MODELING OF IMMUNOLOGICAL REACTIONS

by

Sellapperumage Amarasiri Fernando

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF CHEMISTRY

In Partial Fulfillment of the Requirements
for the degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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The University of Arizona, 1991

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Sellapperumage Amarasiri Fernando

entitled Modeling of Immunological Reactions

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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SIGNED: S. Amarasiri Fernando

To my mother

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Table of Contents

Table of Contents -- continued

Table of Contents -- continued

Table of Contents -- continued

Table of Contents -- continued

page

Table of Contents -- continued

page

Table of Contents -- continued

page

List of Illustrations

List of Illustrations -- continued

List of Illustrations -- continued

List of Illustrations -- continued

List of Illustrations -- continued

List of Illustrations -- continued

Abstract

The optimization of the competitive binding and sandwich immunoassays was investigated in order to examine some commonly encountered experimental aberrations. Experimental and theoretical results were integrated to provide a better understanding of the causes of the "hook" or "prozone" effect.

The "hook" generates ambiguous results for the test sample in an immunoassay. The competitive binding assay manifests a low dose "hook" effect while the sandwich immunoassay demonstrates a high dose "hook" effect. Human growth hormone (hGH) having no repeating epitopes was examined as a model for the "hook" effect in the competitive binding assay. Three model analytes, hGH, the dimeric form of hGH (D-hGH, having a discrete number of repeating epitopes) and ferritin (multiple epitopes) with differing immunological properties were employed.

To elucidate the low dose "hook" effects in the competitive binding assay the interaction of two different monoclonal antibodies with hGH was investigated. The individual monoclonal antibodies show normal behavior in a competitive binding assay, but mixtures of antibodies demonstrate a "hook" attributed to formation of multicomponent complexes in solution. Size exclusion chromatography was employed identify higher molecular weight complexes. The experimental data were supported by theoretical models assuming a circular tetrameric complex formation.

The one-step sandwich immunoassay suffers from the "hook" effect irrespective of the analyte characteristics. Model analytes, hGH, D-hGH, and ferritin offer new insights into general guidelines for assay procedures allowing the analyst to quickly optimize assay conditions without *a priori* knowledge of the

immunological characteristics of the antibody or the antigen. Experimental and theoretical data show that the high capacity solid-phase antibodies shifted the "hook" to relatively higher analyte concentrations. The effect of the concentration of labeled antibody on assay response was examined theoretically.

The cause of the "hook" effect in two-step sandwich immunoassay is attributed to the desorption of the bound analyte most likely due to a conformational change after the labeled antibody interacts with the several epitopes of the analyte, hence the assay for hGH shows no "hook" effect. Two different protocols for D-hGH were implemented. These assays demonstrated a "hook" effect if the labeled antibody was allowed to interact with more than one epitope of the analyte. Multiple epitope interactions with the labeled antibody, as exemplified by ferritin, demonstrate the "hook" effect. The effect of the ferritin concentration, capture antibody and the labeled antibody was examined.

Chapter 1

1.1 Introduction

Immunoassays are rapidly replacing many other methods used to detect or quantitate biologically important substances. The high levels of sensitivity and specificity achieved with immunoassays result from the specific and high affinity binding of antibodies to antigens. In order to develop sensitive and specific assay methods, it is necessary to understand the basic principles and concepts in immunoassays. In this process, mathematical formulations of immunoassay reactions are useful to identify relationships between the various components and thus, to predict optimal procedures. This study resolves some of the fundamental analytical discrepancies related to competitive and sandwich type immunoassays.

An antibody is a member of the family of mildly glycosylated proteins called immunoglobulins. Each immunoglobulin is composed of equal numbers of heavy and light polypeptide chains. The constant regions of both heavy and light chains contain the antigenic markers that determine the isotype (class, subclass or light chain type) of an immunoglobulin. Humans and many common experimental animals such as mice, rabbits and goats contain the light chain isotopes, kappa and lambda. Five heavy chain class isotypes, IgG, IgM, IgD, IgA and IgE have also been identified in these animals of which IgG is the most common. The IgG molecule consists of two identical and independent binding sites and has an approximate molecular weight of 160 kDa. X-ray crystallographic studies carried out by Amit et al. (1985) have shown the antibody binding site to be concave at the interface between the light and heavy chain variable domains. A number of elegant reviews on immunoglobulins have appeared in the literature (Alzari et al., 1988; Barton, 1985; Gally, 1973; Nisonoff et al., 1975).

Monoclonal antibodies are produced by a single clone (Kohler and

Milstein, 1975) and, consequently, have the same specificity. Such antibodies are considered to be homogeneous. The monoclonal mouse IgG is the most common analytical reagent used in immunoassays. Polyclonal antibodies consist of a random distribution of monoclonal antibodies possessing similar but not identical characteristics.

An antigen is a substance to which the antibody binds, which may be as complex as a protein molecule or as simple as a small organic molecule. Antibody molecules recognize patches at the surface of an antigen called antigenic determinants or epitopes (Atassi, 1984). Epitopes are complementary to antibody combining sites or paratopes in shape and chemical properties. The antibody combining site corresponds in size to approximately 6 amino acids (Kabat, 1970) or monosaccharide units (Schechter, 1971). Crystallographic studies of a complex between lysozyme and its specific Fab fragment shows the epitope area to be 2 nm x 3 nm in size (Amit, 1985).

A continuous epitope is a linear peptide fragment of a protein, which binds to antibodies raised against the whole molecule (Van Regenmortel and Daney de Marcillac, 1988). It is known from the folding pattern of many globular proteins that no surface epitope region is likely to contain functionalities from a continuous stretch of residues. Thus, antibody molecules are likely to recognize a set of amino acids in the antigen that are not contiguous in the sequence, and have been brought together by the folding of the peptide chain or by the juxtaposition of two separate chains. Such an antigenic determinant is referred to as a "discontinuous" or "assembled" epitope, or alternatively, as a "conformational" epitope (emphasizing the folded character of the assemblage). It is generally assumed that the antibodies directed against a conformational epitope of a protein will not bind

to the unfolded peptide fragment derived from the corresponding part of a native molecule. However, the division between conformational-dependent and conformational-independent epitopes is artificial, because it is difficult to envision that a paratope would recognize a sequence of residues independently from its conformation. Antigens may have from one to several thousand antigenic determinants, which may be different from each other. Some of the epitopes in an antigen may be similar in sequence and are referred to as repeating epitopes.

Additional information on the binding sites of antigen-antibody complexes has been provided by X-ray crystallography using myoglobin, lysozyme and the influenza virus neuraminidase as model antigens (Colman et al., 1987). The studies on lysozyme revealed that sixteen of the amino acids in the lysozyme and seventeen amino acids of the Fab fragment of an antibody form a tightly packed complex. Since the X-ray structure of lysozyme itself is known, it is possible to verify that no major conformational changes occur in the lysozyme molecule during the interaction with the antibody. The locations of epitopes on lysozyme correlate with the most exposed regions of the protein surface. This interaction has been described as conforming to a "lock and key" model of antigen-antibody interaction, in which no structural changes occur in either the antibody or the antigen, apart from the movements of the amino acid side chains.

Some antigen-antibody interactions tend to exhibit conformational changes in the antigen, and possibly also in the antibody. The conformational changes specifically affect the folding of the antigen or the antibody which can stimulate or inhibit interaction with another molecule, causing either a binding enhancement or an inhibition of the complex. This phenomenon has been called "cooperativity". Recent findings have demonstrated that the initial association of one monoclonal antibody with an antigen gave rise to an increased affinity, so that subsequent

binding of the antigen with another antibody occurs more rapidly. This enhancement has been explained (Holmes and Parham, 1983) by several different mechanisms: (1) Formation of highly stable cyclic complexes of antigen and antibody against different antigenic sites (2) Recognition by the second antibody of an epitope expressed on the first antibody as a consequence of its binding to the antigen (3) Production of an allosteric change in the antigen, resulting from the binding of the first or "enhancing antibody". Such mechanisms modify the epitope recognized by the second antibody.

Antigens as well as antibodies may exist in many different forms so many different types of antigen-antibody interactions are possible. Monovalent haptens usually are well-defined organic molecules or small peptides of low molecular weight. A bivalent antibody can interact with two haptens if they do not interfere with each other during binding, however it is not possible for two antibodies to bind the same hapten. One antibody can exhibit bivalency with large antigen molecules, if the size of the antigen does not prohibit binding two molecules to the same antibody. Furthermore an antigen can bind with two antibodies, only if the two epitopes on the same molecule are sufficiently separated (Tzartos et al., 1981). Interaction between a bivalent antibody and a bivalent antigen can result in the formation of linear as well as cyclic complexes depending very much on the nature of the antigen. Moreover, an oligovalent antigen (typically a macromolecule) can include at least three kinds of interaction. If all epitopes of an antigen are unique, multiple interactions will require a specific antibody for each different epitope used in the reaction. If all the determinants are alike for the antigen (repeating epitopes) a single antibody is sufficient for more than one interaction. Finally, a macromolecule such as ferritin can have more than one type of repeating epitope,

so that a single antibody can react at more than one site and additional antibodies can also react at more than one site. A useful feature of these multiple interactions is that precipitating complexes can be formed.

The strength of the antigen-antibody interactions can be described by the term "affinity". Intrinsic affinity is used to describe the interaction of a monovalent hapten with the corresponding antibody. Functional affinity is used to describe the interaction of the antibody combining sites of an antibody molecule with the antigenic determinants on an oligovalent antigen. Avidity is often synonymously used with affinity to describe the antigen-antibody reactions quantitatively. When additional contributory factors such as antibody valence and/or antigenic valence are involved the term avidity should be applied.

Today, immunoassays enjoy an almost unprecedented popularity as methods of choice for quantitation. They have permeated many branches and disciplines of scientific investigation, especially in biologically related areas. As the use of immunoassays has proliferated, so has the variety.

The factors that have a bearing on the choice of immunoassay procedure include: nature of the analyte; analyte concentration, whether fixed or variable; type of sample; type of test (qualitative or quantitative); availability, quality and cost of reagents; and technical complexity. The quality of the antibody and the characteristics of the analyte are more relevant to the assay performance than the other factors.

