibodies onto a single protein is often observed when a polyclonal antibody is incubated with a protein antigen. The polyclonal antibodies with different binding specificity binds to epitopes on a protein antigen. The large size of a protein molecule allows two or more of antibodies to bind simultaneously onto the molecule. The multiple binding is also observed in the binding of a monoclonal antibody with a protein which has multiple, identical epitopes to the antibody (Deverill *et al.*, 1981).

The formation of an immune complex of proteins-antibodies mixture results from binding reactions between the antibodies with divalent or multi-valent binding sites and protein antigen with multiple epitopes. The intermolecular binding between antibodies and proteins results in a formation of a cross linked network of the proteins-antibodies. The cross linked network is called an immune complex. Figure 2 shows a schematic of this complex. At early stages in binding, the immune complex can be soluble in solution. As the binding

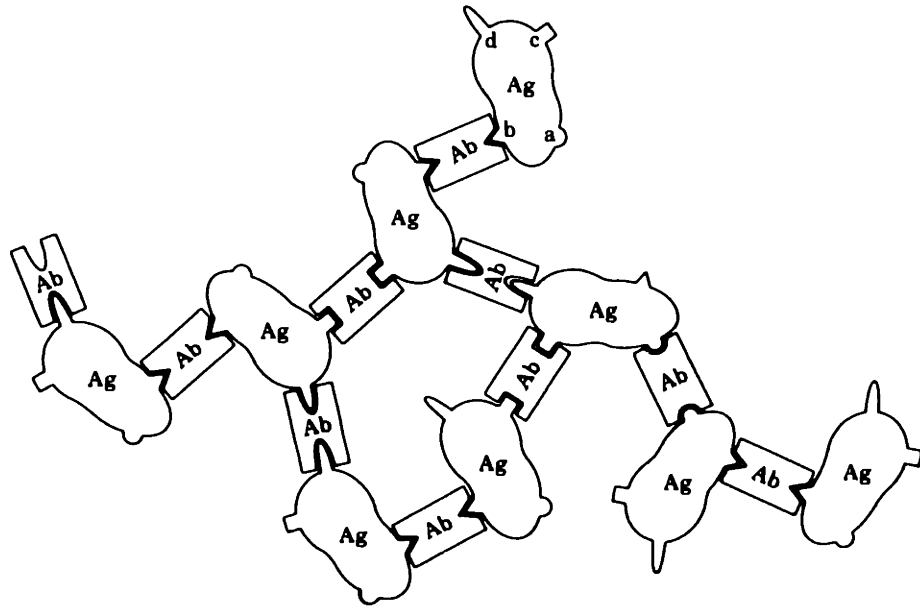


Figure 2. Schematic of immune complex. Formation of immune complex by crosslinking of a soluble antigen (Ag) by antibody. The antigen is represented as having four different determinants (epitopes), labeled a,b,c, and d. The network, or lattice, can grow indefinitely, eventually resulting in precipitation. Note that for crosslinking occur, the antibody and antigen must each have at least two combining sites (Nisonoff, 1985).

reaction progresses the size of the complex increases. As the result, the immune complex precipitates.

Specific binding reaction, multiple binding reaction and immune complex formation are the basis for developing immunoassay for proteins. Application of such assays can be extended to determine other large antigens like cells, viruses, and polysaccharides, whose binding characteristics with antibody are similar to those for proteins.

2.1.2. Assay Types

In this section immunoassays for proteins have been classified by the binding reaction between antigens and antibodies that is employed in the assay procedures. The binding reactions are competitive binding, two-site binding, and intermolecular binding. The assay procedures and common characteristics for the three assay types are discussed. Other possible classification methods are by analyte, by the probe used for labeling, by binding reagents, or by separation method. These can be found in the literature (Kricka, 1985).

2.1.2.1. Assays employing competitive binding

Although an antibody can specifically bind an antigen at low concentration, in many cases the binding reaction itself does not provide any signal to detect easily. Thus, a label was introduced for tagging an antigen. By measuring the label from the bound or unbound antigen, the amount of antigen that binds to antibodies can be determined. Employing a radioisotope labeled antigen, Berson and Yalow (1959) developed a competitive type of immunoassay.

In a competitive type of assay the antigen in the sample is first mixed with a known amount of the labeled antigen, and then the mixture is incubated with antibodies (Yalow and Berson, 1960; Etkins, 1960). During the incubation, the labeled antigen and the unlabeled antigen competitively bind to antibodies which are present in a limiting amount: As a result, the labeled antigen bound to antibody is inversely proportional to amount of antigen in the

sample. By measuring the bound or unbound labeled antigen, one can quantify the concentration of antigen in the sample.

Alternatively, the similar principle can be used for an assay with a labeled antibody (Miles and Hales, 1968). In the assay, a limited amount of antigen immobilized on solid support is mixed with the antigen in a given sample. A known amount of labeled antibody is then incubated with the mixture. After separation, the amount of the labeled antibody in the solid phase is measured and this is proportional to the antigen in the sample.

In most competitive assays, the bound fraction of the labels must be separated from the unbound material in order to measure the signal from the bound labels or unbound labels (Odell, 1983). A common separation method is through precipitation of the antibodies using chemicals or secondary antibodies (Kricka, 1985). The precipitates can be separated by centrifugation, filtration or phase separation. Alternatively, the antibody fractions can be separated by the use of secondary antibodies immobilized on solid support.

The sensitivity of the assay is often determined by the specificity and avidity of the binding of antigen and labeled antigen to the antibodies. The low detection limit of the assay is governed by the equilibrium binding constant of the reaction between the antigens and antibodies, and by the experimental error mainly in the separation steps. Etkins (1980) estimated that the limit does not exceed 10^{-14} mole/L.

Competitive assay offers a narrow dynamic range of measurement and the assay procedures are laborious. Dynamic range of the competitive assays is usually from the low detection limit to 1000 times of the limit. In addition, the assay procedure is often optimized with the use of fixed amount of labeled antigen and antibodies. As a result, each sample must be diluted in a proper ratio of antigen to the labeled antigen. In general a series diluted samples are mixed with the labeled antigen before carrying out binding reaction with antibodies.

2.1.2.2. Assay employing two-site binding

This assay employs two antibodies: labeled antibody and an antibody linked to a solid support (Addison and Hales, 1971). This assay is only applicable for measuring an antigen having at least two binding sites on which antibodies can bind simultaneously. An antigen first binds to the antibody on the solid support. The labeled antibody is then attached to the antibody/antigen complex on the solid support. The signal from the solid phase is proportional to the analyte concentration.

The assay in general employs two incubation and two separation steps. After each incubation, the solid support needs to be washed to minimize the nonspecific binding of the immunochemicals. The solid support introduces a mass transfer resistance for the binding of the antigen or antibody in liquid phase. When the solid supports are porous matrices or microtiter plates, the mass transfer limitation is the rate limiting step for the binding reaction. In addition, if the solid support used is micro beads, then the resuspension of the solid support after separation is required.

The two-site assay was shown to have a wider dynamic range of measurement compared to the competitive assay: this is possible because of the use of labeled antibodies and solid phase antibody in excess which increases the dynamic range up to a certain point. The dynamic range is about from the low detection limit to 10,000 times the limit. For the measurement of a very high concentration of antigen, however, the sample still needs to be diluted. The more detailed discussion on two-site assays can be found in section 2.2.

2.1.2.3. Assays using intermolecular binding

The principle of intermolecular binding assays is the measure of the increase in size of the antibody-antigen complex over the unbound immunochemicals. This assay is only applicable to measure an antigen that can form immune complexes (Deverill and Reeves, 1980). In the assay, the antigen in the sample is mixed with predetermined amount of polyclonal antibodies to the antigen. Through the intermolecular binding, an immune complex

forms, which can be either soluble or insoluble. After a given incubation time, the amount of the immune complex formed depends on the added antigen concentration. Thus by measuring the amount of immune complex formed, one can determine the antigen concentration.

Several techniques have been used for measuring the immune complex. In one technique, the reaction is carried out to form precipitates. The precipitates are then centrifuged and measured by weight (Goettsch and Kendall, 1935). Alternatively, the precipitates can be resuspended in a buffer, and then quantified by measuring the intensity of turbidity or light scattering from the suspension (Heidelberger and Kendall, 1935). Assays without separation have also been developed. The assay reaction is allowed to form a soluble immune complex; the amount of complex is determined by measuring the turbidity and light scattering (Tiffany *et al.*, 1974). To improve the sensitivity, some assays employ particles such as latex beads or cells, which enhance the light scattering or the amount of precipitate.

The results of assays using intermolecular binding are quite susceptible to assay conditions. The formation of immune complex results from the multiple, intermolecular binding. The formation is often interfered by nonspecific protein interaction. Particularly, when the sample is in serum, the complement components in serum will cause non-specific interactions with the antigen or antibody (Collet-Cassart *et al.*, 1981). This interaction leads to the changes in amount of immune complex. In addition, small changes in mixing and incubation procedures while performing assays also affect the assay accuracy.

The assays use a single step of incubation for the binding reaction so that the binding reaction itself can be simple and rapid. However, for each measurement sample and reagents preparations have to be done carefully to obtain reproducible assay results. Such preparation include centrifugation or filtration whose purpose is to remove particles in the samples and reagents (Hudson *et al.*, 1981; Cambiaso *et al.*, 1974).

In addition, sample dilution is often needed to prepare the antigen concentration in the dynamic range of the measurement which is narrow and fixed. The low detection limit of the assay measuring soluble immune complex is in a range of 10^{-9} mole/L which is much

higher than that of RIA or two-site assays. The high detection range of the assay is no more than 100 times the low limit.

2.1.3. Steps affecting assay speed

The procedures in immunoassays can be divided into three major steps: sample and reagent preparation, binding reaction, and signal determination and calculation. How each assay step affects the overall assay speed is described in this section.

Before the binding reactions, many assays require sample preparation for three reasons: (1) to reduce the concentration of compounds that interfere with a binding reaction or with signal detection; (2) to prepare the antigen concentration within the dynamic range of the measurement; (3) to prepare samples for the standard curve for the measurement. Samples are commonly diluted, filtered or extracted, which requires extensive liquid handling. Particularly, for competitive binding assays and intermolecular binding assays, sample preparation is a major time consuming step. In competitive binding assays, samples must be diluted to obtain a proper ratio of the antigen to the labeled reagents. In intermolecular binding assays, the sample must be filtered or diluted to minimize the interferences by particulates.

The binding reaction of immunoassays can also be a time consuming step when the assays use solid supports as a mean of separation. The assay steps then consist of incubation, separation, washing. The solid support often introduces a mass transfer resistance which limits the antigen-antibody binding reaction (Stenberg *et al.*, 1986). When the solid support is beads, they are separated by centrifugation or filtration, which requires transfer of the contents of the reaction container. In addition, the beads need to be resuspended afterward with vortexing. Washing of the solid support is needed to reduce the nonspecific adsorption of the immunochemicals on the solid support. (It is known that use of magnetized beads (Pourfarzaneh and Nargessi, 1981; Schroeder *et al.*, 1986) avoids the requirement of the sample transfer, but the washing and resuspension steps are still needed.)

Signal detection and calculation can be an additional time consuming step. Some labels require the further step of determining their activity. For example, label such as an enzyme needs additional steps including sample and enzyme substrate preparation for the activity measurement. In many immunoassays, the procedures consist of multiple steps so that the reproducibility of assay results depends on how each step can be performed in consistent manners. In many cases, the assays must be simultaneously run with standard samples to obtain a standard curve, from which the assay results are calculated.

As described above, the immunoassay procedures consist of a multiple, complex steps. Thus, it takes a long time to perform these procedures. To develop a simple and rapid assay, the number of assay steps and time required for performing each step must be reduced. In the next section, we discuss how such rapid, simple assays have been developed and what are their advantages and disadvantages for rapid measurement.

2.1.4. Rapid and simple assays

Radioimmunoassay (RIA) developed in 1959 is the first assay which has a high enough sensitivity to measure insulin in serum. Because of the high sensitivity, it has quickly been adopted for measuring other peptide hormones, haptens (small molecular compounds), and proteins. At present time it is still one of the most widely used immunological techniques.

Although the RIA offers high sensitivity, performing the assay is difficult for three reasons. The handling of radioactive materials requires strict regulatory control. Frequent preparation of reagents is also needed because the radioisotope has a short shelf life; for example, the half life of ^{125}I is 60 days, and the half life of ^{131}I is 8 days. In addition, the assay requires a separation of the bound and the free fractions prior to measurement, which is labor intensive.

To avoid some of the drawbacks, nonradioisotope labels have been introduced. Engvall and Perlmann (1971) and Van Weemen and Schuurs (1972) independently described the use of the enzyme as a label. The immunochemicals with enzyme are stable and the activity

of enzyme can be determined with a spectrophotometer which is readily available in many laboratories. Another type of label is a fluorophore whose quantity can be easily determined by fluorescence intensity. Other probes include metals (Cais *et al.* 1977) and chemiluminescent groups (Simpson *et al.*, 1979).

Replacing radioisotopes with these new probes avoids the problems of the regulatory control and frequent reagent preparations, but the performing the assay is still complex and time consuming. This is because the assay procedures are not changed.

The separation step is one of the main time consuming steps. An assay not using separation steps can offer the following advantages. First, the labor intensive step of separation can be avoided so that the assay becomes simpler and faster. Second, the binding reaction and signal determination can be performed in the same sample container, so that it is much easier for non-trained personnel to perform the assay. Lastly, simpler assay lends itself for its use through automation which enhances the number of samples that can be analyzed per given time.

Scientists have taken three main approaches to develop an assay that does not require a separation step. One approach is to measure the modulation of probe when the antigen in sample binds to the antibody. This type of assay is called a homogeneous assay. Another approach is to measure the signals from the known non-separation assays such as intermolecular binding assay with higher sensitivity and reproducibility. The other approach is by the use of an instrument like a flow cytometry which can determine the fraction between the bound antigen and antibody without separation.

Some other assays, although not providing a quantitative result, can be rapid and simple to perform. One example is agglutination of latex particles, whose assay procedure consists of only one incubation step followed by detection with the naked eye. Another example is immunofiltration which uses an adsorbent on which antibodies are immobilized (Anderson *et al.*, 1986). The filtration of the adsorbent can be a quick and simple separation of

liquid so that the time for separation can be substantially reduced. Most such assays give only positive or negative results as to the presence or absence of the antigen.

In the following sections, we describe fundamentals of immunoassays that have been claimed to be rapid and simple. The advantages and disadvantages when using them for protein measurements are described.

2.1.4.1 Homogeneous assays

Homogeneous assays are designed to determine an antigen concentration by the use of signal modulation of a probe when the binding reaction of antigen-antibody complex occurs. Fluorophores and enzymes are common probes used for homogeneous assays. Although homogeneous assays with the other probes have been proposed, the assay procedures are more complex and their sensitivity is substantially less than those of the assay with fluorophores or enzymes.

2.1.4.1.1. Homogeneous assay with fluorophore

The fluorescence characteristics used for the development of homogeneous assays includes enhancement (Smith, 1977), quenching (Zuk, 1981), polarization (Dandliker *et al.*, 1973), and energy transfer (Ullman *et al.*, 1976; Ullman and Khanna, 1981).

Fluorescence enhancement immunoassays are based on the increase of fluorescence signal when the antigen-antibody binding occurs (Smith, 1977). An antigen labeled with fluorescein have a reduced fluorescence quantum yield in the presence of iodine atoms. The binding of antibody to the fluorophore labeled antigen reduces the interference of iodine to the fluorophore, so that the fluorescence intensity increases.

Fluorescence quenching immunoassays are based on the decrease of fluorescence signal as the binding reaction proceeds. To increase the quenching effect, Nargessi *et al.* (1978) used anti-fluorescent antibody to quench effectively unbound fluorescence-antigen in liquid. Fluorescence quenching immunoassays for protein use charcoal

or other particles on which antibodies are immobilized. The particles sterically inhibit the binding of anti-fluorescein antibody to the label bound on the solid support (Ullman *et al.*, 1980).

Fluorescence polarization immunoassays (FPIA) are based on the principle that the bound fluorophore-labeled antigen/antibody have a more polarized emission than the unbound fluorophore label. The unbound fluorophore is depolarized to a higher extent than the bound fluorophore with antibody because the unbound fluorophore rotates more freely (Dandliker *et al.*, 1973; Spences *et al.*, 1973). The practical application of the assay was the introduction of a commercial assay kit by Jolley *et al.* (1981). An automatic analyzer (Abbott TDX system) uses a FPIA kit and the major application is for monitoring haptens including drugs and hormones. The assay principle was applied for small-sized proteins like growth hormone (Levison *et al.*, 1976), human chorionic gonadotropin (Urios *et al.*, 1978) and insulin (Yamaguchi *et al.*, 1982).

Fluorescence excitation transfer immunoassay (FETIA) uses a donor (fluorescein) and acceptor dye (tetramethylrhodmine) which have an overlapped region of the emission and excitation spectrum. Because of the overlapped spectrum, emission from the donor is quenched when the donor and acceptor are close in distance (Ullman *et al.* 1976). When antigen is protein, however, the quenching of fluorescence is the relatively small compared to the total fluorescence intensity.

The application of the homogeneous assay with fluorophore is limited to small size molecules (haptens) and the sensitivity of the assays is limited by the strength of the measured signal. Quite often, background noise interferes with the measured signal. Two reasons account for its limited use for protein measurement. The size difference of protein antigen and antibody is not significant, so that the modulated signal is small. Furthermore, the small signal is easily affected by the nonspecific binding of fluorescent dye to protein in the sample, and the fluorescence intensity is affected by the scattering of either excitation or emission radiation.

2.1.4.1.2. Homogeneous assay employing enzyme

The types of enzyme characteristics used in homogeneous assays include enzyme inhibition and enzyme channeling. Similar to homogeneous fluorescence immunoassays, use of homogeneous enzyme immunoassays is mainly for small molecules at micromoles per liter.

Enzyme inhibition immunoassays is based on the principle that the activity of enzyme label decreases when antigen-antibody binding occurs. Rubenstein *et al.* (1972) conjugated lysozyme with hapten and found that the enzyme activity is inhibited by binding of the anti-hapten antibody. This prevents the macromolecular substrate from reaching the enzyme active site (Schneider *et al.* 1973; Bastiani *et al.*, 1973). The inhibition mechanism for these enzymes is antibody-induced conformational changes in the enzyme (Ullman *et al.* 1979; Ullman and Maggio, 1980). These modulations of enzyme activity are implemented to develop a competitive type of assay technique called "enzyme multiplied immunoassay technique" (EMIT). This is used for numerous haptens (hormone, medicines and drugs).

The same concept was extended for measuring concentration of large molecules. The enzyme-antigen:antibody complex inhibits the enzyme from acting on a large macromolecular substrate. Assays have been developed for IgG using an IgG- β -galactosidase conjugate and a chromogenic 0-nitrophenylgalactosyl substituted dextran (Gibbons *et al.*, 1980), and for ferritin, using a fluorescent umbelliferone-substituted dextran (Ullman, 1981).

The enzyme channelling immunoassay employs the principle that the activity of two enzymes acting in sequence are enhanced when the enzymes are close in space (Goldman and Katchalski, 1971). Litman *et al.* (1980) co-immobilized a human IgG and glucose-6-phosphate dehydrogenase onto agarose beads. When an anti-human IgG-hexokinase conjugate is bound to IgG on the bead, the two enzymes are in close proximity and the rate of conversion of ATP, glucose-6-phosphate, and NAD to ADP, 6-phosphogluconolactone and NADH is accelerated. By competition of IgG in the sample with the IgG on the agarose bead, the amount of bead-bound hexokinase conjugate is reduced. Thus, the overall rate of the coupled

enzyme reaction decreases. The speed of the assay is rather slow; two hours of incubation for binding reaction between antibodies and antigens is needed (Litman *et al.*, 1980). Such slow binding reaction may result from the limited mass transfer of the enzyme-labeled antibody caused by the use of porous matrices.

2.1.4.1.3. Homogeneous assays with other probes

Probes which are conjugates between a hapten and different substances are able to influence the activity of an enzyme. Such probes include cofactors (Carrico *et al.* 1976; Schroeder *et al.* 1976), substrates (Burd 1981; Worah *et al.* 1981) or inhibitors (Finley *et al.* 1980; Dona 1985). The binding of the antibody to the conjugate modulates the activity of the enzyme by inhibiting the effect of the probes. However, enzyme activity is not directly modulated by the antibody-antigen complex and thus their procedure is more complex and less sensitive than EMIT.

2.1.3.1.4. Limitation of homogeneous assays for protein determination

The rapidity and simplicity of homogeneous assays using the modulation of probe have led to a considerable expansion of their use. However, their use are mainly for low molecular weight haptens. Their sensitivity is substantially low compared to RIA or two-site assays.

The design of a homogeneous assay for protein is rather difficult. One of the reasons has roots in the nature of the binding between a protein antigen and antibody. Particularly, the size of the antibody is in the same order as the antigen-antibody complex. Therefore, when the modulation depends on the size difference, the change in signal is not sufficiently high to allow accurate detection. Another reason of the difficulty originates in the binding site which is quite small compared to the size of the protein antigen and antibody molecule. The present immobilizing techniques give only random distribution of probes on protein surface. As a result of the random distribution, a substantial portion of the immobilized

probes are attached on sites remotely located from the binding sites. These probes are not effectively modulated by the binding reaction.

2.1.4.2. Nephelometric and turbidimetric determination

Measurement of light scattering is a simple and rapid detection method. When a light beam is directed at a fluid containing suspended particles, light is absorbed, scattered, reflected and transmitted. The quantification of the residual transmitted light is the basis for turbidimetry while the measurement of scattered light is employed in nephelometry.

The immunoassays using light measurement are divided into two groups: (1) assays that use signal detection from the soluble immune complex and (2) assays that use beads which increases the light intensity. In both assays the conditions of the binding reaction are maintained not to form precipitates, and thus there is no requirement of separation steps.

The use of particles has been introduced to enhance the sensitivity of the assay. The changes of latex particles by turbidimetric measurements (Dezelic *et al.*, 1971), or by nephelometry (Blume and Greenberg, 1975, Cohen and Benedek, 1975) have been reported. To enhance sensitivity of the measurement, Benedek and coworkers have introduced the use of quasi-elastic laser-light scattering spectroscopy (Cohen and Benedek, 1975; von Schultheses *et al.*, 1976). By using angular anisotropy measurements (von Schulthess *et al.*, 1980), the sensitivity was further improved.

To reduce the required incubation time, a difference in signal between two fixed time points was measured, and then the observed rate of change of light scattering was related to the antigen concentration (Beck and Kaiser, 1982); for IgE in serum the assay has a low limit of detection of 24 µg/L.

The advantages of light scattering measurements for protein concentration include the fact that the binding reaction in the assay procedure is simple (Cambiaso *et al.*, 1974). The assay procedure requires a one incubation step and the light signal can be detected without the need of prior separation procedures.

Disadvantages of these assays for a rapid measurements include the requirement of sample preparation by filtration or dilution. Any particles present in the sample interfere with the light scattering from the binding reaction so that the sample and reagents preparations have to be performed to avoid the presence of particles. In addition, sample preparation is needed to minimize the effect of the other compounds on the formation of immune complex or the particle binding reaction. The binding reaction itself is a multiple, competitive reaction, and thus small disturbance in incubation and changes in mixing procedures result in different assay results. Therefore each assay has to be calibrated with a standard procedure which has to be repeated with great care.

2.1.4.3. Detection with instrument

One of the detection methods without separation employed in immunoassays is by using a waveguard surface. The response of a fluorophore labeled protein on a waveguard surface to internally reflected laser light can be used as a signal in an immunoassay (Kronick and Little, 1975). Only fluorescent dye-antibody conjugates bound to antigens on the quartz surface are excited by the incident laser light, and thus the free conjugates can be readily differentiated without physically separating these two fractions. The optical sensing of surface immunoassay reactions, known as interface immunoassays, have been reviewed extensively (Mosbach *et al.*, 1984; Place *et al.*, 1985). This assay has been developed for detecting the antigen continuously, but the application of this assay has been limited because problem associated with the geometry of the assay can not be overcome.

The fluctuation in fluorescent intensity of a fluorophore-labeled protein can be measured (Nicoli *et al.*, 1980; Briggs *et al.*, 1981). The fluorescence carried by small particles can be measured in the presence of free fluorophore. The fraction of antibody bound to labeled antigen was measured by flow-cytometric detection (Lisi *et al.*, 1982), and by fiber optic flow-cytometric detection (Briggs *et al.*, 1985). In all cases, the sensitivity of the measurements

depends on the relative strength of background signal noise. Therefore, practical application is significantly limited.

The principle of particle counting techniques (Cambiaso *et al.*, 1977) is the measure of the number of residual non-agglutinated particles with an equipment designed to count blood cells. The antigens or antibodies are immobilized on micron sized latex particles and the number of the mono dispersed particles changes as a function of the antigen amount added. This assay procedure share all the disadvantages associated with light scattering measurements of latex particles.

2.1.4.4. Simple but semiquantitative assays

2.1.4.4.1. Agglutination

Agglutination assays are based on quantifying the precipitation as the result of the binding reaction of the particles on which antigens or antibodies are immobilized. The particles used in the assay include erythrocytes (Binns *et al.*, 1982), polystyrene beads of micron size (Singer and Plotz, 1956) and inorganic colloidal particles gold sols (Leuvering *et al.*, 1981).

This assay uses only one incubation step which is followed by visual detection with the naked eye. In general, the assay results are semi-quantitative. In the use of colloidal gold sol particles, the light absorption spectrum can be significantly changed by the aggregation. The change in color indicates the level of the antigen in the sample. The nonspecific interaction between proteins cause agglutination which leads to poor sensitivity and reproducibility (Moussebois *et al.*, 1983).

2.1.4.4.2. Immunofiltration

Immunofiltration refers to the detection of an analyte by capturing it from the solution by means of filtration with an adsorbent (Anderson *et al.*, 1986). A layer of membrane over a packed bed of adsorbent is used as a solid support for immobilization of

antibodies. In some case, antibodies are first immobilized on latex particles, and then the particles are entrapped in the membrane (Rubenstein *et al.*, 1986). The large surface area allow the use of high concentration of antibodies per measurement. The high concentration of the reagents and short diffusion distances of the pores in the membrane increase the binding reaction rate. The common assay format is two-site assay using enzyme labeled antibodies. The rapid assay speed, less than 5 minutes for one measurement lead to a wide use in pregnant tests.

The disadvantage of this assay is that the assay procedure requires a series of handling the reagents. The concentration may be estimated from the comparison of a standard sample that can be incorporated on the same membrane, but in most cases the assay results are not quantitative.

2.1.4.5. Limitation of current immunoassays for rapid detection

Although there are many assays developed to achieve a simpler procedure or faster assay speed, for quantitative protein measurement immunoassays are still complex and time consuming. Most of the homogeneous assays are only useful for measurement of small molecules and the light scattering measurement requires a sample preparation steps. Many assay with instruments are not practical.

One of the limitation for designing such a homogeneous assay for protein is that the binding reaction does not give sufficient changes in characteristics of the probes, because of large size of a protein antigen. To render sufficient modulation to allow detection, it may be necessary to develop a novel labeling technique to attach a probe near the binding site specifically.

It seems that the light scattering detection method can be a rapid protein measurement. The binding reaction itself is simple and rapid, but the limitation in speed of the assays is in the sample preparation. Because of the nature of the assays using immune complex formation, the sample preparation steps can not be avoided.

The assays with instruments to avoid separation cannot be practical. In interface immunoassays the binding reaction occurred on the plain surfaces and it takes a long time to obtain a signal which can be meaningful to yield the antigen concentration. The assay using fluctuation of fluorescence can be easily interfered by the assay conditions. Flow cytometry and fiber flow cytometry measurement requires a forceful delivery of the sample and reagents. In addition, the most assays require an expensive instrument.

A quantitative measurement of proteins needs a novel assay which assay steps are substantially reduced. In addition the time for incubation requires to be reduced. In the next section, we discuss the rationale for selection of two-site immunofluorometric assay as a model assay system and the modification to achieve a simpler format is described.

2.2. Two-site immunofluorometric assay

The overall goal of this research is to facilitate the methodology based on an immunological technique for determining a protein's concentration. Particularly we focused on reducing the number of steps and increasing the speed of assay. We tried to achieve this goal by overcoming the procedural complexity in an immunoassay that was already available.

In this research, we chose a two-site immunofluorometric assay as a model assay system. The assay is a quantitative assay for determining protein, and employs two kinds of antibodies; a solid-phase antibody and an antibody labeled with a fluorophore (Hemmila, 1985; O'Donnell and Suffin, 1979; Soini and Hemmila, 1979). This assay is useful only for determining the antigen which has multiple binding sites (epitopes) to antibodies.

2.2.1. Rationale for selection of model assay

A two-site immunofluorometric assay provides several inherent advantages over other immunological methods when rapid results are needed. The first is that the assay does not require a series of sample dilutions with a labeled reagents. In some types of assays, the use of varying amounts of antigen is required to construct a calibration curve from which the antigen concentration is then determined. The sample dilution involves multiple steps of liquid transfer which result in a slow rate of measurements.

The second advantage is that fluorophore-labeled materials have a long shelf-life (more than a year) and their quantity can be directly determined by measuring their fluorescence. For rapid results, a fluorophore has advantages over a radioisotope or an enzyme. A radioisotope provides good sensitivity, but can not be stored for a long period of time. For example, the half life of ^{125}I , a commonly used radioisotope, is only 60 days. In addition, the potential radiation hazard requires additional care for using the isotope. In the case of an enzymatic-linked assay, determining the enzyme activity requires additional procedures (Engvall and Perlmann, 1972).

The third advantage is that instead of consuming purified antigen, the assay employs two different kinds of antibodies. Preparation of the purified antigen is often difficult and expensive. In comparison, the preparation of antibody is readily achieved through monoclonal antibody technology as the results of cell fusion methodologies (Köhler and Milstein, 1975). Therefore, it is possible to produce an unlimited quantity of the antibody whose binding characteristics are known.

2.2.2. Fluorophore

Fluorophores are the molecules which can absorb energy by radiation and the excited state is emitted as photons. The small loss of energy in fluorescence, which is seen as a difference between excitation and emission energy (wave lengths), is defined as the Stoke's shift. In many organic fluorophores, the shift is normally 30 to 50 nm. The ratio between absorbed light and emitted light is defined as the quantum yield. Fluorophores can be characterized by their absorptivity and fluorescence life time. Table 1 lists the properties for some the frequently used probes for immunoassays.

The measurement of fluorescence is often affected by temperature, pH, fluorophore concentration, and quenching molecule concentration (Soini and Hemmila, 1979). This interference is particularly problematic when fluorescence measurement is to be performed in the solution containing the fluorescent compounds. Several probes have been introduced to minimize the interference. Fluorescent rare earth ions, such as europium and terbium are now used because these molecules have large Stoke's shift (200 nm) and long fluorescence life time (millisecond) which can help to reduce the background interference (Yamada *et al.*, 1982). However, the limiting factor for further application is the complex method to label rare earth ions onto the desired molecules. Another example of the probe is phycobiliproteins that are a class of proteins containing bilinprosthetic groups (Kronick, 1986). These phycobiliproteins have ability to efficiently absorb light whose wavelength is far from those of the compounds in

Table 1. Properties of some fluorescent probes used in fluorescence immunoassay (Hemmila, 1985)

Probe	λ abs/exc, nm	ϵ , L/mol	λ emis, nm	Quantum yield, %	τ , ns
Fluorescein (FITC, DTAF)	492	7×10^4	520	0.85	4.5
Rhodamines					
RBITC	550	1.2×10^4	585	0.70	3.0
TMRITC	550	5.0×10^4	580		
RB 200 SC	530, 565		595		1.0
Umbelliferones					
DANS	340	3.4×10^3	450	0.30	14.0
ANS	385		471	0.80	16.0
Fluorescamine	394	6.3×10^3	475	0.10	7
MDFP	390	6.4×10^3	480	0.10	
Pyrene deriv. ^a	340		375, 392		100
Lucifer Yellow VS	430		540		
Porphyrins	400-410		619-633		
Chlorophylls	430-453		648-669		
Phycobiliprotein	550-620	7.0×10^5	580-660	0.50-0.98	
Eu-(β -NTA) ₃	340	2.4×10^6	590, 613		500 000
Tb-EDTA-sulfosalicylic acid	300	3×10^4	490, 545		~150 000
Nd-benzoyltrifluoroacetone	800		900, 1060, 1350		

^a N-(3-Pyrene)-maleimide.

NTA, naphthyltrifluoroacetone; DTAF, dichlorotriazinylaminofluorescein; MDFP, 2-methoxy-2,4-diphenyl-3(2H)-furanone; DANS, dansyl chloride, ANS, anilino-naphthalenesulfonic acid; RBITC, rhodamine B isothiocyanate; TMRITC, tetramethylrhodamine isothiocyanate; RB 200 SC, lissamine rhodamine B sulfonate chloride.

serum and their quantum yield is more than 0.8. The practical application of this protein depends on the method for the coupling with the desired compounds in the assay reagents.

Fluorescein and rhodamine derivatives are the most frequently used probes in an immunoassay because of their high quantum yield. Many chemically reactive reagents such as isothiocyanate for both molecules are available and the labeling procedure onto antigen or antibody is well established (Blakeslee and Baines, 1976; Goding, 1976).

2.2.3. Solid-support

The solid support provides a surface on which antibodies are immobilized. It facilitates the separation of bound fraction from the unbound. The common shapes of solid support used in the fluorometric assays are beads or dipsticks. Other shapes of solid support, such as plastic tubes, polystyrene beads, and microtiter plates are not used because it is difficult to measure fluorescence from their surface. The common materials for beads includes polysaccharide bead (Ekeke *et al.*, 1979) and polyacrylamide bead (Kobayashi *et al.*, 1982), which have low fluorescence and scattering properties. The dipsticks with a polymethylmethacrylate or cellulose acetate-nitrate surfaces are used to facilitate the separation procedure (Tsay *et al.*, 1980, Stewens *et al.*, 1979). The measuring device for the coated surface of the dipstick is a fluorometer that was specially designed.

2.2.4. Conventional assay procedures

A conventional procedure of a two-site immunofluorometric assay includes two incubation steps and two washing steps. Each washing step consists of several times of separation and resuspension of the solid support.

For the first incubation, the solid-phase antibody is mixed with a sample which contains the antigen to be measured. During incubation the solid-phase antibody binds to an antigen and a bound form of antigen/antibody on the solid surface is made. After a certain incubation period, the solid support is washed several times to remove the non-specifically

bound antigen. Then, the fluorophore labeled antibody is added. During a second incubation period, the labeled antibody binds to the other sites of the antigen, and makes a complex of labeled-antibody/antigen/antibody on the solid support. The solid support is washed again to reduce the non-specifically bound labeled antibody, and then the fluorescence intensity from the solid support is determined. The measured fluorescence intensity is correlated to the antigen concentration in the sample.

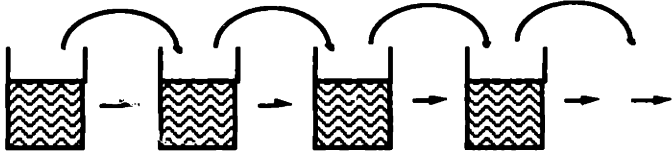
2.2.5 Simplification of the assay

The conventional two-site assay is a slow procedure because of the following reasons (Figure 3). A wide range of initial sample dilutions is required because the assay has a narrow and fixed range of measurement. The assay needs two incubation steps and two washing steps which demand sequential addition of reagents and separation. The incubation takes a long time to reach equilibrium state to ensure a constant output signal. There have been several attempts to reduce the number of assay steps and the time for performing this two-site immunometric assay. In this survey, we included literature regarding the two-site assay with other probes because the basic principle among these assays is the same.

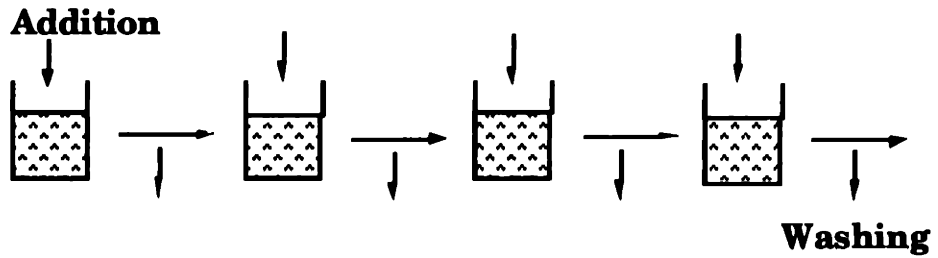
A washing step is avoided by the use of a reverse order of incubation for the assay. Reverse two-site immunoassay consists of two incubation steps and one washing step. The labeled antibody and the sample are mixed for incubation and then the solid-phase antibody is added to the reaction mixture. Piasio *et al.* (1980) demonstrated that the quantitative results were obtained by using polyclonal antibodies for hepatitis associate antigen (HAA) and carcinoembriogenic antigen (CEA). Another example of the procedural reduction is the simultaneous incubation assay. A washing step and an incubation step are eliminated by incubating the antibodies and the sample at a time.

In both reverse and simultaneous assays, the amount of the labeled polyclonal antibody should be maintained at a level which minimizes the formation of the soluble complex; the labeled antibody-antigen. This soluble complex reduces the sensitivity and reproducibility

1. Sample Dilution



2. Addition and Washing



3. Long Incubation

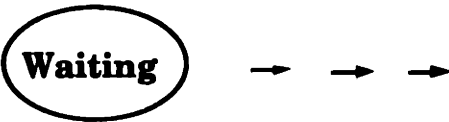


Figure 3. Schematic of the slow procedures of conventional two-site assay.

of the assay. The use of two monoclonal antibodies, which do not interfere with the binding of each other to the antigen, minimizes the formation of the soluble complex (David and Greene, 1983). The use of the antibodies provided accurate and reproducible results for determining proteins (Sevier *et al.*, 1981; Uotila *et al.* 1981; Tanaka *et al.* 1985; Nomura *et al.*, 1983).

The solid supports introduce a mass transfer limitation which slows down the reaction kinetics. Consequently significant time for incubation is normally required to ensure that the reaction goes as far as possible towards completion. In order to reduce the incubation time micron-sized beads were used as a solid support. Jolly *et al.* (1984) used 0.8 micro meter-sized polystyrene beads in a single-step immunofluorometric assay for determining IgG. They used 15 minutes incubation time and the solid phase was collected by filtration. After washing the bead cake, the fluorescence from the solid phase was measured by a front surface fluorometer. The performance between each assay varied with the concentrations of the secondary fluorophore-labeled antibodies as well as the primary antibodies and with the amount of beads used. A light scattering from the solid surface could be one of the reasons for the performance variation.

Because of the narrow dynamic range, samples have to be diluted when one wants to measure higher concentrations over the limited dynamic range. By increasing the amount of antibodies, researchers have tried to increase the dynamic measurement of the assay. In single-step incubation of two-site enzyme immunoassay, Gupta *et al.* (1985) tried to increase the dynamic range of measurement by increasing enzyme-labeled antibodies. In their assay for human chorionic gonadotropin (hCG) in a 96 well microtiter plate, the dynamic range of the assay was 1-31 ng hCG/mL when labeled antibodies were used at 1 μ g/mL. By increasing the labeled antibodies to 10 μ g/mL, the dynamic range of the assay increased to 1-500 ng hCG/mL. Further increase of the labeled antibodies did not affect the dynamic range (Figure 4).