This study is centered on the characteristics of analytes as they affect the performance of the immunoassay. The monoclonal antibodies are selected as predefined and homogeneous reagents to offer considerable control on the performance of an immunoassay. The obvious goal of an immunoassay is the development of a linear dose-response curve to quantitate the amount of the

analyte of interest. Some immunoassays such as competitive and sandwich immunoassays can give rise to a biphasic dose-response curve which is often referred to as the "hook" effect or the "prozone" effect. This biphasic response does increase the technical complexity of the analytical method and introduces possible ambiguities in the result. Chapter Two investigates the biphasic nature of competitive binding assays. Chapter Three studies one-step sandwich immunoassays. Chapter Four examines two-step sandwich immunoassays. Each assay is evaluated with well characterized analytes to develop strategies to reduce or eliminate discrepancies and to optimize the assay system.

Chapter 2

Fundamental Studies of the Low Dose "hook" Effect in a Competitive Homogeneous Immunoassay

2.1 Introduction

Immunoassays have proven to be extremely useful for sensitive and selective detection of analytes. One such technique, the competitive binding assay, has been widely applied in biomedical research and clinical chemistry after its initial introduction by Berson and Yalow (1959). Since then, mathematical and statistical theories for the competitive binding assay have been developed (Rodbard and Bertino, 1973). Some of these theories are, however, not useful in predicting the behavior of practical assays.

Competitive binding assays sometimes exhibit the low dose "hook" effect in which there is a paradoxical increase in the calibration curve of the assay with increasing analyte concentration. As a consequence of this, samples containing very low analyte concentrations give ambiguous results. This phenomenon was observed for the adrenocorticotropic hormone (ACTH) antisera (Matsukura et al., 1971; Matsuyama et al., 1971). Twelve different antihuman ACTH antisera showed this effect (Matsukura et al., 1971). Recently, some other antibodies to biologically active molecules have exhibited this phenomenon: human Chorionic Gonadotropin (hCG), luteinizing hormone-releasing hormone (LH-RH), 3,5,3′-triiodo-L-thyronine (T_3), L-thyroxine (T_4) and Human Ia (Weintraub et al., 1973; Arimura et al., 1973; Carayon and Carella, 1974; Tosi et al., 1981). These reports all indicated that the "hook" was related to the multivalence of the antibody.

The "hook" effect has also been attributed to antigen-induced conformational changes in the antibody binding sites leading to increased antibody

affinity (i.e., cooperativity). These conclusions were drawn by assuming that non-independent binding sites exist on the antibody molecule (Weintraub et al., 1973; Carayon and Carella, 1974). Since then, a more comprehensive understanding of the effect has been obtained using monoclonal antibodies because they provide better system control and can be characterized individually.

Recently monoclonal antibodies for hCG have been used (Ehrlich et al., 1982) as a model for the fundamental understanding of the "hook" effect in competitive binding assays. For the hCG model, it was postulated (Moyle et al., 1983) that formation of a circular tetrameric complex is responsible for this behavior. The circular complex is very stable and therefore changes in conditions, such as the ionic strength of the buffers, are less critical. The complex has been characterized using electrophoresis techniques (Moyle et al., 1983). Some authors concluded that this is a general phenomenon involving mixtures of antibodies which yield multi-antibody-antigen complexes and is not a unique property of hCG (Ehrlich et al., 1983). This effect has been further investigated theoretically (Moyle and Ehrlich, 1983; Kuczek and Moyle, 1985).

Cooperativity has also been invoked to explain interactions of antibodies with HLA-A2 antigen (Holmes and Parham, 1983). The molecular sizes of the resulting complexes have been directly measured using HPLC and found to be consistent with a two antigen-two antibody tetrameric complex. It has been reported (Mazza et al., 1989) that human growth hormone (hGH) exhibits cooperativity, forming a binary complex (ratio hGH:antibody - 1:2) with monoclonal antibodies. The formation of a ternary complex (ratio 1:3) (Mazza et al., 1989) has also been reported. This report suggests that the hGH molecule undergoes a localized conformational change after binding the first monoclonal antibody and the second monoclonal antibody binds to a modified region of the

molecule. More fundamental experiments, however, are necessary to demonstrate and understand this phenomenon in detail.

The present systematic study investigates the nature of the interaction of two monoclonal antibodies against different epitopes of Biosynthetic hGH (hGH) as a model system for polyclonal antisera. The hGH is a biologically active single chain protein and is of sufficient size (22 kDa) to permit multiple interactions. Cooperativity cannot be predicted in advance from the binding characteristics of the individual antibodies, since the effect is the result of concomitant interactions of several antibodies with the antigen. Therefore, some preliminary experiments were devised to predict the behavior of mixtures of antibodies in a competitive binding assay. The titration curve, which is the first step in the optimization of a competitive binding assay, can be used to predict the low dose "hook" effect. Experimental data are also supported by a theoretical model. Size exclusion chromatography has been used to investigate the nature of multicomponent complexes. Experimental results support for the formation of high molecular weight stable complexes. These complexes were plausibly identified by considering the stoichiometry and predicting cooperative interactions. The effect of the concentration of each reactant was studied for multiple component complex formation.

2.2.3 Apparatus

A computer controlled gamma counter (Compugamma 1882-003, Pharmacia LKB Biotech. Inc., Gaithersburg, MD) was used for all radioactivity measurements. Size exclusion chromatography was performed using a Shimadzu LC-6A liquid chromatographic system consisting of a Model SCL-6B controller, Model LC-6A pump, Model SIL-6B auto injector, Model CR4A integrator and Model SPD-6AV UV detector (all from Shimadzu Corporation, Kyoto, Japan). A Shimadzu Model 160 UV/Vis spectrophotometer was used for the spectrophotometric measurements. Separation of antibody complexes was performed using GF-450/GF-250 coupled columns (9.4 mm ID X 25 cm) with a guard column (Du Pont, Chicago, IL).

2.2.4 Methods/Immunoassay

2.2.4.1 Antibody dilution curves

The antibody titration curve is the first step of the optimization of the competitive binding assay. 100 μL of ^{125}I-hGH (approximately 10,000 cpm, 58.2 μCi/μg), 100 μL of affinity purified antibody or antibody mixture, and 300 μL of assay buffer were mixed and incubated overnight at room temperature. The 0% binding control (the amount of ^{125}I-hGH precipitated in the absence of antibody) was used to correct for nonspecific binding. Each calibration curve was run in the 1 pM - 15 nM antibody range. Following overnight incubation, the bound ^{125}I-hGH was precipitated by the addition of 100 μL bovine gamma globulin (0.5% W/V in assay buffer) followed by 600 μL of polyethylene glycol (25% W/V in distilled water). The tubes were vortex-mixed for 10 seconds, allowed to stand at room temperature for 10 minutes, then centrifuged at 2800 rpm for 30 minutes at room temperature. Supernatants were decanted and discarded. The tubes were inverted and allowed to drain for 30 minutes. Residual drops were then wiped

from the outside and rims. The tubes were counted in a gamma-counter. All measurements were made in triplicate.

2.2.4.2 Saturation curves

Saturation curve is an alternative method to compare the binding behavior of single antibody and antibody mixture. Saturation curves were obtained with monoclonal antibodies to achieve a specific ^{125}I-hGH binding of about 30%. 100 μL of monoclonal antibody or antibody mixture, 100 μL of ^{125}I-hGH (10 -150 pg) and 300 μL of assay buffer were incubated overnight at room temperature. The 0% binding control was used for each concentration of ^{125}I-hGH to correct for non-specific binding. Bound ^{125}I-hGH was precipitated and counted using a procedure similar to that for antibody dilution curve.

2.2.4.3 Competitive binding assay

Monoclonal antibodies were diluted to achieve approximately 30% binding of ^{125}I-hGH in the absence of unlabeled hGH. At this dilution 100 μL of antibody samples were incubated with 100 μL ^{125}I-hGH (about 10,000 cpm), 100 μL hGH (10 - 1000 ng/mL) and 200 μL of assay buffer. With each assay, non specific binding was monitored. Separation of the bound ^{125}I-hGH was carried out using a procedure similar to that employed in the antibody titration. The experimental procedures for the mixture of antibodies were similar to those for single monoclonal antibodies.

2.2.5 Methods/Chromatography

Size exclusion chromatography with hGH

A dual column system was used to separate all protein complexes which were monitored at 280 nm. The mobile phase was 0.20 M phosphate buffer, pH = 7.4. The flow rate was 1.0 mL/min. The injection volume was 25 μL for all sam-

ples. All reactions were performed in 0.20 M phosphate buffer, pH = 7.4. Solutions of GHC 101 and GHC 072 antibodies (50 μg/mL) and unlabeled hGH (8 μg/mL) were used for the experiments described below. All samples were diluted to 200 μL unless otherwise specified.

2.2.5.1 Comparison of complexes of individual monoclonal antibodies and 1:1 mixture

100 μL of antibody (50 μg/mL) was mixed with 100 μL of unlabeled hGH (8 μg/mL) to analyze complexes involving individual antibodies. 50 μL of each antibody was mixed with 100 μL of unlabeled hGH (8 μg/mL) to analyze for mixed antibody-antigen complexes. These samples were incubated at 37 °C for one hour.

2.2.5.2 The effect of antibody concentration -- mixture of antibodies

10 μL of hGH (8 μg/mL) was mixed with (20 - 80) μL of each antibody for the preparation of different mixtures. 50 μL of each antibody and 50 μL of hGH were used to obtain the complex with a mixture of the two antibodies for comparison. 20 μL of each antibody was used to observe the chromatographic profile of the antibody mixture. These samples were incubated at 37° C for one hour.

2.2.5.3 The effect of hGH concentration: hGH-GHC 101 complexes

60 μL of GHC 101 (50 μg/mL) was mixed with (10-40) μL of hGH (8.0 μg/mL) and diluted to 100 μL. After incubation for 30 minutes at 37° C samples were analyzed by size exclusion separation.