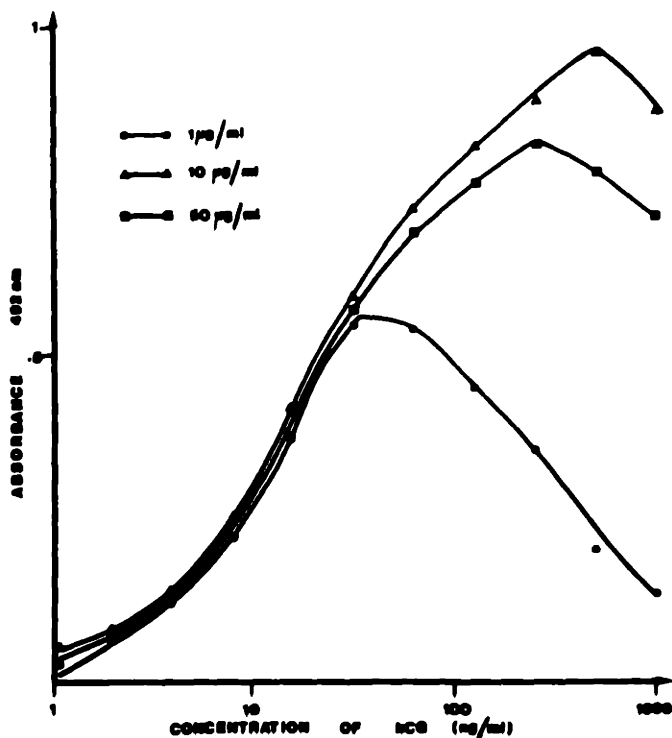


Fig. 4. Effect of monoclonal anti- α -hCG antibody-HRP conjugate concentrations on the dose-response curves of hCG in 'one-step' sandwich enzyme immunoassay. Antibody-enzyme conjugate was used at 3 different concentrations: 1 μ g/ml (●—●), 10 μ g/ml (Δ — Δ) and 50 μ g/ml (\blacksquare — \blacksquare). The background has been subtracted from the values observed.

Figure 4. Limited dynamic range of conventional two-site assay.
(Gupta *et al.*, 1985)

Shields and Turner (1986) observed that the quality of antibodies affects significantly the dynamic range and sensitivity of measurement in a two-site enzyme immunoassay. The affinity-purified antibodies improved 50-100 fold the dynamic range (2-500 ng/mL) of human IgG compared to the antibodies obtained by ion-exchange chromatography.

Expansion of the dynamic range of measurement needs the use of higher concentrations of the solid-phase and labeled antibodies. The use of higher concentration of solid-phase antibody demands a large surface area on which antibodies are immobilized. The porous bead can provide a large surface area but often restricts the binding reaction because of the internal and external mass transfer limitations. On the other hand, smaller nonporous bead such as micron-sized polystyrene bead (Latex) increases the external surface area. However, the small beads generate a quite significant level of light scattering while the fluorescence measurement. In addition because of their smallness in size, the separation and resuspension of the beads are quite laborious procedures.

2.2.6. Proposed single-step immunofluorometric assay

The novel format of the two-site immunofluorometric assay employs a solid-phase antibody and a fluorophore labeled antibody. The assay can be performed with a rapid speed, and with a minimum of operational steps - one incubation and one separation step.

The main concept of the assay development is on the detection of fluorescence signal from the liquid phase. As explained in Figure 5, the use of nonporous beads with a submicron diameter as a solid support minimizes the mass transfer resistance and provides a larger surface area for antibody immobilization. The low mass-transfer limitation and high antibody loading make the binding of antigen and antibodies reach equilibria quickly. This would generate a sufficient change in fluorescence of liquid phase.

In the assay procedure (Figure 6), the premixed antibodies having different binding sites on the antigen are employed to minimize the interference with one another while binding processes. The addition of the antigen yields a complex of the form, labeled-

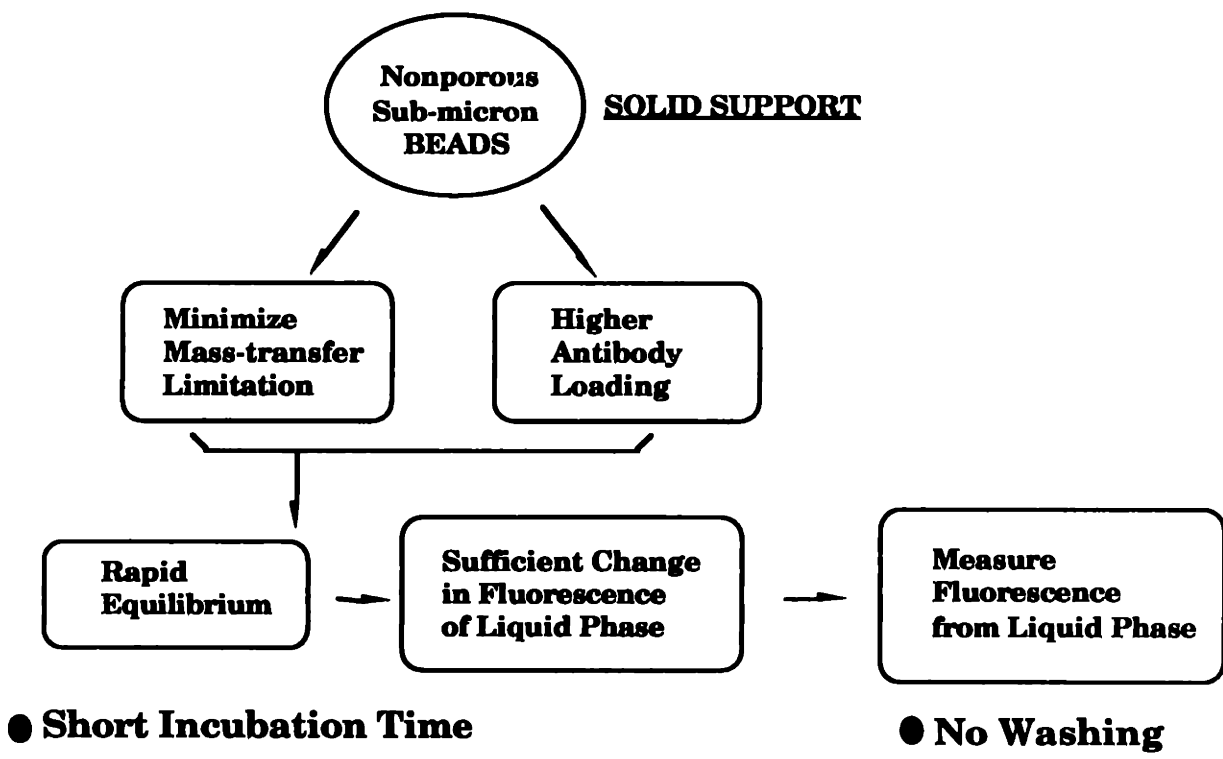


Figure 5. Schematic diagram of the immunofluorometric assay concepts.

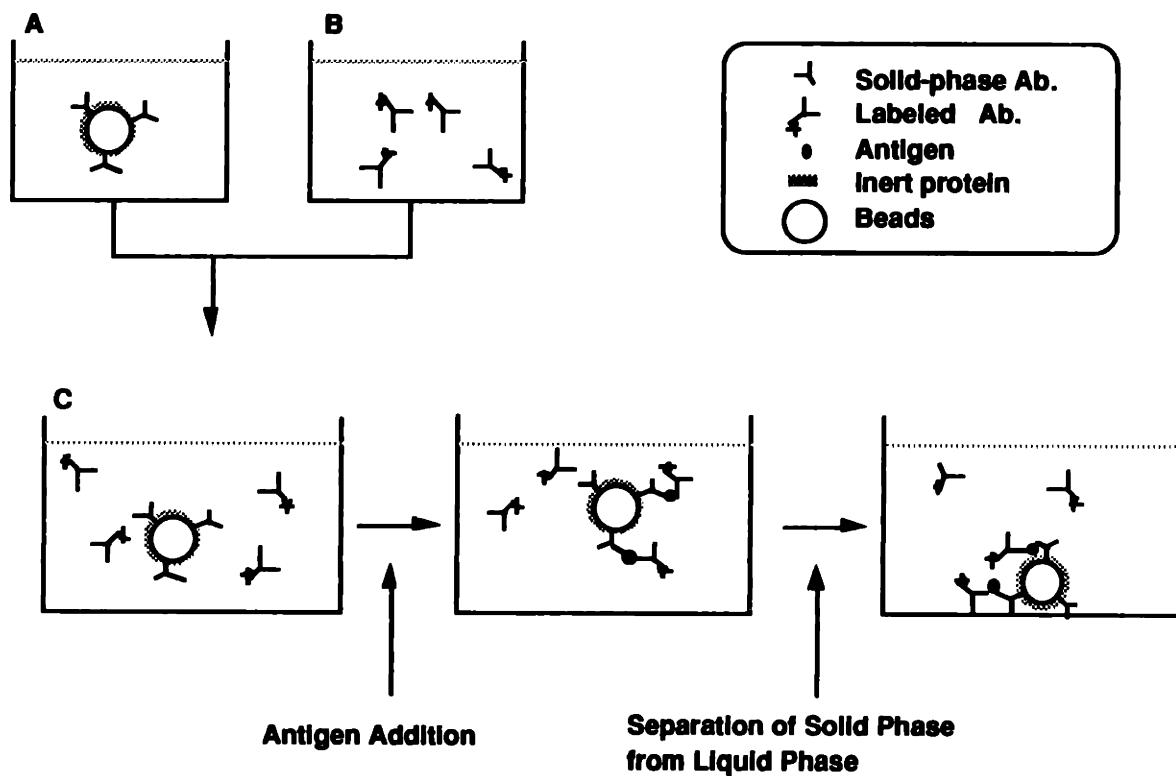


Figure 6. Schematic diagram of the two-site immunofluorometric assay. A: solid-phase antibody preparation B: labeled antibody preparation C: assay mixture. The assay mixture is prepared by mixing the solid-phase and labeled antibodies at varying concentration. An inert protein in the assay buffer was used to reduce nonspecific adsorption of proteins on solid surface. The assay is initiated by adding the sample into the prepared assay mixture. The assay steps only consist of one incubation and one separation before measuring fluorescence from the liquid phase.

antibody/antigen/antibody on the solid support. As a result of this complex formation, the liquid phase fluorescence will decrease proportionally as the antigen concentration in the sample increases. By measuring the residual liquid phase fluorescence, washing and resuspending the beads is eliminated and the interference from light scattered by the solid surface is also avoided. For operational considerations, this assay employs only one incubation step and one separation step prior to the fluorescence measurement. In addition, the use of different amounts of each antibody (solid-phase and labeled) makes it possible to determine an antigen concentration over a wide range. In this manner the initial sample preparation and dilution can be avoided.

The success of signal determination from the liquid phase in the assay is highly dependent upon carrying out the reaction to completion. If an excess amount of labeled antibody is used, the signal change due to antigen-mediated binding to the solid surface is a small fraction of the total unbound fluorophore signal. If the reaction does not go to completion, the ratio of signal to background will be even lower. In addition, to reduce interference in the signal from the liquid phase, purified antibodies should be used.

III. MATERIALS AND METHODS

3.1 Materials

3.1.1 Polyclonal antibodies and their antigens

To develop the proposed assay system, we employed affinity purified polyclonal antibodies and their antigens, human IgG and mouse IgG. For each antigen we selected two antibodies which have different binding sites on an antigen molecule. Such antibody preparations may not interfere with each other during the binding process.

For human IgG measurement, a rabbit antibody (stock no. 309-0508, lot 5166, Jackson ImmunoResearch West Grove, PA) specific to human IgG-Fc fragment was used as the solid-phase antibody, and a dichlorotrizinyl amino fluorescein (DTAF) conjugated rabbit antibody (stock no. 309-506, lot 2524, Jackson ImmunoResearch) specific to human IgG-F(ab')₂ fragment (fluorescein/protein: 7.4 µg/mg) was used as the labeled antibody. For mouse IgG measurement, a goat antibody (stock no. 115-1571, lot 5721, Jackson ImmunoResearch) specific to mouse IgG-Fc fragment was used as the solid-phase antibody, and a DTAF conjugated goat antibody (stock no. 115-1572, lot 5196, Jackson ImmunoResearch) specific to mouse IgG-F(ab')₂ fragment (fluorescein/protein: 15 µg/mg) was used as the labeled antibody. According to the report from the company, these antibodies had been isolated through immunoadsorption by the use of the antigen fragment. The fluorophore conjugated antibodies were prepared by labeling a fluorophore on the purified antibodies. The selected antibodies were used without being further purified.

As antigens, human IgG (stock no. 009-003, lot 6729, Jackson ImmunoResearch) and mouse IgG (stock no. 015-0003, lot 5520, Jackson ImmunoResearch) were used. As provided by the company, the immunoelectrophoresis for the human IgG and for the mouse IgG gives a single precipitin line.

3.1.2. Monoclonal anti-BSA and BSA

Three hybridoma cell lines used for the production of monoclonal anti-BSAs, denoted as 5.1, 6.1 and 9.1, were donated from New England Nuclear Products (Boston, MA); the cell lines were prepared by fusion of the myeloma line sp2/0 and spleen cell from AKR mice immunized with BSA. Figure 7 shows nonoverlapping determinants located on BSA to which the monoclonal anti-BSAs bind; the monoclonal antibody (MAb) 9.1 binds to an epitope located on BSA subdomain 3-C, MAb 6.1 to 1-N, and MAb 5.1 to subdomain 1-C (Morel *et al.*, 1988). The study by Morel *et al.* indicated that the three MAb preparations do not interfere with each other during the binding processes.

BSA (fraction V powder, 98-99 % albumin, remainder mostly globulins, fatty acid free, Sigma Company, St. Louis, MO) and sulfhydryl modified BSA (Pentex monomer standard, ICN, Elkhart, IN) were used as antigens. Monomeric fraction of sulfhydryl modified BSA was prepared by gel permeation chromatography as described in section 3.5.4.

3.1.3. Polystyrene beads

Monodispersed polystyrene beads (0.5 μm , 2.5 % bead suspension, Polysciences, Warrington, PA) were used as the solid support for the polyclonal antibody assay system. For the immobilization of monoclonal anti-BSA, monodispersed polystyrene beads (0.5 μm diameter, 10 % bead suspension, Seragen, Indianapolis, PA) or carboxylated polystyrene beads (0.5 μm , 0.05 milli equivalent/g of polymer, 10% bead suspension, Seragen) were used.

3.1.4. Other materials

Phosphate buffered saline (PBS) used in all experiments was prepared using 0.01M $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, 0.15M NaCl, pH 7.4. Phosphate buffered saline with azide (PBSA) is PBS containing 0.02% sodium azide. The dialysis membrane used for desalting

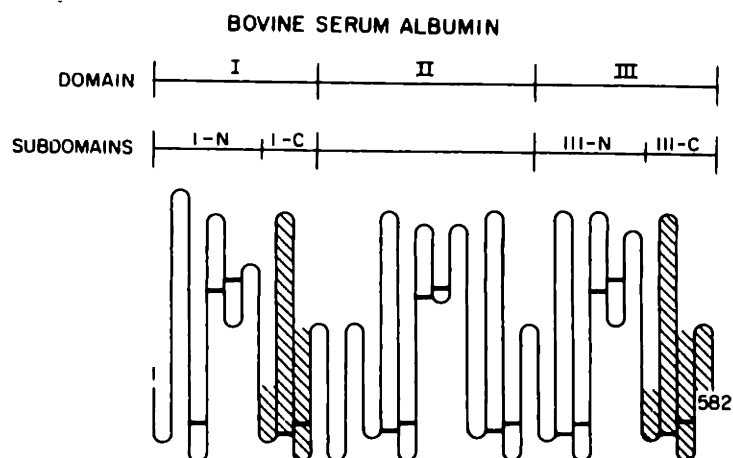


Figure 7. Diagrammatic representation of the structure of BSA. Antigenically active fragments are shown to illustrate the BSA domain overlap pattern (Morel *et al.*, 1988).

Table 2. Immunologic and physicochemical characterization of anti-BSA MAb¹

MAb	Binding specificity (domain)	Isotype	pI
5.1	D1-C	$\gamma 1, \kappa$	8.9–9.1
6.1	D1-N	$\gamma 1, \kappa$	7.6–7.7
9.1	D3-C	$\gamma 1, \kappa$	6.9–7.1

1. Morel *et al.* (1988)

had a molecular weight cut off at 12,000 (Spectra/por2, Spectrum, Los Angeles, CA.). Other chemicals were reagent grade, and water was of Milli Q grade (Millipore, Bedford, MA).

3.2. Methods for material preparation

3.2.1 Preparation of anti-BSA

3.2.1.1. Production of anti-BSA

MAB 5.1, 6.1 and 9.1 in ascites were produced by growing the hybridoma cells in the peritoneal cavities of athymic mice (Misra *et al.*, 1986). Sixty athymic mice (8 week-old, nu/nu, female) were used: 25 mice for MAb 9.1, 25 mice for MAb 5.1 and 10 mice for MAb 6.1. The mice were maintained at the animal facility of the Division of Comparative Medicine at M.I.T. by following the guidelines.

The medium for the growth of hybridomas was Dulbecco's modified Eagle's medium (DMEM), supplemented with 15% horse serum, 4 mM of L-glutamine, 800 units of penicillin and 0.8 mg/mL of streptomycin. Prior to inoculation of the hybridoma cells, the mice were primed for induction of ascites. Twice one week apart we injected 0.5 mL of pristane (2,6,10,14-tetramethylpentadecane) into peritoneal cavities of mice. One week after the second injection, each mouse was injected intraperitoneally with 0.5 mL of the cell suspension (10^6 cells/mL) that was prepared by resuspending the active growing hybridoma cells in DMEM without any supplements.

After the cell injection, the mice were checked every three days for ascites formation. One week after the injection, the mice started to accumulate ascites. The ascites fluids were collected by tapping the mice over the three week periods. The tapping was done by inserting a 1.5 inch 18 gauge hypodermic needle into the abdominal cavity and letting the fluids drip into a container. The collected ascites were centrifuged at 10,000 G, for 30 minutes to remove cells and debris, and then were stored at -70 °C. The ascites production of the each cell line was about 4 mL per mouse.

3.2.1.2. Purification by immunoadsorption

The monoclonal anti-BSA in ascites was isolated through affinity purification by using BSA-agarose gel as an immunoadsorbent. The immunoadsorbent was prepared by immobilizing BSA on cross-linked agarose beads (6%) derivatized with 1,1'-carbonyl-diimidazole (CDI) (Reacti-Gel 6X, Pierce Chemical Company, Rockford, IL)(Bethel *et al.*, 1979). We followed the manufacturer's suggested method. For the immobilization, we mixed the activated gel (10 mL or 60 mL) with the same gel volume of BSA (5g/L of 0.1M of carbonate buffer at pH 8.5) for 24 hours at 4 °C. The gel was washed with 1M NaCl, water, and PBS sequentially. The washed gel was then stored in PBSA at 4 °C.

MAb 9.1 and 5.1 were purified with a column (5 cm diameter, Pharmacia) in which 60 mL of the immunoadsorbents was packed. The frozen ascites was thawed at 4 °C and further dialyzed in PBSA for two to three days. The ascites was then filtered through a 0.22 µm filter (Millex, Millipore) to remove the coagulants in the ascites which formed during dialysis. The filtered ascites (2.5 mL) were pumped onto the column at a flow rate of 0.075 cm/min. PBSA was pumped onto the column to remove the proteins adsorbed non specifically, until the optical density of the effluent to the flow cell at 280 nm (1 cm of light path) was below 0.01. The bound antibodies were then eluted from the column by applying 60 mL of 0.1 M glycine-HCl at pH 2.5. Two milliliter fractions of the eluted sample were collected in 5 mL tubes containing 0.5 mL of 0.1M sodium carbonate to neutralize the pH of the eluted sample. After dialysis in PBSA overnight at 4 °C, the proteins solution was concentrated by membrane filtration with either Centricon YM-30 (Amicon, Danvers, MA) or CentriCell-20 (Polysciences, Warrington, PA). The antibodies at concentrations about 5 - 10 mg/mL were stored in a freezer at -70 °C. The antibody concentration in the stock solution was calculated by using the absorbance measurement at 280 nm and an extinction coefficient of 1.4 mL/(mg cm) for IgG.

After using the column for one antibody, a washing procedure was performed before using the column for the other antibody. The washing procedure consisted of pumping

60 mL 0.1N glycine-HCl at pH 2.5 into the column, and then reequilibrating the column with PBSA.

MAB 6.1 was purified on a column (2.5 cm diameter, BioRad) in which 10 mL of the immunoabsorbents. The purification procedure with 10 mL column was similar to the procedure described above. We used 0.5 mL of filtered ascites per each batch.

3.2.1.3. Preparation of monomeric form antibody

Monomeric forms from the affinity purified antibody was prepared using Suprose 6 gel permeation chromatography column (1 x 30 cm, Pharmacia) or prep-Suprose 6 column (1.6 x 50 cm, Pharmacia) with PBSA, a mobile phase. The fraction corresponding to monomer IgG was collected, and then stored at 4 °C. The homogeneity of the antibody preparation was checked by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) and by Native PAGE. The SDS-PAGE gel shows major staining bands of approximate molecular masses of 27KDa and 53 KDa corresponding to the light and heavy chains of immunoglobulin G. The native-PAGE gel shows a single band corresponding to IgG. The details for the gel electrophoresis are described in section 3.5.4.

3.2.2. Immobilization of antibodies on polystyrene beads

3.2.2.1. Physical adsorption

Physical adsorption of antibodies onto polystyrene beads were carried at room temperature (Pesce *et al.*, 1977). In all immobilizations, a ratio of 40 mg of antibody per mL of beads was maintained. This ratio corresponds to a monolayer of antibody on the 0.5 µm diameter bead surface (Cantarero *et al.*, 1980). The adsorption procedure used polystyrene beads (400 µL, 2.5% bead suspension) suspended in 1.0 mL plastic tubes and centrifuged at 8,000 G for 10 minutes, and 300µL of the supernatant was then replaced with 900µL of PBSA. After vortexing the tubes, the suspended beads were mixed with 400 µg of the antibodies, and incubated for 20 minutes. The beads were washed twice in PBSA buffer and

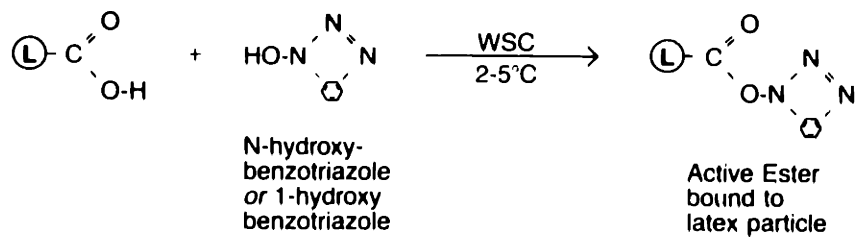
twice in the assay buffer, then further diluted to the proper concentration in the assay buffer. (The assay buffer for anti-IgG was PBSA containing 1.5% BSA, and the buffer for anti-BSA was PBSA containing 1.5 % ovalbumin).

The amount of antibody immobilized per volume of beads was determined indirectly: the difference between the initial total protein and the amount of protein recovered from the waste. The antibody concentrations in the wastes were determined by measuring optical density (O.D.) at 280 nm. Through immobilization, greater than 95% of the initial antibody remained adsorbed after washing twice in PBSA. Therefore, the concentration of immobilized antibody is presented to equal the initial concentration of antibody because less than 5 % was lost during adsorption. The bead concentration was presented as a volume percentage.

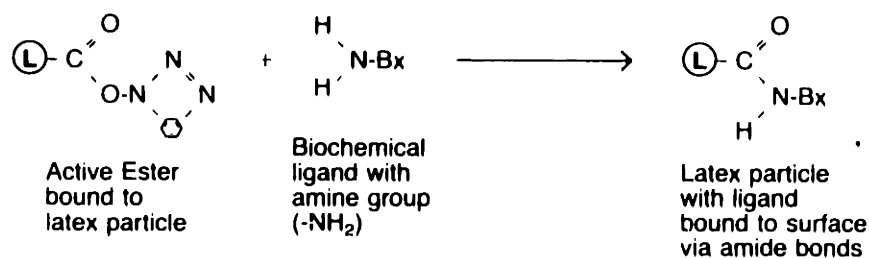
3.2.2.2. Covalent immobilization

MAB. 9.1, in monomeric form, was immobilized on carboxylated styrene beads with active intermediate ester. The active intermediate ester on carboxylated polystyrene beads was prepared by using triazole and carbodiimide (Dorman, 1977). Figure 8 shows the schematic of the immobilization procedure. A triazole solution was prepared by dissolving 24.1 mg of N-hydroxybenzotriazole in 0.4 mL of dimethylformamide, and diluting with water up to 1 mL. The carboxylated polystyrene beads (6.0 mL, 10% solid fraction, 0.05 meq of carboxylate group/g of polymer) and 0.52 mL of the triazole solution were mixed, and then cooled in an ice bath. The mixture of the beads and a carbodiimide solution (12.5 mg of 1-cyclohexyl 3-(2-morpholinoethyl)-carbodiimide metho-p-toluene-sulfonate dissolved in 0.25 mL of water) was incubated for four hours at 4 °C, and then the activated beads were washed four times with 0.1 M NaCl solution (10 mL). The activated beads were further dialyzed in 0.1 M NaCl at 4 °C overnight.

Immobilization of MAB 9.1 on the activated beads was done at 4 °C. The initial antibody concentration used for immobilization was 30 mg of IgG per mL of bead volume. As



Step 1: Formation of Active Ester



Step 2: Coupling Ligand to Active Ester

Figure 8. Immobilization procedure with active intermediate ester.
(from Dorman, 1977)

an example, 18 mg of antibody 9.1 in 6 mL of a phosphate buffer (0.05 M phosphate, 0.1 M NaCl, pH 8.0) was mixed with the activated beads (0.6 mL of bead volume), and then 0.15 mL of the triazole solution was added to the mixture. After five days, the antibody-beads were washed three times with PBSA, then stored at 4 °C.

Beads with different antibody concentrations on the surface were prepared by immobilizing a mixture of MAb 9.1 and mouse IgG (Jackson ImmunoResearch, West Grove, PA) on the beads; the antibodies were 0, 25, 50 and 100% of total protein. For immobilization, 30 mg of protein per mL of bead volume was maintained. The binding capacity (mean \pm standard deviation) for the 25 % antibody beads was $0.01 \pm 0.001 \mu\text{g}$ of ^{125}I -BSA/cm²; for the 50 % antibody beads, 0.019 ± 0.0015 ; for the 100% antibody beads, 0.04 ± 0.0025 , which were measured from duplicate experiments of the equilibrium binding technique (section 3.4.1).

The amount of antibody immobilized was determined indirectly from the difference in protein amount between the initial and the recovered from the wastes. The concentration of protein in the wastes was determined with a IgG standard by Lowry's protein assay (Lowry *et al.*, 1951). The wastes were analyzed after dialysis because of the interference of the triazole interference on the protein measurement. Through immobilization, greater than 99% of the initial protein were absorbed onto the beads.

3.2.3. Conjugation of MAb 5.1 with rhodamine

A fluorescent labeled MAb 5.1 was prepared by tagging the antibody with tetramethyl rhodamine isothiocyanate (TRITC, Molecular Probe, Eugene, OR) at room temperature (Goding, 1976). This procedure consisted of mixing 2.3 mL of monomeric MAb 5.1 (6 mg/mL of 0.3 M carbonate /bicarbonate buffer at pH 9.3) with 0.138 mL of 1 mg/mL of tetramethyl rhodamine isothiocyanate (TRITC) in dimethyl sulfoxide. After 2 hours of incubation, the labeled 5.1 antibody was separated by gel filtration on a Sephadex G-25 column (PD-10, Pharmacia, Piscataway, NJ) with PBSA as elution buffer. We followed the

manufacturer's suggested procedures for the use of the column. The concentration of the labeled MAb 5.1 in the stock solution was determined by Lowry assay with a standard of monomeric MAb 5.1. Greater than 95% of the initial MAb 5.1 amount was in the labeled preparation. The amount of labels on the protein was estimated by measuring the absorbance at 545 nm and 280 nm. The ratio of the optical absorbance of the labeled antibody at 545 nm to at 280 nm was determined to be 0.25. Because of the lack of standard of rhodamine, however, we can not calculate the number of TRITC per molecule of IgG. The purity of the labeled 5.1 antibody was determined by gel filtration with Suprose 6 column (1.0 x 30 cm, Pharmacia) and native PAGE. Following the gel filtration, we observed a single peak at the retention volume of IgG, and the gel electrophoresis of the labeled antibody showed a single band.

3.2.4. Preparation of monomeric BSA and ^{125}I -BSA.

3.2.4.1. Monomeric fraction of sulfhydryl modified BSA.

The monomeric fraction of sulfhydryl modified BSA (ICN) was prepared by gel permeation chromatography on a prep-Suprose 6 column (1.6 x 50 cm, Pharmacia, Piscataway, NJ) to remove the oligomers. The fraction corresponding to monomer BSA was collected, and then stored at 4 °C. The homogeneity of the monomeric fraction of BSA was checked by gel permeation chromatography on a Suprose 6 column (1.0 x 30 cm, Pharmacia) and by native polyacrylamide gel electrophoresis (Native-PAGE) as described in the section of analytical procedure. A single peak on the gel chromatogram was observed at the retention volume corresponding to monomer BSA. In addition, only a single band on the gel was observed.

3.2.4.2. Iodination on sulfhydryl-modified BSA

The monomeric fraction of sulfhydryl-modified BSA prepared by gel filtration was iodinated using the iodine chloride (ICl) exchange procedure at room temperature

(McFarlene, 1958). The reason for the selection of this iodination technique is that it avoids the use of oxidizing agents which may damage the protein. The details of this method can be found in an article by Contreras *et al.* (1983). We performed the iodination procedure in a glove box at a laboratory of the Radiation Protection Office at M.I.T.

The labeling solution for iodination was prepared by mixing 0.7 mL of the ICl₃ (3.5×10^{-4} M) with 0.1 mL of a 10 mCi/mL of NaI¹²⁵ (New England Nuclear, NEZ 033), and subsequently by adding 0.1 mL of glycine buffer (0.2 M, pH 8.5) and 0.8 mL of 0.1 NaOH to the mixture. We adjusted the pH of the labeling solution to about 8.5 by adding a few drops of 0.1 N NaOH while checking the pH with a pH indicator paper. The labelling solution was added drop by drop to 0.7 mL of BSA solution (10 mg/mL in glycine buffer, 0.2 M, pH 8.5) with mixing. The reaction mixture was then dialyzed against borate buffer (0.2 M, 0.2 NaCl, pH 8.0) containing 0.02 M KI. The iodinated protein solution was further dialyzed against the borate buffer, saline solution (0.15 M NaCl) and PBSA, sequentially. The iodinated BSA was stored at 4 °C, and its concentration in the stock solution was calculated by using the measured absorbance at 280 nm and an extinction coefficient of 0.6 mL/(mg.cm) for sulfhydryl modified BSA.

The fraction of the radioactivity on BSA was determined by measuring the precipitate that was formed by the addition of trichloro acetic acid. Briefly, 500 µL of the iodinated BSA (10 µg/mL in a PBSA with 1.5 % OVA) in 1.5 mL tubes was mixed with 20 % (w/v) trichloro acetic acid. After 10 minute incubation, the tubes were centrifuged at 10,000 G for 10 minutes, and the supernatant (700 µL) and the remainder were counted in a gamma counter. Greater than 99 % of the radioactivity of the iodinated BSA was in the precipitate.

3.2.4.3. Determination of reactive fraction of ¹²⁵I-BSA

The percentage of ¹²⁵I-BSA that binds specifically to the immobilized Mab 9.1 was determined by the methods of Lindmo and Bunn, Jr. (1986) in which equilibrium data are extrapolated to infinite antibody excess in order to estimate the fraction of reactive antigen.

^{125}I -BSA (1 $\mu\text{g}/\text{mL}$) was incubated with the MAb 9.1- beads (30 μg of MAb 9.1 /mL of bead volume) at varying concentrations from 0.05 % to 0.8 % of bead. After a two hour incubation, the reaction mixture (250 μL) tubes were centrifuged and 200 μL of supernatant were removed. Supernatant and sediment fractions were counted in a gamma counter, and the amount of the labeled BSA bound onto the beads was calculated.

When only a fraction (r) of antigen is immunoreactive, the concentration of reactive antigen is $r[T]$, where $[T]$ is the concentration of total antigen. The equilibrium concentration of bound antibody-antigen complex $[B]$ can be expressed as

$$[B] = K_a [Ab] (r[T] - [B]) \quad (1)$$

where K_a is the association constant and $[Ab]$ is the unbound antibody and $(r[T] - [B])$ represents the unbound reactive antigen.

A transformation of the above equation results in a linear relationship of $[T]/[B]$ as a function of $1/[Ab]$:

$$[T]/[B] = 1/r + 1/r K_a [Ab] \quad (2)$$

The data ($[T]/[B]$) obtained from the equilibrium experiments in duplicate were plotted as a function of $1/[Ab]$. The intercept on the Y axis represented the reciprocal value of active fraction of the labeled antigen.

3.2.5. Dispersion of beads by sonication

After the immobilization of antibody on the carboxylated polystyrene beads, we observed with a microscope the beads aggregated to larger sizes (5 - 10 μm). These aggregated beads were dispersed to yield a monomeric suspension by sonication. Five milliliters of beads in PBSA with 1.5% ovalbumin were placed in a metal tube in a ice-water

bath, and the bead suspension was sonicated for 15 seconds at one minute intervals. The sonication step was repeated three or four times. The sonicator (Model W-350, Heat system-Ultrasonics, Farmingdale, NY) was operated at the power level three with 50% continuation of the power. The binding capacity and equilibrium constant of the sonicated beads were the same as those of the non-sonicated beads. This observation indicates that after sonication the most of antibodies on the beads remained intact.

3.3. Immunofluorometric assay

3.3.1. Assay buffer, solid-phase antibody and labeled antibody

The buffer for the assay for human IgG and mouse IgG was PBSA with 1.5 % of bovine serum albumin (BSA). The buffer for the assay for BSA was PBSA with 1.5% ovalbumin (OVA, Sigma Company). BSA or OVA was included in buffer to reduce the non-specific adsorption of proteins on the solid support. The lists of the solid-phase antibody and labeled antibody are in Table 3. For the polyclonal antibody assay system, the antibodies specific to IgG-Fc were used as the solid-phase antibodies; the antibodies specific to IgG-F(ab')₂ labeled with DTAF was used as labeled antibody. For the monoclonal antibody assay system, MAb 9.1 and 6.1 were used as the solid-phase antibodies; MAb 5.1 used as the labeled antibody after being tagged with rhodamine.

3.3.2. Standard procedures of assay

All assays for human IgG, mouse IgG and BSA were carried out at 25 °C. In the assay procedure, the concentrations of antibodies and antigen reported were based on the final volume after addition of all three components. Figure 9 shows the schematics of assay procedures. The reaction volume of assay was 500 μL and some of assay's volume was 250 μL. For each antigen, the solid-phase and labeled antibodies were diluted to the proper concentrations in the assay buffer. The assay mixture was prepared by mixing the solid-phase

Table 3. Lists of the components for the two-site immunofluorometric assay systems.

A. Polyclonal antibody assay system:

	human IgG	mouse IgG
Solid-phase antibody ¹	Anti-human IgG-Fc	Anti-mouse IgG-Fc
Labeled antibody	DTAF-anti-human IgG-F(ab') ₂	DTAF-anti-mouse IgG-F(ab') ₂
Assay buffer	PBSA with 1.5% BSA	PBSA with 1.5% BSA
Incubation time	30 minutes	30 minutes
Analyte	human IgG	mouse IgG

1. The solid -phase antibodies were prepared by immobilizing the antibodies on the polystyrene beads (0.5 μm) by physical adsorption.

B. Monoclonal antibody assay system for BSA

Solid-phase antibody ²	MAb 9.1 MAb 6.1
Labeled antibody	TRITC labeled MAb 5.
Assay buffer	PBSA with 1.5% ovalbumin
Incubation time	30 minutes
Analyte	monomeric BSA

2. The solid-phase antibody for MAb 9.1 was prepared by physical adsorption and covalent immobilization of the antibody on the solid supports. The solid-phase antibody for MAb 6.1 was prepared by physical adsorption.

ASSAY PROCEDURE

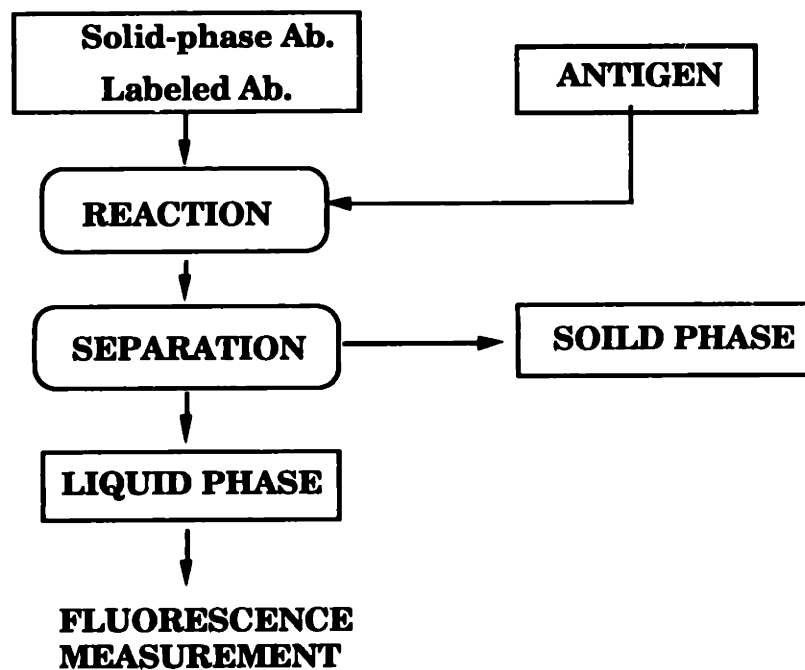


Figure 9. Two-site immunofluorometric assay procedure. The assay mixture is prepared by mixing the solid-phase antibody and labeled antibodies at various concentrations. The antibodies and antigen were prepared in the assay buffers.

antibodies (100 μL) and labeled antibodies (200 μL) together in a 1.0 mL plastic tube. The assay was started by adding 200 μL of the antigen at various concentration to the reaction mixture, or 200 μL of assay buffer for the control. After a 30-minute incubation without mixing, the liquid fraction of the reaction mixture was separated from the solid phase by centrifugation at 8,000 G for 10 minutes, and the fluorescence intensity from the liquid phase was determined. The fluorescence intensity of DTAF was measured in a fluorometer with a 200 μL cuvette. The intensity of DTAF was measured at wavelength of 494 nm excitation and 520 nm emission; the intensity for rhodamine, at wavelength of 545 nm for excitation and 570 nm for emission.

3.3.3. Dose response curve

A standard curve for the assay was developed; the curve describes the relationship between the added antigen concentration and the fluorescence signal. Here, the fluorescence signal is defined as the following equation:

$$\text{Fluorescence Signal} = \frac{F_c - F_1}{F_0} C_L \quad (3)$$

where F_c is the fluorescence intensity of the control, F_1 the fluorescence intensity of the sample, and F_0 is the fluorescence intensity of the labeled antibody, C_L is the concentration of the labeled antibody used at each set of assay. $F_c - F_1$ is the changes in fluorescence intensity caused by the binding of the labeled antibody to the complex of antigen/solid-phase antibody specifically. Thus, the fluorescence signal represents the concentration of the labeled antibody that specifically bound to the complex. A dynamic range of measurement was defined as a range of antigen concentration over which the fluorescence signal changes proportionally with antigen concentration.

3.3.4. Assay using freeze-dried reaction mixture

To permit advance reaction mixture preparation and storage for a long period of time, we prepared the antibodies in a freeze-dried form. The reaction mixture of the solid-phase antibody (20 μ g/mL, 0.05% of bead concentration) was prepared by physical adsorption and the labeled antibody (40 μ g/mL) against mouse IgG was frozen in liquid nitrogen and then freeze-dried. In conducting the assay, the prepared freeze-dried reaction mixture was dissolved by adding the antigen solution, and the fluorescence signal was determined.

3.3.5. Incubation time

The change of fluorescence intensity at varying incubation time was measured to determine the standard incubation time for the assay. For the polyclonal antibody assay, the solid-phase antibody prepared by physical adsorption (40 μ g/mL, 0.1% of bead concentration) was mixed with the DTAF-labeled antibody (10 μ g/mL or 40 μ g/mL), and then incubated with 2.4 μ g/mL of human IgG. At fixed incubation times, the liquid phase was separated from the solid phase by centrifugation for 10 minutes, and then the fluorescence from the liquid phase was then measured. For the monoclonal antibody assay, sulfhydryl modified BSA (4.8 μ g/mL) was added to the mixture of the solid-phase MAb 9.1 (80 μ g/mL, 0.2 % of bead concentration, prepared by physical adsorption) and 10 μ g/mL of the labeled antibody (TRITC-Mab 5.1). Because of the rapid binding reaction the separation was performed by filtration with a 0.22 μ m filter which took less than three seconds.

3.4. Kinetic study

For the study of intrinsic binding kinetics of immobilized antibody and BSA, we used MAb 9.1 after being immobilized on the carboxylated polystyrene beads. All binding reactions were performed in duplicate at 25 °C, unless otherwise stated. The immobilized MAb 9.1 and BSA were diluted to proper concentrations in a buffer (PBSA with 1.5 % OVA) which

was filtered with a 0.22 μm filter. The concentrations of antibody, bead, and antigen reported were based on the final volume of the reaction mixture. The surface protein concentration for the immobilized antibody is 0.25 μg of protein/ cm^2 which represents that 75 % of the bead surface was adsorbed with protein as a monolayer. We assumed that the antibody binding sites have the same binding characteristics to active, uniform ^{125}I -BSA. For the calculation of the kinetics constants, we used molecular weights of 68,000 for ^{125}I -BSA and 160,000 for MAb 9.1.

3.4.1. Equilibrium binding

To determine the binding capacity of the antibody-beads and the equilibrium constant of MAb 9.1 immobilized, we incubated the antibody-beads ranging from 0.0032 to 0.2 % and ^{125}I -BSA at varying concentration for 2 hours. After separating the liquid from the reaction mixture (300 μL) by centrifuging at 8,000 G for 10 minutes, we counted the radioactivity from both the supernatant (200 μL) and the sedimented fraction in a gamma counter. The concentration of immobilized BSA in the beads which was free (not bound to MAb) was calculated from this data. And the ratio of bound ^{125}I -BSA to the unbound was calculated.

The data obtained from the experiments in duplicate were fit to

$$\frac{B}{U} = K (Q_{\text{max}} - B) \quad (4)$$

where B is a bound ^{125}I -BSA in $\mu\text{g}/\text{mL}$, U is an unbound ^{125}I -BSA in $\mu\text{g}/\text{mL}$, K is an equilibrium association constant in $\text{mL}/\mu\text{g}$ of ^{125}I -BSA, and Q_{max} is the antibody binding capacity in μg of ^{125}I -BSA / mL (Scatchard,1949). The best fit of the data to Equation (4) was determined by finding the values of binding capacity (Q_{max}) and equilibrium constant (K) which gave the minimum sum of squared residuals from an iterative numerical technique. The

nonspecific binding of ^{125}I -BSA on the solid support was determined from the control experiment as follows: the mouse IgG-beads (0.1 % bead suspension) were incubated with ^{125}I -BSA (0.25 - 8 $\mu\text{g}/\text{mL}$) for two hours, and the bound ^{125}I -BSA was determined as described above. From this experiment, the nonspecific binding was found to be minimal.

3.4.2. Association reaction

The association reaction was performed by incubating the antibody-beads (400 μL) with ^{125}I -BSA (400 μL) in 1.0 or 2.0 mL tubes for various fixed times. After separating the liquid from the reaction mixture by filtration with a 0.22 μm filter, we counted the radioactivity of the filtrate (400 μL) in a gamma counter. The bound fraction of ^{125}I -BSA was calculated from the total added amount and the amount remaining in the liquid.

The association rate constant (k_1) was calculated from the data for the first 40 seconds of incubation. Using Equation (5) the association rate constant (k_1) is shown to be a function of the unbound antigen (C_B). Equation (5) was derived from the second order association rate in combination with a mass balance on the antigen, as described in section 4.3.

$$k_1 = \frac{-V}{t_k} \frac{1}{(SA_0 - VC_{B0})} \text{Ln} \left[\frac{(C_B/C_{B0})}{\{1-(VC_{B0}/SA_0)(1- C_B/C_{B0})\}} \right] \quad (5)$$

where antibody at the solid surface is A_0 , the initial antigen concentration is C_{B0} , the bead surface area per unit volume is S/V .

The initial association rate was calculated using the data during the early incubation period. The procedures were; (1) the average association rate at time (t) was calculated from the equation of $(C_{B0} - C_B)/t$, where the C_B is the antigen concentration in the bulk solution at time (t). (2) the average rate was then multiplied by $2C_{B0}/(C_{B0} + C_B)$ which represents the ratio of the initial antigen concentration to the average during the incubation.

3.4.3. Dissociation reaction

To study the dissociation kinetics, we first prepared the complex of ^{125}I -BSA/MAb 9.1 by incubating the antibody-beads with ^{125}I -BSA for one hour. After adding 1000 fold excess of BSA to the reaction mixture, at fixed times, we sampled the reaction mixture (1 mL) with 1 mL syringe, and the liquid from the mixture was separated by using a 0.22 μm filter. The radioactivity from the liquid (500 μL) was counted in a gamma counter. The volume change by the addition of non-labeled BSA was accounted for in the calculation.

For the calculation of the dissociation rate constant, as the total bound ^{125}I -BSA, we considered the amount of the ^{125}I -BSA desorbed within 30 minutes. The natural logarithm of the fraction of the bound ^{125}I -BSA to the total bound was plotted as a function of time, and from the slope during the early dissociation period the dissociation rate constant (k_2) was thus calculated.

3.5. Analytical methods

3.5.1. Lowry assay

Lowry assay (Lowry *et al.*, 1951) for protein measurements was performed as follows. The color development reagent was prepared by mixing 50 ml 2% Na_2CO_3 in 0.1N NaOH with 0.5 mL 0.1% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 0.5 mL 2% Na-K-Tartrate. After 2 mL of the color development reagent were added to 0.4 ml of the sample with mixing, the reaction mixture was incubated for 15 minutes at room temperature. After the incubation, 0.2 mL of 1 N Folin & Ciocalteu's phenol reagent (Sigma) was added into each tubes with mixing, and further incubated for 40 minutes. An optical density of the developed color was measured at 540 nm and the protein concentration was determined from the standard curve.

3.5.2. Polystyrene bead concentration

The concentration of dispersed beads was determined by using the absorbance read at 500 nm wave length with a standard, the same carboxylated beads.

3.5.3. Gel permeation chromatography

All analytical gel permeation chromatography were carried by use of a Fast Protein Liquid Chromatography (FPLC) apparatus (Pharmacia). The chromatogram was perform on a Suprose 6 column (1.0 cm diameter and 30 cm length, Pharmacia) with a mobile phase, PBSA, at a flow rate of 0.1 mL/minute.

3.5.4. Gel electrophoresis

All gel electrophoresis was perform by the use of Phast system (Pharmacia). For the SDS PAGE, the sample (0.1 - 0.5 $\mu\text{g}/\text{mL}$ of PBS containing 2.5 % SDS and 5 % β -mercaptoethanol) was boiled for 10 minutes, and then loaded onto an 10 - 15 % of polyacrylamide gel (Pharmacia). Protein was visualized by silver staining by following the manufacture's suggested procedure (Heukeshoven and Dernick, 1985). The size of protein was estimated by comparison with molecular standard from Pharmacia. For the native PAGE the sample (0.1 - 0.5 $\mu\text{g}/\text{mL}$) was loaded on the gel (10-15 %), and then the gel was visualized by the silver staining.

IV. RESULTS AND DISCUSSION

4.1. Polyclonal antibody assay system

As described in section 2.2.6, we proposed a simplified format of a two-site immunofluorometric assay whose procedure includes only one incubation and one separation before measuring fluorescence from the liquid phase. Like a conventional two-site assay, the novel format also employs a solid-phase antibody and a labeled antibody, but does not require any sample dilution or preparation. This is because the dynamic range of measurement is increased by the use of a higher concentration of antibodies. In addition, the use of submicron non-porous beads as a solid support can reduce incubation times by decreasing the mass transfer limitations (Stenberg *et al.*, 1986; Li, 1985). It is expected, therefore, one can perform the assay rapidly and with minimal efforts. To test the feasibility of this concept, we developed an assay employing polyclonal antibodies and the results are reported in this section.

For a model assay, human IgG and mouse IgG were selected as analytes. They were selected because of the ease of handling, stability and commercial availability. For each IgG, we have chosen one antibody that is specific to IgG-Fc fragment and one fluorophore labeled antibody that is specific to IgG-F(ab')₂ fragment. By choosing antibodies having different binding sites on an IgG, we minimized the binding interference with each other. A solid-phase antibody was prepared through immobilizing an antibody on 0.5 μm diameter polystyrene beads through physical adsorption. For the labeled antibody, we used the labeled DTAF antibodies without any further purification. Solutions of antibodies and antigen were prepared in PBSA with 1.5% OVA. The concentrations of antibodies and antigen are based on the final volume after addition of all three components, or unless otherwise stated. Further details of the components of the assay and its procedures were described in the Materials and Methods section.

To test the feasibility of the polyclonal antibody assay system the following four areas were considered: (1) the standard incubation time for assay, (2) the expansion in dynamic

ranges of measurement, (3) the effects of the environmental factors such as pH, ionic strength, protein serum concentration and serum, and (4) the use of freeze-dried antibodies.

4.1.1. Incubation Time

To determine a standard incubation time we measured changes in fluorescence intensity from the liquid phase at various lengths of incubation. In the first experiment, we added 2.4 $\mu\text{g/mL}$ of human IgG to a mixture of solid-phase anti-human IgG-Fc (40 $\mu\text{g/mL}$, 0.1% beads, prepared by physical adsorption) and DTAF labeled anti-human IgG-F(ab')₂ (10 $\mu\text{g/mL}$). In the second experiment, we added 2.4 $\mu\text{g/mL}$ of human IgG to a mixture of 40 $\mu\text{g/mL}$ of each of the antibodies. For both experiments, the binding reaction was carried out in 1.5 mL plastic tubes with a 500 μL working volume. The liquid phase was separated from the solid phase by centrifugation at 8,000 G for 10 minutes at time intervals ranging from 1 to 120 minutes, and then the fluorescence from the liquid was measured in a fluorometer. The measured fluorescence intensity was divided by the specific fluorescence activity for labeled antibody to calculate the concentrations of unbound labeled antibody. As a control for nonspecific adsorption of labeled antibody, we measured the extent of binding of DTAF labeled antibody alone on the solid support. For this experiment, we added PBSA with 1.5 % OVA to the antibody mixtures (solid-phase and labeled) instead of the human IgG preparation. After incubating for 120 minutes the fluorescence intensity from the liquid was measured.

In Figure 10, fluorescence intensity shown as the concentration of the unbound labeled antibody is plotted as a function of incubation time. With both concentrations (10 and 40 $\mu\text{g/mL}$) of labeled antibody, the fluorescence intensity decreased rapidly after the reactions started, and reached constant values in less than 20 minutes, and remained constant up to 120 minutes of incubation. The change in fluorescence intensity results from the formation of a complex of labeled antibody/antigen/solid-phase antibody on the solid supports. The control experiment for both labeled antibody concentrations showed that the fluorescence intensity after a 120-minute incubation is the same as that at the initial. This result indicates that the non-

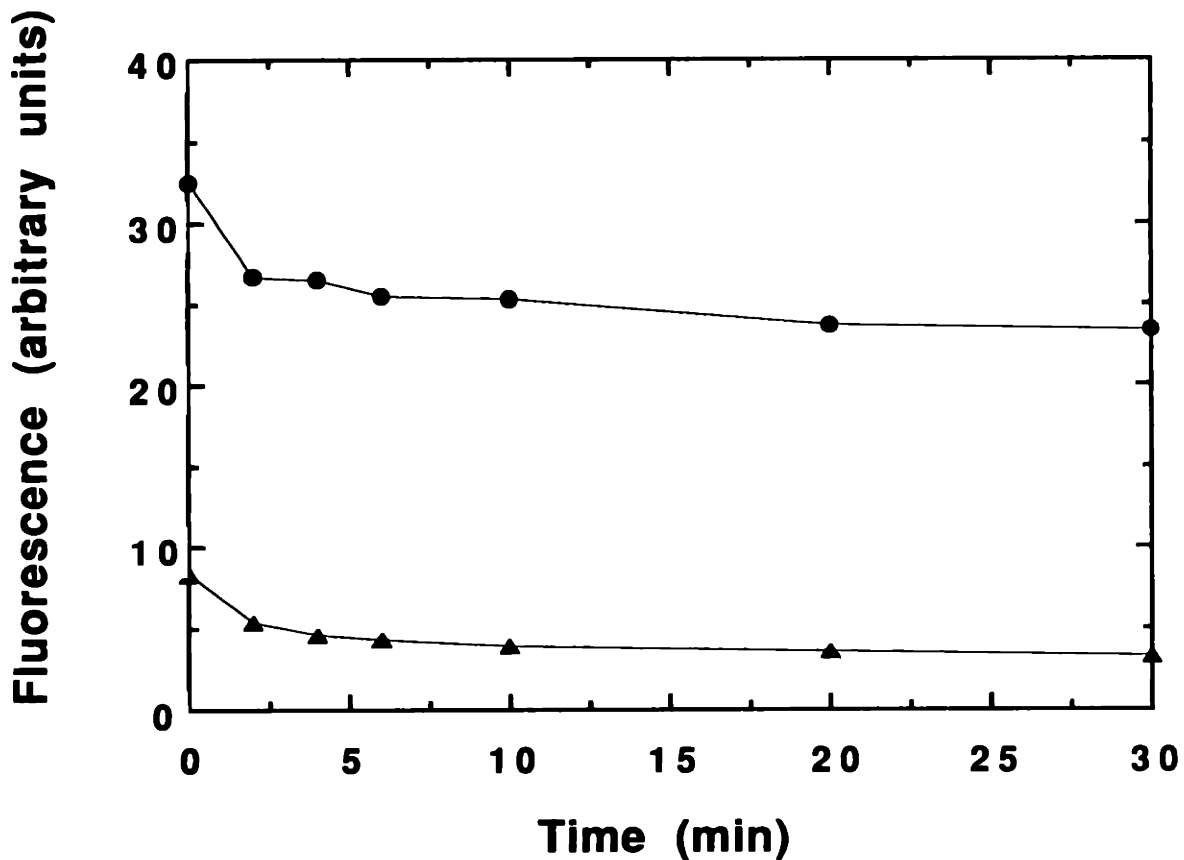


Figure 10. Changes of fluorescence intensity at varying incubation time for the polyclonal antibody assay system. The data represent the results of duplicate experiments by using two combinations of the solid-phase antibody and labeled antibody ($\mu\text{g/mL}$, $\mu\text{g/mL}$; 40, 10 \blacktriangle ; 40, 40 \bullet) with 2.4 $\mu\text{g/mL}$ of human IgG. The error bars (standard deviations) smaller than the size of symbols are not shown. The data for incubation time up to 30 minutes are shown. Less than 1 % nonspecific adsorption of the labeled antibody was noted for the each assay. The lines were drawn to enhance the view.

specific adsorption of the labeled antibody on the solid support is minimal. From the observation above, we chose 30 minutes as a standard incubation time for the assay.

4.1.2. Assay performance and dynamic ranges

4.1.2.1 Human IgG measurement

For the assay for human IgG, we employed a solid-phase anti-human IgG-Fc (prepared by physical adsorption) and DTAF labeled anti-human IgG-F(ab)₂ with a 30-minute incubation. Fluorescence signal (bound labeled antibody concentration) was calculated from the measured fluorescence intensity from the liquid and the intensity of the control using Equation 1. To obtain a dose-response curve, the fluorescence signal was plotted as a function of human IgG concentration added.

Typical results of the assay for human IgG with 10 µg/mL of each antibody (solid-phase and labeled) are shown in Figure 11. The dose-response curve shows a distinguished increase in fluorescence signal at 0.2 µg/mL of human IgG compared to that in the absence of antigen. This behavior limits this assay to 0.2 µg/mL or less of human IgG. The fluorescence signal increased with increasing IgG concentration in the range of 0.2 - 3.2 µg/mL, which represents the dynamic range of measurement. After reaching a value of 3.2 µg/mL, fluorescence signal starts to decrease as the antigen is increased; at 6.0 µg/mL of human IgG, the fluorescence signal was lower than that at 3.2 µg/mL of human IgG. This inflection (other researchers call it as 'hook effect') indicates that only a fraction of the antigen/labeled antibody in the solution was bound to the solid-phase antibody. The binding sites of the solid-phase and labeled antibody are being saturated by the antigen at the higher concentrations so that less complex (labeled antibody/antigen/solid-phase antibody) forms. The inflection in the assay's dose-response curve can lead to false assay results because a measured signal can yield two different concentrations.

To eliminate the inflection, we performed the assay with an increased concentration of the solid-phase antibody (40 µg/mL) while maintaining the labeled antibody at

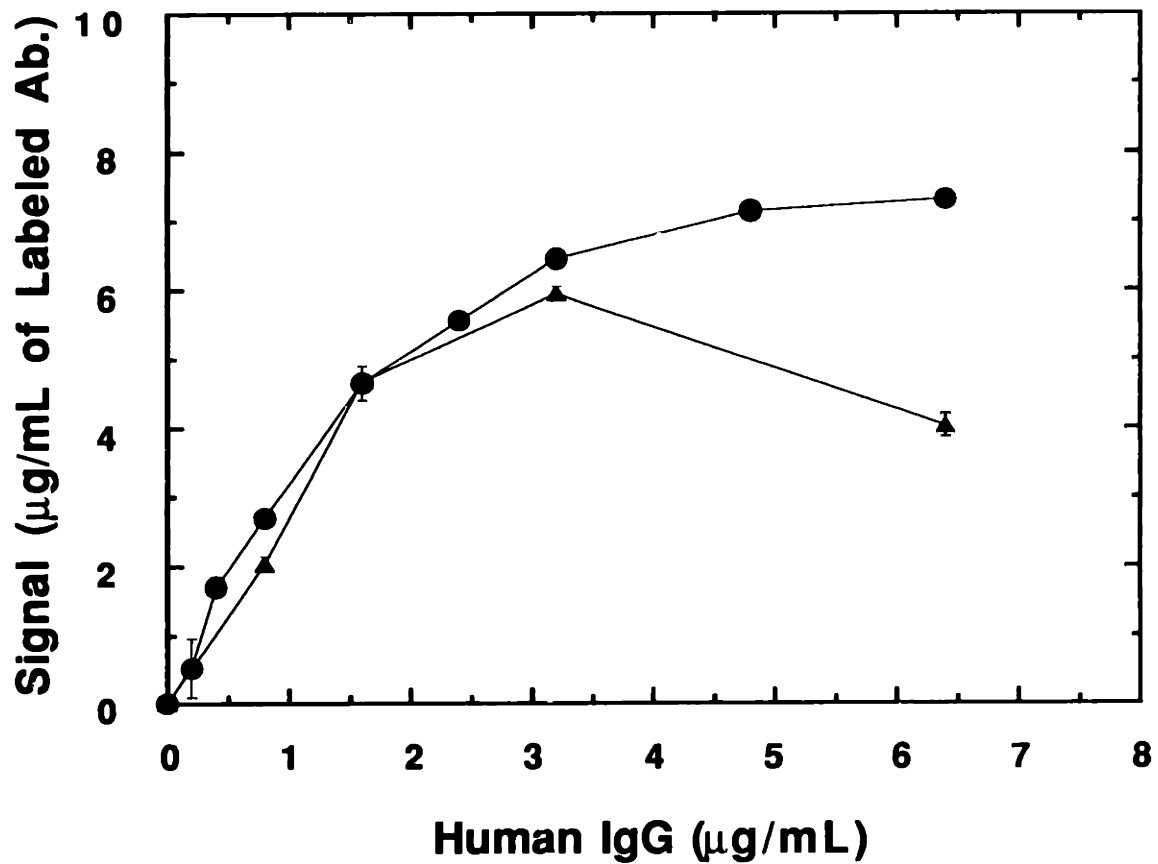


Figure 11. Standard curve for the polyclonal antibody assay for human IgG. The assays were performed with 10 µg/mL of the labeled antibody and either 10 µg/mL (▲) or 40 µg/mL of solid-phase antibody (●). The fluorescence signal is plotted as a function of antigen concentration added. The data represent the results of duplicate experiments and error bars are standard deviations. Error bars smaller than the size of symbols are not shown. The lines were drawn to enhance the view.

10 $\mu\text{g/mL}$. The dose-response curve of the assay shows no sharp inflection up to 6.4 $\mu\text{g/mL}$ of human IgG (Figure 11). Although the solid-phase antibody concentration was increased four times, the dynamic range of the measurement did not increase at all. This is probably because the labeled antibody was exhausted at 3.2 $\mu\text{g/mL}$ of human IgG, and thus using higher concentration of IgG does not increase the fluorescence signal.

Use of higher concentration of both antibodies increases the dynamic range of measurement. Figure 12 shows that the assay with 40 $\mu\text{g/mL}$ of each antibody gives a dynamic range of 0.2 - 6.4 $\mu\text{g/mL}$ of human IgG, which is about twice of the dynamic range for the assays in Fig. 4.2. Increasing the solid-phase antibody concentration to 80 $\mu\text{g/mL}$ and the labeled-antibody concentration to 160 $\mu\text{g/mL}$ further expanded the dynamic range up to 40 $\mu\text{g/mL}$ of human IgG (Figure 12). These results indicate the dynamic ranges can be expanded by the use of higher concentrations of the antibodies.

4.1.2.2. Mouse IgG measurement

To study whether the assay concept can be applied to measure other IgG molecules, we developed an assay for mouse IgG. The assay consists of a solid-phase anti-mouse IgG-Fc (immobilized on the solid support), DTAF-labeled anti-mouse IgG-F(ab)₂, and assay buffer of PBSA with 1.5% of OVA. These reagents were developed in the same manner for the human IgG assay.

A similar assay performance for mouse IgG was observed as in the assay for human IgG. Typical results of the assay for mouse IgG using 20 $\mu\text{g/mL}$ of solid-phase antibody and 40 $\mu\text{g/mL}$ of DTAF-labeled antibody are presented in Figure 13. The dose-response curve shows a dynamic range of 0.2 - 6.4 $\mu\text{g/mL}$ (Figure 13). Increasing the concentration of the antibodies to 40 $\mu\text{g/mL}$ of the solid-phase antibody and 160 $\mu\text{g/mL}$ of labeled antibody expanded the dynamic range further up to 40 $\mu\text{g/mL}$ of mouse IgG (Figure 13).

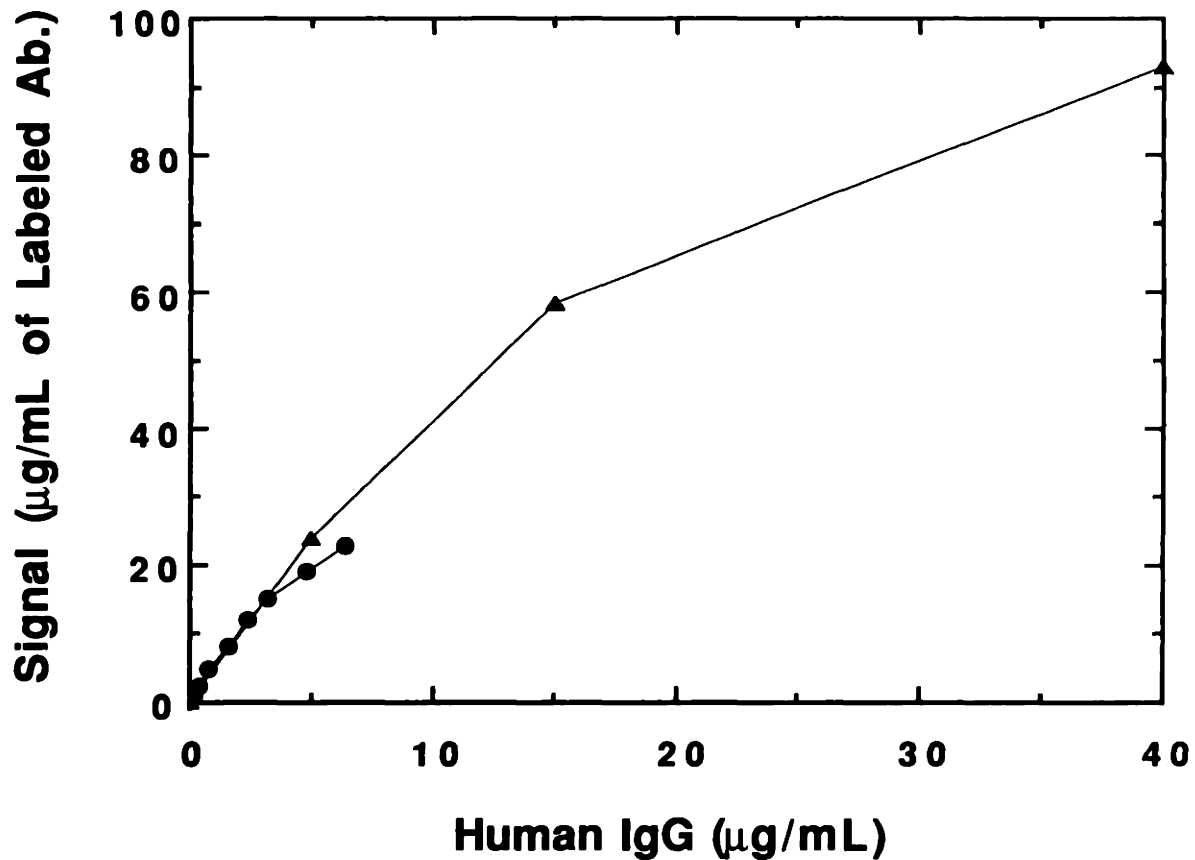


Figure 12. Effects of antibody concentration on dose-response curve. The concentrations of solid-phase antibody and labeled antibody were; (40 µg/mL, 40 µg/mL ● ; 80 µg/mL, 160 µg/mL ▲). The data are presented as described in Figure 11.

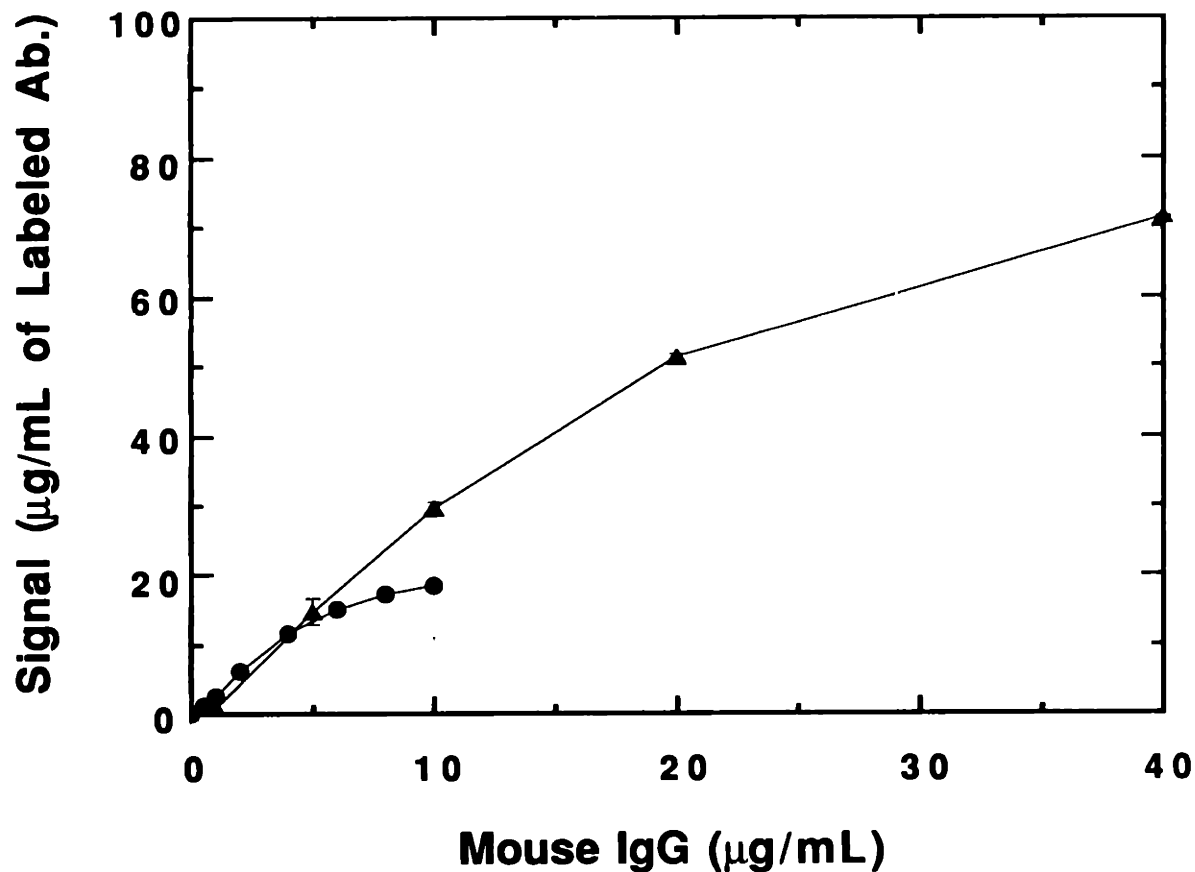


Figure 13. Standard curve for the polyclonal antibody assay for mouse IgG. The concentrations of solid-phase antibody and labeled antibody were; (20 µg/mL, 40 µg/mL ● ; 20 µg/mL, 160 µg/mL ▲). The data are presented as described in Figure 11.

4.1.3. Effects of ionic strength, pH and protein concentration

When an assay is used for measurement with an undiluted sample, other components in the sample may change the fluorescence intensity, and this could lead to false results. To investigate these effects, the assay was carried out at various conditions of ionic strength, pH, and protein concentration.

To test the effect of ionic strength in the assay, phosphate buffer and sodium chloride at various concentrations were examined. The solid-phase and labeled antibodies to human IgG in four different buffer concentrations were prepared, ranging from 10 mM phosphate buffer (150 mM NaCl) to 40 mM phosphate buffer (600 mM NaCl). All buffers contain 1.5 % OVA. Forty $\mu\text{g/mL}$ of each of the solid-phase and the labeled antibodies were incubated with 2.4 $\mu\text{g/mL}$ of human IgG for 30 min and the fluorescence from the liquid phase was measured. Table 4. shows the results where the intensities of the fluorescence at all conditions are in the range of 76 - 79% of the control without antigen.

To determine the effects of the pH on the assay performance, the binding of solid-phase antibody (40 $\mu\text{g/mL}$) and labeled antibody (40 $\mu\text{g/mL}$) to human IgG was studied over a pH range of 6-9. After 30 minutes of incubation of the antibody mixture with human IgG (2.4 $\mu\text{g/mL}$), the fluorescence intensity from the liquid was measured. Figure 14 shows the observed fluorescence intensity was sensitive to the pH of the solution between 6 and 8. However, it is known that fluorescein is sensitive to pH changes; thus, we calculated the relative ratio of the measured fluorescence intensity of the sample to the fluorescence intensity of fluorescein at each pH (Steinbach and Mayersbach,1976). The relative ratio at each pH was the same, and we therefore conclude that the fluorescence change was the result of fluorescein sensitivity to pH and not due to the assay *per se*.

The effects of BSA and serum on assay performance were done by measuring the interference of these components on fluorescence intensity from labeled antibody. BSA did not interfere with the fluorescence measurement up to a concentration of 8%. Calf serum (100 %) alone will yield a fluorescence signal of 4.4 units. Under typical assay conditions (40

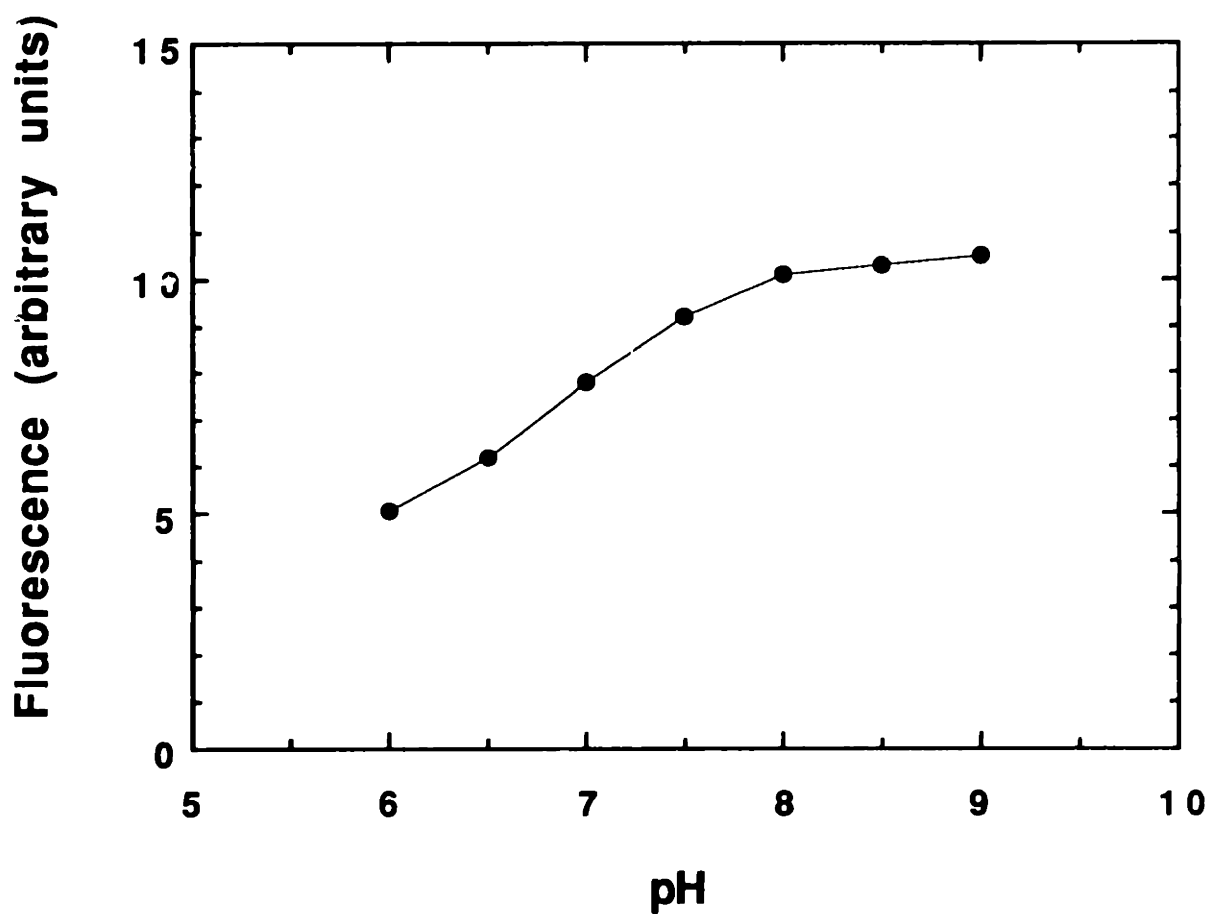


Figure 14. Effects of the pH on the assay performance. The solid-phase antibody (40 $\mu\text{g/mL}$), labeled antibody (40 $\mu\text{g/mL}$), and human IgG (2.4 $\mu\text{g/mL}$) were prepared in an assay buffer over a pH range of 6 - 9. After a 30-minute incubation, the fluorescence intensity from the liquid was measured.

Table 4. Effects of ionic strength on assay¹

Phosphate, NaCl (mM) (mM)	Fluorescence	Relative Intensity (%)
10, 150	23.7	78
20, 300	23.2	76
30, 450	23.5	77
40, 600	24.1	79
Control (without Antigen)		
10, 150	30.4	

1. The solid-phase and labeled antibodies to human IgG in four different buffer concentrations were prepared. All buffers contain 1.5 % OVA. Forty $\mu\text{g/mL}$ of each of the solid-phase and the labeled antibodies were incubated with 2.4 $\mu\text{g/mL}$ of human IgG for 30 min and the fluorescence from the liquid phase was measured. The relative intensity is the ratio of the measured fluorescence to the control.

$\mu\text{g/mL}$ of labeled antibody to human IgG), the fluorescence reading in the absence of antigen was 33 units. Therefore, we can conclude that the noise introduced by background serum fluorescence is minimal.

4.1.4. Assay with freeze-dried reaction mixture

To permit the reaction mixture to be prepared in advance and to allow for long term storage, we prepared a freeze-dried form of antibody mixture; the antibody preparation was DTAF labeled anti-mouse IgG-F(ab')₂ (40 $\mu\text{g/mL}$) and anti-mouse-IgG-Fc (20 $\mu\text{g/mL}$) physically adsorbed to the beads. The assay performance with the freeze dried mixture was compared with non freeze-dried samples. Figure 15 shows that the sensitivity at low concentration of antigen in the freeze-dried mixture was lower than the non freeze-dried materials. However, both systems provided a similar dynamic range for the measurement. Nonspecific adsorption of labeled antibody for both assay systems was at the same level.

4.1.5. Discussion

We have shown a simplified two-site immunofluorometric assay where the operational steps include only one incubation and one separation step. This simplified assay does not require any washing and resuspending of the solid support. The ability to expand dynamic range and its insensitivity to sample condition avoids the need for sample dilution or preparation. Use of submicron size non-porous beads reduces the required incubation time substantially.

The reaction time of the solid-phase and labeled antibodies with the antigen is the major time contributor in the overall assay. The preparation of the mixture of antibodies requires multiple steps and contributes to the time required. When rapid assay results are needed, however, time can be reduced by preparing the mixture in advance. The separation of the solid requires a mechanical procedure such as centrifugation or filtration. Such procedures

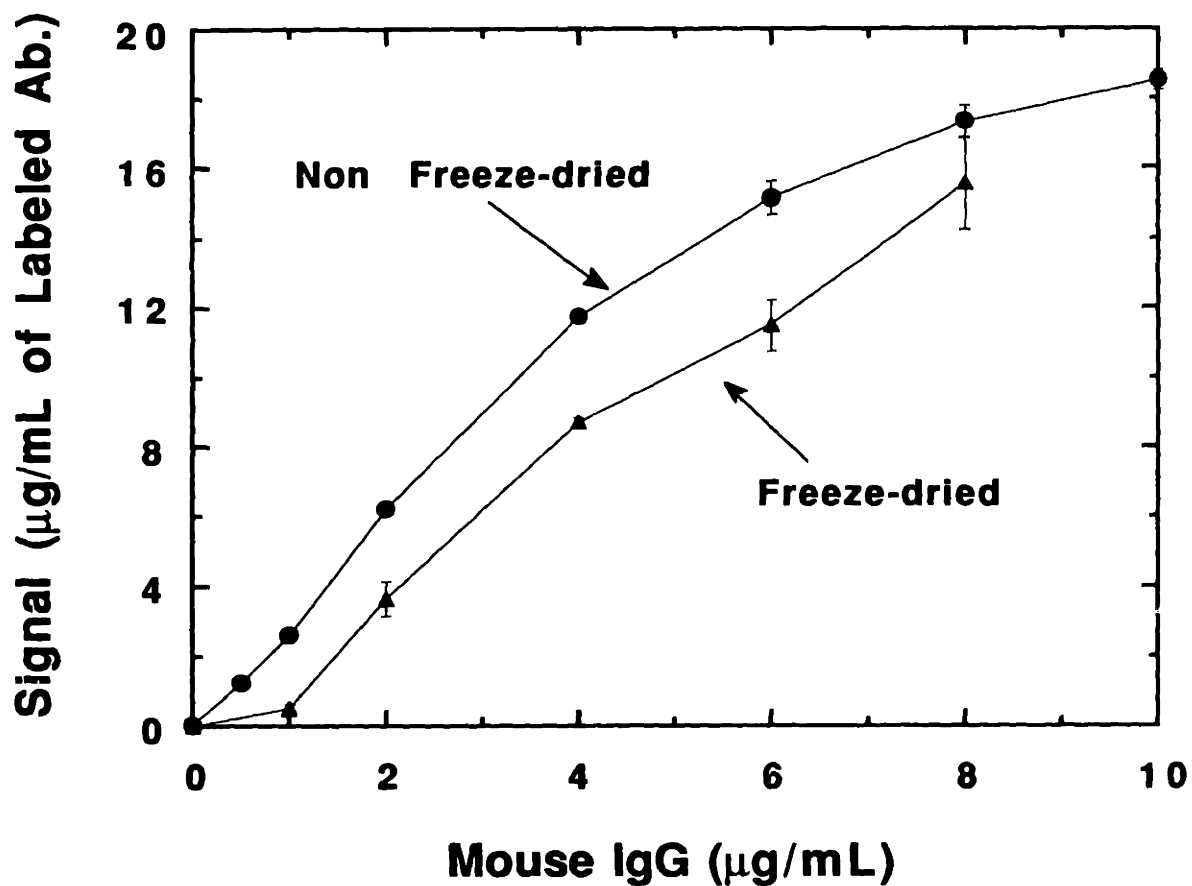


Figure 15. Comparison of assay performance: assay with the freeze dried mixture vs. with non freeze-dried. The antibody preparations were the labeled anti-mouse IgG ($40 \mu\text{g/mL}$) and the solid-phase anti-mouse IgG ($20 \mu\text{g/mL}$) physically adsorbed to the beads; freeze-dried (\blacktriangle) and nonfreeze-dried (\bullet). Less than 1 % nonspecific adsorption of the labeled antibody was noted for the both assays. The lines were drawn to enhance the view.

can be done rather quickly. For example, filtration of reaction mixture with a 0.22 μm filter takes only several seconds which is much less as compared to the incubation time.