2.2.5.4 The effect of hGH concentration -- mixture of antibodies

80 μL of each antibody (100 μg/mL) was mixed with 10-40 μL of hGH (8.0 μg/mL) and diluted to 200 μL. Samples were incubated for 30 minutes at 37° C. These samples were then separated by size exclusion chromatography.

2.2.5.5 Identification of components in the mixture -- 1

60 μL of each antibody (50 μg/mL) was mixed with 10 μL hGH (8.0 μg/mL) and diluted to 200 μL at room temperature. After the chromatographic data were obtained, the following steps were carried out for the mixture. After each step, the mixture was monitored chromatographically. The chromatograms were determined for the following solutions:

(a) 5.0 μL of GHC 101 (560 μ/mL)

(b) 5.0 μL of hGH (200 μg/mL)

(c) 5.0 μL of GHC 101 (560 μg/mL).

2.2.5.6 Identification of components in mixture -- 2

60 μL of each antibody (50 μg/mL) and 10 μL of hGH (8.0 μg/mL) were diluted to 200 μL at room temperature. The chromatographic profile for this mixture was obtained. Then the following steps were carried out to the above sample:

(a) Equal volumes of (3.0 μL) GHC 101 (560 μg/mL) and GHC 072 (500 μg/mL) were added

(b) The reagents in (a) was added to the mixture

(c) 5.0 μL of hGH (200 μg/mL) was added.

2.2.5.7 Identification of the components in the mixture -- 3

60 μL of each antibody (50 μg/mL) was mixed and diluted to 200 μL of the mixture was injected to the column. The following reagents were added to the above sample:

(a) 5.0 μL of hGH (200 μg/mL); (b) Equal volumes (2.0 μL) of antibodies GHC 101 (560 μg/mL) and GHC 072 (500 μg/mL).

2.2.6 Size exclusion chromatography with D-hGH

2.2.6.1 Complexes with D-hGH and GHC 101

60 μL of GHC 101 (50 μg/mL) was mixed with 10 - 40 μL of D-hGH (100 μg/mL) and diluted to 200 μL at room temperature. Samples were chromatographed. Then the sample containing 40 μL D-hGH was added 2.0 μL of GHC 101, mixed and rechromatographed.

2.2.6.2 Complexes with D-hGH and GHC 072

60 μL of GHC 072 (50 μg/mL) was mixed with 10 - 40 μL of D-hGH (100 μg/mL) and diluted to 200 μL at room temperature. Samples were applied to size exclusion separations. 60 μL of GHC 072 diluted to 200 μL and monitored the chromatographic behavior for comparison.

2.2.6.3 Complexes with D-hGH and mixture of antibodies

60 μL of each antibody (50 μg/mL) was diluted to 200 μL and monitored the chromatographic behavior. After data was obtained, the following steps were carried out for the mixture. At each step the mixture was monitored for the chromatographic data. The added reagents and concentrations at each step are given below:

(a) 5.0 μL of D-hGH (500 μg/mL)

(b) 5.0 μL of D-hGH (500 μg/mL)

(c) Equal volumes (3.0 μL) of GHC 101 (560 μg/mL) and GHC 072 (500 μg/mL).

2.3 Results and discussion

2.3.1 General discussion

The schematic diagram of the reaction resulting in the standard curve for a competitive binding assay of a monoclonal antibody (Case I) is shown in Figure 2.1A. A polyclonal antibody population represented by two non-interacting antibodies in the mixture (Case II) is shown in Figure 2.1B. In these assays, varying concentrations of hGH are incubated with fixed amounts of ^{125}I-hGH and antibody. The hGH competes with ^{125}I-hGH for a fixed and limiting concentration of antibody. The Curve in Figure 2.1A represents the typical behavior of the competitive binding assay for a monoclonal antibody in which the response is progressively reduced with increasing hGH. Also, using a similar procedure, the response for a polyclonal antibody is approximately the same under normal conditions and a hypothetical dose-response curve is shown in Figure 2.1B (Case II). B_o is the response with only ^{125}I-hGH present. X_1 corresponds to a very low concentration of hGH. The response decreases with the addition of hGH because of competition for a fixed, limited amount of antibody. A typical curve representing the low dose "hook" effect is shown in Figure 2.1C (Case III). This effect can be simulated using a mixture of two monoclonal antibodies which can interact synergistically to facilitate the formation of a circular complex. B_o is again the response before hGH is added. If circular complexes are formed, then at low concentrations of added hGH, ^{125}I-hGH is incorporated into the circular complex. In case III, the concentration of hGH at X_1 leads to maximum incorporation of ^{125}I-hGH. X_1 corresponds to the same hGH concentration for all cases; I, II and III. The response decreases at higher concentrations of hGH, such as X_2, resulting from replacement of ^{125}I-hGH with hGH in complexes and from dissolution of

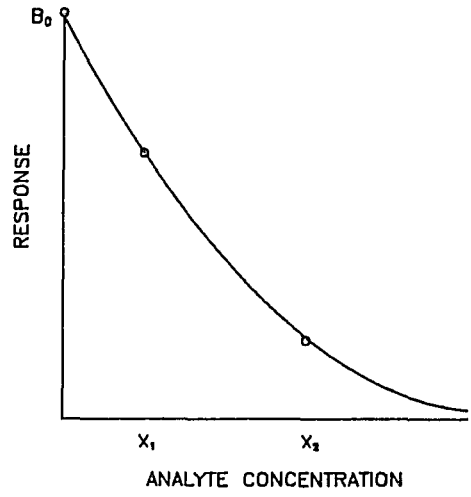

Figure 2.1A

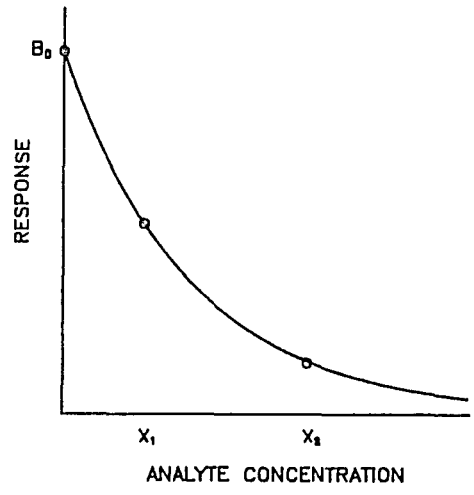

Figure 2.1B

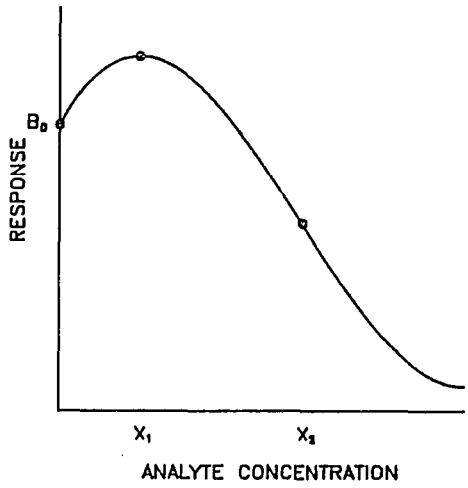

Figure 2.1C

Figure 2.1A Schematic for competitive binding assay: Case I -- single monoclonal antibody

Figure 2.1B Schematic for competitive binding assay: Case II -- two monoclonal antibodies: A model for polyclonal antibodies (no cooperative interactions)

Figure 2.1C Schematic for competitive binding assay: Case III -- two monoclonal antibodies, assuming that circular complexes are formed due to concomitant interactions of an antigen and two antibodies.

complexes. Figure 2.2 shows schematically the possible complexes formed for Case I and Case II without distinguishing the labeled and unlabeled antigen. Symbols A and B symbols represent the monoclonal antibodies. H represents the antigen. In both Cases I and II, A or B can be monovalent and/or divalent. In Case II, antigen, H, interact with an individual antibody (A and B) then the antigen prohibit reacting with the second antibody. Case III can be represented by Figures 2.2 and 2.3. Figure 2.3 accounts for the formation of linear and circular complexes. Case III assumes the formation of the circular complex, -HABA-.

Theoretical models can help to identify and predict cooperative effects. To make the models compatible with the experimental data, the following assumptions are made: (1) ^{125}I-hGH and hGH have similar affinities for each antibody (2) Single monoclonal antibodies, GHC 101 and GHC 072 can be either as monovalent or divalent (3) All reactions reach equilibrium (4) Bound and free fractions can be separated without disturbing the equilibrium. Based on these general assumptions, several models have been developed in order to simulate the binding behavior. Other relevant assumptions are clearly mentioned in the description of each model. The symbols used are used for all of the models.

2.3.2 Model 1: System containing single antibody and antigen -- a model for titration and saturation curves

The binding reaction between the labeled antigen and antibody is shown in the following equation:

$$L + A \rightleftharpoons LA$$

where L is the labeled antigen (^{125}I-hGH), and A is the monoclonal antibody (GHC 101 or GHC 072). The resulting antigen-antibody complex is LA. At equilibrium the binding constant K_1 is a function of the relative concentrations of

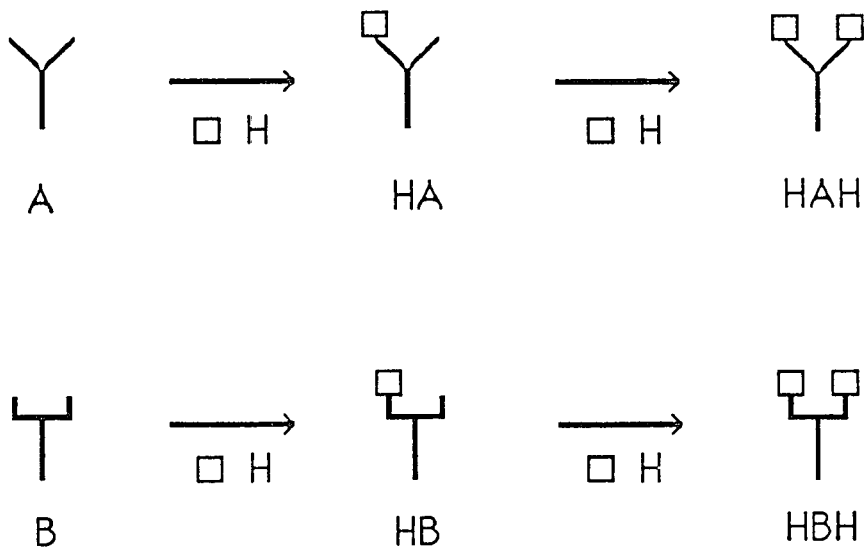

Figure 2.2 Schematic representation of complexes of monoclonal antibodies and hGH A: GHC 101, B:GHC 072 and H: hGH.

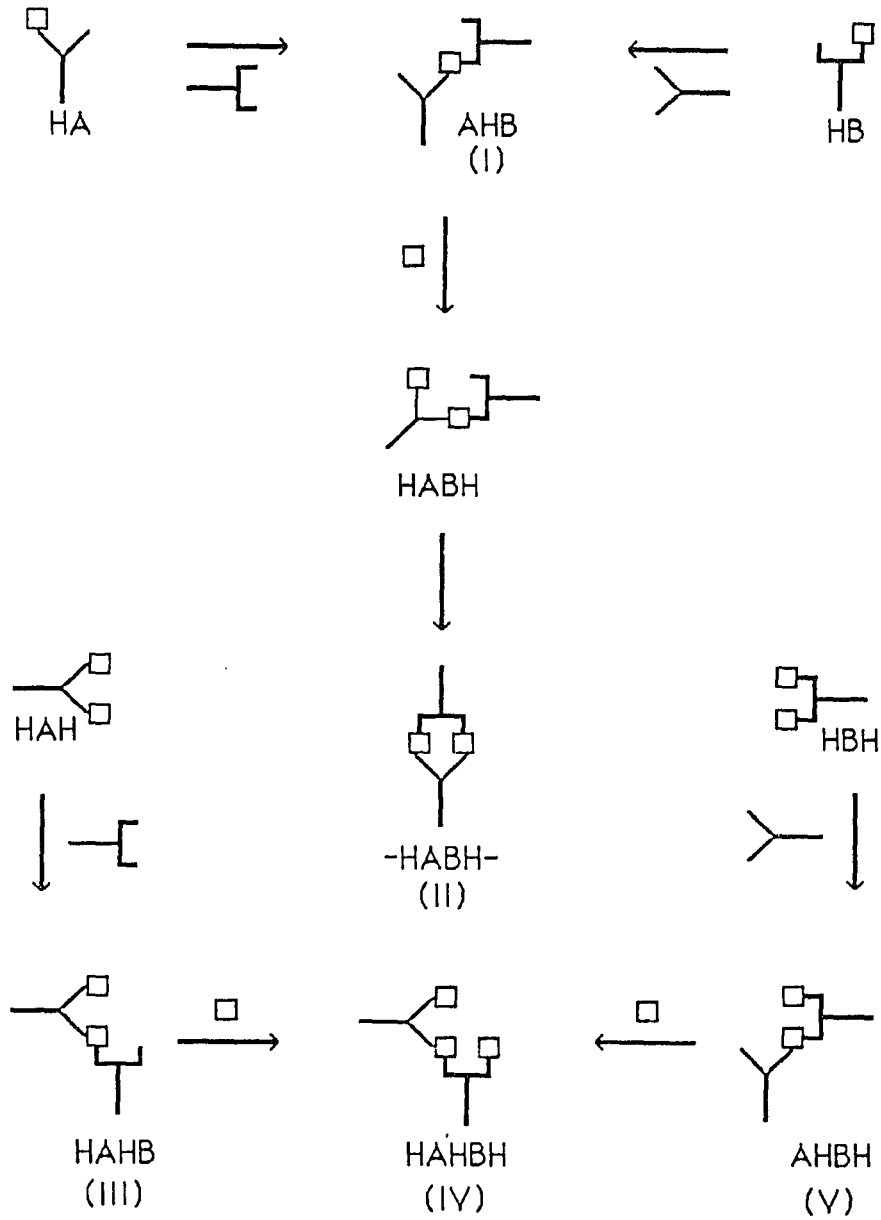

Figure 2.3 Schematic representation of tetrameric complexes with hGH and mixture of antibodies. A:GHC 101, B:GHC 072 and H:hGH.

reactants and products is given by the following expression:

$$K_1 = [LA]/[L][A]$$

where [LA] is the concentration of antigen-antibody complex, [L] is the free antigen concentration and [A] is the concentration of free antibody. According to conservation of mass it is possible to relate the concentration of each species to the total concentration of reactants:

$$l = [L] + [LA]$$

$$a = [A] + [LA]$$

where l and a are the total labeled antigen and antibody concentrations, respectively. This model can be used to obtain the theoretical binding data for titration and saturation curves consisting of single antibodies. In the experimental procedure for the titration curve a fixed concentration of the labeled antigen (L) and variable concentrations of antibody (A) are used. A fixed concentration of antibody and variable concentrations of labeled antigen is used for the saturation curves.

2.3.3 Model 2: System containing a mixture of two non-interacting monoclonal antibodies -- a model for titration and saturation curves

This is analogous to the interaction of ^{125}I-hGH with GHC 101 and GHC 072 assuming that the two antibodies do not interact with each other when bound to the antigen. The experimental procedure is similar to that described in Model 1. Similar to Equation 1 in Model 1, the following equation can be written for this model:

$$L + A + B \rightleftharpoons LA + LB$$

In addition to the symbols explained in Model 1, B is the second monoclonal antibody. K_2 is the binding constant for the interaction of L and B, b is the total concentration of antibody, B. The following relationships can be developed for this

model:

$$K_1 = [LA]/[L][A]$$

$$K_2 = [LB]/[L][B]$$

$$l = [L] + [LA] + [LB]$$

$$a = [A] + [LA]$$

$$b = [B] + [LB]$$

Similar to Model 1, this model can be used to generate titration and saturation curves for the mixture of antibodies.

2.3.4 Model 3: System containing single antibody -- a model for the competitive binding assay

This model corresponds to Case I, Figure 2.1A. H represents hGH. L represents ^{125}I-hGH. As assumed earlier, the binding reaction for the antibody B is similar to A. The following equation shows the fundamental competitive binding reaction for the antibody A:

$$L + H + A = HA + LA$$

Again $K_1 = [HA]/[H][A] = [LA]/[L][A]$

If the total labeled antigen concentration is *l*, the following equations will relate the concentration of each species to the total concentration:

$$l = [L] + [LA]$$

$$h = [H] + [HA]$$

$$a = [A] + [LA] + [HA]$$

Model 3 is used to generate theoretical dose-response curves for assays involving single antibodies.

2.3.5 Model 4: System containing a mixture of two non-interacting monoclonal antibodies -- a model for competitive binding assay

This is the model for two non-interacting monoclonal antibodies (Case II, Figure 2.1B). Model 4 is analogous to Model 3 and the only difference is the mixture of antibodies. The reaction between the antigen and the mixture of antibodies is given in the following equation using same set of symbols:

$$H + L + A + B \rightleftharpoons HA + HB + LA + LB$$

Similar to other models the following mass balance equations can be written:

$$K_1 = [LA]/[L][A] = [HA]/[H][A]$$

$$K_2 = [LB]/[L][B] = [HB]/[H][B]$$

$$l = [L] + [LA] + [LB]$$

$$h = [H] + [HA] + [HB]$$

$$a = [A] + [LA] + [HA]$$

$$b = [B] + [LB] + [HB]$$

Model 4 generates dose-response curves for a mixture of two antibodies.

2.3.6 Model 5: Cooperative interactions

In this model the mixture of two antibodies is assumed to form a circular tetrameric complex. Case III, Figure 2.1C represents the hypothetical curve for the competitive binding assay. This model presumes the circular tetrameric complex presumably to be the most stable (Moyle et al., 1983; Schumaker, 1973). This model can be expanded into four sub models to simulate the theoretical curves for titration, saturation and competitive binding assay experiments, respectively. Model 5a and 5b could generate theoretical curves for titration and saturation experiments. In Model 5a only linear complexes are considered. Circular complex formation is explained in Model 5b. Model 5b also includes all possible linear

the competitive binding assay.

2.3.7 Model 5a: System containing two interacting monoclonal antibodies: formation of higher molecular weight linear complexes -- a model for titration and saturation curves

A model similar to Model 5a has been described by Moyle et al. (1983). This model assumes the interaction of two antibodies with the labeled antigen. Both antibodies are assumed to be bivalent. Antibodies GHC 101 and GHC 072 are known to bind to two spatially different epitopes, therefore the following relationships will account for the formation of possible linear complexes with ^{125}I-hGH (L):

$$C_1 = [LA] = 2K_1[L][A]$$

$$C_2 = [LB] = 2K_2[L][B]$$

$$C_3 = [LAL] = K_1^2[L]^2[A]$$

$$C_4 = [LBL] = K_2^2[L]^2[B]$$

$$C_5 = [ALB] = 4K_1K_2[L][A][B]$$

$$C_6 = [LALB] = 4K_1^2K_2[L]^2[A][B]$$

$$C_7 = [ALBL] = 4K_1K_2^2[L]^2[A][B]$$

where $C_1, C_2, C_3, C_4, C_5, C_6$ and C_7 represent the concentration of each species. The mass balance equations can be written as follows:

$$l = [L] + 2\sum_{i}^{7} C_i - (C_1 + C_2 + C_5)$$

$$a = [A] + \sum_{i}^{7} C_i - (C_2 + C_4)$$

$$b = [B] + \sum_{i}^{7} C_i - (C_3 + C_5)$$

2.3.8 Model 5b: System containing two interacting monoclonal antibodies: formation of mixture of linear and circular complexes -- a model for titration and saturation curves

This model deals with an additional step in which a circular tetrameric complex formation has been generalized. All the complexes discusses in Model 5a are also formed. In addition to Model 5a, LALB and ALBL are permitted to form circular complexes. Both complexes have at least one unreacted combining site. The unbound antibodies in these complexes will further combine with appropriate epitopes of hGH to form ring complexes. The following reactions account for the cyclization:

$$\text{ALBL} \rightleftharpoons \text{-LALB-}$$

$$\text{LALB} \rightleftharpoons \text{-LALB-}$$

The concentration of the cyclic complex, C_8 is given by the following relationship:

$$C_8 = [\text{-LALB-}] = 4K_rK_1^2K_2^2[L]^2[A][B]$$

where K_r is the ring formation constant for the circular complex. The units for K_r are moles/L. The following mass balance equations can be used to estimate concentrations of each species.