The standard time for the incubation was 30 minutes, which is equivalent to one-tenth of the incubation time for the conventional two-site assay in a microtiter plate (Li, 1985), and is comparable to that for an assay employing 0.8 μm polystyrene beads (Jolly *et al.*, 1984). This reduction of incubation time was possible because the small-sized nonporous beads offer very low mass-transfer resistance and large surface area per unit volume. The surface area of beads in the assay mixture was 120 cm^2/mL (0.1 % bead concentration) or 240 cm^2/mL (0.2 % bead concentration). This is much greater than the surface area of a well (6 mm diameter, 200 μL assay volume) in a microtiter plate; 15 cm^2/mL . The large surface area allows us to use more antibodies per measurement; the high concentration of the solid-phase antibody enhances the binding reaction rate.

The required incubation time for the polyclonal antibody assay was 20 minutes. This is probably the result of multiple binding reactions between the polyclonal antibodies and the antigen. The polyclonal antibodies are a mixture of antibodies having various binding specificities and affinities. The overall binding reaction consists of multiple reactions that differ in equilibria, and competitive reactions in which antibodies interfere with each other. Thus the multiple, competitive binding will require a long time to reach equilibrium. In this study we did not attempt to determine the controlling step in the overall binding reaction rate because of the complexity of the polyclonal assay system.

The detection range of the assays was 0 - 40 $\mu\text{g}/\text{mL}$ of human IgG or 0 - 40 $\mu\text{g}/\text{mL}$ of mouse IgG, and further expansion in range by using higher concentrations of antibodies is expected. This range is much greater than other two-site assays; ranges of 0 - 500 ng/mL of human chorionic gonadotropin (hCG, a molecular weight of 45,000) (Gupta *et al.*, 1985), and 0 - 3 $\mu\text{g}/\text{mL}$ of mouse IgG (Jolly *et al.*, 1984) were reported in the literature. Such a wide detection range can eliminate the necessity of dilution that is often required when measuring a high antigen concentration with the other assays.

Preliminary estimates of number of labeled antibody bound to the solid-support per IgG addition indicated that over the dynamic range, several molecules of labeled antibody can bind to a single molecule of IgG. For example, for human IgG at 10 $\mu\text{g/mL}$ with both antibodies as shown in Figure 11, the calculated number of the labeled antibodies per human IgG is 2.5. This magnitude could be increased by using increased concentrations of the labeled-antibody. In using 40 or 160 $\mu\text{g/mL}$ of the labeled antibody, there was five labeled-antibody per antigen molecules, which is twice the value at 10 $\mu\text{g/mL}$.

The dynamic ranges expand as the antibody concentration is increased, but the expansion is not a linear function of the concentration. This is because polyclonal antibodies can bind at multiple sites of a single antigen. The ratio of the bound labeled antibody to antigen depends on the antibody concentration.

With a given assay mixture, one can measure a higher concentration of antigen by using a smaller sample volume. During incubation, the binding reaction of the antibodies with the antigen reaches equilibrium, and the final concentration of the antigen determines the magnitude of the signal of the assay. Since the sample was diluted with the reaction mixture by the factor of the ratio of the total assay volume to the sample volume, to measure a high concentration of the antigen, therefore, one can use a small sample volume which leads to high dilution with the reaction mixture.

It is generally recognized that the fluorescence intensity can be altered by the sample conditions. From our experimental results, the ionic strength and the presence of other proteins did not interfere with fluorescence measurement from the liquid. The pH, however, did indeed change the fluorescence intensity due to the fluorophore. This interference, however, can be minimized by employing a fluorophore which is not affected in pH changes. Alternatively, one can use a solution that has a strong buffering capacity over the pH range encountered in the assay.

With the freeze-dried reaction mixture, the sensitivity for the low concentration of antigen is less than in the soluble form. A possible reason for this is that the solid-phase

antibody detaches from the beads during freeze-drying and enters in liquid reaction mixture. The detached antibody in the liquid can bind the antigen/labeled antibody complex so that a reduced amount of the labeled antibody could bind to the solid support. In addition, various levels of the detached solid-phase antibody may give scattered results at one concentration of antigen. This detaching of the antibody can be overcome by covalent immobilization to the solid phase.

4.2 Monoclonal antibody assay system

To understand the performance of the novel format for the two-site assay, we developed an assay with monoclonal antibodies to BSA. As a model assay system, two sets of monoclonal antibodies have been chosen: one is MAb 9.1 and 5.1; the other is MAb 6.1 and 5.1. The antibodies in the each set have different binding sites on a BSA molecule, so that they do not interfere with each other during the binding processes. For the solid-phase antibodies, MAb 9.1 and 6.1 were used; the antibodies were immobilized on 0.5 μm of polystyrene beads by physical adsorption. For some assays, covalently immobilized MAb 9.1 on carboxylate polystyrene (0.5 μm diameter) was used instead of physical adsorption. This is because of possible long-term problems with detachment of the antibody which might interfere with quantitative studies. For labeled antibody, MAb 5.1 was used after being tagged with TRITC, as described in the Materials and Methods section. All solutions for the antibodies and BSA were prepared in PBSA with 1.5% OVA. The reported concentrations of antibodies and antigen are based on the final volume after addition of all three components. A detailed description of the assay can be found in the Materials and Methods section.

4.2.1. Nonspecific adsorption of labeled antibody on beads.

To determine the extent of nonspecific adsorption of labeled antibody on solid support, we incubated TRITC-labeled MAb 5.1 (10 $\mu\text{g}/\text{mL}$) with the polystyrene beads (0.2 % volume fraction of total reaction mixture) on which either MAb 9.1 or MAb 6.1 was immobilized by physical adsorption. After a 30-minute incubation, we separated the liquid phase by centrifugation at 8,000 G for 10 minutes, and then measured fluorescence intensity from the liquid. Table 5 shows the results for both antibodies from the triplicate experiments; the fraction of nonspecific adsorption for MAb 9.1 is 5.2% of the total; for MAb 6.1, 3.6 %.

Table 5. Nonspecific adsorption of TRITC labeled MAb 5.1 on solid support¹.

Sample #	MAb 9.1		MAb 6.1	
	Incubation	Control	Incubation	Control
1	18.5	19.4	18.6	19.3
2	18.4	19.4	18.6	19.3
3	18.2	19.3	18.4	19.1
Average Value	18.367	19.367	18.533	19.233
Nonspecific Adsorption (%)		(5.2%)		(3.6%)

1. TRITC labeled MAb 5.1 (10 µg/mL) was incubated with the polystyrene beads (0.2 % volume fraction of total reaction mixture) on which either MAb 9.1 or MAb 6.1 was immobilized by physical adsorption.

4.2.2. Incubation Time

The change in fluorescence intensity at various incubation times was measured in order to determine a standard incubation time. Two different experiments were performed. For the first experiment, BSA (4.8 $\mu\text{g/mL}$) was added to the mixture of the solid-phase antibody (MAb 9.1) prepared by physical adsorption (80 $\mu\text{g/mL}$, 0.2 % of solid concentration) and TRITC labeled antibody (MAb 5.1, 10 $\mu\text{g/mL}$). At fixed times ranging from 0.33 to 90 minutes, the liquid phase was separated by filtration with a 0.22 μm filter. This filtration required less than three seconds. The liquid phase fluorescence was then measured in a fluorometer. For the second experiment, the BSA was incubated with TRITC labeled MAb 5.1 for 30 minutes and then the solid-phase antibody (MAb 9.1) was added. The fluorescence intensity from the liquid was determined by the same manner above. Figure 16 shows the results for the both experiments; within 2 minutes the fluorescence intensity reached a constant value in both cases. Although the change of fluorescence was much faster than for the polyclonal antibody assay, we chose 30 minutes as a standard incubation time.

4.2.3. Assay performance with solid-phase MAb 9.1 and 6.1

To demonstrate the feasibility of the assay with monoclonal antibodies, we carried out a standard assay with TRITC-labeled MAb 5.1 (10 $\mu\text{g/mL}$) and two different kinds of solid-phase antibody (80 $\mu\text{g/mL}$), MAb 9.1 and MAb 6.1, prepared by physical adsorption. For the control, the labeled MAb 5.1 and the solid-phase antibody were incubated without adding BSA to determine nonspecific adsorption of labeled antibody. For the dose-response of assay, concentration of specifically bound labeled antibody was plotted as a function of the concentration of BSA added.

A typical assay performance with 80 $\mu\text{g/mL}$ of the solid-phase MAb 9.1 and 10 $\mu\text{g/mL}$ of the labeled MAb 5.1 is shown in Figure 17. The fluorescence signal increased linearly with added sulfhydryl modified BSA in the range of 0 - 6.4 $\mu\text{g/mL}$, remaining constant

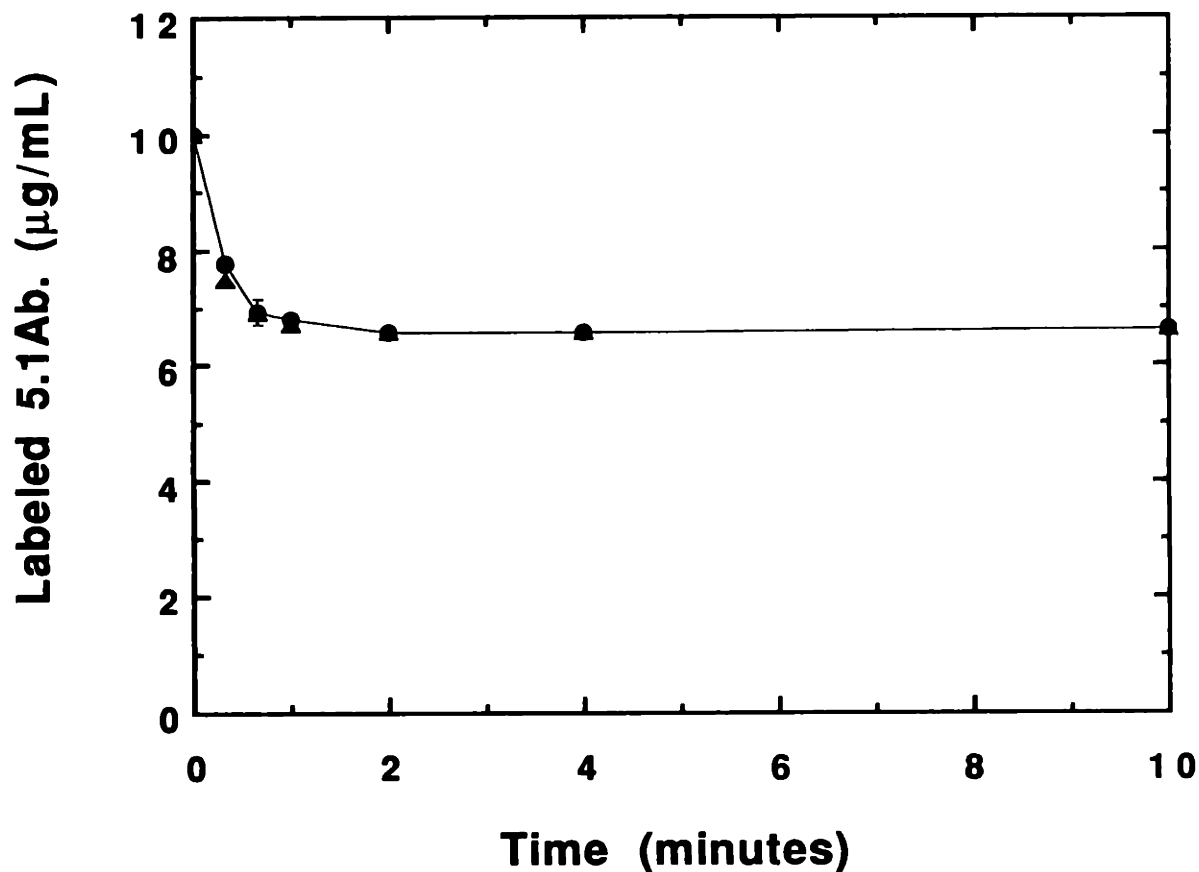


Figure 16. Changes of fluorescence intensity at varying incubation time for monoclonal antibody assay. The solid-phase MAb 9.1 prepared by physical adsorption (80 µg/mL, 0.2 % of solid concentration) was added to the antigen (4.8 µg/mL) which was previously incubated with the TRITC labeled antibody (10 µg/mL) for 30 minutes (▲). The same antigen was added to the mixture of the antibodies at same concentrations (●). The data represent the results of duplicate experiments and error bars are standard deviations. The error bars smaller than the size of symbols are not shown. The lines were drawn to enhance the view.

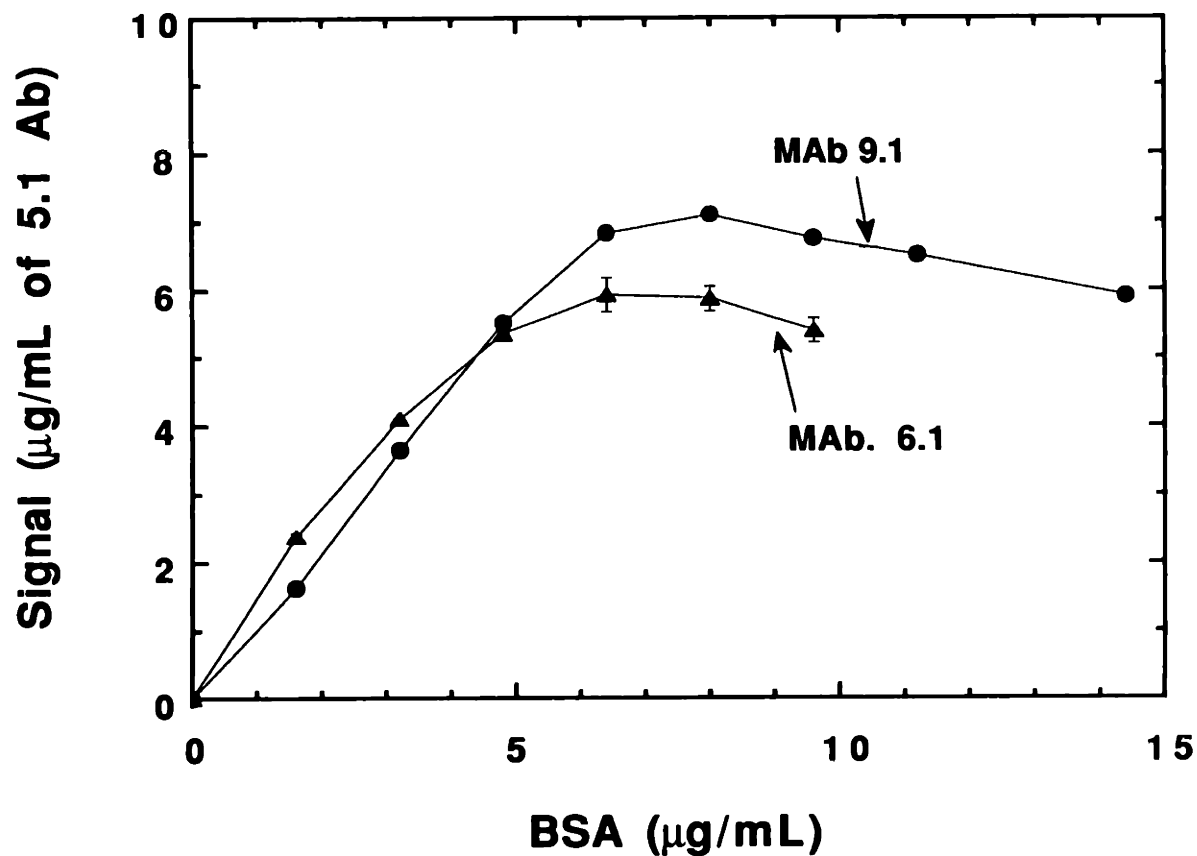


Figure 17. Standard curve for the monoclonal antibody assay. The dose-response relations of assay were obtained with TRITC-labeled MAb 5.1 (10 µg/mL) and two different kinds of solid-phase antibody (80 µg/mL); MAb 9.1 (▲) and MAb 6.1 (●). The solid-phase antibodies were prepared by physical adsorption. The data represent the results of duplicate experiments and error bars are standard deviations. Error bars smaller than the size of symbols are not shown. The lines were drawn to enhance the view.

at a BSA concentration ranging from 6.4 to 14.4 $\mu\text{g/mL}$. Over the dynamic range of measurement (0 - 6.4 $\mu\text{g/mL}$), the slope of the line is 1.1 $\mu\text{g/mL}$ of antibody per $\mu\text{g/mL}$ of BSA; this value corresponds to 0.47 mole of labeled antibody per mole of BSA and is calculated using a molecular weight of 160,000 for immunoglobulin G and 68,000 for BSA. The linear dose-response over dynamic range indicates that the formation of complex occurs with high affinity.

Assay with solid-phase MAb 6.1 also yielded similar quantitative results, but its dose-response is quite different from that of MAb 9.1. Unlike the linear dose-response for MAb 9.1, the dose-response for MAb 6.1 increased sharply initially with BSA concentration, and reached a maximum of 5.5 $\mu\text{g/mL}$ of the labeled antibody. As concentration of added BSA is increased further, the fluorescence signal starts to decrease. The signal change per added BSA over the initial range of 0 - 1.6 $\mu\text{g/mL}$ of BSA was 0.65 in mole of labeled antibody/mole of BSA, and this value decreased to 0.1 over the BSA concentration ranging from 4.8 to 6.4 $\mu\text{g/mL}$.

4.2.4. Dynamic ranges

To determine the changes in dynamic range of measurement by antibody concentration we performed assays with three sets of solid-phase antibody (MAb 9.1) and labeled antibody (MAb 5.1) concentrations (160 $\mu\text{g/mL}$, 10 $\mu\text{g/mL}$; 80 $\mu\text{g/mL}$, 20 $\mu\text{g/mL}$; 160 $\mu\text{g/mL}$, 20 $\mu\text{g/mL}$); the solid-phase antibody used was prepared by physically absorbing MAb 9.1 on polystyrene beads. Figure 18 shows the results for duplicate experiments. When the solid-phase antibody was increased to 160 $\mu\text{g/mL}$, the assay was sensitive from 0 to 8 $\mu\text{g/mL}$ of antigen. At antigen concentrations from 8 $\mu\text{g/mL}$ to 12.8 $\mu\text{g/mL}$, the fluorescence signal remained at the same level. This result indicates that the amount of the labeled antibody is not sufficient to bind all the antigen in the reaction mixture. On the other hand, when the labeled antibody concentration was increased to 20 $\mu\text{g/mL}$, similar assay performance was obtained; this result confirms that the amount of solid-phase antibody in the previous case was

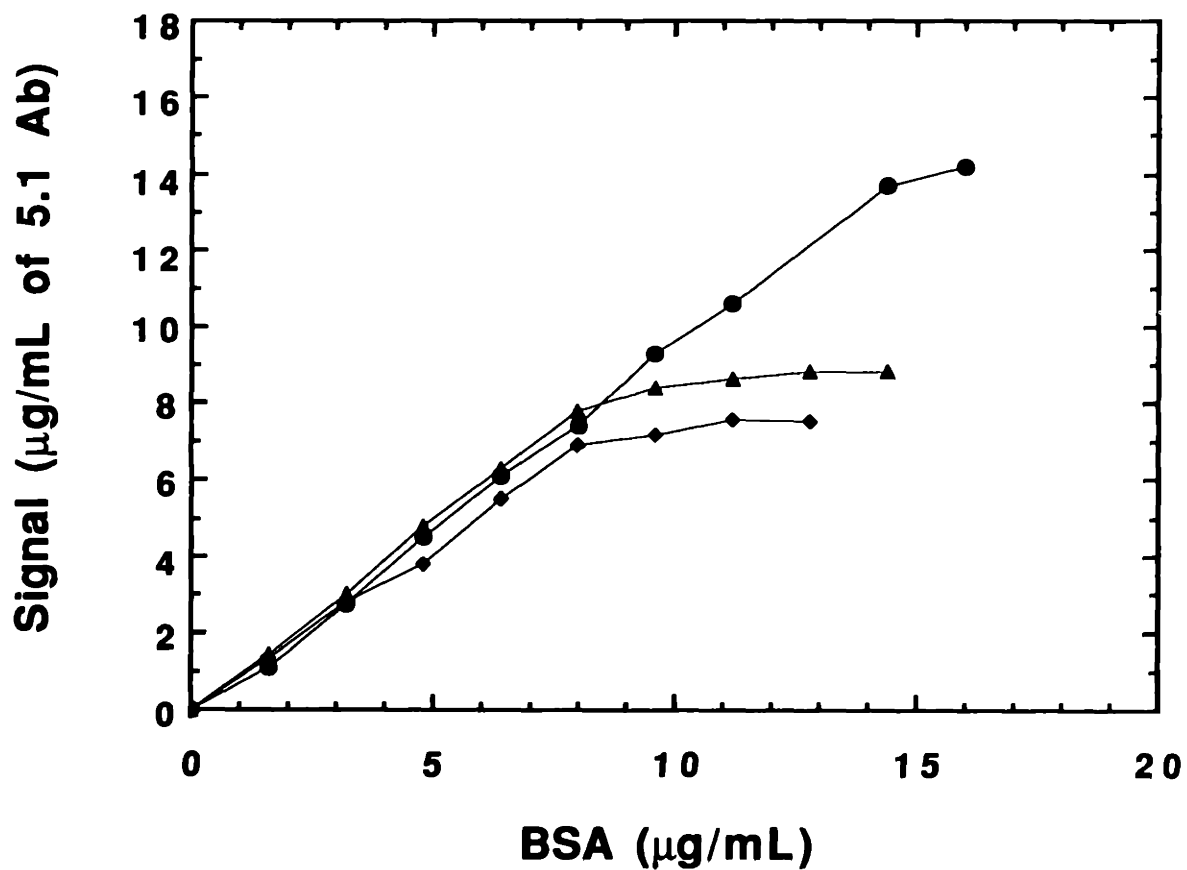


Figure 18. Assay performance at various antibody concentrations. Concentrations of solid-phase antibody and labeled antibody are: ($\mu\text{g/mL}$, $\mu\text{g/mL}$; 160, 10 \blacklozenge ; 80, 20 \blacktriangle ; 160, 20 \bullet). The solid-phase antibodies were prepared by physical adsorption. The data represent the results of duplicate experiments and error bars are standard deviations. Error bars smaller than the size of symbols are not shown. The lines were drawn to enhance the view.

insufficient to bind all the antigen added. In contrast, when both antibodies were doubled, the dynamic range was nearly doubled to the range of 0 - 14 $\mu\text{g/mL}$. Therefore both antibodies have to be present in sufficient quantities in order to expand the dynamic range. One observation worth noting is that in all assays the magnitude of the change in fluorescence signal with added antigen in the dynamic range remained at 0.44 mole per mole of BSA; this result represents the binding of two BSA molecules to one labeled-antibody molecule. This slope is the same for all antibody concentrations.

4.2.5. Assay performance with covalently bound MAb 9.1

To understand the assay performance more quantitatively, we carried out a standard assay by using the covalently immobilized MAb 9.1. The monomeric fraction of the sulfhydryl modified BSA was used as an antigen. Figure 19 shows a typical performance of the assay with 10 $\mu\text{g/mL}$ of labeled-MAb 5.1 and 30 $\mu\text{g/mL}$ of the MAb 9.1 (the bead concentration is 0.1%). The fluorescence increased linearly up to a BSA concentration of 3.2 $\mu\text{g/mL}$, but for higher concentration, the fluorescence signal remained at the same value. When the concentration of the solid-phase antibody was doubled to 60 $\mu\text{g/mL}$, the dynamic range of the assay was also expanded two-fold (6.4 $\mu\text{g/mL}$). Over the dynamic range the fluorescence signal changes with added antigen with a constant slope, 0.44 mole of labeled antibody per mole of antigen. This is the same value as that for the assay with the physically absorbed antibody.

4.2.6. Reproducibility and accuracy

Reproducibility and accuracy of the assay were determined by measuring the inter- and intra-assay variability for assay with covalently immobilized antibody. We performed the assay with the solid-phase antibody (60 $\mu\text{g/mL}$, 0.2% solid suspension) and labeled MAb 5.1 (10 $\mu\text{g/mL}$). Variability is a ratio (as a percentage) of the standard deviation

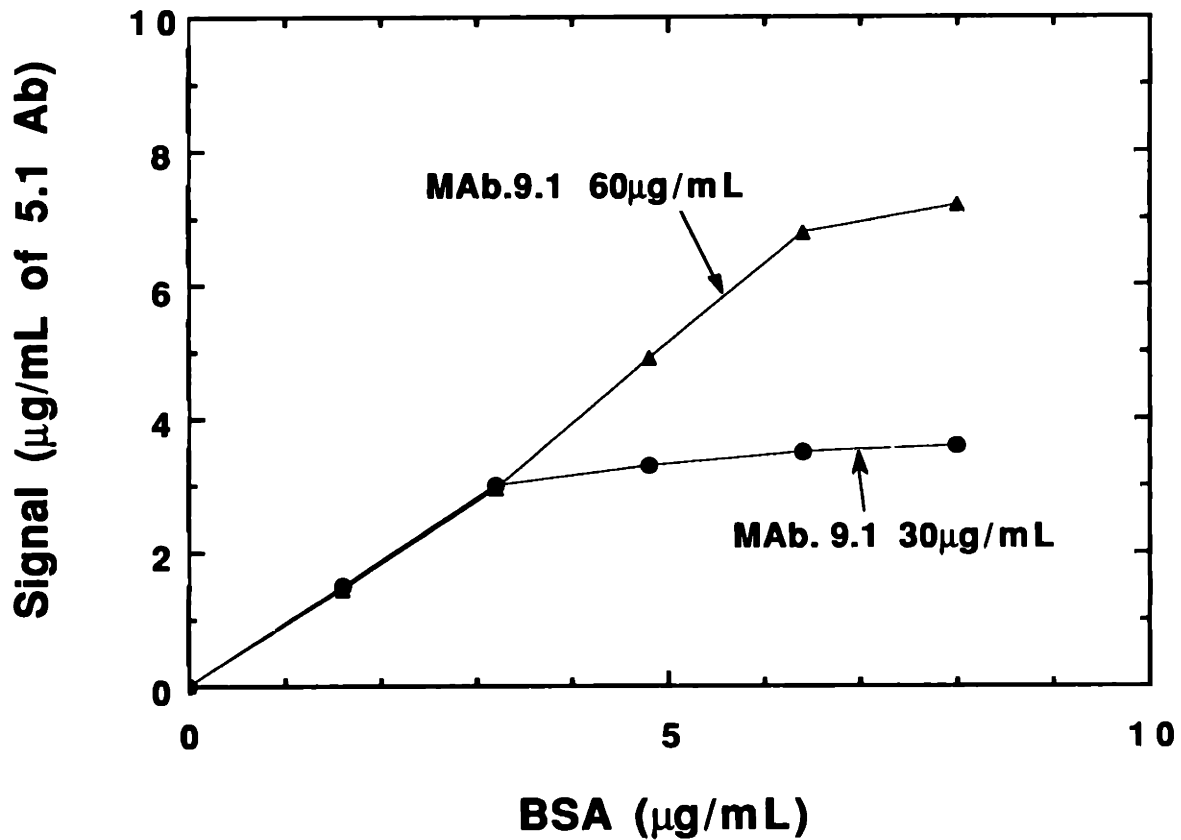


Figure 19. Assay performance with covalently bound MAb 9.1. MAb 9.1 (30 µg/mL ● , 60 µg/mL ▲) and labeled MAb 5.1 (10 µg/mL) were used. The binding capacity of the beads (30 µg/mL of antibody) is 6 µg/mL of BSA. The data represent the results of duplicate experiments, and standard deviations are smaller than the size of symbols. The lines were drawn to enhance the view.

to the mean value of the antigen concentration. The inter-assay variability was obtained from the six sets of assay for standard BSA samples (0.8, 2.0 and 4.0 $\mu\text{g/mL}$). Table 6 shows that the inter-assay variability is 1.9% at 4.0 $\mu\text{g/mL}$ of BSA. At lower BSA concentrations, a higher inter-assay variability was observed, while the standard deviations remained relatively constant. (0.07 - 0.1 $\mu\text{g/mL}$) From four separate experiments with 3.2 $\mu\text{g/mL}$ of BSA, an intra-assay variability measured was 2.3%.

4.2.7. Discussion

The two-site immunofluorometric assay was developed by using two sets of monoclonal anti-BSA (MAb 6.1 & 5.1; MAb 9.1 & 5.1). The assay can be carried out with one two-minute incubation, without any washing and sample dilution. From statistical studies of the assay performance with covalently immobilized 9.1 antibody and 5.1 labeled antibody, it was found that the assay performance is accurate and reproducible.

The required incubation time for the monoclonal antibody assay is only 2 minutes, which is the time required for the rapid binding reaction of antibodies and antigen to reach an equilibrium state. In order to compare these results with a theoretical calculation, the measured fluorescence change was normalized to the maximum change observed. Figure 20 shows that the fluorescence change (X) falls to zero within 2 minutes. We also observed that if we change the order of addition and incubated the antigen with the mixture of the solid-phase and labeled antibody, the change in normalized fluorescence still follows the same curve.

The observed binding kinetics were then compared with calculated the external mass transfer rate to determine the rate-limiting step. If the reactions were controlled by the external mass-transfer rate, the incubation time (t) is then equal to $-\{(V r)/(S D)\} \ln (X)$. The overall incubation time at a specific normalized fluorescence (X) is mainly governed by the available surface area per unit volume (S/V), radius of bead ($r = 0.5 \mu\text{m}$), and diffusivity of the complex, antigen/labeled antibody. If we assume the diffusivity of the complex to be $4.0 \times$

Table 6. Assay variability¹

BSA ($\mu\text{g/mL}$)	Measured Mean Value ($\mu\text{g/mL}$)	Standard Deviation ($\mu\text{g/mL}$)	Variability (%)
Inter-assay			
0.8	0.69	0.072	10.4
2.0	1.8	0.092	5.1
4.0	4.0	0.076	1.9
Intra-assay			
3.2	3.14	0.071	2.3

1. The inter-assay variability was obtained from the measurement of six sets of the standard BSA samples; the intra-assay variability, from the four separate measurements of a standard of BSA. The variability represents the ratio (as a percentage) of the standard deviation to the mean value of the antigen. For the assay, the covalently immobilized MAb 9.1 (60 $\mu\text{g/mL}$) and labeled MAb 5.1 (10 $\mu\text{g/mL}$) were used.

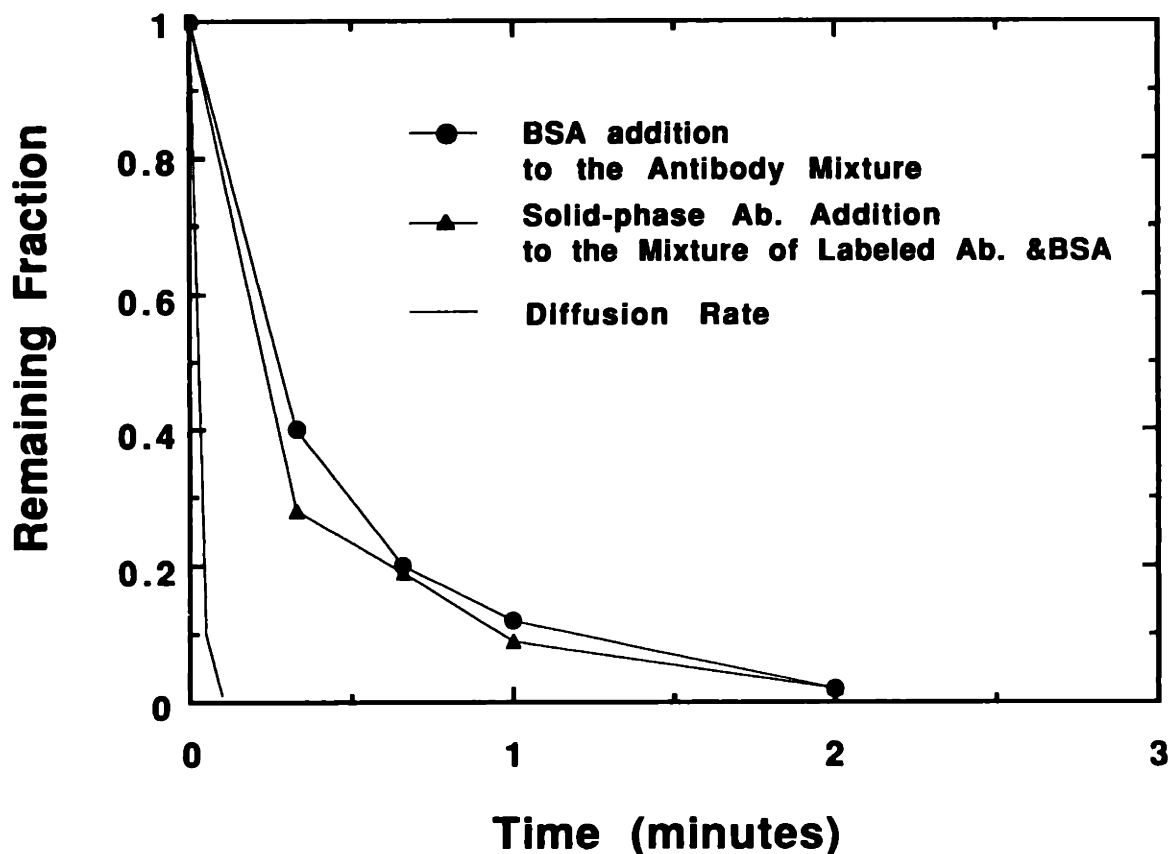


Figure 20. Normalized fraction of the remaining labeled antibodies in the liquid at various incubation times. The experiment data are the same in Figure 16. The solid-phase MAb 9.1 prepared by physical adsorption ($80 \mu\text{g/mL}$, 0.2 % of solid concentration) was added to the antigen ($4.8 \mu\text{g/mL}$) which was previously incubated with the TRITC labeled antibody ($10 \mu\text{g/mL}$) for 30 minutes (▲). The same antigen was added to the mixture of the antibodies at same concentrations (●). The data represent the results of duplicate experiments and error bars are standard deviations. The error bars smaller than the size of symbols are not shown. The lines were drawn to enhance the view.

10^{-7} cm²/second (Wank *et al.*, 1983), we would observe 99 % completion of the binding reaction within 5 seconds. In our experiments, we observed a much longer incubation time, and therefore this observation indicates that the binding reaction of the assay is kinetically controlled.

The detection range of the assays was 0 - 14 µg/mL of BSA, and further expansion in range by using higher concentrations of antibodies is expected. The dynamic ranges linearly expand as the antibody concentration is increased. The linear expansion was observed in both assays with the physically adsorbed and covalently bound antibodies.

The inter-assay variability for the assay with MAb 9.1 & 5.1 is in the range of 2 - 10 % which is comparable to that of the other two-site assays (Uotila *et al.*, 1981; Shields and Turner, 1986; MacCrimble *et al.*, 1985). The monoclonal antibody assay, however, is less sensitive to measure a low concentration of antigen than the other assays (Gupta *et al.*, 1985; Jolly *et al.*, 1984). When the minimum detectable concentration is defined as the antigen concentration corresponding to two standard deviations of the fluorescence measurement from the control, the detectable concentration is 0.2 µg/mL (3×10^{-9} M) of BSA, calculated from the data in Table 6. This value is at least 10 times higher than that of the other conventional two-site assays. This insensitivity may be due to the fluorescence signal at a low antigen concentration which is only a small fraction of the labeled antibody in the liquid. When the signal is less than the error from the control, the antigen concentration can not be determined.

For the assay with MAb 9.1 and 5.1, the molecular ratio of the bound antigen to the labeled antibody was two to one over the dynamic range; this ratio was independent from changes in the concentration of antigen and antibodies. One explanation of the above observation is that antibodies 5.1 and 9.1 form a circular⁹ complex on the solid support. Circular complex formations have been observed: for example, BSA with monoclonal antibodies 5.1 and 9.1 (Murphy *et al.*, 1988), HCG with its antibodies (Moyle *et al.*, 1983), and HLA-A₂ with its monoclonal antibodies (Holmes and Parham, 1983). In a circular complex, each antibody binds with two antigens through two attachment points, so that the

avidity of the binding is increased (Ehrlich *et al.*, 1982). Because of this high avidity, the fluorescence signal changes linearly as the antigen is added. In designing the assay, therefore, it can be advantageous to select monoclonal antibodies which form a circular complexes with antigen because these antibodies can extend the linear range of the assay performance.

More than 70 % of activity of initial MAb 9.1 was lost during immobilization, and only a fraction of the remaining active solid-phase antibody may contribute to the binding of the complex of labeled antibody and BSA. For the assay with MAb 9.1 (30 $\mu\text{g/mL}$) and 5.1 (10 $\mu\text{g/mL}$), the observed dynamic range was 0 - 3.2 $\mu\text{g/mL}$ of BSA, where the amount of solid-phase antibody was limited. This means that the effective binding capacity of the solid-phase antibody is 3.2 $\mu\text{g/mL}$ of BSA bound with labeled antibody. This value is substantially low compared to the measured binding capacity of the same solid-phase antibody (30 $\mu\text{g/mL}$); the binding capacity of the solid-phase antibody (30 $\mu\text{g/mL}$) measured by the equilibrium binding experiment with ^{125}I -BSA was 6 $\mu\text{g/mL}$ of ^{125}I -BSA.

The physically adsorbed antibody gave a similar assay performance as the covalently bound antibody, but the preparation of the physical adsorbed antibody demanded extensive washing of the beads. In our assay system, to minimize the amount of the detached solid-phase antibody in the assay procedure, we washed the physically adsorbed beads four times before carrying out the assay. We also observed that if the beads were not washed completely, the response of the assay was less sensitive to the added antigen (Figure 21).