$$l = [L] + 2\sum_{i}^{5} C_i - (C_1 + C_2 + C_5)$$

$$a = [A] + \sum_{i}^{5} C_i - (C_2 + C_4)$$

$$b = [B] + \sum_{i}^{5} C_i - (C_3 + C_5)$$

Note that the mass balance equations differ from Model 5a by accounting for the concentration of circular complex, C_8.

$$D_7 = [AHBH] = 4K_1K_2^2[H]^2[A][B]$$

All mixed complexes containing L and H:

$$E_1 = [HAL] = 4K_1^2[H][L][A]$$

$$E_2 = [HBL] = 4K_2^2[H][L][B]$$

$$E_3 = [HALB] = 4K_1^2K_2[H][L][A][B]$$

$$E_4 = [LAHB] = 4K_1^2K_2[H][L][A][B]$$

$$E_5 = [AHBL] = 4K_1K_2^2[H][L][A][B]$$

$$E_6 = [ALBH] = 4K_1K_2^2[H][L][A][B]$$

The following mass balance equations account for the total concentrations of A, B, C and L which are denoted as a,b,c and *l* respectively.

$$l = [L] + 2\sum_{1}^{7} C_i + \sum_{1}^{6} E_i - (C_1 + C_2 + C_5)$$

$$h = [H] + 2\sum_{1}^{7} D_i + \sum_{1}^{6} E_i - (D_1 + D_2 + D_5)$$

$$a = [A] + \sum_{1}^{7} C_i + \sum_{1}^{7} D_i + \sum_{1}^{6} E_i - (C_2 + C_4 + D_2 + D_4 + E_2 + E_4)$$

$$b = [B] + \sum_{1}^{7} C_i + \sum_{1}^{7} D_i + \sum_{1}^{6} E_i - (C_3 + C_5 + D_3 + D_5 + E_3 + E_5)$$

2.3.10 Model 5d -- System containing two interacting monoclonal antibodies: formation of mixture of linear and circular complexes -- a model for competitive binding assay

This model accounts for circular complex formation in addition to the linear complexes. However, Model 5b deviates from Model 5d in which mixed labeled and unlabeled complexes are also involved. The following cyclization reactions are involved in Model 5d:

HALB = -HALB-

ALBH = -HALB-

LAHB = -LAHB-

AHBL = -LAHB-

HAHB = -HAHB-

LALB = -LALB-

The concentration of all possible cyclic complexes are given below:

$$C_8 = [\text{-LALB-}] = 4K_rK_1^2K_2^2[L]^2[A][B]$$

$$D_8 = [\text{-HAHB-}] = 4K_rK_1^2K_2^2[H]^2[A][B]$$

$$E_7 = [\text{-HALB-}] = 4K_rK_1^2K_2^2[H][L][A][B]$$

$$E_8 = [\text{-LAHB-}] = 4K_rK_1^2K_2^2[H][L][A][B]$$

The concentrations of cyclic complexes are denoted as C_8, D_8, E_7, and E_8, respectively. The following equations account for the total concentrations of L, H, A and B respectively:

$$l = [L] + 2\sum C_i + \sum E_i - (C_1 + C_2 + C_5)$$

$$h = [H] + 2\sum D_i + \sum E_i - (D_1 + D_2 + D_5)$$

$$a = [A] + \sum C_i + \sum D_i + \sum E_i - (C_2 + C_4 + D_2 + D_4 + E_2 + E_4)$$

$$b = [B] + \sum C_i + \sum D_i + \sum E_i - (C_3 + C_5 + D_3 + D_5 + E_3 + E_5)$$

Model 5d simulates the theoretical curves for the competitive binding assay by accounting for circular complex formation.

2.3.11 Titration curves

In the normal course of optimizing conditions for the competitive binding assay, the first step is to generate the antibody titration curve. The antibody titration curve involves the incubation of a fixed amount of radiotracer with serial dilution of the antibody or antibody mixture to establish the optimal concentration for the assay. This experiment was carried out for monoclonal antibodies GHC 101, GHC 072 and a 1:1 mixture of these antibodies at the same total concentration. These data are shown in Figure 2.4. According to these data, GHC 101 has a higher affinity for hGH than does GHC 072. A 1:1 mixture of antibodies at the same total antibody concentration should result in a titration

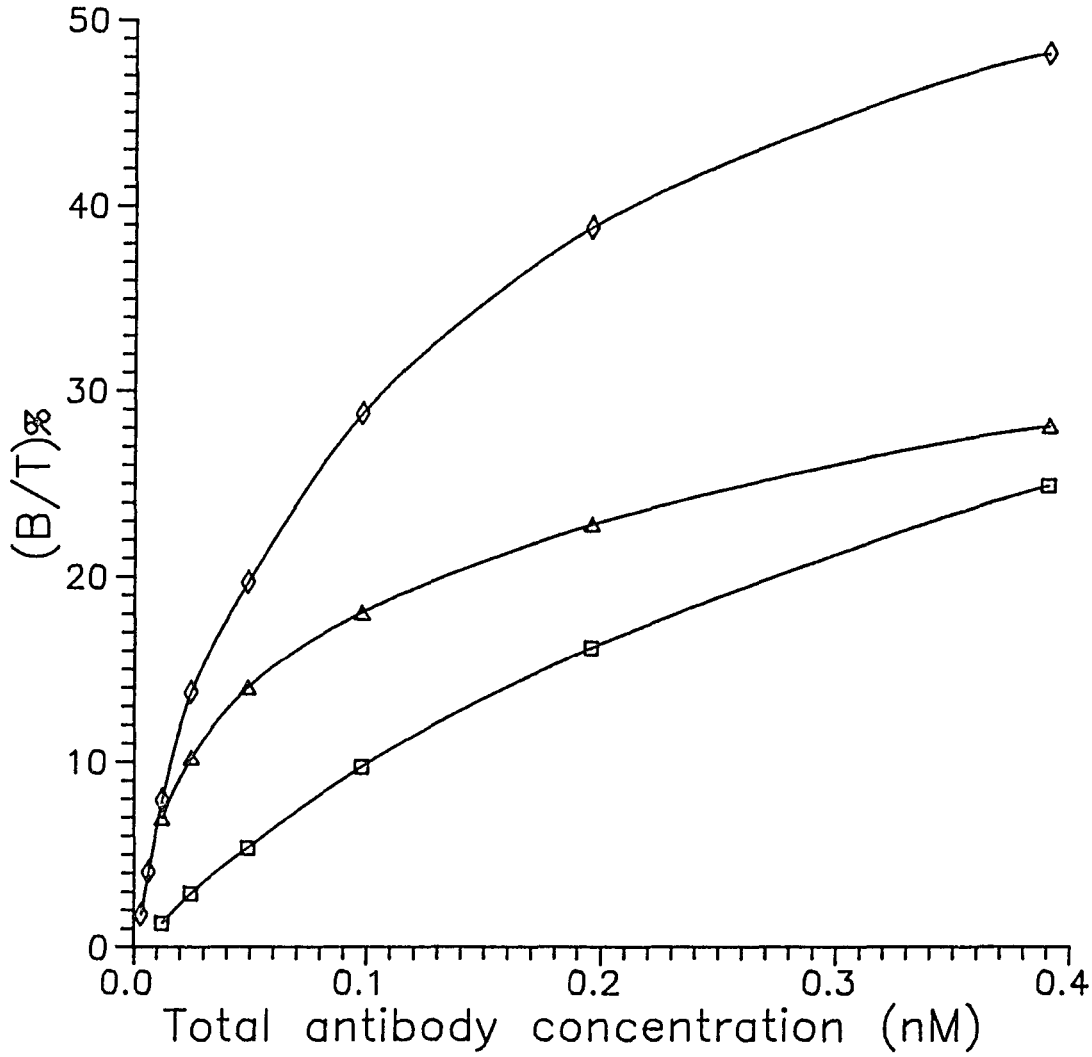

Figure 2.4 Titration data -- Comparison of individual antibodies and the mixture. Antibodies: GHC 101 (△), GHC 072 (□) and 1:1 mixture (◇). The concentration range for antibodies is 10 pM - 0.4 nM.

curve between those of GHC 101 and GHC 072 if the antibodies do not interact each other. However, data reflecting much higher affinity was observed for the mixture. GHC 101 antibody show a saturation of the response at approximately 0.4 nM. The titration data, (B/T)%, at 12.5 nM concentrations of GHC 101 and GHC 072 and the 1:1 antibody mixture show about 26, 64 and 69 respectively indicate that the less binding response of GHC 101 at higher antibody concentrations. To learn theoretical insight into the binding behavior of individual monoclonal antibodies and the mixture, computer simulated curves were generated for Models 1, 2, 5a and 5b. These theoretical curves are shown in Figure 2.5. The necessary binding parameters used for these simulations are compatible with the experimental data (Figure 2.4). The experimental values for the affinities of GHC 072 and GHC 101 are 1.1 nM^{-1} and 3.8 nM^{-1} respectively (Sportsman et al., 1989). Curves C and E represent the theoretical titration curves for the individual antibodies. Affinity of GHC 101 approximately 4 fold less than the experimental value. Affinity of GHC 072 is almost same as the experimental value. It will be noted that the theoretical titration curve for the mixture of antibodies, generated using Model 2 is in between the curves for individual antibodies. The experimental data for the 1:1 mixture of antibodies are not compatible with the theoretical Curve D suggesting that these antibodies do not bind to overlapping epitopes of the antigen (cf: Figure 2.4 and 2.5). Calculated values in Curves A (Model 5a) and B (Model 5b) do show that the formation of higher molecular weight complexes could explain the higher binding response for the mixture of antibodies (Figure 2.4). Curves A and B were generated using comparable affinity values for GHC 072 (same as the experimental value) and GHC 101 (approximately 4 fold less than the experimental value).The enhancement of the response in Curves A and B is due to the formation of mixture of higher molecular weight linear and circular complexes,

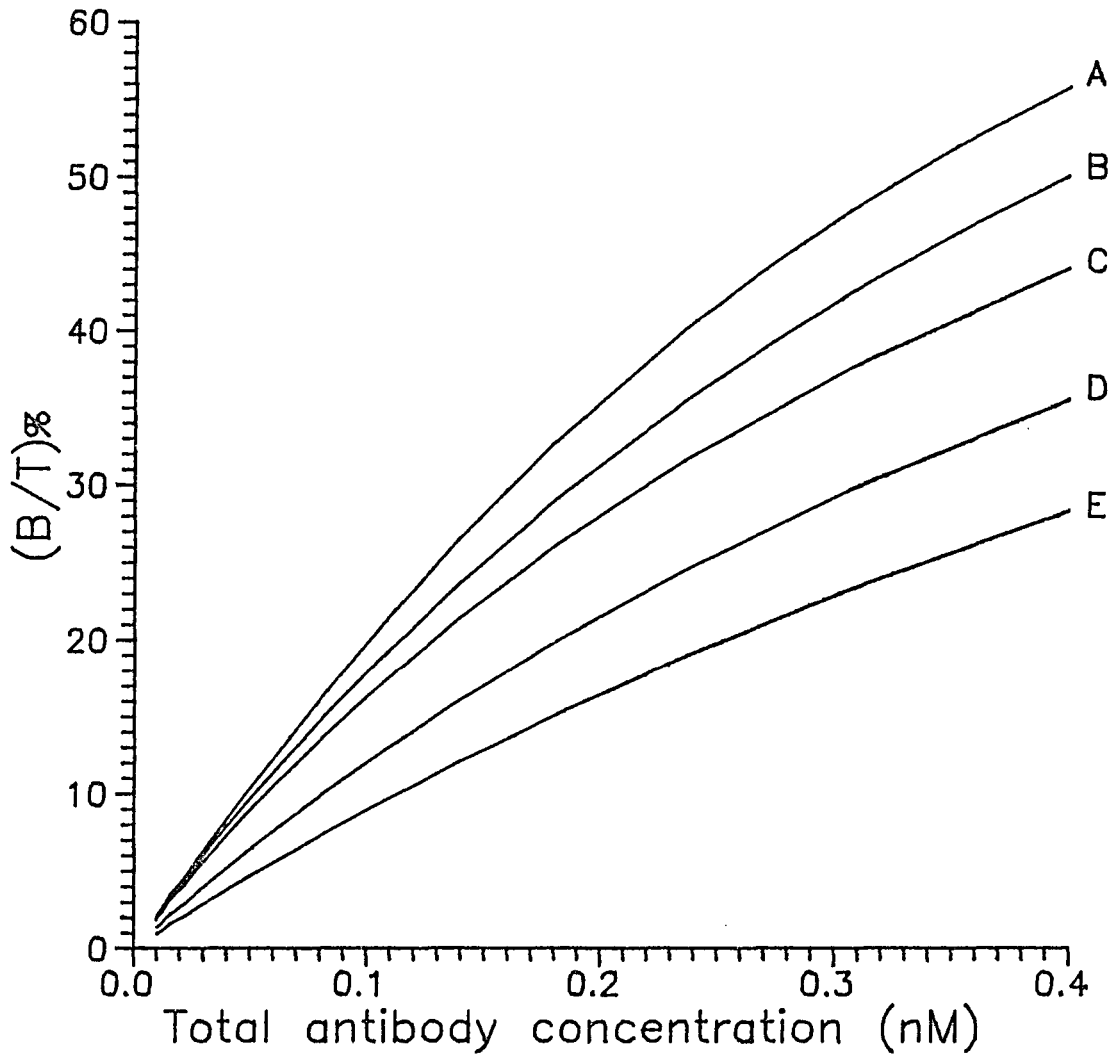

Figure 2.5 Theoretical titration curves. Theoretical Curves A, B and D correspond to the experimental data for mixture of antibodies. Theoretical Curves C and D correspond to the experimental data for GHC 101 and GHC 072 respectively. Simulated titration curves: *Curve A -- Model 5b:* K_1 = 1.1 nM^{-1}; K_2 = 1.0 nM^{-1}; K_r = 100 nM; l = 16 pM. *Curve B -- Model 5a:* K_1 = 1.1 nM^{-1}; K_2 = 1.0 nM^{-1}; l = 16 pM. *Curve C -- Model 1:* K_1 = 2 nM^{-1}; l = 16 pM. *Curve D -- Model 2:* K_1 = 2 nM^{-1}; K_2 = 1 nM^{-1}; l = 16 pM. *Curve E -- Model 1:* K_2 = 1 nM^{-1}; l = 16 pM.

respectively. Theoretical results for Model 5a indicate that the enhancement in binding for the titration experiment could also be attributed to the formation of linear complexes. Moreover the enhancement in binding can also be explained assuming that LA, LB, LAL, LBL and ALB are formed with antibody mixtures (L is the labeled antigen and A and B represent GHC 101 and GHC 072 monoclonal antibodies). As shown in Curve A (Model 5b) the enhancement in binding for the mixture of antibodies could also be attributed to concomitant interactions. However, the titration curves with fixed ^{125}I-hGH could not directly distinguish whether the enhancement in binding for the mixture of antibodies is due to the formation of higher molecular weight linear or cyclic complexes.

Different ratios of the mixtures of antibodies were also compared with the 1:1 ratio of the mixture while keeping the total concentration constant (Figure 2.3). There is no significant effect of antibody concentration ratio on bound concentration of ^{125}I-hGH. The higher affinity for the mixture of antibodies is presumably due to the formation of multicomponent complexes, but the data do not directly confirm cooperativity behavior.

As shown in previous titration data (Figure 2.4), it is necessary to clarify that the mixture of antibodies forms circular complexes thus enhancing the binding response. In order to investigate the influence of the hGH (H) concentration on formation of complexes, titration experiments for individual antibodies and 1:1 mixtures were performed at higher total (unlabeled (H) and labeled (L)) hGH concentrations. It would be expected that the unlabeled antigen could incorporated into the higher molecular weight complexes facilitating formation of circular complexes. Otherwise the addition of excess unlabeled antigen should result decrease in assay response due to displacement of the labeled antigen. The

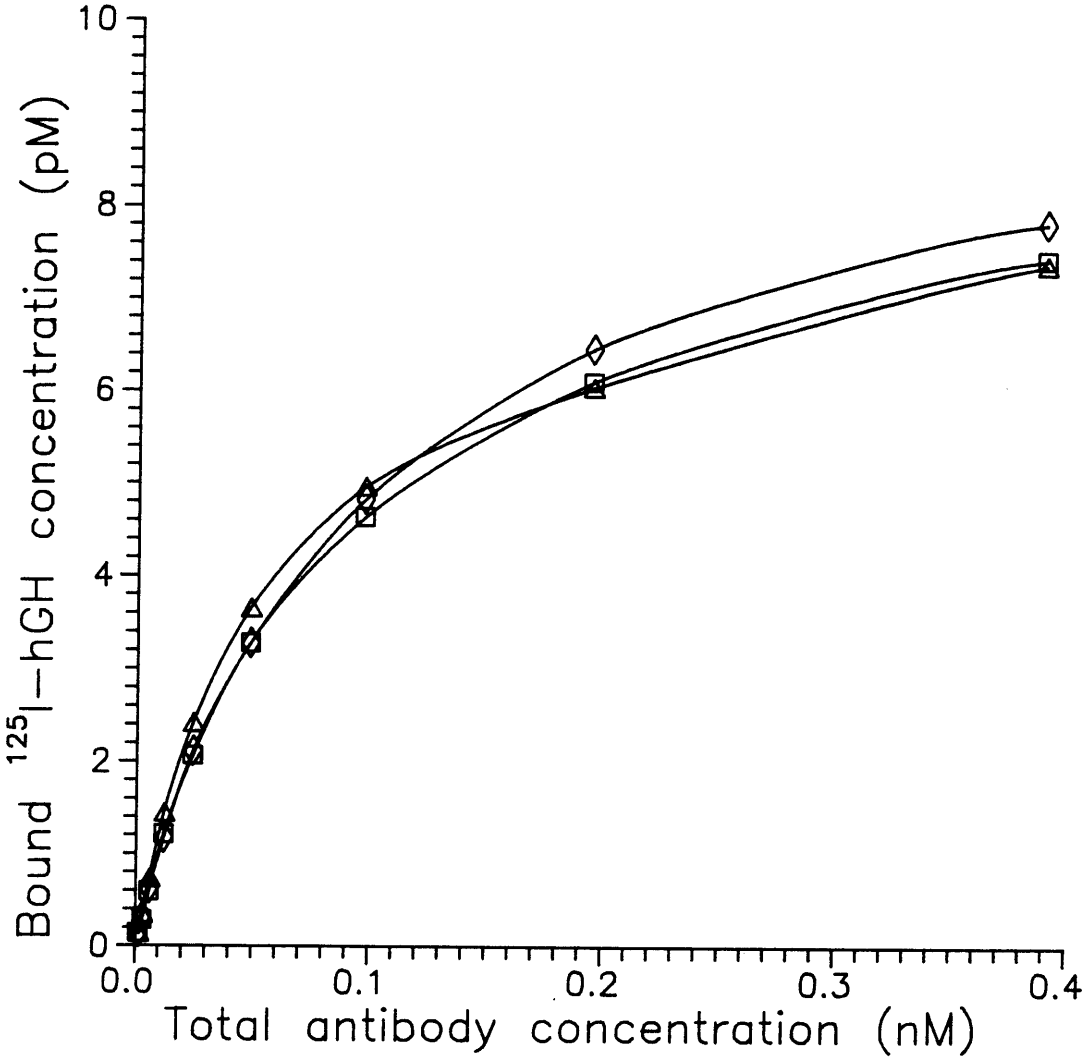

Figure 2.6 Titration data -- Effect of the ratio of antibodies on response. 1:1 mixture (□), 2:1 mixture (◊) and 1:2 mixture (△).Concentration range for antibodies is 1.0 pM - 0.4 nM.

concentrations of unlabeled hGH for the experiments were 6 fold (0.09 nM) and 12 fold (0.18 nM) excess over ^{125}I-hGH, respectively. The titration curve data are shown in Figure 2.7. The data for the 1:1 mixture of antibodies containing only ^{125}I-hGH show lower binding than that with added unlabeled hGH. If the mixture of antibodies react with overlapping epitopes of the labeled antigen (Model 2) the addition of unlabeled hGH will displace the ^{125}I-hGH resulting in a decrease in binding of ^{125}I-hGH. On the contrary, the reverse is observed. To further illustrate the binding behavior, it is assumed that only LA, LB, LAL, LBL and ALB complexes are formed in the titration curve generated with ^{125}I-hGH (L) for the antibody mixture. In such a case the addition of excess hGH (H) should replace ^{125}I-hGH and result in lower response. A higher response cannot be explained by incorporating hGH to ALB to form mixed complexes such as HALB, ALBH. The higher affinity for the mixture can only be attributed to the formation of mixed cyclic complexes such as -ALBH- and -HALB-. The experimental data support the hypothesis that ^{125}I-hGH and unlabeled hGH form mixed complexes with a mixture of monoclonal antibodies whereas individual antibodies, which bind to only one epitope on the molecule and do not form such complexes with the antigen.