In designing the assay, several important factors must be considered. One important consideration is the use of appropriate amounts of the solid-phase and labeled antibody. Because the signal change is a small fraction of the total unbound fluorophore signal, the use of excess labeled antibody may reduce the sensitivity at low antigen concentrations. Second, in order to reduce the total unbound fluorophore, the preparation of labeled-antibodies should be purified so that inactive labeled contaminants are held to a minimum. Third, to facilitate the operational steps in the assay, one can prepare the solid-phase antibody by immobilizing the antibody on magnetized beads so that the separation can be

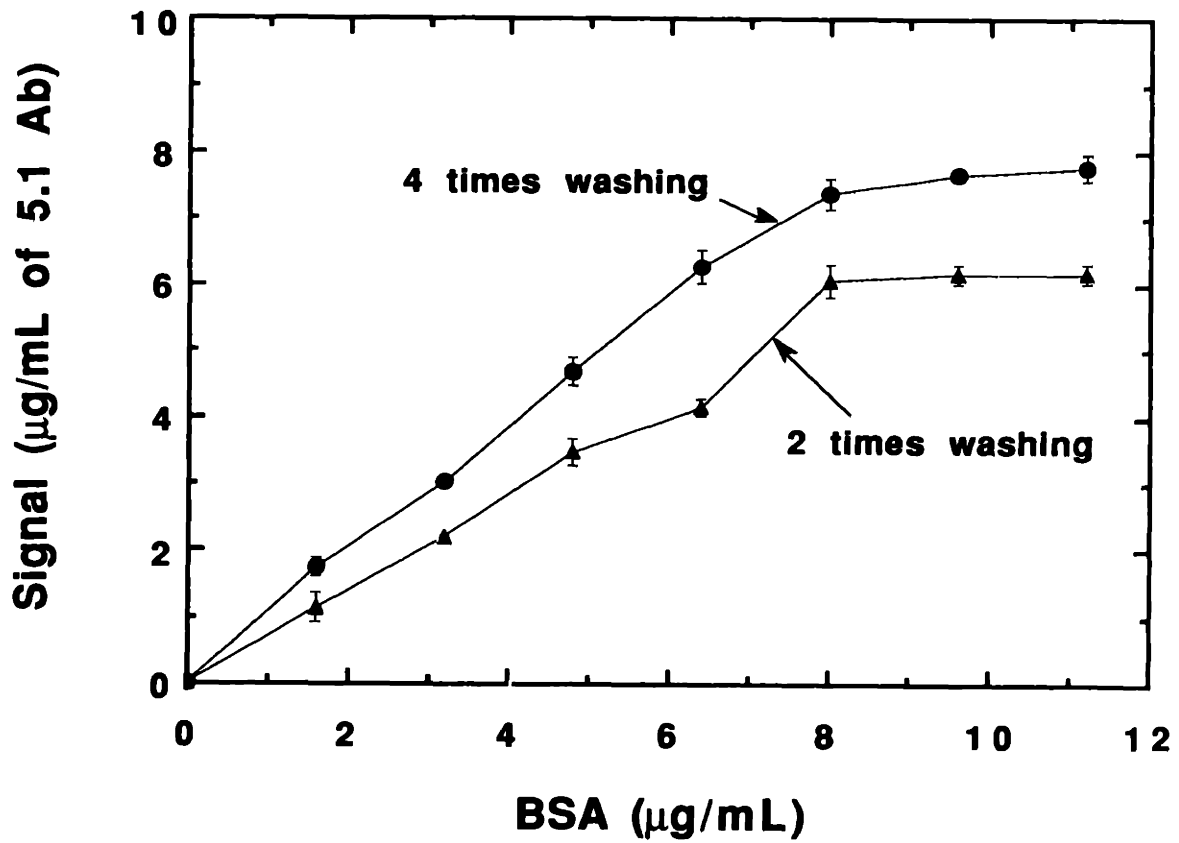


Figure 21. Effects of washing procedures in the preparation of solid-phase antibody on dose-response curve. The dose-response relations of assay were obtained with TRITC-labeled MAb 5.1 (10 µg/mL) and solid-phase antibody (80 µg/mL); 4 times washing (●), two times washing (▲).

done by magnetic force rather than centrifugation or filtration. Preparation of the reaction mixture in a frozen form also leads to further simplification of the assay system.

The assay principle can be applied in the measurement of any proteins in a rapid and reproducible way. The potential applications of this type of assay are numerous. Such an assay in general lends itself to the automation of the assay procedures, which enables to perform larger number of assays per given time. Another major application for such an immunoassay is the monitoring of a protein whose concentration changes over a period time. This behavior, for example, is encountered in many therapeutic proteins during production by cultured cells in bioreactors or in purification processes following production. Monitoring of these processes demands quick measurement of the concentration of protein when that concentration varies rapidly.

4.3 Binding kinetics

In our assay system, monoclonal antibodies (MAb) specific to BSA have been immobilized on solid supports to use their selective binding ability as a solid-phase antibody. Binding affinities and rates for protein-antibody interactions are useful for selecting an antibody and for designing assay with a given antibody.

Several studies have examined protein-immobilized antibody interactions, but the observed kinetics are often inconsistent with a bimolecular reaction (reversible with a second order association reaction and with a first order dissociation reaction). For immobilized antibodies on porous beads, the equilibrium constants determined by rate measurements are often in disagreement with the equilibrium constants measured by equilibrium binding (Fowell and Chase, 1986; Budd and Smith, 1986). In addition, significant reduction in binding rates by mass transfer limitations was observed for antibody binding in a well of a microtiter plate for immunoassay (Stenberg *et al.*, 1986; Werthén and Hygren, 1988; Stenberg and Hygren, 1988).

A few studies have been done to measure intrinsic binding rates. A mathematical expression derived by using diffusion and binding rates was developed, but it is only useful for qualitative estimation (Li, 1985). Using small porous beads (13 μm diameter) and non-porous beads (1 μm diameter) as a solid support, kinetic parameters for BSA-immobilized anti-BSA antibodies have been measured (Olson *et al.*, 1989). However, whether the binding is kinetically controlled under the experimental conditions used must be validated.

In this study, binding kinetics with nonporous beads (0.5 μm) as a solid support was studied. After confirming that the binding reaction is kinetically controlled, we measured the association rate, dissociation rate and equilibrium constant at various temperatures. By using these measurements we determined the order of the binding reaction and the intrinsic values of the kinetic constants.

4.3.1 Theory: effect of mass-transfer resistance on association rate

In this analysis, we will first describe equations for the mass transfer and association rate of antigen to the immobilized antibody. Using these equations, we then calculated the effects of the mass transfer on the overall association rate. This information was used to select a bead size which is small enough to reduce the mass-transfer effects on the rate measurements.

A schematic diagram of antigen binding to an immobilized antibody on a single bead is shown in Figure 22. We assumed that the binding reaction is performed in a static fluid, and the concentration of the antigen at the bead surface is at pseudo-steady state with the antigen concentration in the bulk solution.

If the association reaction of antigen and the immobilized antibody is of second order and if at initial binding the association rate is much greater than the dissociation rate, the association rate of antigen can be written as :

$$\frac{dA}{dt} = k_1 C_S (A_0 - A) \quad (7)$$

where A represents the antibody concentration bound by antigen in moles/cm², and k_1 is the association rate constant in cm³/mole/sec, and C_S represents the antigen concentration at the bead surface in moles/cm³, and A_0 is the initial antibody concentration on the solid surface in moles/cm². Here, the antibody concentration represents a concentration of active antibody binding sites on solid surface.

On the other hand, the mass transfer of antigen, N (moles/cm²/sec), at the bead surface can be written as :

$$N = h (C_B - C_S) = D/r (C_B - C_S) \quad (8)$$

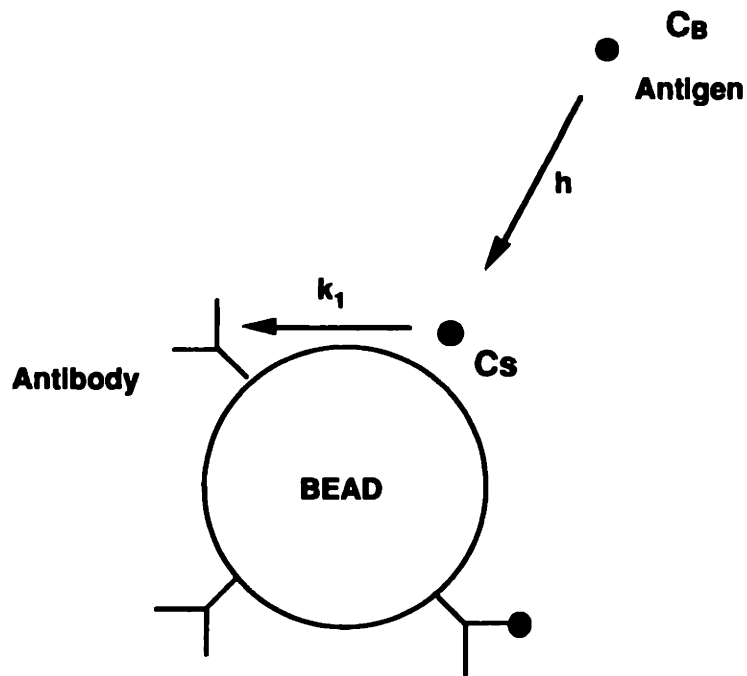


Figure 22. Schematic of antigen binding onto the antibodies on a single bead. The antigen first diffuses to the solid surface before binding. C_B is the antigen concentration in bulk solution and C_S is the antigen concentration at bead surface, and k_1 is the association rate constant.

where h is the mass transfer coefficient of antigen in cm/sec, C_B is the concentration of antigen in the bulk solution in moles/cm³. The mass transfer coefficient (h) in a static fluid is D/r where D is the diffusivity of antigen in cm²/sec, and r is the radius of the bead in cm (Bird *et al.*, 1960).

The change of the antigen in the bulk solution will be equal to the rate of antigen association reaction at the surface of the beads. This can be written as :

$$\frac{V dC_B}{dt} = \frac{S dA}{dt} \quad (9)$$

where V is the bulk volume in cm³, and S is the total surface area of the beads in cm².

When the association reaction is kinetically controlled, the reaction time can be obtained as a function of C_B from the association rate and a mass balance on the antigen. From Equations (7) and (9), the reaction time (t) at an antigen concentration (C_B) can be written as follows:

$$t = \frac{-V}{k_1} \frac{1}{(SA_0 - VC_{B0})} \ln \left[\frac{(C_B/C_{B0})}{\{1 - (VC_{B0}/SA_0)(1 - C_B/C_{B0})\}} \right] \quad (10)$$

From Equation 10, the reaction time is a function of the initial concentration of antibody at the solid surface (A_0), the initial antigen concentration (C_{B0}), the bead surface area per unit volume (S/V), and the association rate constant (k_1).

On the other hand, when the association reaction is controlled by the mass transfer of antigen, from Equations (8) and (9), the reaction time (t) at an antigen concentration (C_B) can be written as follows:

$$t = \frac{-V r}{S D} \ln (C_B / C_{B0}) \quad (11)$$

From Equation 11 the reaction time (t) is a function of the bead surface area per unit volume (S/V), radius of bead (r), and diffusivity of antigen (D), and the initial antigen concentration (C_{B0}).

A comparison of the kinetic rate with the mass transfer rate was performed by calculating the Damköhler number (Da) using association rate constants (k₁) ranging from 10⁷ to 10⁹ cm³/mole/sec (Fowell and Chase, 1986; Olson *et al.*, 1989; Wank *et al.*, 1983) and a value for the maximum antibody binding sites on surface as 2 x 10⁻¹² moles/cm². The antibody concentration was estimated using a 50% recovery of antibody activity after the antibody was immobilized as a monolayer (Cantarero *et al.*, 1980).

$$Da = \frac{k_1 C_{B0} A_0}{h C_{B0}} = \frac{k_1 A_0 r}{D} \quad (12)$$

For the binding of BSA with an antibody immobilized on 0.5 μm diameter beads, the value of Da calculated using the diffusion coefficient for BSA, 3.26 x 10⁻⁷ cm²/sec (Wagner and Scheraga, 1956) is in a range of 0.0025 - 0.25. Given these assumptions, the mass transfer rate of the antigen is much faster than its kinetic rate, and thus it can be assumed that the effects on the association rate by the mass transfer are minimal.

4.3.2. Characterization of ¹²⁵I-BSA

The fraction of ¹²⁵I-BSA that binds specifically to the immobilized MA b 9.1 was determined by the methods of Lindmo and Bunn, Jr (1986). The ¹²⁵I-BSA was prepared by iodinating on monomeric fraction of sulfhydryl-modified BSA prepared by gel filtration by the use of the iodine chloride (ICl) exchange procedure, as described in the Materials and Method section. The percentage of the radioactivity on protein was greater than 99% as determined by measuring the precipitate that was formed by the addition of trichloroacetic acid. From the equilibrium experiment described in Materials and Methods, ratios of total ¹²⁵I-BSA

to bound ^{125}I -BSA at various solid-phase MAb 9.1 concentration were measured. Figure 23 shows the ratio obtained from the equilibrium experiments performed in duplicate were plotted as a function of $1/[\text{Ab}]$. The intercept on the Y axis represented the reciprocal value of active fraction of the labeled antigen which was determined to be 93.3 %. This fraction was accounted for in calculating equilibrium and kinetic parameters.

4.3.3. Nonspecific adsorption of ^{125}I -BSA on solid-phase MAb 9.1

The nonspecific binding of ^{125}I -BSA on the solid support was determined by performing two sets of experiment. For the first experiments, the covalently immobilized mouse IgG on polystyrene (0.0125% bead suspension) was incubated with ^{125}I -BSA (0-8 $\mu\text{g}/\text{mL}$). For the second experiment, the covalently immobilized MAb 9.1 on polystyrene beads (0.0125% beads suspension) was incubated with a mixture of ^{125}I -BSA (0-8 $\mu\text{g}/\text{mL}$) and unlabeled BSA (15 mg/mL). After a two hour incubation, the reaction mixture (250 μL) tubes were centrifuged and 200 μL of supernatant were removed. Supernatant and sediment fractions were counted in a gamma counter, and the amount of the labeled BSA bound onto the beads was calculated. For the both experimental results, ^{125}I -BSA did not bind to the beads.

4.3.4. Determination of the rate-governing step

To determine whether the association is controlled kinetically, we measured the initial rate of antigen binding onto the antibody-beads, which have a binding capacity ranging from 0.01 to 0.04 μg of ^{125}I -BSA/ cm^2 , as described in the Materials and Methods. For the rate measurements, 0.25 $\mu\text{g}/\text{mL}$ of ^{125}I -BSA were incubated with the antibody-beads (0.025%). In Figure 24, the bound fraction of ^{125}I -BSA was plotted as a function of incubation time. At all surface concentrations the bound fraction rapidly increases, and reaches a constant value within 3 minutes of incubation. The initial association rate was calculated by using the data for the first 40 seconds of incubation, and then plotted as a function of the surface concentration of antibody. Figure 25 shows that the initial rate increases as the surface

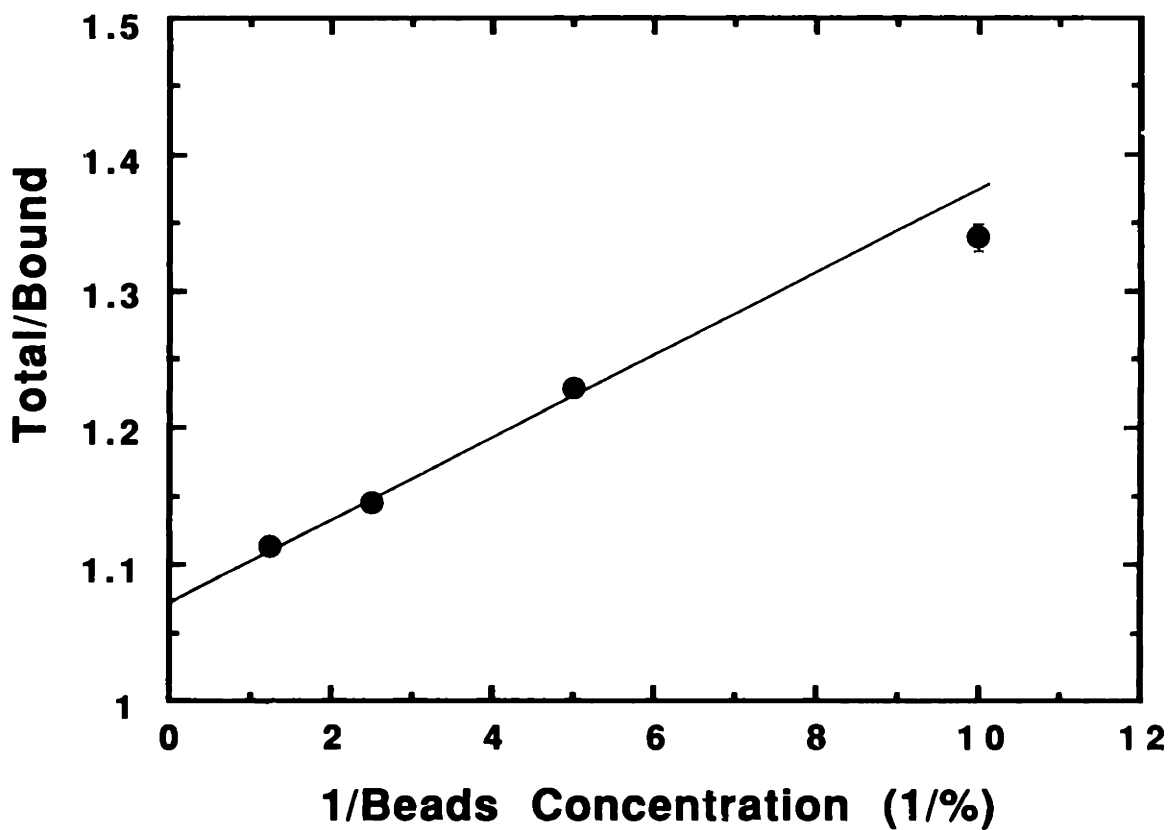


Figure 23. Fraction of active ^{125}I -BSA. ^{125}I -BSA ($1\ \mu\text{g}/\text{mL}$) was incubated with the MAb 9.1- beads ($30\ \mu\text{g}$ of MAb 9.1 /mL of bead volume) at varying concentrations from 0.05 % to 0.8 % of bead. After a two hour incubation, supernatant and sediment fractions were counted in a gamma counter, and the amount of the labeled BSA bound onto the beads was calculated.

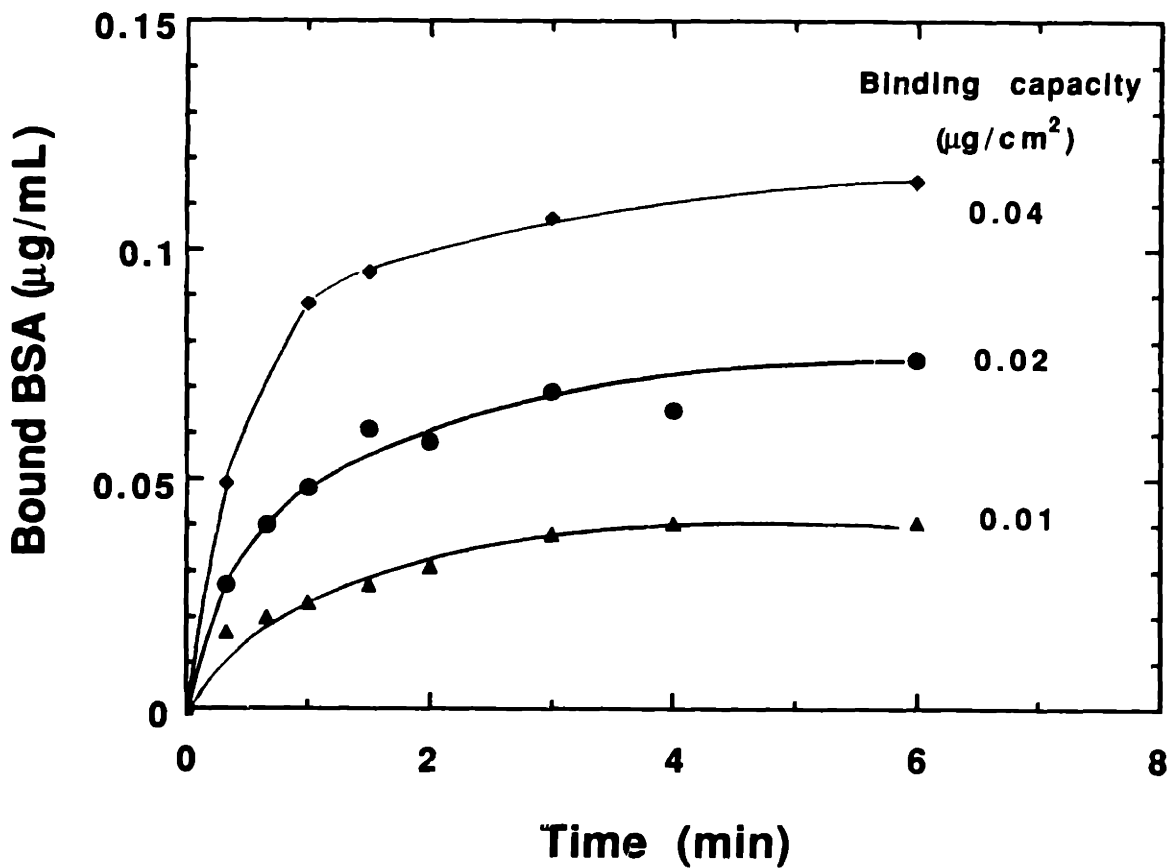


Figure 24. Effects of surface binding site concentration on association. ^{125}I -BSA ($0.25\mu\text{g}/\text{mL}$) was mixed with the antibody-bead (0.025%) which has an binding capacity of 0.01 , 0.019 , or $0.04\mu\text{g}$ of ^{125}I -BSA/ cm^2 of bead surface. The bound ^{125}I -BSA was plotted as a function of incubation time.

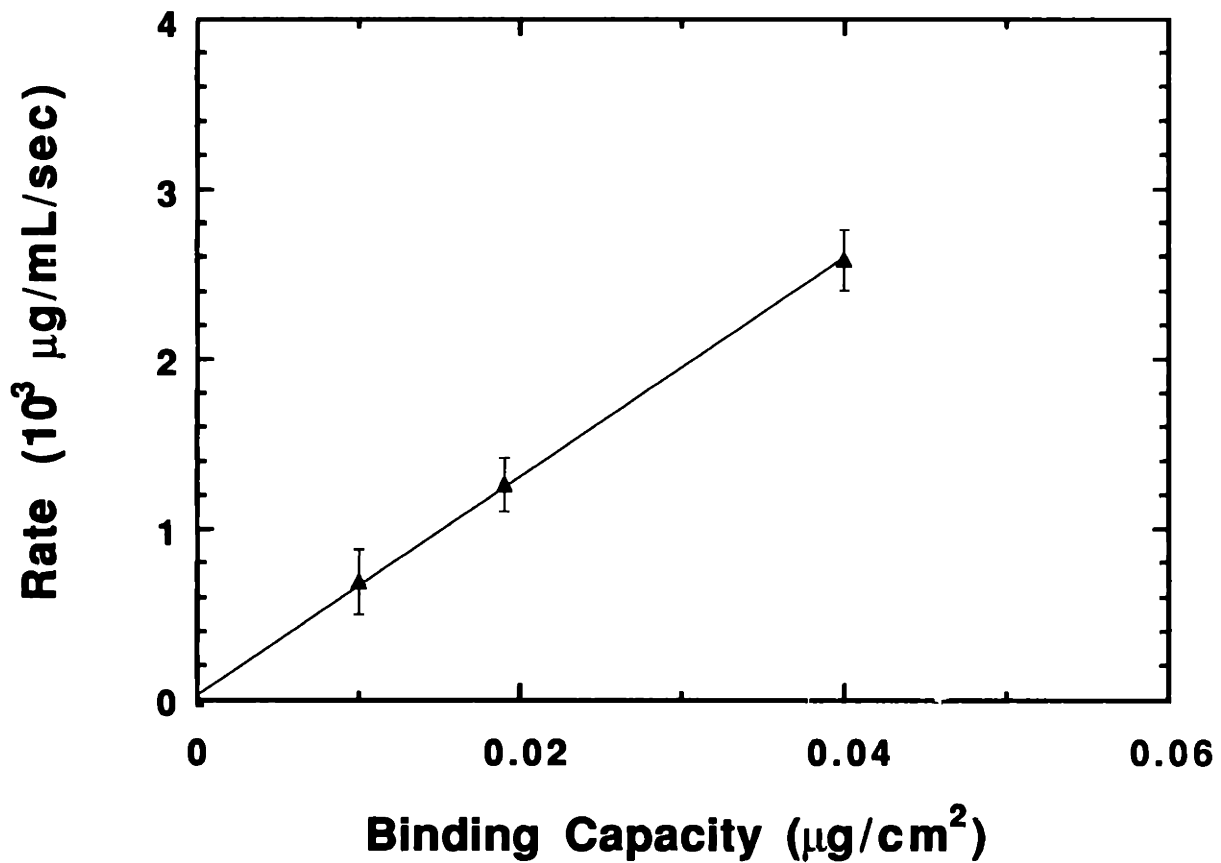


Figure 25. Plot of initial association rates versus the surface concentration of antibody binding sites. The initial rates was determined as described in Materials and Methods with data for the first 40 seconds incubation from Figure 24. Error bars represent a standard deviation.

concentration increases. This linear relationship indicates that the kinetic association is the governing step in the association of the antigen to the immobilized antibody, as expected from the Equations 7 & 9.

In addition, we compared the measured association rate with the calculated mass transfer rate. If the reaction is controlled by the external mass-transfer rate, we will observe 90 % for reaction to be completed within 5 seconds. Based on Equation 11. Figure 24 shows, however, the binding reaction at all surface concentrations reached equilibrium at about 3 minutes. Because the measured association rate is much slower than the mass transfer rate, and the initial association rate linearly depends on the antibody concentration on the surface, we can therefore conclude that the binding reaction is kinetically controlled.

4.3.5. Order of association reaction

The order of association reaction was determined with the method analysing the initial rates (Levenspiel, 1972). To determine the reaction order with respect to the antigen, we incubated ^{125}I -BSA (ranging 0.031 to 0.25 $\mu\text{g}/\text{mL}$) with the antibody-beads (0.014%). The bound fraction of ^{125}I -BSA was measured at various reaction times. At all concentrations of ^{125}I -BSA, we found that the bound fraction of ^{125}I -BSA reached a constant value in less than 4 minutes (Figure 26). By using the data at 20 seconds of incubation, we calculated the initial association rates, and then plotted the logarithm of the initial rates as a function of the logarithm of the antigen concentration (Figure 27). The data yield a straight line with a slope of 1. This result indicates the reaction is of first order with respect to antigen concentration.

Alternatively, to determine the reaction order with respect to the antibody-beads we incubated 0.25 $\mu\text{g}/\text{mL}$ of ^{125}I -BSA with the antibody-beads at concentrations ranging from 0.063% to 0.05% (Figure 28). In Figure 29, the logarithm of the initial rate was plotted as a function of the logarithm of the antibody-beads concentration. The results show first order reaction kinetics. At high bead concentrations, greater than 0.025 %, the initial association rate is less than predicted. This difference may be caused by the dissociation of the

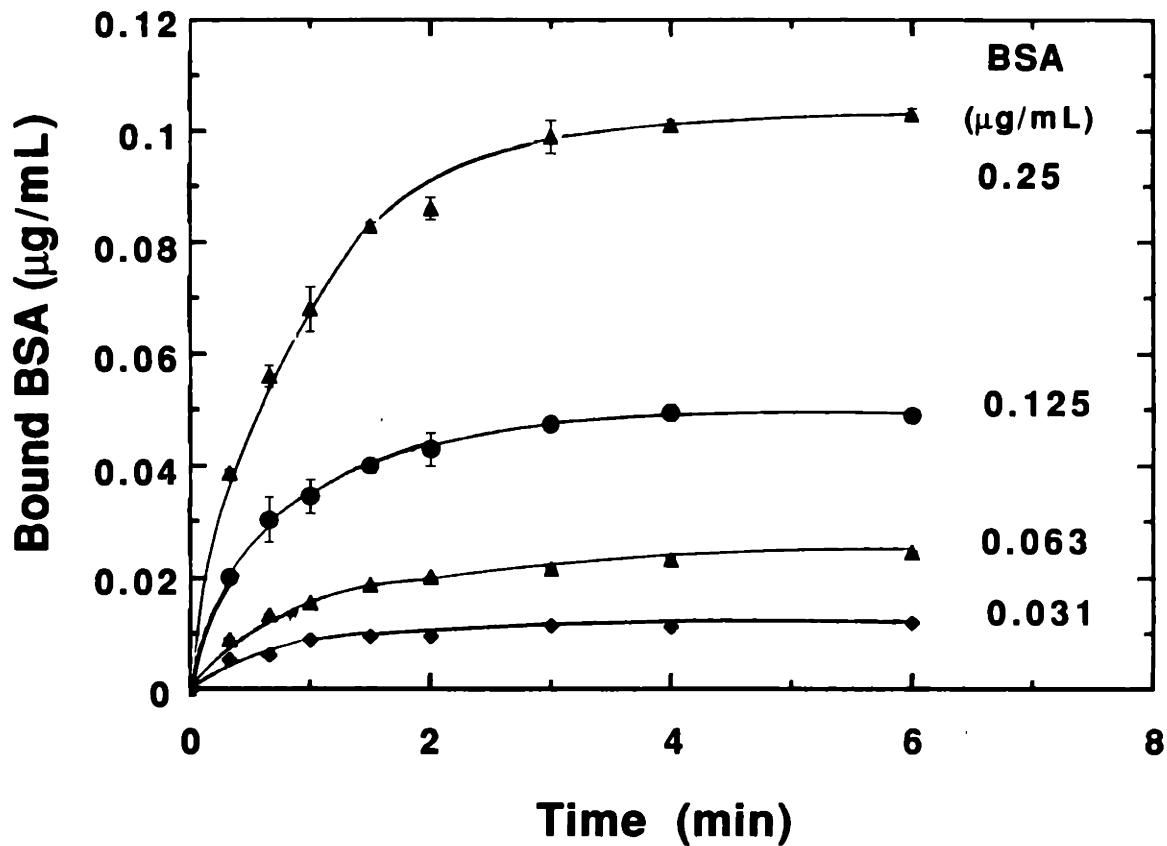


Figure 26. Association reaction order with respect to antigen; plot of bound ^{125}I -BSA versus time. ^{125}I -BSA ranging from 0.031 to 0.25 $\mu\text{g/mL}$ was incubated with 0.014% of bead (the binding capacity; 0.86 μg of ^{125}I -BSA /mL). The lines were drawn to enhance the view. Standard deviations are represented as error bars or are smaller than the size of symbols.

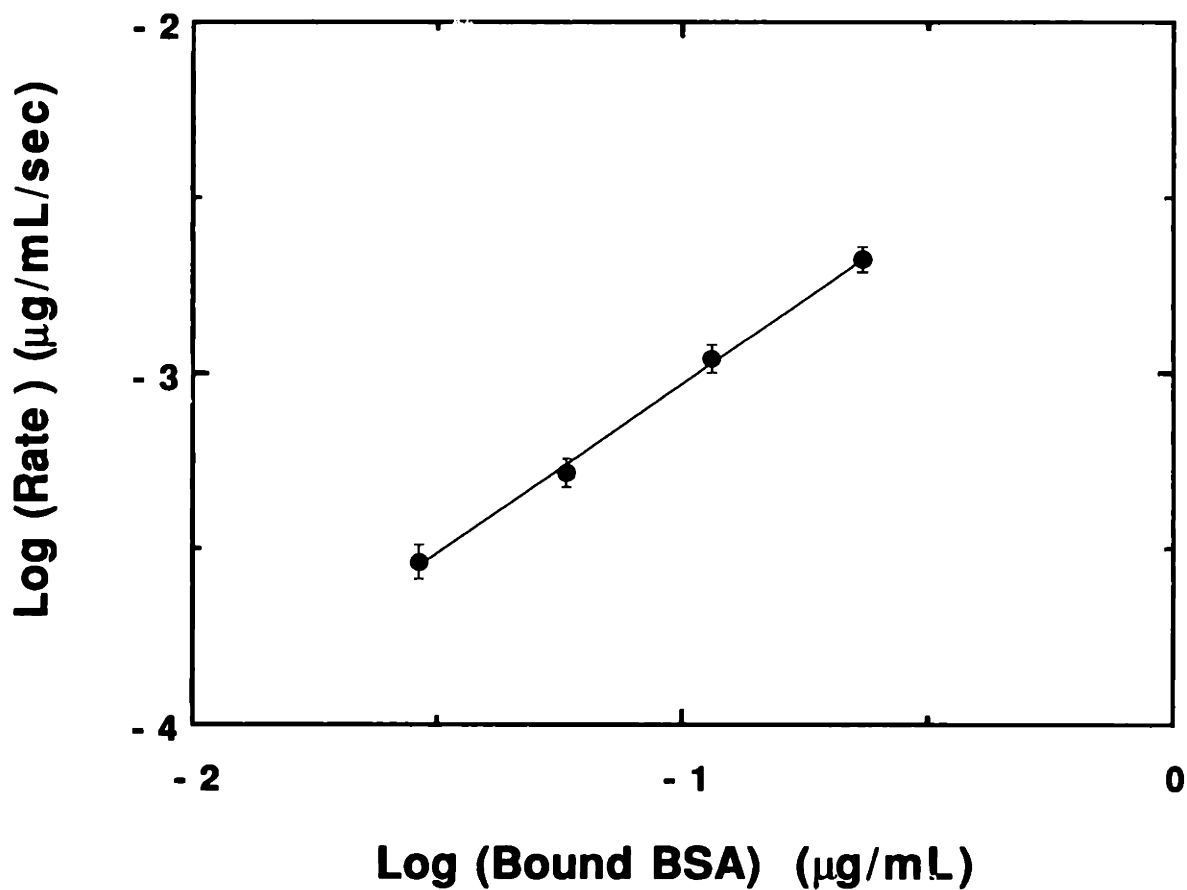


Figure 27. Association reaction order with respect to antigen; plot of log (initial association rate) versus log (^{125}I -BSA). The each data for rate was obtained from four data points in Figure 26. The line represents the first order kinetics, and the error bars represent standard deviations.

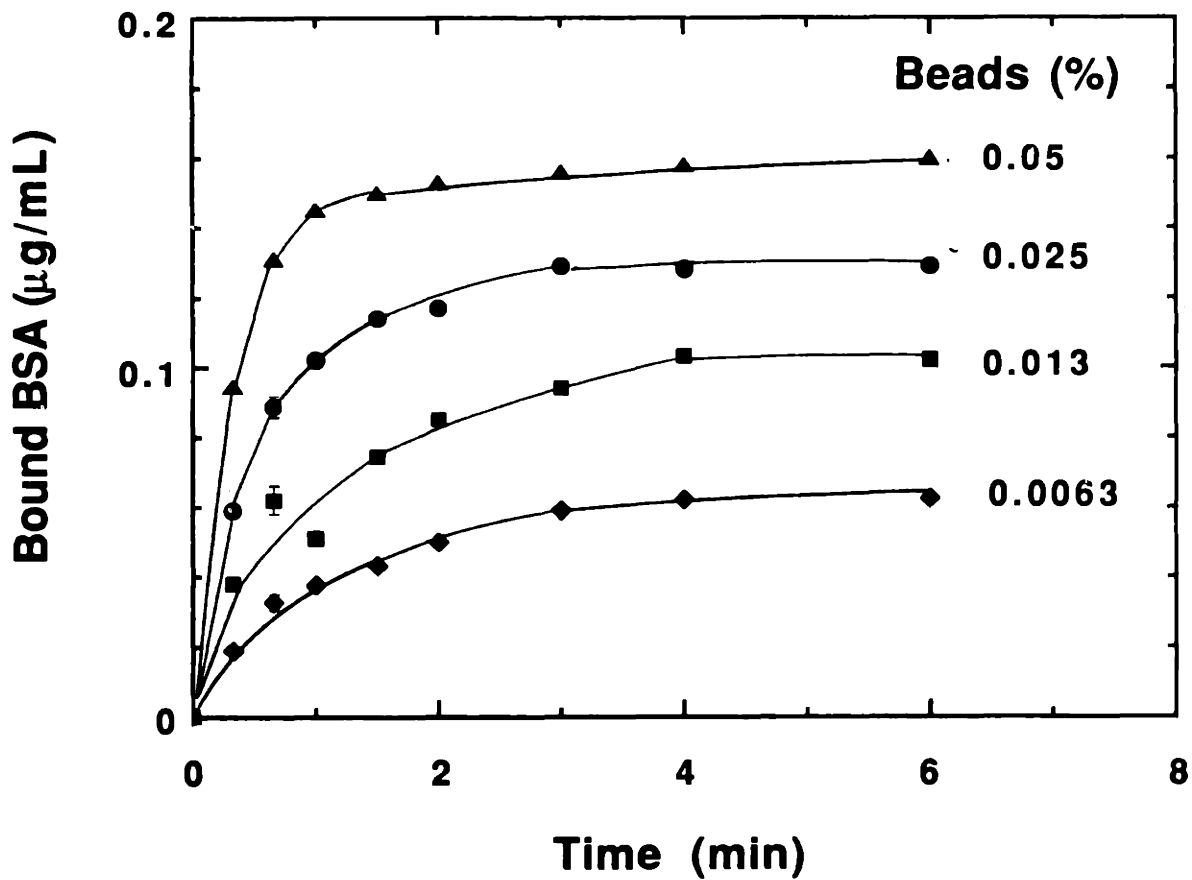


Figure 28. Association reaction order with respect to antibody-beads; plot of bound ^{125}I -BSA versus time. Antibody-beads ranging from 0.032 to 0.25 % was incubated with $0.25 \mu\text{g/mL}$ of ^{125}I -BSA. The lines were drawn to enhance the view. Standard deviations are represented as error bars or are smaller than the size of symbols.

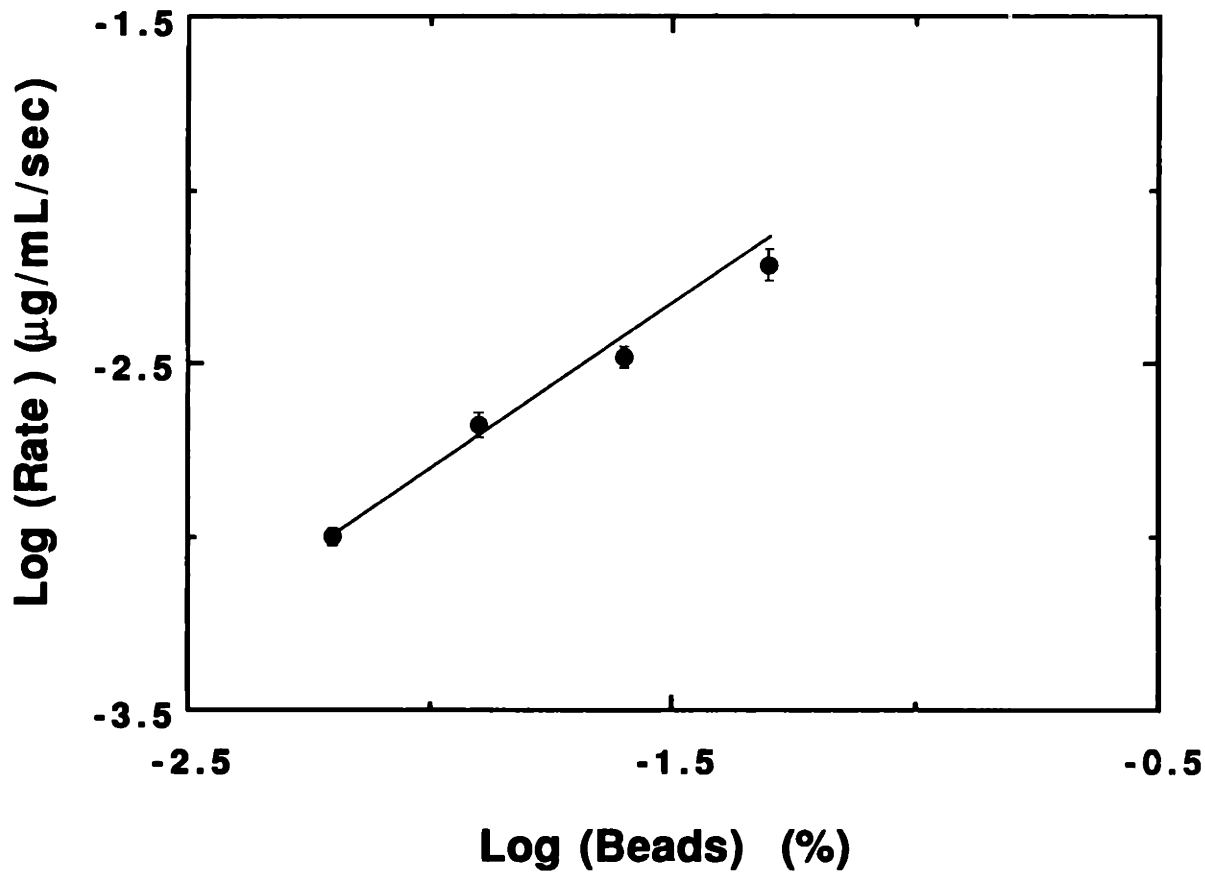


Figure 29. Association reaction order with respect to antibody-beads: plot of log (initial association rate) versus log (antibody-beads). The each data for rate was obtained from four separately measured data. The line represents the first order kinetics, and error bars represent standard deviations.

antibody/antigen complex during the incubation. In general the linear relationship indicates that the reaction is of first order with respect to the antibody-beads. We can conclude from these results that the association reaction is of second order overall.

4.3.6. Order of dissociation reaction

The order of dissociation was determined by fitting the data of dissociation kinetics into the equation for a first order reaction. We measured the dissociation of ^{125}I -BSA from the complex of ^{125}I -BSA-MAb 9.1 which was prepared by preincubating beads (0.027%) with 0.5 $\mu\text{g}/\text{mL}$ of ^{125}I -BSA. After addition of the unlabeled BSA, the bound ^{125}I -BSA started to desorb, and the desorbed ^{125}I -BSA reached a constant value (Figure 30). There appears to be very tightly bound ^{125}I -BSA, occupying about 7 % of the total binding sites, which was not detached even after two hours of dissociation time. For the calculation of the dissociation rate constant, we considered the amount of the ^{125}I -BSA desorbed within 30 minutes as the total bound ^{125}I -BSA. Figure 31 shows the logarithm of the fraction of the bound ^{125}I -BSA to the total bound as a function of incubation time. The data yield a straight line during early incubation. Therefore, the initial rate of dissociation suggests that the dissociation reaction is of first order.

4.3.7. Binding capacity of beads and equilibrium constant

The binding capacity of the antibody-beads and the equilibrium constant for MAb 9.1, was determined using three different antibody-beads concentrations (0.0031, 0.0062 and 0.0125 %). The antibody-beads were incubated for two hours with ^{125}I -BSA ranging 0.031 - 2.0 $\mu\text{g}/\text{mL}$. Figure 32 shows a plot of the bound BSA on the beads as a function of the unbound. At all antibody-beads concentrations we obtained the same value of the binding capacity per bead volume and the equilibrium constant. In a Scatchard plot (Figure 33), the data at all bead concentrations yield straight lines with the same slope. The binding capacity

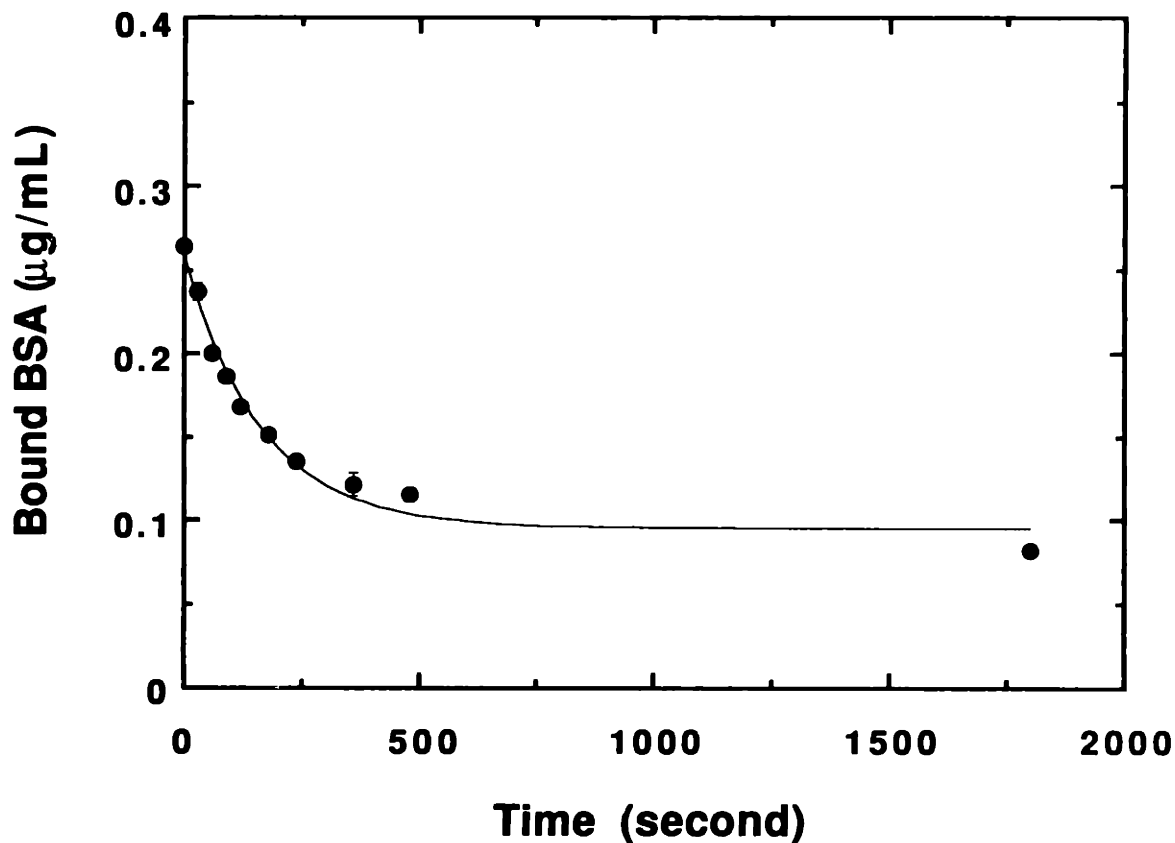


Figure 30. Dissociation kinetics; plot of the bound fraction of ^{125}I -BSA versus time. The line represents the first order kinetics, $y = a \exp(-k_2t) + b$, where $a = 0.182 \mu\text{g/mL}$, $b = 0.082 \mu\text{g/mL}$, and $k_2 = 0.0062/\text{sec}$. a represents the total bound which was considered the amount of the ^{125}I -BSA desorbed within 30 minutes incubation. b represents the remaining fraction at 30 minutes incubation. Standard deviations are represented as error bars or are smaller than the size of symbol.

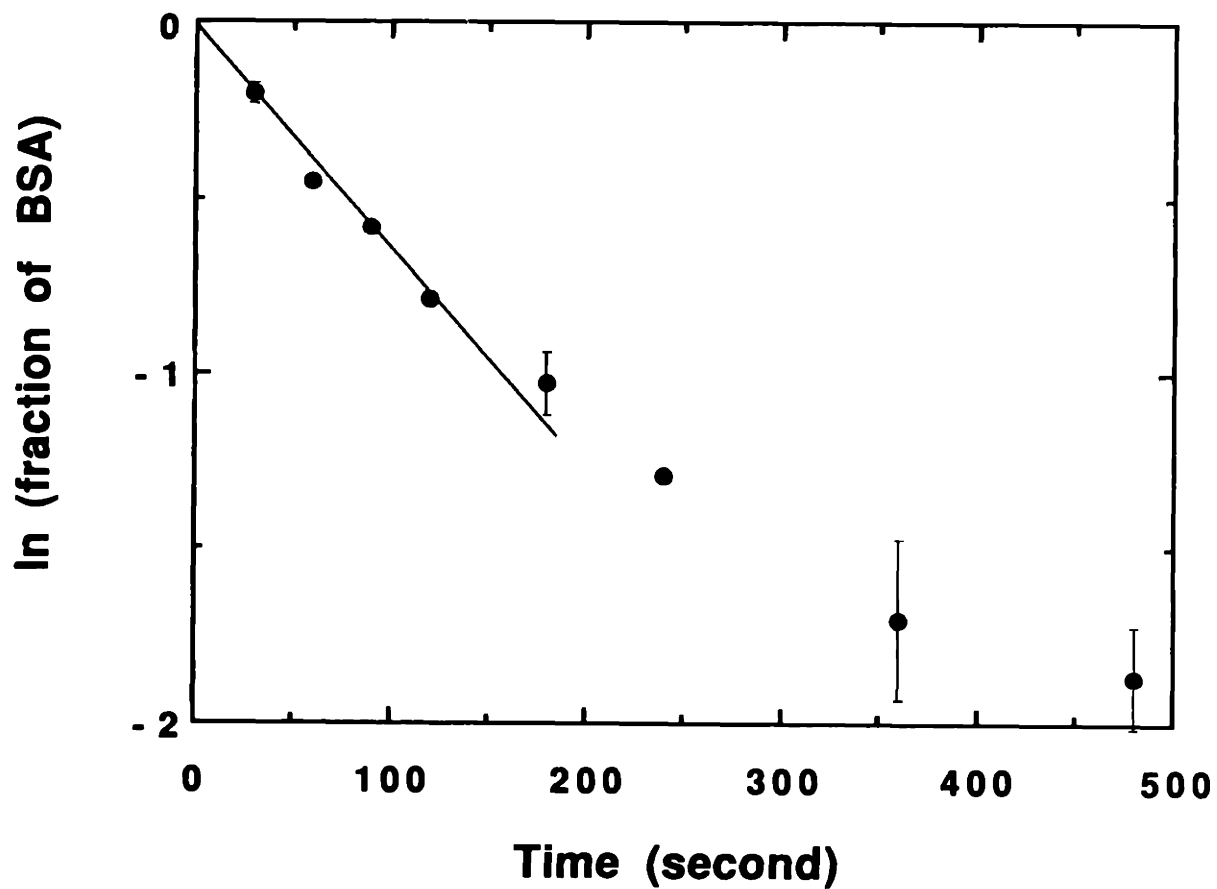


Figure 31. Dissociation kinetics; plot of natural logarithm of the fraction of the bound ^{125}I -BSA to the total bound versus time. Standard deviations are represented as error bars or are smaller than the size of symbol. The line represents the dissociation rate constant, $k_2 = 0.0062/\text{sec}$.

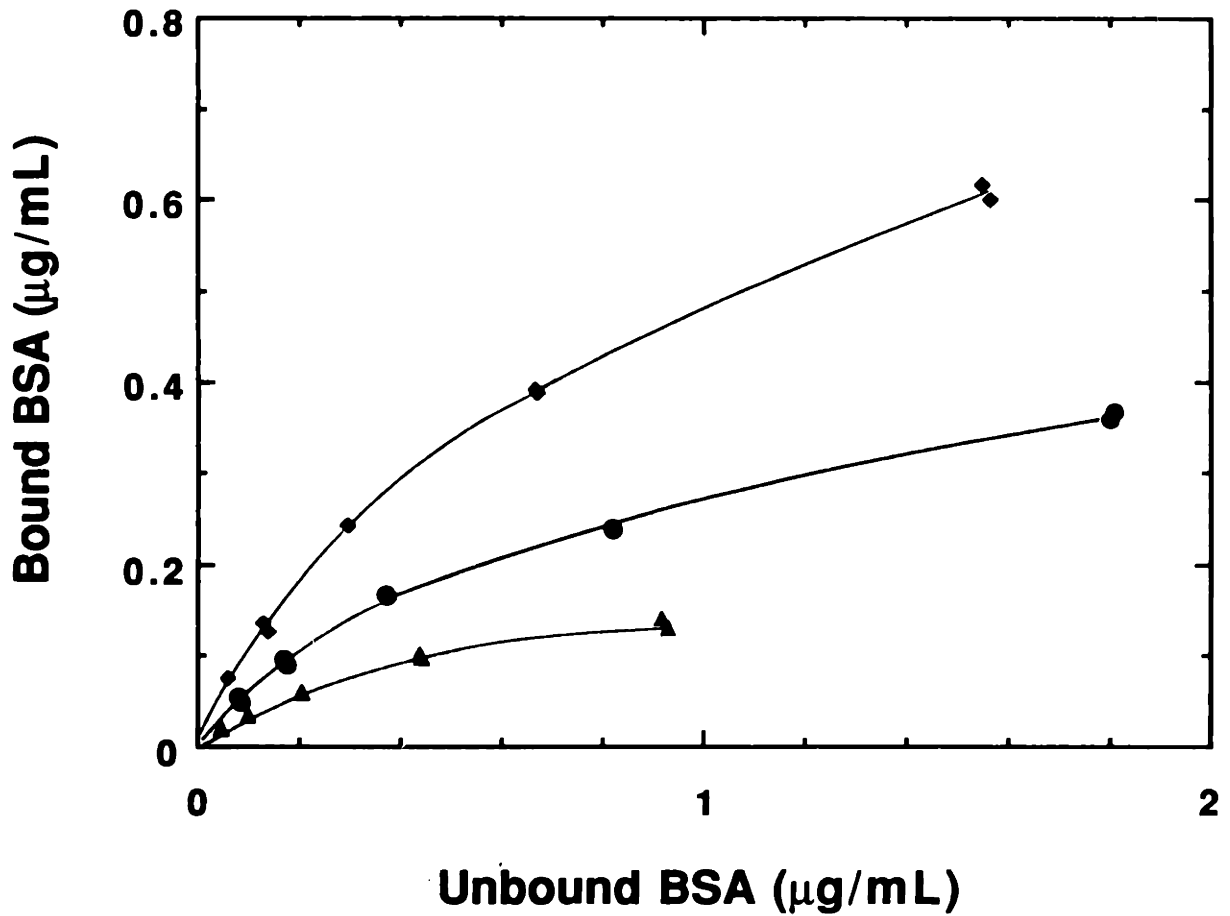


Figure 32. Equilibrium reaction with the antibody-beads at various concentration. The antibody-beads (0.0031% ▲ , 0.0062% ● , 0.0125% ◆) were incubated for two hours with ^{125}I -BSA ranging 0.031 - 2.0 $\mu\text{g}/\text{mL}$.

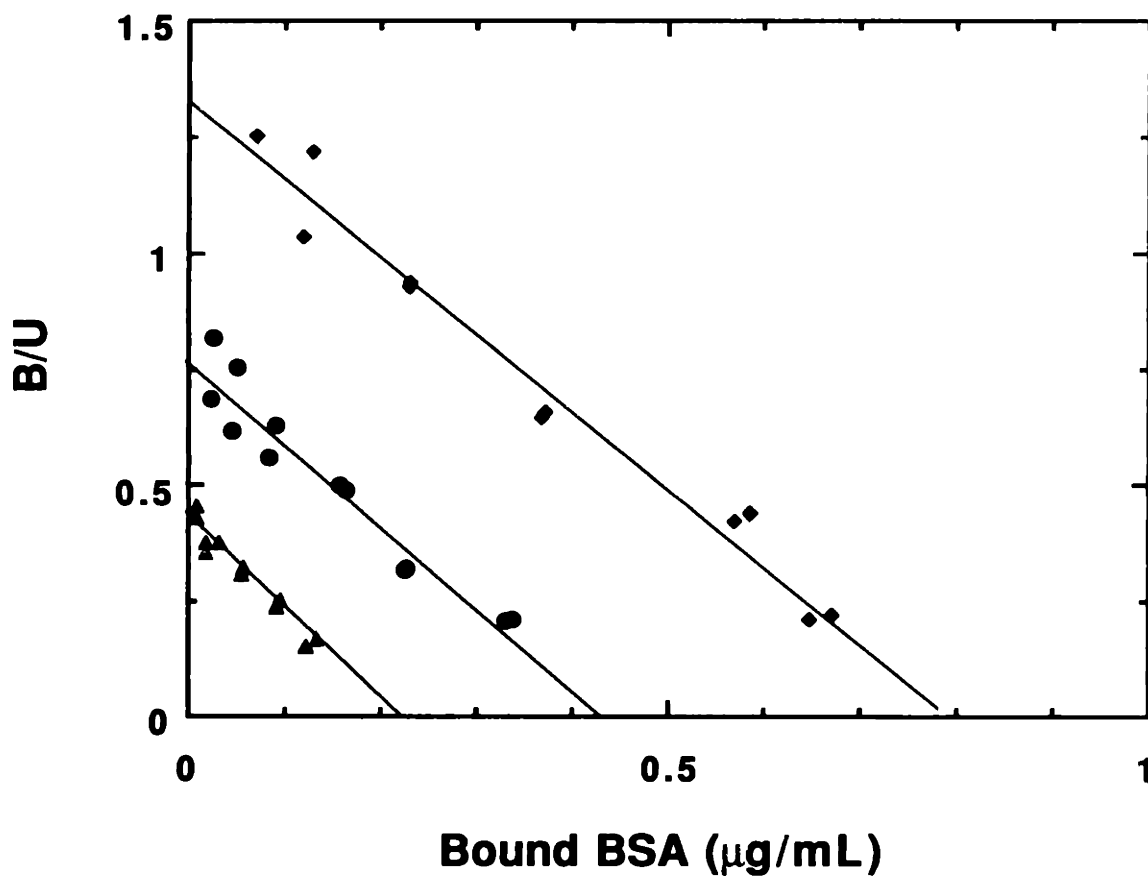


Figure 33. Scatchard plot for the equilibrium reaction with the antibody-beads; (0.0031% ▲ , 0.0062% ● , 0.0125% ◆). B is the bound fraction on the beads and U is the unbound fraction in the solution. The lines are obtained by least-square regression of the data from the experiment in duplicate.

determined from the intercepts of the X axis linearly increases from 0.19 to 0.78 $\mu\text{g/mL}$ of BSA as the bead concentration increases from 0.0031 to 0.0125%. The average binding capacity was determined to be 6 mg of ^{125}I -BSA per mL of bead volume. In addition, from the slopes at all bead concentrations the equilibrium association constant (mean \pm standard deviation) was found to be $1.2 \pm 0.06 \times 10^8$ L/mole.

4.3.8. Intrinsic values of kinetic constants at varying temperature

In order to know how the changes in temperature affect the kinetics and their parameters, we measured the equilibrium and the rate constants at 4, 15, 25 and 37 $^{\circ}\text{C}$. Equilibrium binding was performed using the same concentration of antibody-beads (0.0125%) at all temperatures. Figure 34 shows that at all temperatures the binding capacity of the antibody-beads was the same, 0.75 $\mu\text{g/mL}$ of ^{125}I -BSA with 0.0125% of beads. The equilibrium constant (K_a) calculated from the slopes increases from 0.33 to 1.3×10^8 L/mole as the reaction temperature increases from 4 to 37 $^{\circ}\text{C}$. Table 7 shows that the measured association rate constant at these temperatures ranges from 1.7 to 8.6×10^5 L/mole/second, and dissociation rate constants also varied from 4.7 to 6.7×10^{-3} second^{-1} . At all temperatures, the measured equilibrium association constant was in accord with the value calculated from the ratio of association to dissociation rate constants, based on the second order kinetics.

The activation energies for association and dissociation was determined by using Arrhenius equation, $k = k_0 \exp(-E/RT)$, where R is the gas constant and T is the absolute temperature and k_0 is the frequency factor. In Figure 35, the natural logarithms of the rate constant for both association and dissociation were plotted as a function of $(1/T)$. The slope for the association rate constant yields an activation energy of 9 Kcal/mole. In contrast to the strong temperature effect on the association rate, the effect on dissociation rate was rather small. The activation energy for the dissociation constant was 2 Kcal/mole.

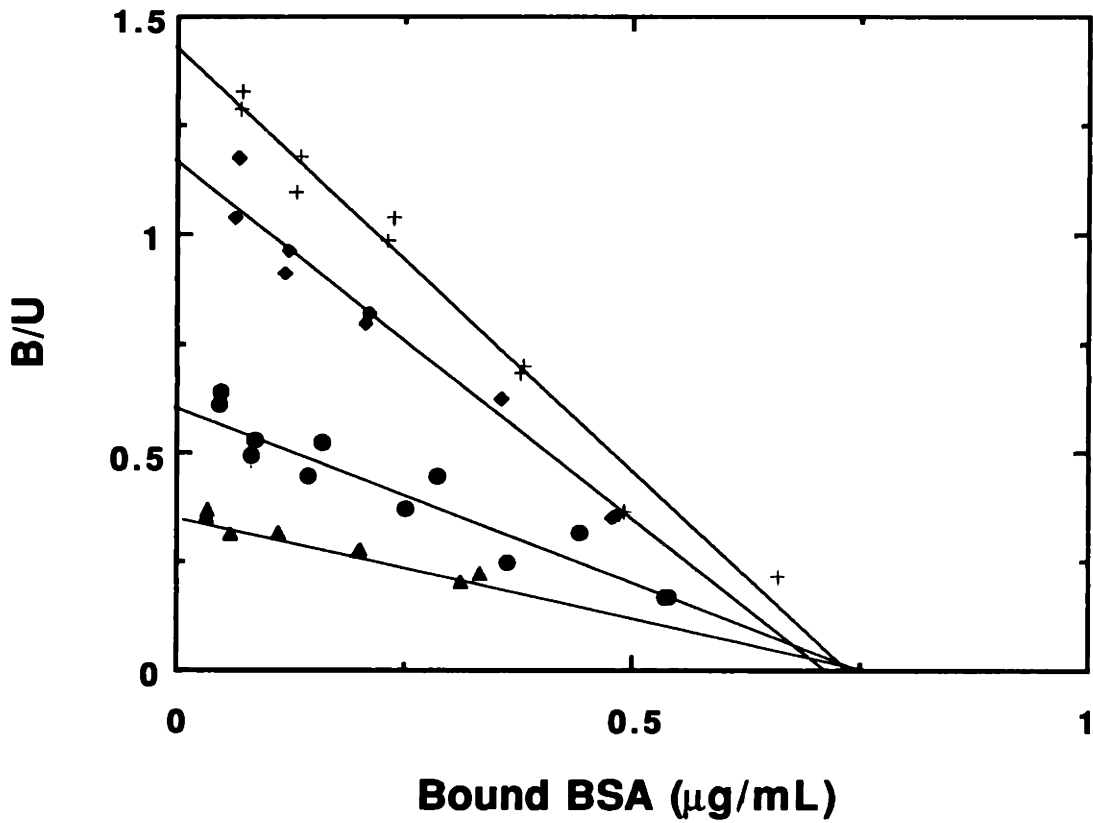


Figure 34. Scatchard plot for equilibrium binding reaction at varying temperature; 4 (▲), 15 (●), 25 (◆), 37 °C (⊕). Antibody-beads (0.0125%) was incubated with ¹²⁵I-BSA. The lines are obtained by least-square regression of the data from the experiment in duplicate.

Table 7. Intrinsic kinetic parameters at varying temperature.

Temperature °C	Rate Constant		Equilibrium association constant	
	Association (10^{-5} L/mole/sec)	Dissociation (10^3 1/sec)	Calculated (10^{-8} L/mole)	Measured (10^{-8} L/mole)
4	1.7 ± 0.05	4.8 ± 0.53	0.35	0.33 ± 0.028
15	3.2 ± 0.12	5.5 ± 0.72	0.58	0.54 ± 0.037
25	6.7 ± 0.21 ^a	6.2 ± 0.39	1.10	1.12 ± 0.051 ^a
37	8.6 ± 0.25	6.7 ± 0.47	1.28	1.31 ± 0.083

All data (mean ± standard deviation) represent the results of duplicate experiments, except the data marked with ^a which represent the results of four times experiments.

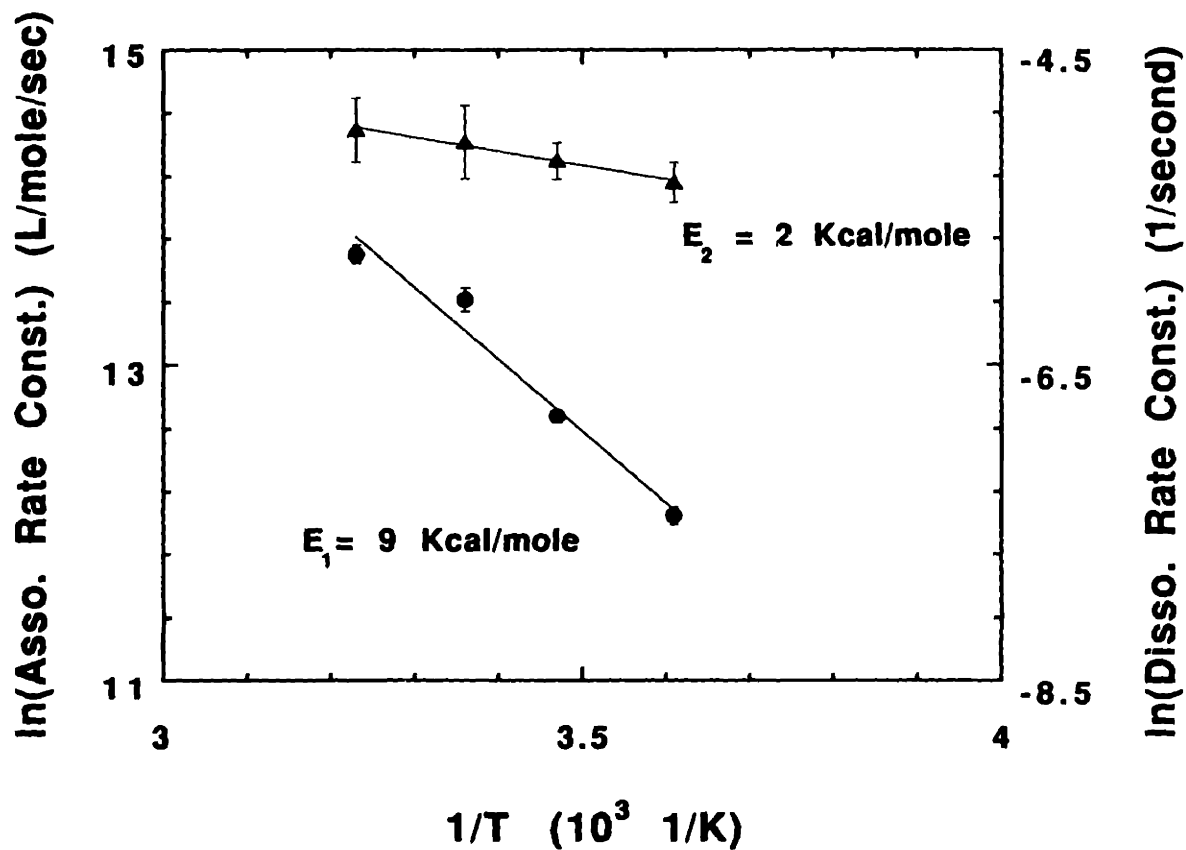


Figure 35. Arrhenius plot for rate constants; (association ● and dissociation ▲) The plots were obtained by using the data in Table 7. Standard deviations are represented as error bars or are smaller the size of symbols. The lines are obtained by least-square regression of the mean value of data. Activation energies were determined from the slopes. E_1 is the activation energy for association rate constant; E_2 , for dissociation rate constant.

4.3.9. Discussion

The observed reaction rates show that binding between ^{125}I -BSA and immobilized MAb 9.1 is kinetically controlled. The initial association rate increased with increases in surface antibody concentration, as expected for a kinetic controlled reaction, and the association rates were at least 100 times slower than the calculated antigen mass-transfer rate. In addition, the calculated value of Da with the measured antibody binding capacity and association rate constant at 25 °C is 0.036, which means the kinetic binding is the rate controlling step. Similar to the association rate, the dissociation rate is also at least 100 times slower than the antigen mass transfer rate. Thus, the both association and dissociation reactions are kinetically controlled.

To perform the kinetically controlled reactions we ensured the beads were dispersed. We dispersed the aggregated beads to yield a monomeric bead suspension, which were then used for the binding experiments. In addition, we confirmed by microscopic observations that the beads remained in dispersion during the binding experiments.

The present study of the reaction rates and equilibrium illustrates the binding reaction of ^{125}I -BSA to immobilized MAb 9.1 generally follows bimolecular reaction kinetics. The initial association reaction is of second order; the rate was proportionally dependent on the antibody concentration as well as antigen concentration. The initial rate of the dissociation followed first order kinetics. The equilibrium constant determined by Scatchard analysis agrees well with the calculated from the association and dissociation rate constants which were measured independently. Therefore, the bimolecular reaction kinetics can be used to determine the rate and equilibrium constants for the intrinsic binding reaction.

The association rate constant at temperature from 4 to 37 °C was in the range of $1.7 - 8.6 \times 10^5$ L/mole/second which is similar in magnitude to other kinetically controlled binding reactions; for examples, the binding of ovalbumin and polyclonal antibody in solution (Dandliker and Levison, 1967), the binding of rabbit brain acetylcholinesterase and monoclonal antibody in solution (Brimijoin *et al.*, 1985) and the binding between an antibody and its

receptor on cells (Wank *et al.*, 1983; Mason and Williams, 1980; Roe *et al.*, 1985). On the other hand, the measured association rate constant, 6.7×10^5 L/mole/second, at 25 °C was greater than 1.3×10^5 L/mole/second at 23 °C determined for the same antibody immobilized on the porous beads (Olson *et al.*, 1989). This difference indicates that association rates depend on mass transfer of antigen introduced by solid supports or immobilization methods for antibody, or both.

A fraction of the bound ^{125}I -BSA was not desorbed in the dissociation experiments and this fraction at 25 °C represents about 7 % of the total antibody binding sites. To obtain a complete rate equation for the dissociation, we need a more extensive study of the nature of this desorption. In our control experiments, the non-specific binding of ^{125}I -BSA to the solid surface is nearly nil, therefore this fraction could be bound to some portion of the antibody specifically. This non-dissociation (or slow dissociation) has been reported for dissociation of antigen/immobilized antibodies (Olson *et al.*, 1989). However, the nature of this dissociation is still unknown.

The measured activation energy for the association rate constant was 9 Kcal/mole. This compares well with the 12 Kcal/mole activation energy measured for polyclonal antibody-ovalbumin binding in solution (Levison *et al.*, 1968) but is higher than the 4 Kcal/mole reported for hapten systems (Day *et al.*, 1963). The fact that the activation energy is greater than that for diffusional processes, which are characterized by activation energies of less than 4 Kcal/mole, supports the conclusion that BSA-MAb 9.1 binding is kinetically controlled.

The dissociation of the antigen/antibody complex has an activation energy of 2 Kcal/mole, and is insensitive to changes in temperature. Temperature insensitivity is also observed for dissociation of some IL-2/monoclonal anti-IL-2 antibody systems (Budd and Smith, 1986). However, dissociation rates for other IL-2/ anti-IL-2 antibody systems markedly increase with temperature (Budd and Smith, 1986). Antibody/receptor (on cells) system (Mason and Williams, 1980) also have temperature sensitive dissociation rates. The

variability in the effect of temperature on dissociation indicates that dissociation characteristics depends on the nature of the binding site.

In conclusion, we demonstrated the small size non-porous beads offer binding which is essentially free of mass transfer limitations. The observed reaction rates show that the binding between ^{125}I -BSA and immobilized MAb 9.1 is kinetically controlled. The measured activation energy for association indicates the reaction is kinetically controlled. From the measurements of intrinsic binding rates and constants, the binding of ^{125}I -BSA and immobilized MAb 9.1 at solid surface followed a bimolecular reaction kinetics. This system is useful to study the intrinsic binding characteristics between a protein antigen and an antibody immobilized on solid surface.

NOMENCLATURE for Chapter 4.3.

A	Concentration of antibody binding site bound by antigen (moles/cm ²)
A₀	Initial concentration of antibody binding site on surface (moles/cm ²)
C_S	Antigen concentration at bead surface (moles/cm ³)
C_B	Antigen concentration in bulk solution (moles/cm ³)
C_{B0}	Initial antigen concentration in bulk solution (moles/cm ³)
D	Diffusivity of antigen (cm ² /sec)
Da	Damköhler number
f	Fraction of ¹²⁵ I-BSA that binds specifically with an antibody
k	Rate constant
k₀	Frequency factor
k₁	Association rate constant (cm ³ /mole/sec)
k₂	Dissociation rate constant (1/sec)
K	Equilibrium association constant (mL per μg of ¹²⁵ I-BSA)
K_a	Equilibrium association constant(L/mole)
h	Mass transfer coefficient of antigen (cm/sec)
N	Mass transfer of antigen at bead surface (moles/cm ² /sec)
r	Radius of bead (cm)
R	Ideal gas constant (1.987 cal/°K-mole)
S	Total surface area of the beads (cm ²)
t	Reaction time (second)
T	Absolute temperature (°K)
V	Bulk volume (cm ³)

4.4. Modelling of assay performance

The proposed two-site immunofluorometric assay has been demonstrated to be a simple, rapid analytical tool, and the measurements using the assay are quantitative and reproducible. To study the performance of the assay more quantitatively we developed a mathematical model which describes its dose-response. The model by Ehrlich *et al.* (1983) employs a series of binding relations between the solid-phase antibody, labeled antibody and antigen to describe the multiple equilibrium reactions. The model we developed is similar to Ehrlich's model except that we also used a polymerization factor for the formation of complexes which consist of more than two antibodies.

Our objective in this study is two-fold. With the model we try to explain the possible binding reaction mechanisms which govern the dose-response of assay with MAb 9.1 and MAb 5.1; particularly, to determine the factor which makes the dose-response over the dynamic range of measurement linear. The other purpose of the model development is to obtain simulated results for assay performance at various levels of parameters including the affinity of each antibody for antigen and concentration of antibody. From these simulations the sensitivity and dynamic range of an assay at a given condition can be estimated.

Several conditions and assumptions have been used in developing the model. We considered that the antibody for solid-phase and labeled antibodies is monoclonal. The prepared solid-phase and labeled antibodies only bind to one specific site on an antigen and can simultaneously bind to a single molecule of the antigen. The binding reaction between the antibodies and antigen is considered to be a bimolecular reaction. Formation of a cyclic complex, consisting of MAb 5.1 and MAb 9.1 and two BSA molecules has been reported (Murphy, 1989), so that the binding reaction was accounted in the model. We also considered the binding reaction of antigen with monovalent antibody in solid-phase and labeled antibodies.

4.4.1. The model

In this section, we develop a model that describes equilibria of binding of solid-phase and labeled antibody with antigen. We assume that nonspecific interaction of antibodies and antigen with the solid support does not occur. Also we assume that all binding species are in reversible equilibrium at all times. The relations at equilibrium can be written as a set of equations. The list of symbols is in Table 8.

When divalent solid-phase antibody and divalent labeled antibody bind to antigen, the concentration of species at an equilibrium state can be described by the following equations:

$$[AG] = 2 K_1 [A] [G] \quad (13)$$

$$[BG] = 2 K_2 [B] [G] \quad (14)$$

where AG is the complex of solid-phase antibody (A) and antigen (G), and BG is the complex of labeled antibody (B) and antigen. The square brackets represent the concentration of each species in M. K_1 is the intrinsic equilibrium association constant for formation of an A-G bond in M^{-1} ; K_2 , for formation of a B-G bond in M^{-1} . The statistical factor 2 for the both equations is required to account for the two possible binding sites of antibodies.

The addition of a second antigen to AG and BG is described by the following equations:

$$[GAG] = 1/2 K_1 [AG][G] = K_1^2 [A][G]^2 \quad (15)$$

$$[GBG] = 1/2 K_2 [BG][G] = K_2^2 [B][G]^2 \quad (16)$$

where GAG is the complex of solid-phase antibody with two antigens, and GBG is the complex of labeled antibody with two antigens. We considered that the presence of a bound

Table 8. List of symbols for the modelling

Symbol	Definition
G ;	Antigen (mole/L)
G₀ ;	Total concentration of antigen (mole/L)
A ;	Divalent solid-phase antibody (mole/L)
A₀ ;	Total concentration of divalent solid-phase antibody (mole/L)
B ;	Divalent labeled antibody (mole/L)
B₀ ;	Total concentration of divalent labeled antibody (mole/L)
W ;	Monovalent solid-phase antibody (mole/L)
W₀ ;	Total concentration of monovalent solid-phase antibody (mole/L)
L ;	Monovalent labeled antibody (mole/L)
L₀ ;	Total concentration of monovalent labeled antibody (mole/L)
K₁ ;	Equilibrium association constant of solid-phase antibody (divalent and monovalent) (M⁻¹)
K₂ ;	Equilibrium association constant of labeled antibody (M⁻¹)
A_k ;	Cyclization factor (M)
a₁ ;	Polymerization factor for complex with two antibodies
a₂ ;	Polymerization factor for complex with more than two antibodies
a_w ;	Polymerization factor for complex with monovalent solid-phase antibody

antigen does not affect on the binding of a second antigen to an antibody. The statistical factor $1/2$ accounts for the single association site versus the two dissociation sites.

The binding of labeled antibody to antigen that is already bound to a solid-phase antibody can be described by the use of an intrinsic association constant and polymerization factor which represents the reduction in affinity. Because the solid-phase antibodies are immobilized on the surface, only one molecule of the antibody can join in the formation of complexes.

$$[AGB] = 2 a_1 K_2 [AG] [B] = 4 a_1 K_1 K_2 [A][B][G] \quad (17)$$

$$[GAGB] = K_1 [AGB] [G] = 4 a_1 K_1^2 K_2 [A][B][G]^2 \quad (18)$$

$$[AGBG] = K_2 [AGB] [G] = 4 a_1 K_1 K_2^2 [A][B][G]^2 \quad (19)$$

$$[GAGBG] = K_1 [AGBG] [G] = 4 a_1 K_1^2 K_2^2 [A][B][G]^3 \quad (20)$$

$$[BGAGB] = 1/2 2 a_1 K_2 [GAGB] [B] = 4 a_1 a_2 K_1^2 K_2^2 [A][B]^2 [G]^2 \quad (21)$$

$$[GBGAGB] = 2 K_2 [BGAGB] [G] = 8 a_1 a_2 K_1^2 K_2^3 [A][B]^2 [G]^3 \quad (22)$$

$$[GBGAGBG] = 1/2 K_2 [GBGAGB] [G] = 4 a_1 a_2 K_1^2 K_2^4 [A][B]^2 [G]^4 \quad (23)$$

where a_1 is the polymerization factor when a labeled antibody binds to the antigen bound to the solid-phase antibody, and a_2 is the polymerization factor when a labeled antibody binds to the antigen bound to complex that contains a solid-phase antibody and a labeled antibody. We assumed that binding affinity of an antigen onto an antibody in these complexes is the same as that onto the antibody alone.

A linear complex of AGBG or GAGB can form a cyclic complex. The equilibrium constant for cyclization is given by the equilibrium association constant multiplied by a cyclization factor. The equilibrium equations for the complex are

$$[cAGBG] = A_{ka} K_1 [AGBG] = 4 A_{ka} a_1 K_1^2 K_2^2 [A][B][G]^2 \quad (24a)$$

$$[cGAGB] = A_{kb} K_2 [GAGB] = 4 A_{kb} a_1 K_1^2 K_2^2 [A][B][G]^2 \quad (24b)$$

where $cAGBG$ is the cyclic tetramer complex, and A_{ka} and A_{kb} are the cyclization factors M . Because these two expressions must be equal, it follows that $A_{ka} = A_{kb} = A_k$.

To account for the presence of monovalent solid-phase antibody, we described the following binding reactions between monovalent solid-phase antibody and divalent labeled antibody:

$$[WG] = K_1[W][G] \quad (25)$$

$$[WGB] = 2a_w K_1 K_2 [W][B][G] \quad (26)$$

$$[WGBG] = 2a_w K_1 K_2^2 [W][B][G]^2 \quad (27)$$

where WG is the complex of monovalent solid-phase antibody (W) with antigen (G), and a_w is the polymerization factor when a labeled antibody binds to the antigen bound to the monovalent solid-phase antibody.