2.3.12 Saturation curves

In practice, this investigation can be extended by setting up the second experiment with a fixed amount of antibody or mixture of antibodies. In these experiments a fixed amount of antibody was mixed with different concentrations of ^{125}I-hGH (1.5 - 15.4 pM). The results are similar to a calibration curve for ^{125}I-hGH. The general shape of the curve depends on the concentrations of antibody and ^{125}I-hGH. In all of these experiments the antibody concentration was selected to yield approximately 30% bound ^{125}I-hGH at a concentration of 15.4

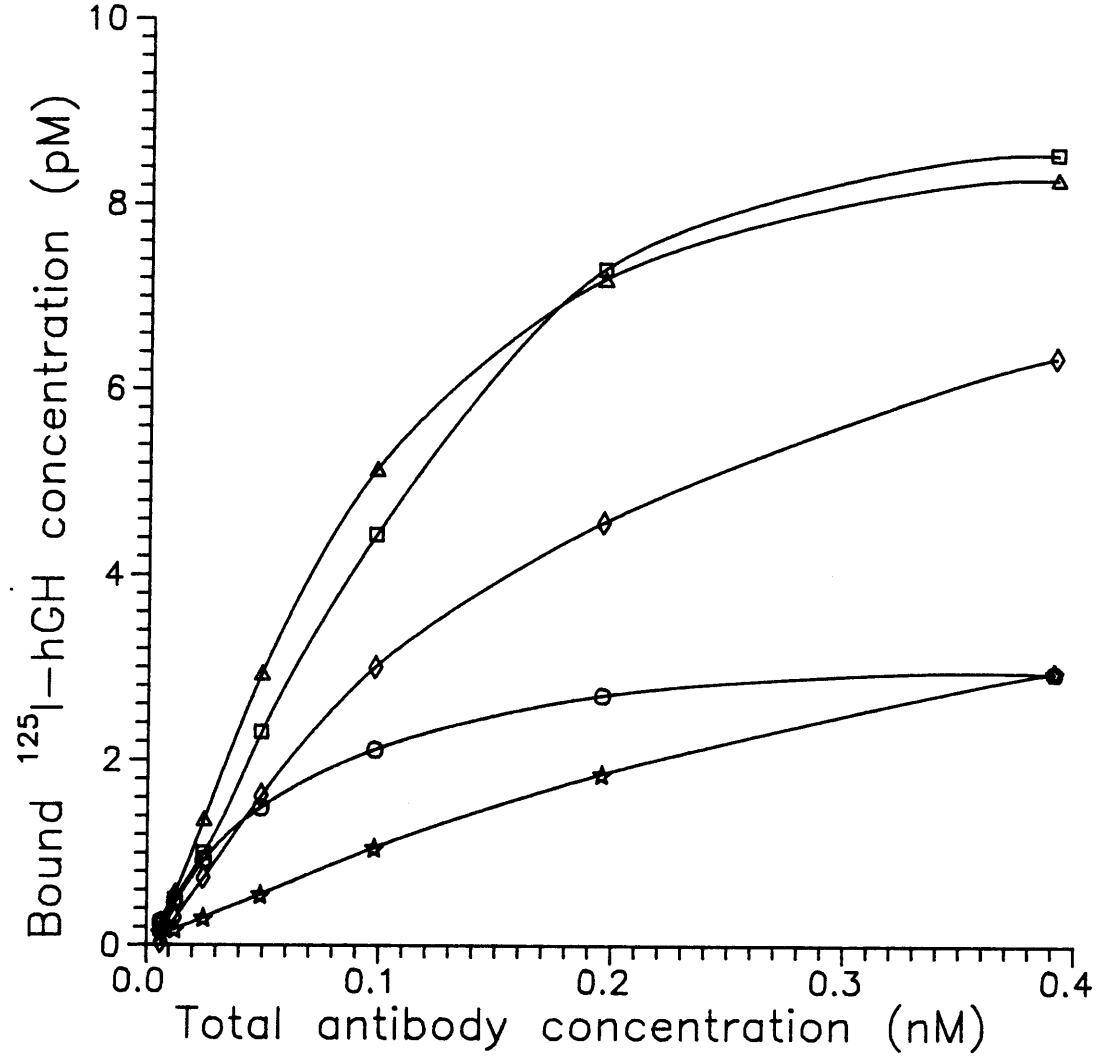

Figure 2.7 Titration data -- Effect of unlabeled hGH on binding response.

GHC 072 (*) - 0.09 nM unlabeled hGH

GHC 101 (o) - 0.09 nM unlabeled hGH

Mixture 1:1 (◊) - no unlabeled hGH

Mixture 1:1 (△) - 0.09 nM unlabeled hGH

Mixture 1:1 (□) - 0.18 nM unlabeled hGH.

pM. However, higher concentrations of individual monoclonal antibodies (0.8 nM for GHC 072 or 0.6 nM for GHC 101) are necessary to obtain the same bound ^{125}I-hGH (Figure 2.8). A very low concentration of the mixture of antibodies is sufficient to increase the bound ^{125}I-hGH concentration to a level comparable to that of the individual antibodies. The experimental data for Curves C and D represent the simulated saturation curves for individual antibodies indicating that the experimental data correlate with the theory. But the experimental data for GHC 101 antibody does deviate from the linearity between 10 - 15.4 pM ^{125}-hGH concentration range. Curve E represents the simulated results corresponding to Model 2 (Case II, Figure 2.1B) for the mixture of antibodies. It will be noted that the theoretical curve for Model 2 shows a very low response compared to the experimental data suggesting that two antibodies are not likely to bind to non-overlapping epitopes of hGH. The theoretical Curve A was generated using Model 5b assuming that circular complexes are formed (Case III, Figure 2.1C). Without considering circular complexes the Curve B is obtained. Note that similar responses are shown in Curves A and B. The experimental data for the mixture of antibodies correlate with the theoretical curves if antibodies are assumed to form higher molecular weight linear or circular complexes. The experimental and the theoretical predictions simply indicate that hGH shows a different binding behavior in the presence of a mixture of antibodies (Figure 2.8). Further, 0.09 nM and 0.18 nM of unlabeled hGH (6 and 12 fold excess, respectively) were added to variable concentrations of ^{125}I-hGH in a 1:1 mixture of antibodies and compared for the bound ^{125}I-hGH (Figure 2.9). The mixtures with higher total concentrations of hGH have higher slopes. But the curve for 0.18 nM unlabeled hGH shows less bound ^{125}I-hGH because hGH is in 12 fold excess. This result is roughly comparable with the unlabeled hGH concentration (X_2) in the competitive

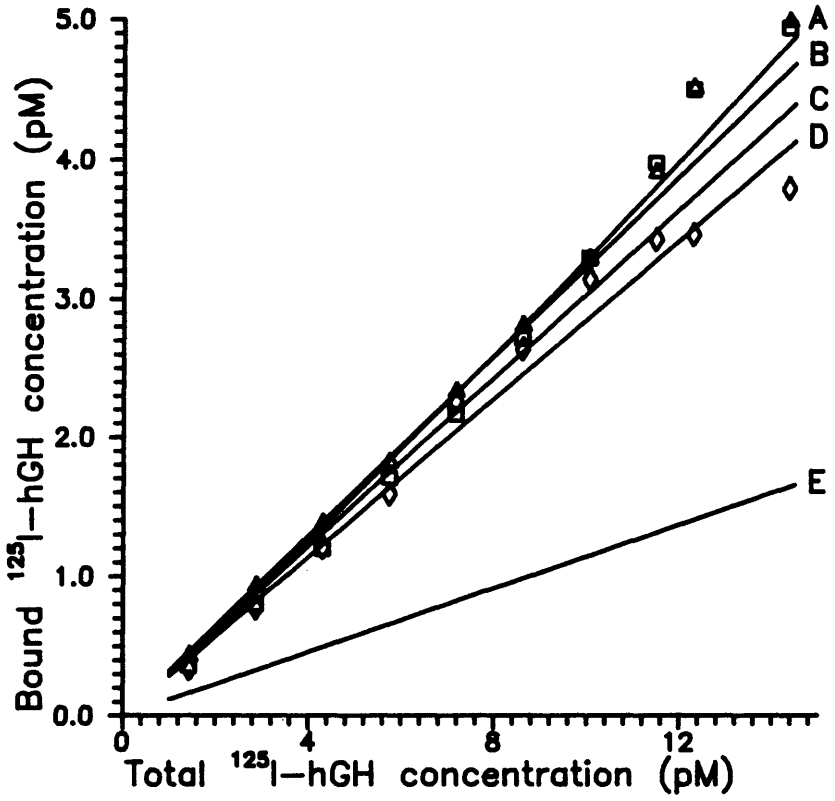

Figure 2.8 Effect of ^{125}I-hGH on binding response for constant antibody mixture. Concentrations of antibodies GHC 072 (△) - 0.8 nM, GHC 101 (◇) - 0.6 nM and 1:1 mixture (□) - 0.2 nM (total). Concentration range for ^{125}I-hGH is 1.0 pM - 14.5 pM.