We also considered the binding reactions with monovalent labeled antibody (L). When the same polymerization factors as that for the divalent case are employed, the binding equilibria involving a monovalent labeled antibody are described as follows:

$$[GL] = K_2[G][L] \quad (28)$$

$$[AGL] = 2 a_1 K_1 K_2 [A][L][G] \quad (29)$$

$$[GAGL] = 2 a_1 K_1^2 K_2 [A][L][G]^2 \quad (30)$$

$$[LGAGL] = 1/2 a_1 K_1^2 K_2^2 [A][L]^2 [G]^2 \quad (31)$$

$$[BGAGL] = 4 a_1 a_2 K_1^2 K_2^2 [A][B][L][G]^2 \quad (32)$$

$$[GBGAGL] = 4 a_1 a_2 K_1^2 K_2^3 [A][B][L][G]^3 \quad (33)$$

$$[WGL] = a_w K_1 K_2 [W][L][G] \quad (34)$$

Assuming the total amounts of antibodies and antigen remain constant during the reaction, we can describe the following equations for the conservation of mass of antigen,

divalent solid-phase antibody, divalent labeled antibody, monovalent solid-phase antibody and monovalent labeled antibody:

for antigen,

$$\begin{aligned}
 [G_0] = & [G] + [AG] + [BG] + [AGB] + 2([GAG] + [GBG] + [GAGB] + [AGBG] + \\
 & [BGAGB] + c[AGBG]) + 3([GAGBG] + [GBGAGB])+ 4 [GBGAGBG] + [WG] + [WGB] \\
 & + 2[WGBG] + [GL] + [AGL] + [GAGL] + 2[LGAGL] + 2[BGAGL] + 3[GBGAGL] \\
 & + [WGL]
 \end{aligned} \tag{35}$$

for solid-phase antibody (divalent)

$$\begin{aligned}
 [A_0] = & [A] + [AG] + [AGB] + [GAG] + [GAGB] + [AGBG] + [BGAGB]+ c[AGBG] \\
 & + [GAGBG] + [GBGAGB] + [GBGAGBG] + [AGL] + [GAGL] + [LGAGL] \\
 & + [BGAGL] + [GBGAGL]
 \end{aligned} \tag{36}$$

for labeled antibody (divalent)

$$\begin{aligned}
 [B_0] = & [B] + [BG] + [AGB] + [GBG] + [GAGB] + [AGBG]+ c[AGBG] + [GAGBG] \\
 & + 2[BGAGB] + 2[GBGAGB] +2 [GBGAGBG] + [WGB] + [WGBG] \\
 & + [BGAGL] + [GBGAGL]
 \end{aligned} \tag{37}$$

for solid-phase antibody (monovalent)

$$[W_0] = [W] + [WG] + [WGB] + [WGBG] + [WGL] \tag{38}$$

for labeled antibody (monovalent)

$$\begin{aligned}
 [L_0] = & [L] + [GL] + [AGL] + [GAGL] + 2[LGAGL] + [BGAGL] + [GBGAGL] \\
 & + [WGL]
 \end{aligned} \tag{39}$$

Concentrations of each species were obtained by using a Newton-Raphson iteration procedure with the total concentration of antigen and each antibody taken to be the

initial value of the free concentration. Appendix A describes the basics of Newton-Raphson method and the computer program. To help the convergence of the calculated values, we took the absolute value of species after one iteration. If the absolute value is larger than the possible maximum value with the given antigen and antibody concentrations, we replaced it with the maximum for the next iteration. We considered the iteration to be converged when the absolute values of the difference between the calculated values of each species was less than 10^{-14} moles/L, and when the sum of the absolute values of the difference between the initial and calculated values of each antibody and antigen was less than 10^{-14} moles/L. The model was programmed in FORTRAN and the program was run on an MicroVax 2000 computer. In most cases the calculation converged after 3-7 iterations.

Simulation results are presented by plotting the amount of labeled antibodies bound to the solid support as a function of antigen concentration added. The bound amount was calculated the difference between total labeled antibody (monovalent and divalent) minus the labeled antibody in the liquid. The relation is described as follows:

$$[\text{Bound Labeled antibody}] = [L_0] + [B_0] - [L] - [LG] - [B] - [BG] - [GBG] \quad (40)$$

4.4.2. Model prediction vs. experimental data

To determine whether the model has the ability to predict assay's dose-response, we compared the simulation results with experimental data. The experimental data were from the assay results with solid-phase MAb 9.1 (8.8×10^{-8} moles/L or 17.6×10^{-8} moles/L) and labeled MAb 5.1 (6.25×10^{-8} moles/L) which are presented in Figure 19. Here, the solid-phase antibody (MAb 9.1) is presented by a concentration of active binding sites on beads which was determined with the equilibrium binding experiments in section 4.3.

For the simulation, the model requires values for equilibrium binding parameters. We obtained them from the independent measurements and from the literatures, which are listed in Table 9. For example, the equilibrium association constant for solid-phase

Table 9. List of parameters and their values for simulation¹

$$(2 \times A_0 + W_0) = 8.8 \times 10^{-8} \text{ M or } 17.6 \times 10^{-8} \text{ M}$$

$$(B_0 + L_0) = 6.5 \times 10^{-8} \text{ M}$$

$$K_1 = 1.1 \times 10^8 \quad \text{M}^{-1}$$

$$K_2 = 1.4 \times 10^8 \quad \text{M}^{-1}$$

$$A_k = 7.6 \times 10^{-8} \quad \text{M}$$

$$a_1 = 0.1$$

$$a_2 = 0.18$$

$$a_w = 0.1$$

1. Value for kinetic parameters including K_2 , A_k , a_1 and a_2 are from the work of Murphy (1989).

MAb 5.1 ($1.1 \times 10^8 \text{ M}^{-1}$) was obtained from an equilibrium binding experiment. We assumed that half of binding sites of solid-phase MAb 9.1 is monovalent; justification of this assumption is described in the discussion section. The equilibrium association constant for the labeled MAb 5.1 ($1.4 \times 10^8 \text{ M}^{-1}$) was considered to be the same as that from the equilibrium binding reaction of unlabeled MAb 5.1 with BSA (Murphy, 1989); we assumed that after being tagged with a fluorophore the labeled MAb 5.1 is 100 % active and divalent.

For further simplification in the simulation, we assumed that the value of the polymerization factor (a_w) for monovalent antibody is the same as that (a_1) of divalent antibody. The value of polymerization factors ($a_1 = 0.1$ and $a_2 = 0.18$) and of the cyclization factor ($A_k = 7.6 \times 10^{-8} \text{ M}$) used were from the binding reaction of the same antibodies (MAb 5.1 and 9.1) with BSA in the liquid (Murphy, 1989).

Simulation results with the above parameters indicate that the model predicts the assay's dose-response well, and the observed linear dose-response may have resulted from the cyclic complex formation. In Figure 36, with value for cyclization factor of $A_k = 7.6 \times 10^{-8} \text{ M}$ and solid-phase antibody concentration of $8.8 \times 10^{-8} \text{ M}$, a dose-response curve from the simulation generally predicts the experimental data. However, the curve does not give a linear response with a slope of 0.44 M/M over the dynamic range that we observed in the experiments in section 4.2. This discrepancy may be because the value of cyclization factor used ($7.6 \times 10^{-8} \text{ M}$) may differ from the actual value. It has been reported that values of cyclization factor for other systems of antibody/protein binding on solid surface are higher than the one we used: those values in the literatures are in the range of 10^{-6} M to 10^{-5} M (Archer and Krakauer, 1977; Schweitzer-stenner *et al.*, 1987). With these higher values the simulation better fits the experimental data over the dynamic range (Figure 36), but at high concentration of antigen the model predicts more bound labeled antibody than that is observed experimentally. Figure 37 shows a simulation result using the same kinetic parameters but with the amount of solid-phase antibody, doubled to $17.6 \times 10^{-8} \text{ M}$. The model with high cyclization factor (10^{-6} M and 10^{-5} M) predicts linear response over the dynamic range which

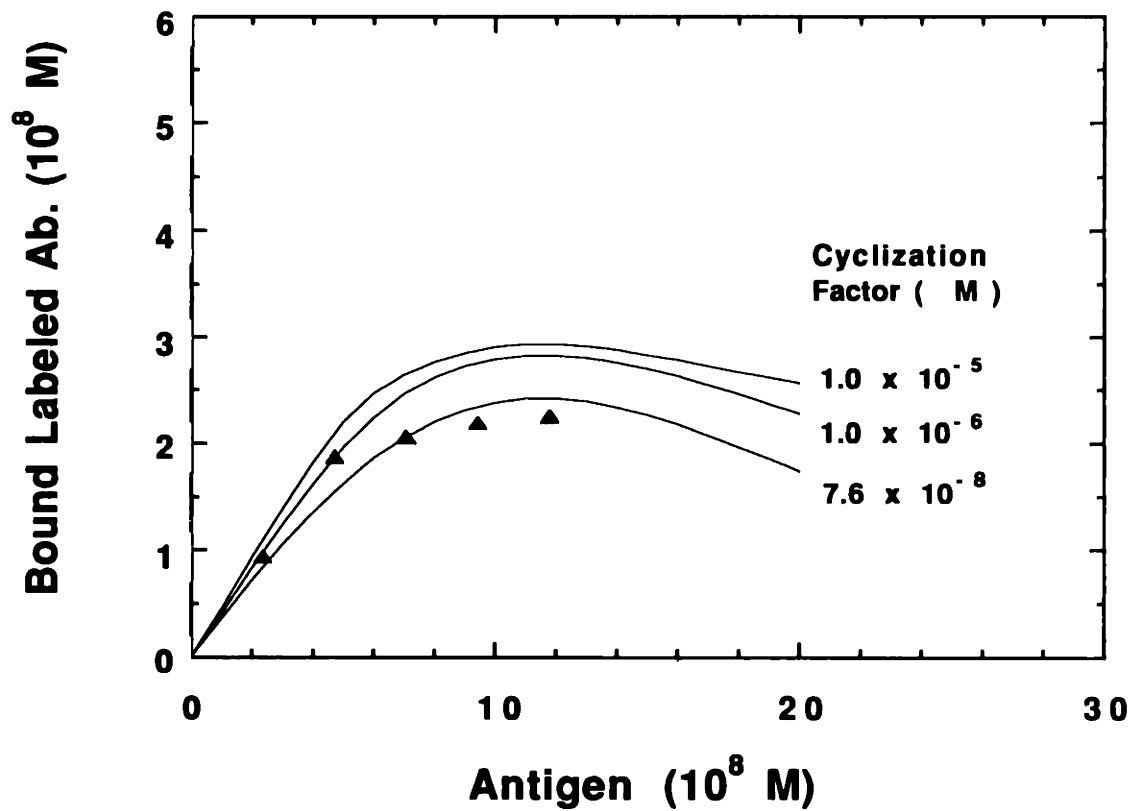


Figure 36. Model prediction vs. experimental data, with an assay system employing solid-phase MAb 9.1 (8.8×10^{-8} M) and labeled MAb 5.1 (6.25×10^{-8} M). Values for equilibrium binding parameters for the modelling are listed in Table 9. Experimental data (▲) were from the assay result in Figure 19.

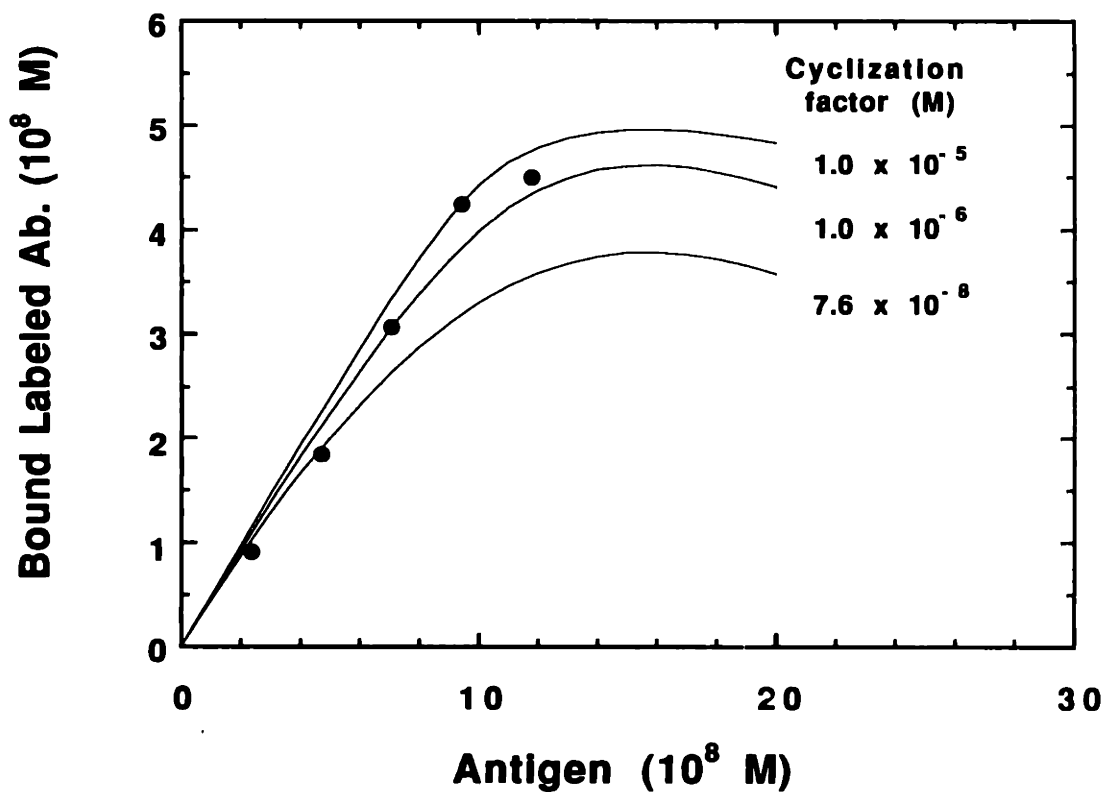


Figure 37. Model prediction vs. experimental data, with an assay system employing solid-phase MAb 9.1 (17.6×10^{-8} M) and labeled MAb 5.1 (6.25×10^{-8} M). Values for equilibrium binding parameters are listed in Table 9. Experimental data (●) were from the assay result in Figure 19.

fits the experimental data in a good agreement. In addition, the simulation fits the data in the regime of excess antigen concentration.

4.4.3. Characterization of assay performance by simulation

Simulations with the model have been performed to identify the effects on assay performance by various factors. Our objective is to predict dose-response, dynamic range and sensitivity at a given assay condition.

4.4.3.1 Effects on dose-response by cyclization factor

In the previous simulation in section 4.4.2., we observed that the extent of cyclic complex formation significantly affects the shape of assay's dose-response. This observation leads us to investigate the effects of different values of A_k (cyclization factor) on assay dose-response. For the simulations, we used the same values for equilibrium binding parameters as those listed in Table 9. Concentrations of bound labeled antibody at a range of A_k were calculated when both solid-phase and labeled antibodies have an equal equilibrium constant of 10^9 or 10^8 M^{-1} .

The effects of increased value of cyclization factor on dose-response depends on the level of antibody affinity. Using a value of 10^9 M^{-1} for the equilibrium constants K_1 and K_2 , the simulation results with $A_k = 0$ (this is, the case with no cyclization) has a dose-response curve that is parabolic and concave downward (Figure 38). At low concentration of antigen, the ratio of bound labeled antibody to added antigen is close to 1 because of the high affinity; most added antigen makes a univalent linkage between a labeled antibody and solid-phase antibody. As the antigen concentration increases the ratio of bound labeled antibody to the added antigen decreases, because the binding sites of solid-phase and labeled antibodies are getting saturated. When added antigen concentration is in excess compared to the binding sites of antibodies, the concentration of bound labeled antibody decreases as the antigen

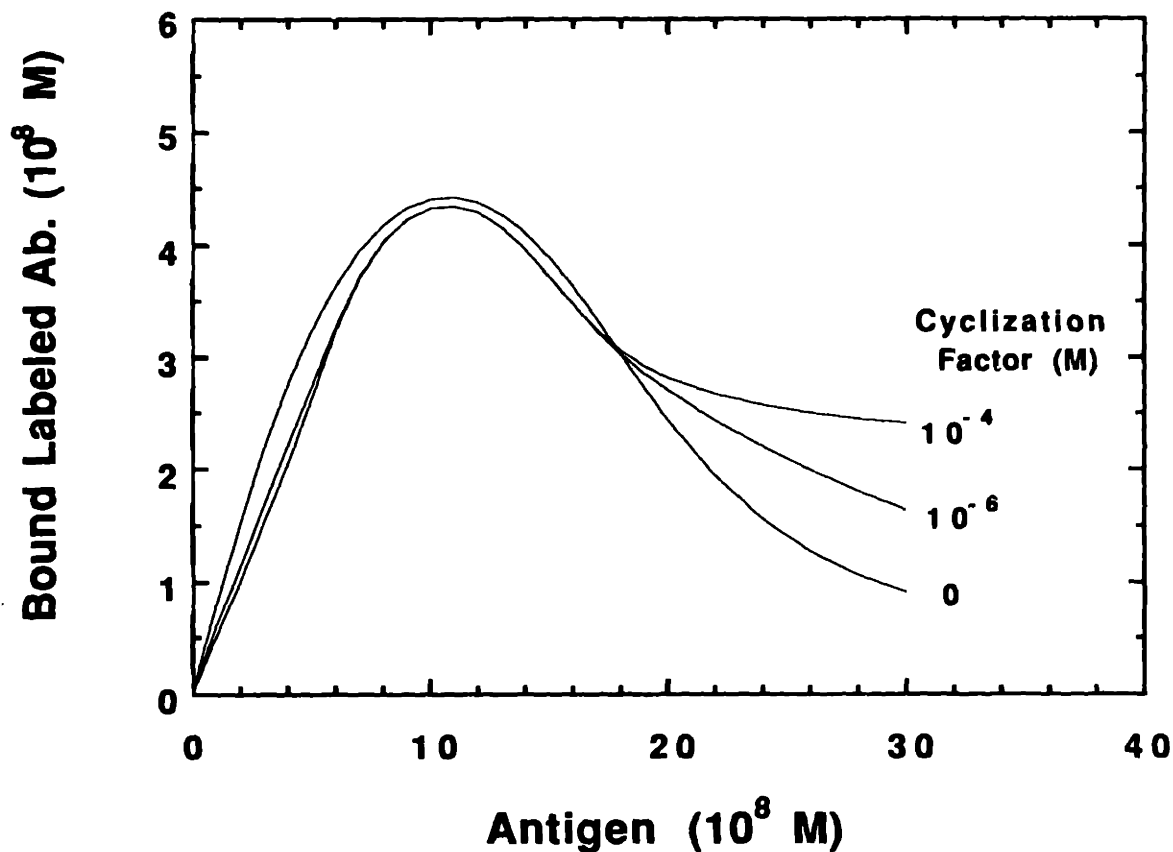


Figure 38. Effects of the value of cyclization factor on dose-response at a high value of equilibrium constants. K_1 and K_2 are 10^9 1/M. The assay system employs a solid-phase antibody (8.8×10^{-8} M) and labeled antibody (6.25×10^{-8} M). The used values for other equilibrium binding parameters were the same as those listed in Table 9.

concentration increases. This is because the saturation in binding sites of antibodies with antigen reduces the formation of complex, solid-phase antibody/antigen/labeled antibody.

Increasing A_k leads to a linear dose-response over the dynamic range with a slope of 0.5 (Figure 38). At high A_k , most bound labeled antibody is in the form of a cyclic complex, in which the ratio of labeled antibody to antigen is 0.5. In addition, the amount of bound labeled antibody at high values of A_k was more than that at low A_k in the region of excess antigen concentration. This is because the stronger binding in cyclic complex formation leads to a higher value of labeled antibody on the solid support.

At an equilibrium constant of 10^8 M^{-1} and at $A_k = 0$, the bound labeled antibody per added antigen is small. Because of low binding affinity only a small portion of the added antigen is involved in the binding of labeled antibody on solid support (Figure 39). As A_k increases, the ratio of bound labeled antibody to added antigen proportionally increases to 0.5. At a high value of A_k , most of added antigen forms cyclic complex with antibodies because of the increased avidity, and the bound labeled antibody amount at excess antigen remains fairly constant (Figure 39).

The above simulation results indicates that a higher value of cyclization factor leads to more labeled antibody bound to solid-phase antibody as a cyclic complex at both high and low antibody affinities. As a result, the dose-response became linear at low antigen concentrations with a slope close to 0.5. The value of cyclization factor significantly affects the slope of dose-response, but it did not change the dynamic range.

4.4.3.2. Effects of affinity of antibody on assay performance

The effect of different values of K_1 and K_2 on the dose-response of the assay was investigated. The concentrations of bound labeled antibody at a range of values of K_1 and K_2 were calculated from the model in two different cases: when a cyclic complex forms ($A_k = 10^{-5} \text{ M}$) and when a cyclic complex does not form ($A_k = 0 \text{ M}$).

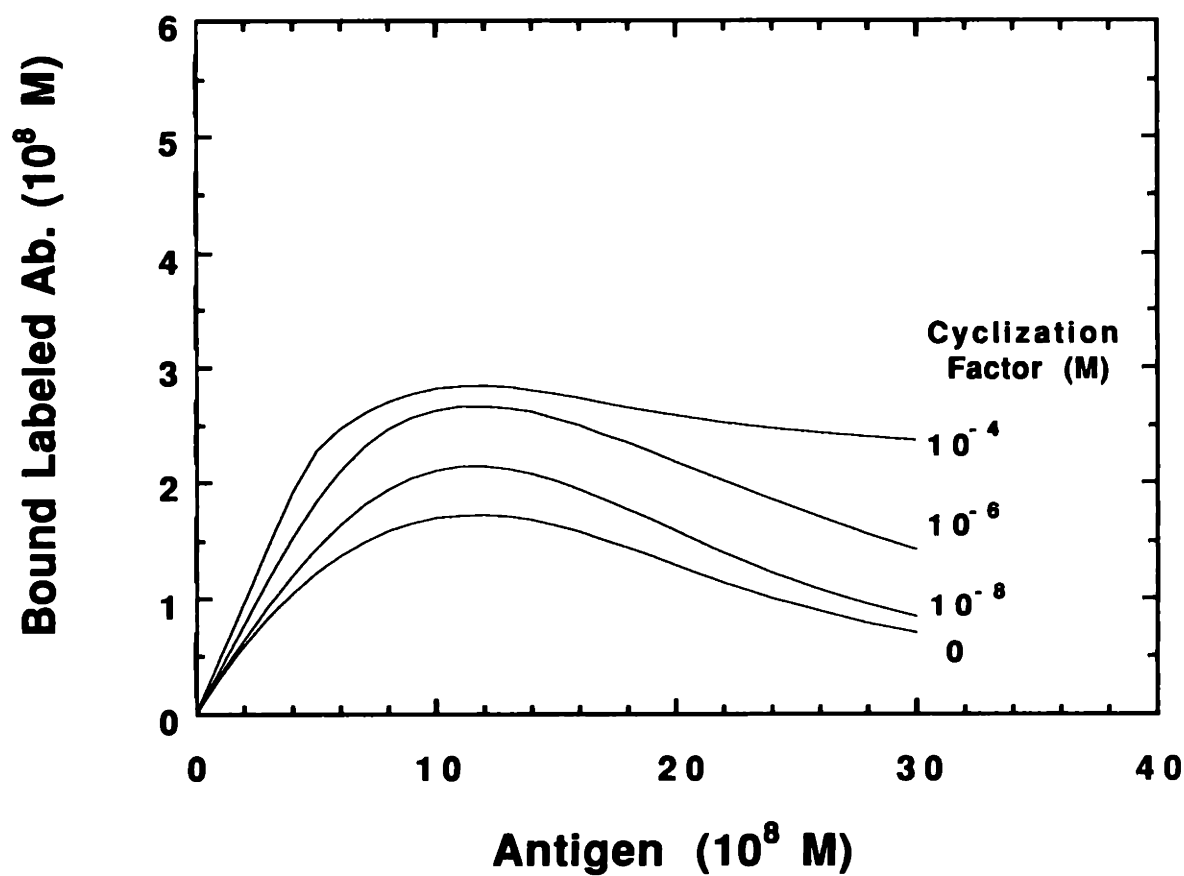


Figure 39. Effects of the value of cyclization factor on dose-response at a low value of equilibrium constants. K_1 and K_2 are 10^8 M^{-1} . Others are the same as in Figure 38.

Figure 40 shows the results of the $A_K = 10^{-5}$ M. A higher value of antibody affinity gives higher signal changes at a given antigen concentration. As K_1 and K_2 increase, the bound labeled antibody per added antigen increases to 0.5. At all values of antibody affinity, the concentration of bound labeled antibody at excess antigen reaches about 2.2×10^{-8} M, which is the concentration of the divalent solid-phase antibody used for this simulation. This is because of the high avidity of formation of the cyclic complex.

Simulation results at $A_K = 0$ show effects of the changes in antibody affinity similar to those at $A_K = 10^{-5}$ M, except that the concentration of bound labeled antibody reaches zero more quickly as the antigen concentration increases (Figure 41). As K_1 and K_2 increase, the ratio of bound labeled antibody to the added antigen proportionally increases. The slope of dose-response at low concentration strictly depends on the value of antibody affinity, because the binding reactions themselves mainly depend on their affinities. At all values of affinity, no changes in dynamic range were observed (Figure 41).

4.4.3.3. Effects of monovalent antibody on assay performance

Effects of the value of monovalent labeled antibody fraction on the assay performance was also investigated when cyclic complex forms with high affinity ($A_K = 10^{-5}$ M). With 50% of monovalent solid-phase antibody, the bound labeled antibody at various values of monovalent labeled antibody fraction was calculated. When the monovalent fraction of labeled antibody is less than 40 %, the assays offer an almost identical shape of dose-response to that from the assay employing no monovalent labeled antibody (Figure 42). The shape of the assay's dose-response seems to mainly depend on the extent of formation of cyclic complex. When the concentration of divalent labeled antibody is higher than of divalent solid-phase antibody, the simulation gives almost identical dose-response. As the monovalent fraction further increases, the ratio of bound labeled antibody dramatically decreases because of the lower concentration of divalent antibody. As the result, the region of linear dose-response also slightly decreases.

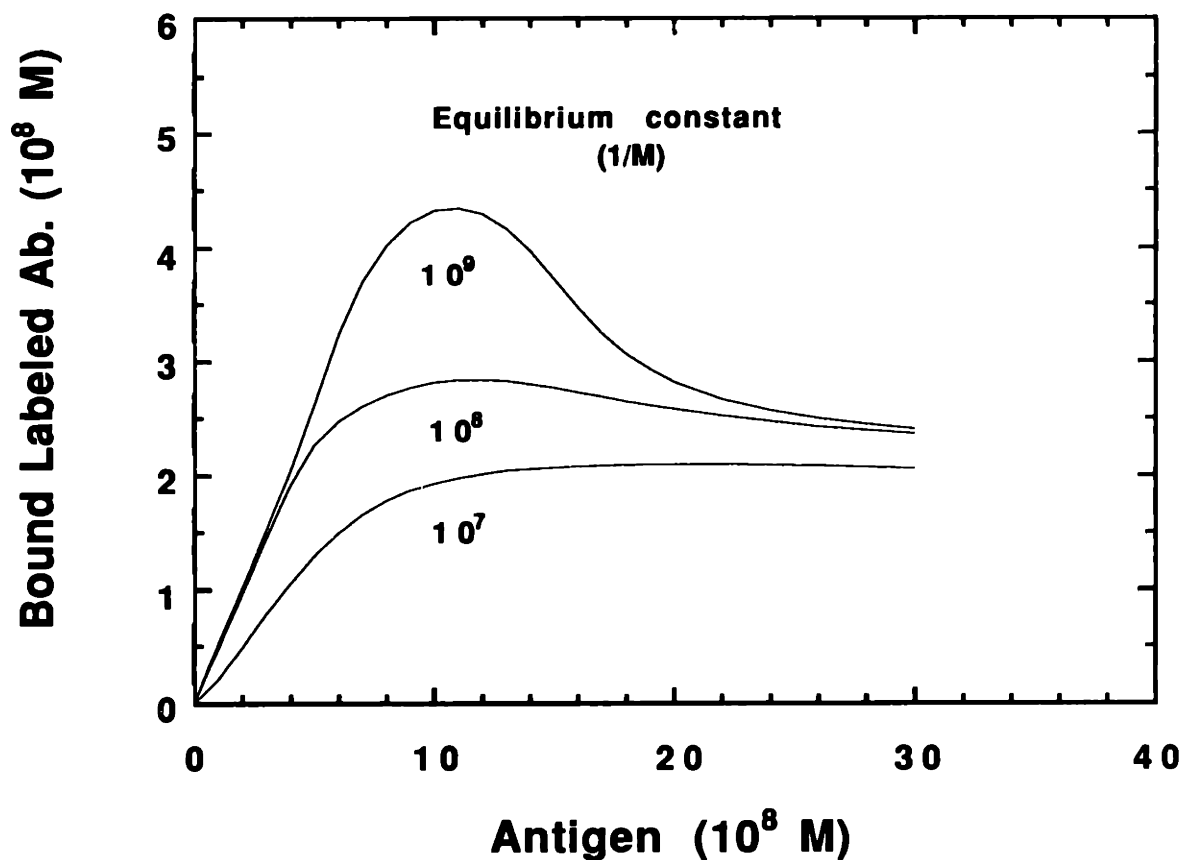


Figure 40. Effects of the value of equilibrium constant on assay performance when the cyclic complex forms with a high degree. The cyclization factor (A_k) is 10^{-5} M. The assay system employs solid-phase antibody (8.8×10^{-8} M) and labeled antibody (6.25×10^{-8} M). The used values for other equilibrium binding parameters were the same as those listed in Table 9.

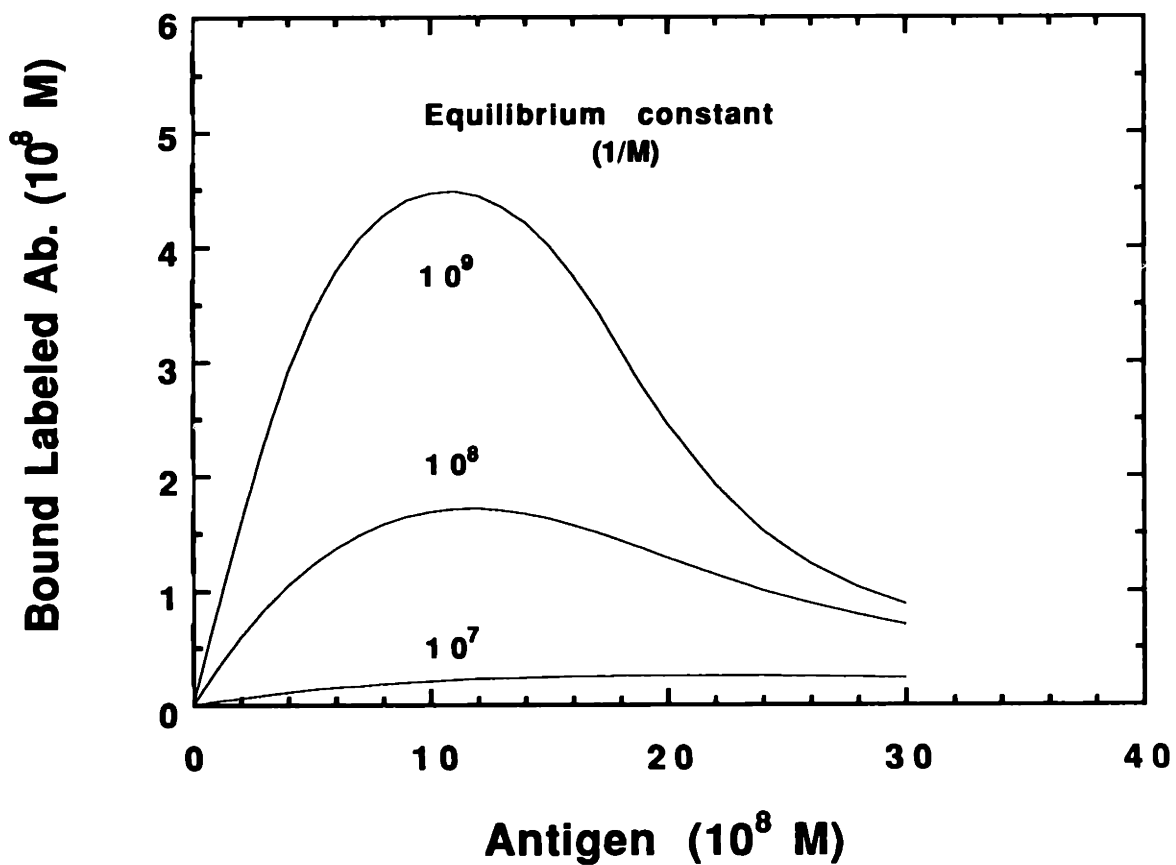


Figure 41. Effects of the value of equilibrium constant on assay performance when no cyclic complex forms. The cyclization factor (A_k) is 0 M. The assay system employs solid-phase antibody (8.8×10^{-8} M) and labeled antibody (6.25×10^{-8} M). The used values for other equilibrium binding parameters were the same as those listed in Table 9.

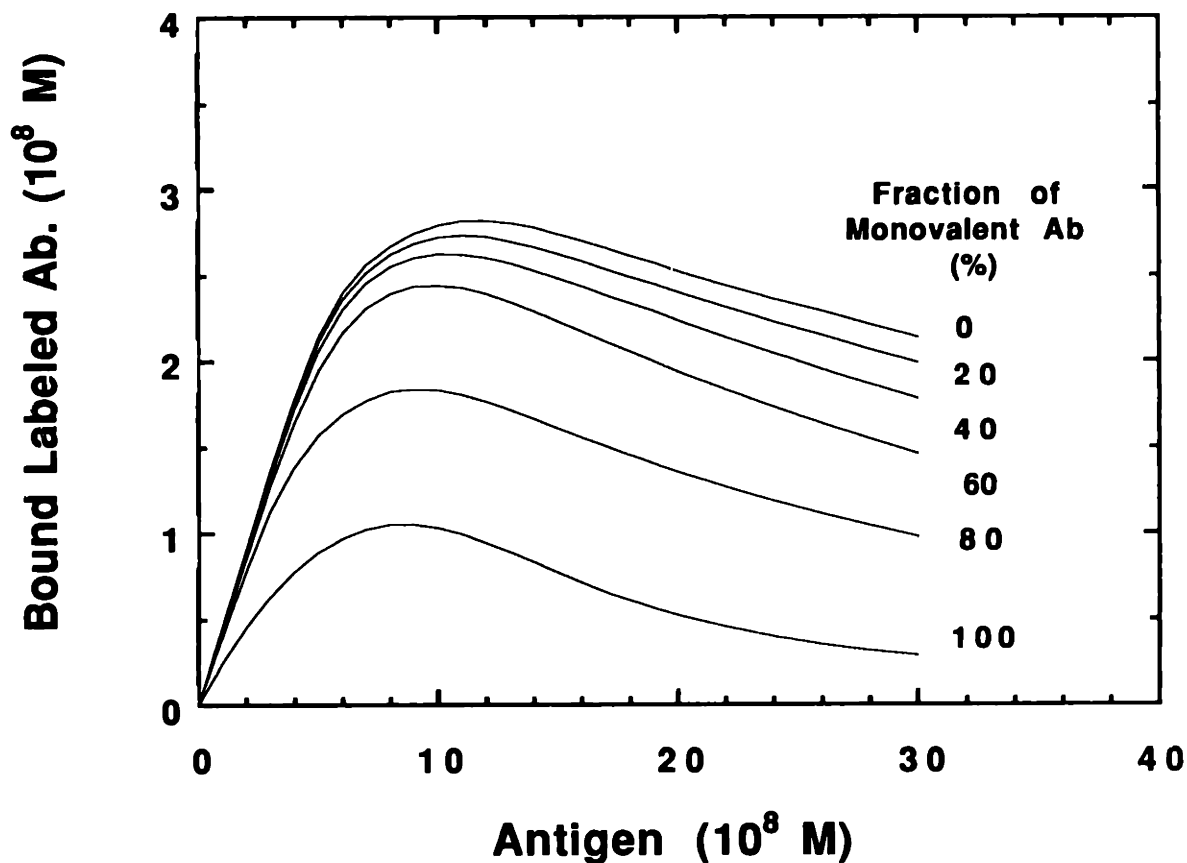


Figure 42. Effects of monovalent fraction in the labeled antibody on the assay performance. The cyclization factor (A_K) is 10^{-5} M. The assay system employs a solid-phase antibody (8.8×10^{-8} M) with 50% of monovalent fraction and a labeled antibody (6.25×10^{-8} M). The used values for other equilibrium binding parameters were the same as those listed in Table 9.

4.4.3.4. Sensitivity of measurement

The estimation of low detection limit at a given assay condition was investigated. Sensitivity of assay is defined as the lowest concentration of antigen at which the measured signal is bigger than the standard deviation of error from the control. The errors are associated with experimental procedures, including the measurement of fluorescence from liquid. At low antigen concentration, the bound labeled antibody concentration is obtained by subtracting two large numbers (total and free concentration) from each other, so that the lowest antigen detection limit depends on the error in fluorescence measurement.

A mathematical expression for the lowest detection limit is developed. We assumed that the ratio of standard deviation to the total fluorescence is constant at all concentrations of labeled antibody. The detection error at one antibody concentration can be described as the follows:

$$\text{detection error} = \Delta s \times B_0 \quad (41)$$

where Δs is the standard deviation of measurement in %, and B_0 is the labeled antibody in M. The signal changes, represented as the concentration of bound labeled antibody on solid support, at antigen concentration of G_L is also written as follows:

$$\text{Signal Changes} = q \times G_L \quad (42)$$

where q is the slope of dose-response at G_L in M/M.

If a measured signal change at a given antigen is n times larger than the detection error, we consider that the value is detectable. From the Equations. 41 and 42, the lowest concentration detectable can be written as follows:

$$G_L = n \Delta s B_0 / q \quad (43)$$

This expression indicates that the limit of lowest detection depends on the concentration of labeled antibody, the ratio of bound labeled antibody to added antigen at low antigen concentration, and the relative standard deviation. Higher antibody affinity usually offers higher value of q so that the assay with an antibody with high affinity can detect a lower limit of antigen concentration. On the other hand, effects of the concentration of labeled antibody on the sensitivity depends on the assay conditions. If an increase in antibody concentration increases q to a ratio of greater than 1, then the sensitivity increases. Otherwise, there is no increase in sensitivity with increasing antibody concentration.

4.4.4 Discussion

In this work, a mathematical model has been developed to better understand assay performance. This model which incorporates the formation of cyclic complex in the binding reaction, predicts the linear response which is observed in the dose response of the assay with MAb 5.1 and MAb 9.1. In assays with two different solid-phase antibody concentrations, the model in general predicts the assay's dose-response in agreement with the experimental data.

The value for cyclization factor (A_k) that offers a good fit with experimental data may be at best only a crude estimate because the simulation was performed with a value of polymerization factor that was obtained from the binding reaction in liquid. The value may be different from that of the binding reaction on a solid surface because the solid surface may induce a steric hinderance effect on the antibody/antigen binding.

If divalent antibodies attached to the surface form cyclic complexes with a high tendency, then the polymerization constant of the monovalent antibodies on the surface may be less than that of the divalent antibodies. To form a cyclic complex, the position of two binding sites of two antibodies must be close in space to each other. Because one of the Fab fragments of the monovalent antibody is immobilized on the solid support, the binding of a labeled antibody on to the BSA already bound to the other binding site can be inhibited by space

restriction. Because the inactive binding site of monovalent antibody is located close to the solid surface, one of the binding sites of the labeled antibody may also be forced to come close to the surface. The closeness to the solid surface may cause a space restriction which results in reduced overall affinity in the binding reaction.

The assumption of the presence of a fraction of monovalent antibodies in the solid-phase and in the population of labeled antibody comes from the possibility of denaturation of the antibody during its modification of immobilization and fluorophore labelling. It is also known that some fraction of monoclonal antibodies has a myeloma light chain so that the antibody become monovalent. It is known that MAb 9.1 and 5.1 are divalent (Murphy, 1989). During immobilization, however, the activity of one IgG binding site can be lost so that a fraction of solid-phase antibody become monovalent. The same mechanism can cause the formation of monovalent labeled antibody.

It is probable that half of the solid-phase antibody binding sites are monovalent (Figure 43). This fraction can be calculated with the following assumptions: (1) the active antibody on solid support is attached by a single covalent linkage, (2) the possibility of the presence of a linkage on the one of Fab (P_{ab}) or Fc (P_c) chains is one third; because the size of Fab and Fc is almost same, the randomness of immobilization leads to an equal probability for each fragment. (3) the binding activity on Fab is lost when the Fab fragment is linked on the solid support; as a result, the antibody molecule bound to solid support with Fab is monovalent ($N_{ab} = 1$) while the antibody bound to solid support with Fc is divalent ($N_c = 2$). The fraction of monovalent antibody can be described as $(2 \times N_{ab} \times P_{ab}) / (2 \times N_{ab} \times P_{ab} + N_c \times P_c)$, which results in a value of 0.5.

Effects of the extent of cyclic complex formation on assay performance depends on the affinity of the antibodies in the assay. At low antibody affinity, formation of a cyclic complex increases the ratio of bound labeled antibody per added antigen, so that the measured concentration of antigen can be more accurate. In addition, formation of a cyclic complex increases the sensitivity so that the lower concentration of antigen can be determined. On the

Antibody on Beads

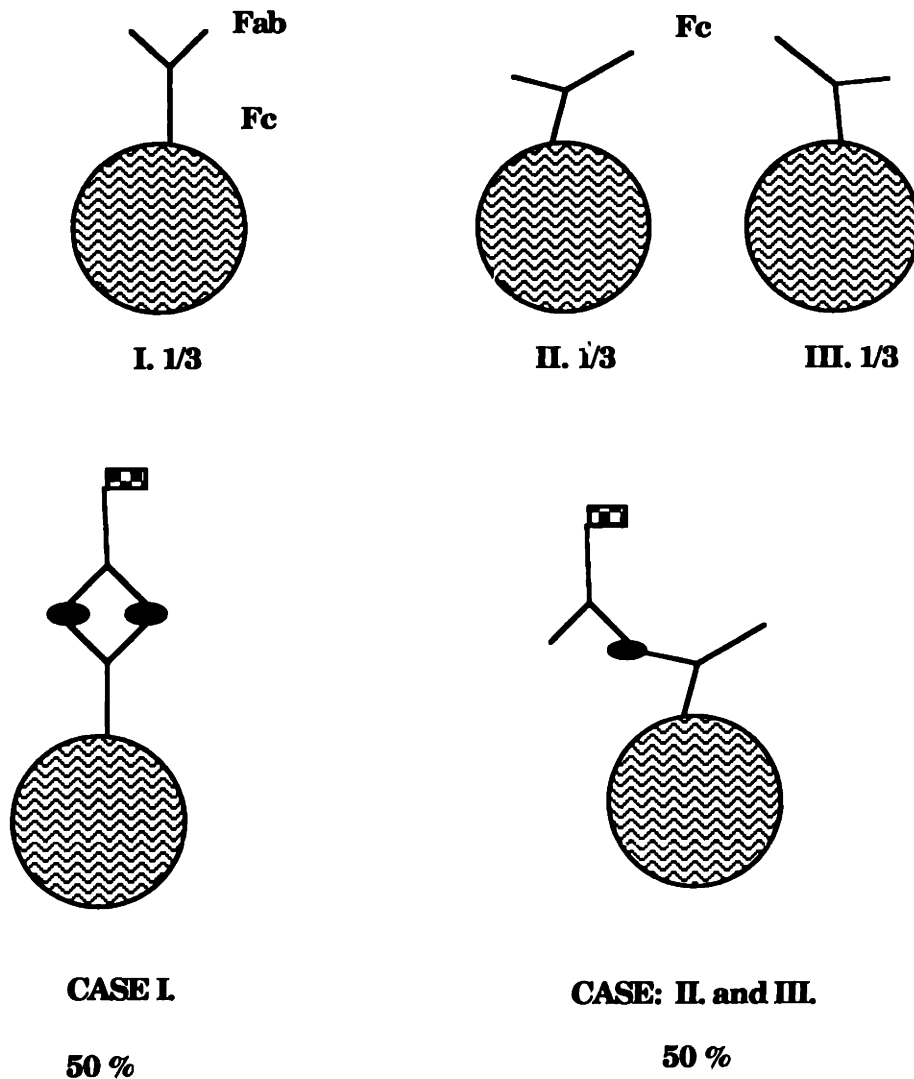


Figure 43. Schematic of the presence of monovalent and divalent antibody on the solid support. A. The fraction of the divalent antibody is one third and the monovalent fraction is two third. B. The each fraction represents 50% of binding sites.

other hand, at high antibody affinity, the complex formation leads to a decrease in ratio of labeled antibody to added antigen, and therefore lower sensitivity and accuracy is expected.

Our model did not account for the possibility that some of the antigen and labeled antibodies may be nonspecifically adsorbed on the solid support. The nonspecific interaction of the labeled antibody is less than 1 % of the total labeled antibody; this value was observed during three different sets of control experiments for fluorescein labeled antibody. On the other hand the nonspecific interaction of the rhodamine labeled antibodies was found to be about 3 - 6 %. These nonspecifically bound antibodies contribute to the background noise of the assay.

The dynamic range of the assay at a given condition depends mainly on the amount of antibody. Results of the simulation indicate that the dose response of assay depends on the degree of cyclization, affinity and presence of monovalent antibody. However, such parameters have almost no effect on the changes in dynamic range. The dynamic range in general proportionally increases as the amount of antibody when the other antibody (solid-phase or labeled) is in sufficient amount. Particularly, when the cyclic complex formation dominates the binding mechanism of labeled antibody on to the solid-phase antibody, the dynamic range is linear as a function of antibody concentration.

In selection of the antibodies for the assays, we must consider whether the antibodies form a tetramer with antigens if the available antibodies are low in affinity. At low affinity the formation of tetramer increases the binding avidity of the labeled antibody on to the solid-phase antibody because of two binding linkages. The formation of cyclic complex formation can increase the sensitivity of the assay and possibly extend the dynamic ranges linearly. The greater the tendency to form cyclic complexes, the more sensitive the assay, and the greater the concentration of antigen required to reach the region of antigen excess.

In summary, the assay performance of two monoclonal antibodies to an antigen depends on a variety of factors, including the intrinsic association constant for the binding, the cyclization factor, and the relative ratio of the cyclization factor to the equilibrium constant.

The dynamic range does not change with changes in the equilibrium parameters. The bound labeled antibody concentration is obtained by subtracting two large numbers (total and free concentration) from each other, so that the lowest antigen detection depends on the error in the fluorescence measurement.

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V. SUMMARY AND CONCLUSIONS

A novel immunoassay procedure, which is easier to use than existing ones, has been studied for its use in protein measurement. A two-site (sandwich) immunofluorometric assay that was already available was selected and a novel format of the assay in which the number of assay steps and time were substantially reduced was developed. The assay is based on using small-sized nonporous beads (0.5 μm diameter) as a solid support and measuring the unbound fraction of labeled antibody in the liquid. These simplified assay procedures include only one incubation and one separation before measuring fluorescence from the liquid phase. The ability to expand dynamic range avoids the need for sample dilution or preparation. It is expected, therefore, one can perform the assay rapidly and with minimal effort.

Feasibility Studies with polyclonal antibody assay system

To test the feasibility of the novel format of two-site immunofluorometric assay, we developed an assay employing polyclonal antibodies for human IgG and mouse IgG, and the results are:

1. The observed results of the assay after a standard incubation time of 30 minutes are reproducible and accurate, as the fluorescence signal reaches constant values after that incubation time. Nonspecific adsorption of the labeled antibody on the beads is less than 1 % of the initial concentrations of the fluorescein labeled antibody. The changes in fluorescence intensity from the liquid, therefore, results from the formation of a complex of labeled antibody/antigen/solid-phase antibody on the solid supports.

2. The dynamic range of the assays is increased by the use of higher concentration of both antibodies. The dynamic range was 0 - 40 $\mu\text{g}/\text{mL}$ of human IgG or 0 - 40 $\mu\text{g}/\text{mL}$ of mouse IgG with a detection limit of 0.2 $\mu\text{g}/\text{mL}$ of IgG or less, and the further expansion of the range is feasible. Such a wide detection range eliminates the dilution that is often required when measuring a high antigen concentration with the other assays.

3. Environmental factors such as pH, ionic strength, protein concentration and serum do not affect the binding reaction of the assay. Ionic strength at concentrations up to 40 mM phosphate buffer (600 mM NaCl), BSA concentration up to a concentration of 8%, and calf serum up to 100% did not interfere with fluorescence measurement from the liquid. The pH, however, did indeed change the fluorescence intensity due to the sensitivity of fluorescein to pH.

4. The reaction mixture of antibody in a freeze-dried form offered reproducible, quantitative results as did the non freeze-dried form. This indicates that it is possible to prepare the reaction mixture in advance and to allow it for long term storage. The dose-response with the freeze-dried reaction mixture was similar to that with the non-freeze-dried mixture, but the sensitivity for the low concentration of antigen is less than in the freeze-dried case.

In summary we have shown that the simplified two-site immunofluorometric assay offers quantitative, reproducible results. The operational steps include only one incubation and one separation step. This simplified assay does not require any washing and resuspending of the solid support. Use of submicron size non-porous beads reduces the required incubation time substantially. The ability to expand dynamic range and its insensitivity to sample condition reduces the need for sample dilution or preparation. The freeze-dried form of the assay mixture offers reproducible, quantitative results. It is possible to prepare the reaction mixture in advance and to allow for long term storage. Our next objective was to develop the assay with monoclonal antibodies and characterize it more quantitatively.

Monoclonal antibody assay system

As a model assay system, monoclonal antibodies to BSA which have different binding specificity on a BSA molecule have been chosen. MAb 9.1 and 6.1 were used as the solid-phase antibody after being immobilized on the polystyrene beads. MAb 5.1 was used as the labeled antibody, after being tagged with TRITC. The results for the assay performance showed that:

1. The required incubation time for the assay is two minutes, which is the time required for the rapid binding reaction of the antibodies and antigen to reach an equilibrium state. From a comparison with the calculation for diffusion rate, the binding reaction of the assay is apparently kinetically controlled. The rapidity in binding reaction is because of the use of submicron size non-porous beads which reduce the mass-transfer limitation and because of the rapid intrinsic association.

2. Assays with both solid-phase MAb 9.1 and MAb 6.1 yield quantitative results. The detection range of the assays was 0 - 14 $\mu\text{g/mL}$ of BSA, and further expansion in range is expected with both solid-phase and labeled antibodies present in sufficient quantities. The inter-assay variability for assay with MAb 9.1 and 5.1 was in a range 1.9 % to 10.4%; the intra-assay variability measured was 2.3%. The estimated detection limit of the assay is 0.2 $\mu\text{g/mL}$ of BSA (3×10^{-9} M).

3. For the assay with MAb 9.1 and 5.1, the molecular ratio of the bound antigen to the labeled antibody was two to one over the dynamic range. This ratio was independent from changes in the concentration of antigen and antibodies. One explanation of the above observation is that MAb 9.1 and 5.1 form a circular complex on the solid support. In the circular complex the antibodies and the antigen are bound each other with a high avidity.

4. The physically adsorbed antibody gave a similar assay performance as the covalently bound antibody, but the preparation of the physical adsorbed antibody demanded extensive washing of the beads. If the beads were not washed completely, the response of the assay was less sensitive to the added antigen.

The two-site immunofluorometric assay was developed by using two sets of monoclonal anti-BSA (MAb 6.1 & 5.1; MAb 9.1 & 5.1). The assay can be carried out with one two-minute incubation, without any washing and sample dilution. From statistical studies of the assay performance with covalently immobilized 9.1 antibody and 5.1 labeled antibody, it was found that the assay performance is accurate and reproducible. We expanded the scope of

the study to better understand the general mechanisms of binding of an immobilized antibody with a protein antigen.

Binding kinetics

In this study, binding kinetics with nonporous beads (0.5 μm) as a solid support was studied. After confirming that the binding reaction is kinetically controlled, we measured the association rate, dissociation rate and equilibrium constant at various temperatures. By using these measurements we determined the order of the binding reaction and the intrinsic values of the kinetic constants.

1. The observed reaction rates show that binding between ^{125}I -BSA and immobilized MAb 9.1 is kinetically controlled. The initial association rate increased with increases in surface antibody concentration, as expected for a kinetic controlled reaction, and the association rates were at least 100 times slower than the calculated antigen mass-transfer rate. In addition, the calculated value of Da with the measured antibody binding capacity and association rate constant at 25 °C is 0.036, which means the kinetic binding is the rate controlling step.

2. The present study of the reaction rates and equilibrium illustrates the binding reaction of ^{125}I -BSA to immobilized MAb 9.1 generally follows bimolecular reaction kinetics. The initial association reaction is of second order; the rate was proportionally dependent on the antibody concentration as well as antigen concentration. The initial rate of the dissociation followed first order kinetics. The equilibrium constant determined by Scatchard analysis agrees well with the calculated from the association and dissociation rate constants which were measured independently.

3. The measured activation energy for the association rate constant was 9 Kcal/mole. The dissociation of the antigen/antibody complex has an activation energy of 2 Kcal/mole, and is insensitive to changes in temperature. The fact that the activation energy is greater than that for diffusional processes, which are characterized by activation energies of

less than 4 Kcal/mole, supports the conclusion that BSA-MAb 9.1 binding is kinetically controlled.

In this study, we demonstrated the small size non-porous beads offer binding which is essentially free of mass transfer limitations. The observed reaction rates show that the binding between ^{125}I -BSA and immobilized MAb 9.1 is kinetically controlled. The measured activation energy for association indicates the reaction is kinetically controlled. From the measurements of intrinsic binding rates and constants, the binding of ^{125}I -BSA and immobilized MAb 9.1 at solid surface followed a bimolecular reaction kinetics. The theoretical prediction on the assay performance was performed in order to provide predictive conditions for assays with other proteins.

Modelling of assay performance

To study the performance of the assay more quantitatively we developed a mathematical model which describes its dose-response. The model employs a series of binding relations between the solid-phase antibody, labeled antibody and antigen to describe the multiple equilibrium reactions. The model also includes a polymerization factor for the formation of complexes which consist of more than two antibodies. The results are:

1. This model, which incorporates the formation of cyclic complex in the binding reaction, predicts the linear response which is observed in the dose response of the assay with MAb 5.1 and MAb 9.1. In assays with two different solid-phase antibody concentrations, the model in general predicts the assay's dose-response in agreement with the experimental data. The value for cyclization factor (A_K) that offers a good fit with experimental data may be at best only a crude estimate because the simulation was performed with a value of polymerization factor that was obtained from the binding reaction in liquid.

2. Effects of the extent of cyclic complex formation on assay performance depends on the affinity of the antibodies in the assay. At low antibody affinity, formation of a cyclic complex increases the ratio of bound labeled antibody per added antigen, so that the

measured concentration of antigen can be more accurate. In addition, formation of a cyclic complex increases the sensitivity so that the lower concentration of antigen can be determined. On the other hand, at high antibody affinity, the complex formation leads to a decrease in ratio of labeled antibody to added antigen, and therefore lower sensitivity and accuracy is expected.

3. The dynamic range of the assay at a given condition depends mainly on the amount of antibody. Results of the simulation indicate that the dose response of assay depends on the degree of cyclization, affinity and presence of monovalent antibody. However, such parameters have almost no effect on the changes in dynamic range. The dynamic range in general proportionally increases as the amount of antibody when the other antibody (solid-phase or labeled) is in sufficient amount. Particularly, when the cyclic complex formation dominates the binding mechanism of labeled antibody on to the solid-phase antibody, the dynamic range is linear as a function of antibody concentration.

4. At low affinity the formation of tetramer increases the binding avidity of the labeled antibody on to the solid-phase antibody because of two binding linkages. The formation of cyclic complex formation can increase the sensitivity of the assay and possibly extend the dynamic ranges linearly. The greater the tendency to form cyclic complexes, the more sensitive the assay, and the greater the concentration of antigen required to reach the region of antigen excess.

In summary, the assay performance of two monoclonal antibodies to an antigen depends on a variety of factors, including the intrinsic association constant for the binding, the cyclization factor, and the relative ratio of the cyclization factor to the equilibrium constant. The dynamic range does not change with changes in the equilibrium parameters. The bound labeled antibody concentration is obtained by subtracting two large numbers (total and free concentration) from each other, so that the lowest antigen detection depends on the error in the fluorescence measurement.

In conclusions, we have shown that the simplified two-site immunofluorometric assay using small sized nonporous beads as a solid support offers quantitative, reproducible results. The operational steps including only one incubation and one separation step can be performed simply and rapidly. The binding between BSA and immobilized MAb 9.1 at the surface is kinetically controlled, and follows a bimolecular reaction kinetics. The model employing a series of bimolecular binding relations between the solid-phase antibody, labeled antibody and antigen predicts the observed assay performances well.

VI. RECOMMENDATIONS FOR FUTURE STUDY

We have demonstrated that the simplified two-site immunofluorometric assay offers quantitative, reproducible results and the assay procedures can be performed rapidly and simply. One research area is the use of the assay concepts to measure proteins other than IgG and BSA. In that assay development, the feasibility of the use of one kind of polyclonal antibody for the both solid-phase and labeled antibodies needs to be explored. This is because some proteins with no subunits or distinctive domains can not produce polyclonal antibodies which only bind to distinctive sites on the proteins. Other variation of the assay is the use of polyclonal antibody as solid-phase antibody and monoclonal antibody with labeled antibody.

The environmental factors such as pH, ionic strength, proteins and serum in general do not affect the binding reaction of the polyclonal antibody assay system. The assay systems, however, have not been tested with 'real' samples including nonpretreated blood, urine and fermentation broth. Whether the current assay will produce accurate and reproducible results under 'real' conditions is yet to be determined.

The assay results from using the reaction mixture of antibody in a freeze-dried form showed that the sensitivity for the low concentration of antigen is less than in the soluble form. A possible reason for this is that the physically adsorbed antibodies detach from the beads during freeze-drying and enter the liquid reaction mixture. Ways to avoid this detachment of the antibody through freeze-drying need to be studied.

The antibody concentration required for each measurement is much higher than the antigen to be measured. In addition, after reagent preparations only fraction of antibodies is active, particularly for solid-phase antibody; more than 70 % of activity of initial antibody was lost during immobilization. For the economic antibody usage, therefore, more efficient immobilizing technique of antibodies on the solid supports is needed.

The study of the reaction rates and equilibrium illustrates that the binding reaction of ^{125}I -BSA to immobilized MAb 9.1 at the solid surface generally follows bimolecular reaction kinetics. These experiments, however, were done in an one specific condition: one monoclonal antibody and one immobilization method. To verify whether these bimolecular kinetics govern other immobilized antibody binding with a protein antigen, further studies are needed. One is to measure the reaction rate and equilibrium with an immobilized antibody prepared with different immobilization techniques. This study will clarify whether the measured binding kinetics is an intrinsic property of the antibody independent of the immobilization method used. Further, in the study of dissociation experiments, we observed that about 7% of the total antibody binding sites was not desorbed in the dissociation. The nature of this dissociation needs to be investigated.

The automation of the developed assay for the rapid detection of one or multiple proteins is another research area. The automated assay may be particularly useful for monitoring purpose in processes. The automation would require an auto sampler combined with a detector. In addition, possibility of the multiple analyte detection needs to be explored.

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APPENDIX A.

Numerical program

Program PARK.FOR was used for model simulations in chapter 4.4. The program uses a Newton-Raphson iterative technique (Press *et al.*, 1986) to solve Equations (13) - (39) which describe the equilibrium relationships and mass balances simultaneously. At a given initial antigen concentration, the concentrations for antibodies, antigen and complexes in the equilibrium relationships were calculated. Basics of Newton-Raphson method (Press *et al.*, 1986) and PARK.FOR are described.

Functions with variables x_i , $i = 1, 2, \dots, N$ are described as follows:

$$f_i(x_1, x_2, \dots, x_N) = 0 \quad i = 1, 2, \dots, N. \quad (44)$$

If X denote the entire vector of values x_i , each of the functions f_i is expanded in Taylor series

$$f_i(X + \delta X) = f_i(X) + \sum_{j=1}^N \frac{\partial f_i}{\partial x_j} \delta x_j + O(\delta X^2) \quad (45)$$

By neglecting terms of order δX^2 and higher, we obtain a set of linear equations for the correction δX that move each function closer to zero simultaneously;

$$\sum_{j=1}^N \alpha_{ij} \delta x_j = \beta_i \quad (46)$$

where

$$\alpha_{ij} \equiv \frac{\partial f_i}{\partial x_j} \quad \text{and} \quad \beta_i \equiv -f_i \quad (47)$$

Values of δx_i can be obtained from the matrix equation (46) by the use of the inversion of the Jacobian matrix, $|\alpha_{ij}|$. The new values of the variables (x_i) are obtained by adding values of δx_i to the old value of x_i .

$$x_i^{\text{new}} = x_i^{\text{old}} + \delta x_i, \quad i = 1, 2, \dots, N. \quad (48)$$

The process above is iterated to convergence.

In the simulations with PARK.FOR, the total concentration of antigen and each antibody was taken to be the initial value of the free concentration. We considered the iteration to be converged when the absolute values of the difference between the calculated values of each species was less than 10^{-14} moles/L, and when the sum of the absolute values of the difference between the initial and calculated values of each antibody and antigen was less than 10^{-14} moles/L.

Program for model simulations

Program name: PARK.FOR

```
C-----*
C   FILE NAME = ANTIGEN.FOR : APR 1990      *
C-----*
C-----*
C-----*
C
C   Required Input Parameters:              *
C
C   K1;   Equilibrium association constant of solid-phase antibody (divalent *
C         and monovalent) in 1/M          *
C   K2;   Equilibrium association constant of labeled antibody (divalent and *
C         monovalent) in 1/M             *
C   AK;   Cyclization factor in M        *
C   A0;   Total concentration of divalent solid-phase antibody in M        *
C   B0;   Total concentration of divalent labeled antibody in M           *
C   G0;   Total concentration of antigen in M                             *
C   W0;   Total concentration of monovalent solid-phase antibody in M      *
C   L0;   Total concentration of monovalent labeled antibody in M         *
C   BK;   Polymerization factor for complex with two antibodies:          *
C         the same as  $a_1$  and  $a_w$       *
C   CK;   Polymerization factor for complex with more than two antibodies: *
C         the same as  $a_2$               *
C-----*

C-----*
C   List of variables                      *
C
C   X(1) ; G                               *
C   X(2) ; A                               *
C   X(3) ; B                               *
C   X(4) ; AG                             *
C   X(5) ; BG                             *
C   X(6) ; GAG                             *
C   X(7) ; GBG                             *
C   X(8) ; AGB                             *
C   X(9) ; GAGB                             *
C   X(10) ; AGBG                             *
C   X(11) ; GAGBG                             *
C   X(12) ; BGAGB                             *
C   X(13) ; GBGAGB                             *
C   X(14) ; GBGAGBG                             *
C   X(15) ; cAGBG                             *
C   X(16) ; W                               *
C   X(17) ; WG                              *
C   X(18) ; WGB                              *
C   X(19) ; WGBG                             *
C   X(20) ; L                               *
C   X(21) ; GL                              *
```

```

C      X(22) ; AGL                      *
C      X(23) ; GAGL                     *
C      X(24) ; LGAGL                    *
C      X(25) ; BGAGL                    *
C      X(26) ; GBGAGL                   *
C      X(27) ; WGL                      *
C
C      G; antigen                       *
C      A; Divalent solid-phase antibody *
C      B; Divalent labeled antibody     *
C      W; Monovalent solid-phase antibody *
C      L; Monovalent labeled antibody   *
C-----*

```

PROGRAM PARK.FOR

```

PARAMETER (NP=27)
REAL*8 X(NP)
REAL*8 K1,K2,AK,A0,B0,W0,BK,L0,CK
CHARACTER TITLE*80

```

```

READ(5,'(A80)') TITLE
READ(5,*)
READ(5,*) K1,K2,AK,A0,B0,W0,BK,L0,CK
READ(5,*)
READ(5,*) N,MAXIT,TOLX,TOLF

```

```

C-----*
C      N; Number of variables           *
C      MAXIT; Maximum number of iteration *
C      TOLX; Summed variable increments *
C      TOLF; Summed fuction values      *
C-----*

```

```

READ(5,*)
READ(5,*) (X(I),I=1,N)

```

```

WRITE(*,'(A80)') TITLE
WRITE(*,'(5X," K1 = ",1PE12.5)') K1
WRITE(*,'(5X," K2 = ",1PE12.5)') K2
WRITE(*,'(5X," AK = ",1PE12.5)') AK
WRITE(*,'(5X," A0 = ",1PE12.5)') A0
WRITE(*,'(5X," B0 = ",1PE12.5)') B0
WRITE(*,'(5X," W0 = ",1PE12.5)') W0
WRITE(*,'(5X," BK = ",1PE12.5)') BK
WRITE(*,'(5X," L0 = ",1PE12.5)') L0
WRITE(*,'(5X," CK = ",1PE12.5)') CK

```

```

WRITE(6,'(A80)') TITLE
WRITE(6,'(5X," K1 = ",1PE12.5)') K1
WRITE(6,'(5X," K2 = ",1PE12.5)') K2
WRITE(6,'(5X," AK = ",1PE12.5)') AK

```

```

WRITE(6,'(5X," A0 = ",1PE12.5)') A0
WRITE(6,'(5X," B0 = ",1PE12.5)') B0
WRITE(6,'(5X," W0 = ",1PE12.5)') W0
WRITE(6,'(5X," BK = ",1PE12.5)') BK
WRITE(6,'(5X," L0 = ",1PE12.5)') L0
WRITE(6,'(5X," CK = ",1PE12.5)') CK

```

```

WRITE(*,('(" NUMBER OF DATA? ")')
READ(1,*) NDATA
WRITE(*,('(" INITIAL ANTIGEN? ")')
READ(1,*) GX

```

```

WRITE(6,'(/5X," NUMBER OF DATA = ",I3)')NDATA
WRITE(6,'(/5X," INITIAL ANTIGEN(G0) = ",1PE12.5)')GX
WRITE(*,('(/1X," RESULTS "/",1X," ===== "/)')
WRITE(6,'(/1X," RESULTS "/",1X," ===== ")')

```

```

C WRITE(*,*) (X(I),I=1,N)

```

```

DO 11 J=1,NDATA

```

```

G0=GX*J

```

```

CALL MNEWT(MAXIT,X,N,TOLX,TOLF,K1,K2,AK,A0,B0,G0,W0,BK,L0,CK,IT)

```

```

WRITE(*,*)
WRITE(*,'(5(1X,1PE12.3))') (X(I),I=1,N)
WRITE(6,*)

```

```

C WRITE(6,'(5(1X,1PE12.3))') (X(I),I=1,N)

```

```

F=X(3)+X(5)+X(7)+X(20)+X(21)

```

```

WRITE(*,'(5X," G0 = ",1PE12.5,5X," F = ",1PE12.5,I10)')G0,F,IT
WRITE(6,'(5X," G0 = ",1PE12.5,5X," F = ",1PE12.5)') G0,F

```

```

11 CONTINUE

```

```

STOP
END

```

```

C=====
SUBROUTINE MNEWT(NTRIAL,X,N,TOLX,TOLF,K1,K2,AK,A0,B0,G0,W0,BK,
*L0,CK,IT)

```

```

C-----*
C Given an initial guess X for a root in N dimension, take NTRIAL *
C Newton-Raphson steps to improve the root. Stop if the root converges in either *
C TOLX or TOLF. *
C-----*

```

```

PARAMETER (NP=27)

```



```

REAL*8 X(NP),ALPHA(NP,NP),BETA(NP),INDX(NP)
REAL*8 K1,K2,AK,A0,B0,G0,W0,BK,L0,CK

IT=0
DO 13 K=1,NTRIAL
  CALL USRFUN(X,ALPHA,BETA,K1,K2,AK,A0,B0,G0,W0,BK,L0,CK,N)
  ERRF=0.
  DO 11 I=1,N
    ERRF=ERRF+DABS(BETA(I))
11  CONTINUE
  IF(ERRF.LE.TOLF)RETURN
  CALL LUDCMP(ALPHA,N,NP,INDX,D)
  CALL LUBKSB(ALPHA,N,NP,INDX,BETA)
  ERRX=0.
  DO 12 I=1,N
    ERRX=ERRX+DABS(BETA(I))
    X(I)=X(I)+BETA(I)
12  CONTINUE
  IF(ERRX.LE.TOLX)RETURN

C  WRITE(*,*) (X(I),I=1,N)

C-----*
C To help the convergence of the calculated values, we took the absolute value of species *
C after one iteration. If the absolute value is larger than the possible maximum value with *
C the given antigen and antibody concentrations, we replaced it with the maximum *
C for the next iteration. *
C-----*

DO 111 I=1,N
  X(I)=DABS(X(I))
111 CONTINUE

IF(X(1).GT.G0) X(1)=G0
IF(X(2).GT.A0) X(2)=A0
IF(X(3).GT.B0) X(3)=B0

IF(X(4).GT.A0) X(4)=A0
IF(X(4).GT.G0) X(4)=G0

IF(X(5).GT.B0) X(5)=B0
IF(X(5).GT.G0) X(5)=G0

IF(X(6).GT.A0) X(6)=A0
IF(X(6).GT.0.5*G0) X(6)=0.5*G0

IF(X(7).GT.B0) X(7)=B0
IF(X(7).GT.0.5*G0) X(7)=0.5*G0

IF(X(8).GT.B0) X(8)=B0
IF(X(8).GT.G0) X(8)=G0
IF(X(8).GT.A0) X(8)=A0

IF(X(9).GT.B0) X(9)=B0

```

IF(X(9).GT.0.5*G0) X(9)=0.5*G0
IF(X(9).GT.A0) X(9)=A0

IF(X(10).GT.B0) X(10)=B0
IF(X(10).GT.0.5*G0) X(10)=0.5*G0
IF(X(10).GT.A0) X(9)=A0

IF(X(11).GT.B0) X(11)=B0
IF(X(11).GT.G0/3.) X(9)=G0/3.
IF(X(11).GT.A0) X(11)=A0

IF(X(12).GT.0.5*B0) X(12)=B0
IF(X(12).GT.0.5*G0) X(12)=0.5*G0
IF(X(12).GT.A0) X(12)=A0

IF(X(13).GT.G0/3.) X(13)=G0/3.
IF(X(13).GT.0.5*B0) X(13)=0.5*B0
IF(X(13).GT.A0) X(13)=A0

IF(X(14).GT.0.5*B0) X(14)=B0
IF(X(14).GT.0.25*G0) X(12)=0.25*G0
IF(X(14).GT.A0) X(14)=A0

IF(X(15).GT.B0) X(15)=B0
IF(X(15).GT.0.5*G0) X(15)=0.5*G0
IF(X(15).GT.A0) X(15)=A0

IF(X(16).GT.W0) X(16)=W0

IF(X(17).GT.W0) X(17)=W0
IF(X(17).GT.G0) X(17)=G0

IF(X(18).GT.W0) X(18)=W0
IF(X(18).GT.G0) X(18)=G0
IF(X(18).GT.B0) X(18)=B0

IF(X(19).GT.W0) X(19)=W0
IF(X(19).GT.0.5*G0) X(19)=0.5*G0
IF(X(19).GT.B0) X(19)=B0

IF(X(20).GT.L0) X(20)=L0

IF(X(21).GT.L0) X(21)=L0
IF(X(21).GT.G0) X(21)=G0

IF(X(22).GT.L0) X(22)=L0
IF(X(22).GT.A0) X(22)=A0
IF(X(22).GT.G0) X(22)=G0

IF(X(23).GT.L0) X(23)=L0
IF(X(23).GT.0.5*G0) X(23)=0.5*G0
IF(X(23).GT.A0) X(23)=A0

IF(X(24).GT.A0) X(24)=A0

```
IF(X(24).GT.0.5*G0) X(24)=0.5*G0
IF(X(24).GT.0.5*L0) X(24)=0.5*L0
```

```
IF(X(25).GT.A0) X(25)=A0
IF(X(25).GT.0.5*G0) X(25)=0.5*G0
IF(X(25).GT.B0) X(25)=B0
IF(X(25).GT.L0) X(25)=L0
```

```
IF(X(26).GT.A0) X(26)=A0
IF(X(26).GT.G0/3.) X(26)=G0/3.
IF(X(26).GT.B0) X(26)=B0
IF(X(26).GT.L0) X(26)=L0
```

```
IF(X(27).GT.W0) X(27)=W0
IF(X(27).GT.G0) X(27)=G0
IF(X(27).GT.L0) X(27)=L0
```

```
C  WRITE(*,*) (X(I),I=1,N)
C  WRITE(*,*)
   IT=IT+1
13 CONTINUE
   RETURN
   END
```

```
C=====
```

```
  SUBROUTINE LUDCMP(A,N,NP,INDX,D)
```

```
  PARAMETER (NMAX=1000,TINY=1.0E-20)
  REAL*8 A(NP,NP),INDX(N),VV(NMAX)
```

```
  D=1.
  DO 12 I=1,N
    AAMAX=0.
    DO 11 J=1,N
      IF (ABS(A(I,J)).GT.AAMAX) AAMAX=ABS(A(I,J))
11  CONTINUE
    IF (AAMAX.EQ.0.) PAUSE 'Singular matrix.'
    VV(I)=1./AAMAX
12  CONTINUE
  DO 19 J=1,N
    DO 14 I=1,J-1
      SUM=A(I,J)
      DO 13 K=1,I-1
        SUM=SUM-A(I,K)*A(K,J)
13  CONTINUE
      A(I,J)=SUM
14  CONTINUE
  AAMAX=0.
  DO 16 I=J,N
    SUM=A(I,J)
    DO 15 K=1,J-1
      SUM=SUM-A(I,K)*A(K,J)
```

```

15  CONTINUE
    A(I,J)=SUM
    DUM=VV(I)*ABS(SUM)
    IF (DUM.GE.AAMAX) THEN
        IMAX=I
        AAMAX=DUM
    ENDIF
16  CONTINUE
    IF (J.NE.IMAX)THEN
        DO 17 K=1,N
            DUM=A(IMAX,K)
            A(IMAX,K)=A(J,K)
            A(J,K)=DUM
17  CONTINUE
        D=-D
        VV(IMAX)=VV(J)
    ENDIF
    INDX(J)=IMAX
    IF(A(J,J).EQ.0.)A(J,J)=TINY
    IF(J.NE.N)THEN
        DUM=1./A(J,J)
        DO 18 I=J+1,N
            A(I,J)=A(I,J)*DUM
18  CONTINUE
    ENDIF
19  CONTINUE
    RETURN
    END

```

C=====

```

SUBROUTINE LUBKSB(A,N,NP,INDX,B)

REAL*8 A(NP,NP),INDX(N),B(N)
II=0
DO 12 I=1,N
    LL=INDX(I)
    SUM=B(LL)
    B(LL)=B(I)
    IF (II.NE.0)THEN
        DO 11 J=II,I-1
            SUM=SUM-A(I,J)*B(J)
11  CONTINUE
        ELSE IF (SUM.NE.0.) THEN
            II=I
        ENDIF
    B(I)=SUM
12  CONTINUE
DO 14 I=N,1,-1
    SUM=B(I)
    IF(I.LT.N)THEN
        DO 13 J=I+1,N
            SUM=SUM-A(I,J)*B(J)
13  CONTINUE

```

```

    ENDIF
    B(I)=SUM/A(I,I)
14  CONTINUE
    RETURN
    END

```

```

C=====
  SUBROUTINE USRFUN(X,J,BETA,K1,K2,AK,A0,B0,G0,W0,BK,L0,CK,N)

```

```

  PARAMETER (NP=27)
  REAL*8 X(NP),J(NP,NP),BETA(NP)
  REAL*8 K1,K2,AK,A0,B0,G0,W0,BK,L0,CK

```

```

C-----*
C   COMPONENTS OF JACOBIAN MATRIX   *
C-----*

```

```

  DO 9 I=1,N
    DO 9 II=1,N
      J(I,II)=0.
9  CONTINUE

  J(1,1)=-2.*K1*X(2)
  J(1,2)=-2.*K1*X(1)
  J(1,4)=1.

  J(2,1)=-2.*K2*X(3)
  J(2,3)=-2.*K2*X(1)
  J(2,5)=1.

  J(3,1)=-0.5*K1*X(4)
  J(3,4)=-0.5*K1*X(1)
  J(3,6)=1.

  J(4,1)=-0.5*K2*X(5)
  J(4,5)=-0.5*K2*X(1)
  J(4,7)=1.

  J(5,2)=-2.0*K1*X(5)*BK
  J(5,5)=-2.0*K1*X(2)*BK
  J(5,8)=1.

  J(6,4)=-1.*K1*X(5)*BK
  J(6,5)=-1.*K1*X(4)*BK
  J(6,9)=1.

  J(7,4)=-1.*K1*X(5)*BK
  J(7,5)=-1.*K1*X(4)*BK
  J(7,10)=1.

  J(8,4)=-2.*K1*X(7)*BK
  J(8,7)=-2.*K1*X(4)*BK
  J(8,11)=1.

```

$J(9,5)=-0.5*K1*X(8)*CK$
 $J(9,8)=-0.5*K1*X(5)*CK$
 $J(9,12)=1.$

$J(10,7)=-2.*K1*X(8)*CK$
 $J(10,8)=-2.*K1*X(7)*CK$
 $J(10,13)=1.$

$J(11,7)=-1.*K1*X(10)*CK$
 $J(11,10)=-1.*K1*X(7)*CK$
 $J(11,14)=1.$

$J(12,4)=-1.*AK*K1*K2*X(5)*BK$
 $J(12,5)=-1.*AK*K1*K2*X(4)*BK$
 $J(12,15)=1.$

$J(13,1)=1.$
 $J(13,4)=1.$
 $J(13,5)=1.$
 $J(13,6)=2.$
 $J(13,7)=2.$
 $J(13,8)=1.$
 $J(13,9)=2.$
 $J(13,10)=2.$
 $J(13,11)=3.$
 $J(13,12)=2.$
 $J(13,13)=3.$
 $J(13,14)=4.$
 $J(13,15)=2.$
 $J(13,17)=1.$
 $J(13,18)=1.$
 $J(13,19)=2.$

$J(13,21)=1.$
 $J(13,22)=1.$
 $J(13,23)=2.$
 $J(13,24)=2.$
 $J(13,25)=2.$
 $J(13,26)=3.$
 $J(13,27)=1.$

$J(14,2)=1.$
 $J(14,4)=1.$
 $J(14,6)=1.$
 $J(14,8)=1.$
 $J(14,9)=1.$
 $J(14,10)=1.$
 $J(14,11)=1.$
 $J(14,12)=1.$
 $J(14,13)=1.$
 $J(14,14)=1.$
 $J(14,15)=1.$

* +X(21)+X(22)+2*X(23)+2*X(24)+2*X(25)+3*X(26)+X(27))
 BETA(14)=A0-((X(2)+X(4)+X(8)+X(6)+X(9)+X(10)+X(11)+X(12)
 * +X(13)+X(14)+X(15))
 * +X(22)+X(23)+X(24)+X(25)+X(26))
 BETA(15)=B0-((X(3)+X(5)+X(7)+X(8)+X(9)+X(10)+X(11)
 * +2*(X(12)+X(13)+X(14))+X(15)+X(18)+X(19))
 * +X(25)+X(26))

C-----*
 C MONOVALENT SOLID-PHASE ANTIBODY *
 C-----*

BETA(16)=K1*X(16)*X(1)-X(17)
 BETA(17)=2*BK*K2*X(17)*X(3)-X(18)
 BETA(18)=K2*X(18)*X(1)-X(19)
 BETA(19)=W0-((X(16)+X(17)+X(18)+X(19))
 * +X(27))

C-----*
 C LABELED MONOVALENT ANTIBODY *
 C-----*

BETA(20)=K2*X(1)*X(20)-X(21)
 BETA(21)=2*K1*X(2)*X(21)*BK-X(22)
 BETA(22)=K1*X(1)*X(22)-X(23)
 BETA(23)=0.5*K2*X(20)*X(23)*CK-X(24)
 BETA(24)=2*K2*X(3)*X(23)*CK-X(25)
 BETA(25)=K2*X(5)*X(23)*CK-X(26)

BETA(26)=BK*K2*X(16)*X(21)-X(27)

BETA(27)=L0-(X(20)+X(21)+X(22)+X(23)+2*X(24)+X(25)+X(26)+X(27))

RETURN
 END