Theoretical Curves A, B and E correspond to the experimental data for the 1:1 mixture (□). Theoretical Curves C and D correspond to the experimental data for GHC 072 (△) and GHC 101 (◇) respectively. Simulated response curves: *Curve A* -- *Model 5b:* K_1 = 1 nM^{-1}; K_2 = 1 nM^{-1}; K_r = 100 nM; a = 0.1 nM; b = 0.1 nM. *Curve B* -- *Model 5a:* K_1 = 1 nM^{-1}; K_2 = 1 nM^{-1}; a = 0.1 nM; b = 0.1 nM. *Curve C* -- *Model 1:* K_2 = 0.5 nM^{-1}; b = 0.8 nM. *Curve D* -- *Model 1:* K_1 = 0.8 nM^{-1}; a = 0.6 nM. *Curve E* -- *Model 2:* K_1 = 0.8 nM^{-1}; K_2 = 0.5 nM^{-1}; a = 0.1 nM; b = 0.1 nM.

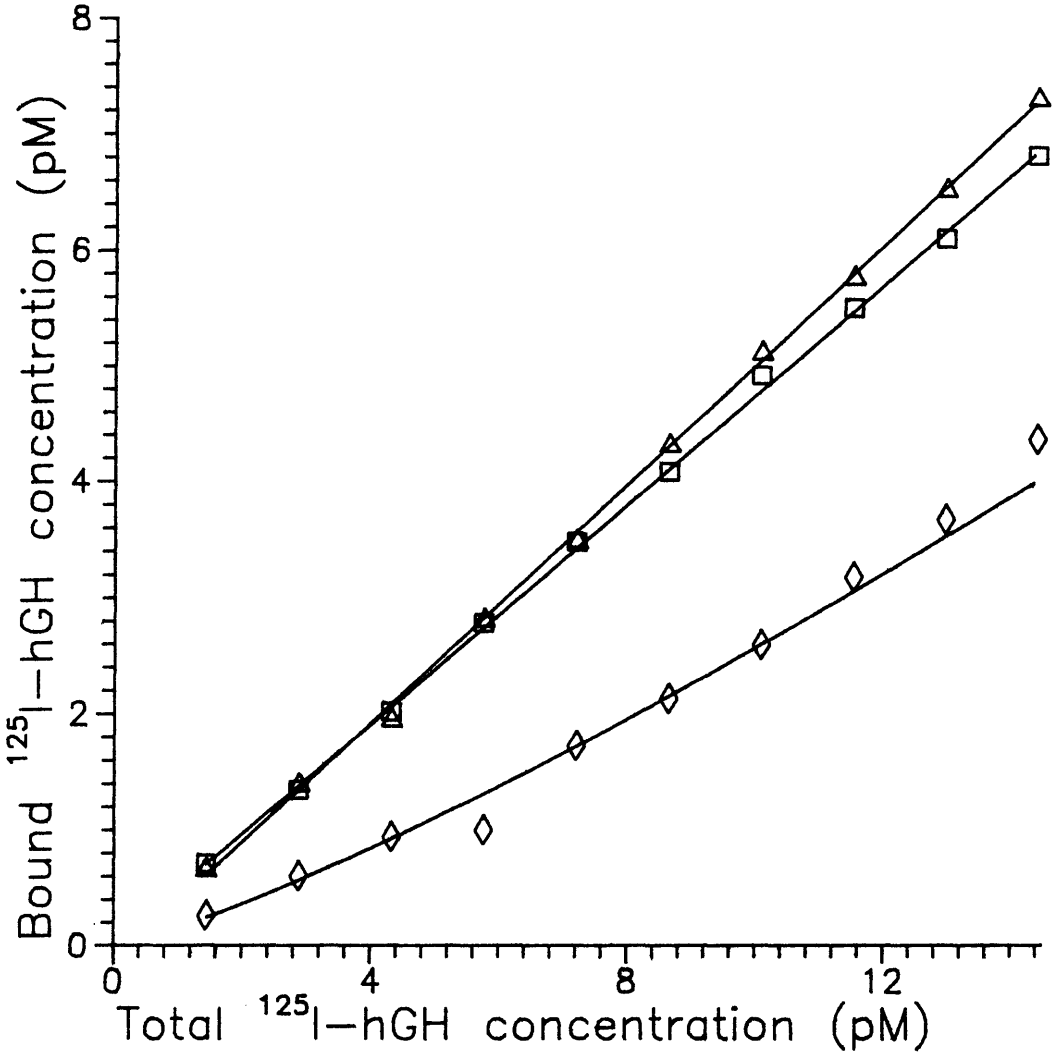

Figure 2.9 Effect of ^{125}I-hGH and hGH on binding response for constant antibody mixture. 1:1 mixture of GHC 101 and GHC 072 (concentration 0.2 nM) was used.

Without hGH (◊)

0.09 nM hGH (△)

0.18 nM hGH (□).

binding assay (Case III, Figure 2.1C). The highest concentration of ^{125}I-hGH, 15.4, pM is in excess of total antibody or antibody mixtures. In such an experiment the addition of hGH is expected to inhibit the binding of ^{125}I-hGH and should result in a lower slope. Therefore the data for the saturation curves probably indicate that the higher slopes are caused by the formation of highly stable mixed circular complexes such as -ALBH- and -HALB- in addition to -ALBL- circular complex.

2.3.13 Competitive binding assay

If a mixture of antibodies interact concurrently with hGH to form circular complexes the dose-response curve expected to demonstrate a low dose "hook" as suggested in Case III, Figure 2.1C. The titration and saturation experiments have already indicated that the mixture of antibodies does bind cooperatively. To elucidate the low dose "hook" in the competitive binding assay, the rising and the descending limbs of the dose-response curves are discussed separately. The experimental data for a 1:1 mixture of antibodies shows a low dose "hook" in the 0 - 300 pM hGH concentration range. The data are shown in Figure 2.10. It will be noted that the simulated curve generated using Model 5c (Curve B) fits the ascending limb of the experimental data for the mixture of antibodies. These data suggest that the multiple interactions of antigen and antibodies to form the circular complex should explain the binding behavior. The size of the major circular complex was assumed tetrameric, however it should be noted that some amounts of other circular complexes such as octameric can be formed. Curve B was generated assuming about 4 fold (GHC 101) and 1.5 fold (GHC 072) lower values for binding constants K_1 and K_2 compared with the experimental values.

To exclude linear complexes as responsible for the ascending limb of the "hooked" response for the mixture of antibodies, theoretical curve A was generated

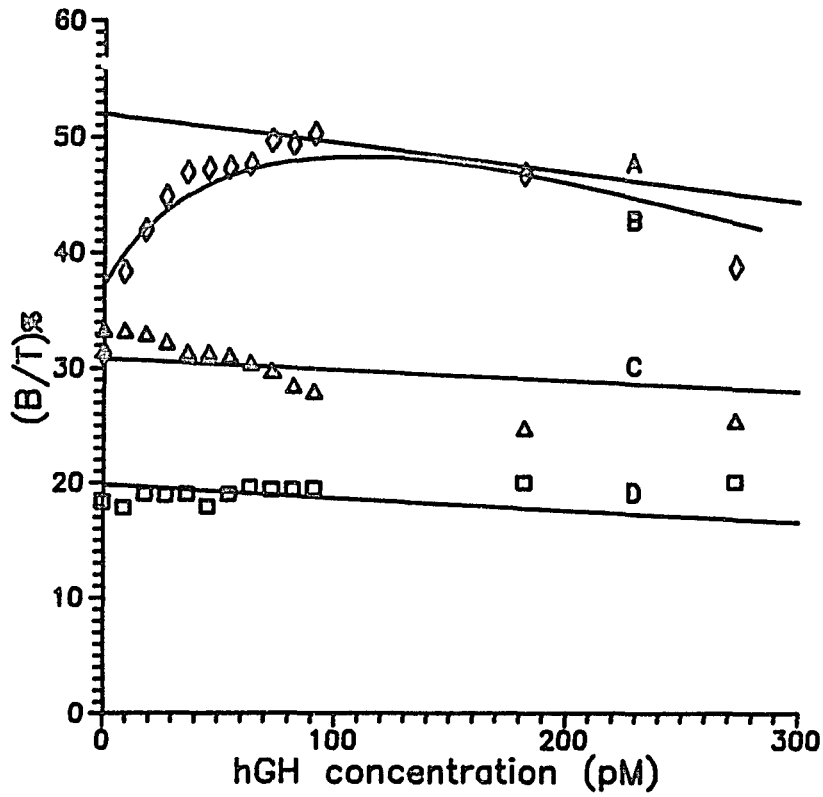

Figure 2.10 Comparison of experimental and theoretical results of competitive binding assay for hGH: *low* analyte concentration range. Antibody concentrations: monoclonal antibodies, GHC 101 (□) - 0.25 nM, GHC 072 (△) - 0.64 nM and 1:1 mixture (◊) - 0.20 nM. hGH concentration range is 10 - 300 pM.

Theoretical Curves A and B correspond to the experimental data for the 1:1 mixture of antibodies. Theoretical Curves C and D correspond to the experimental data for GHC 072 and GHC 101 respectively. Theoretical binding curves: *Curve A -- Model 5c:* K_1 = 1 nM^{-1}; K_2 = 3.8 nM^{-1}; a = 0.1 nM; b = 0.1 nM; *l* = 16 pM. *Curve B -- Model 5d:* K_1 = 0.9 nM^{-1}; K_2 = 1 nM^{-1}; K_3 = 300 nM; a = 0.1 nM; b = 0.1 nM; *l* = 16 pM. *Curve C -- Model 3:* K_1 = 0.7 nM; a = 0.64 nM; *l* = 16 pM. *Curve D -- Model 3:* K_1 = 1 nM^{-1}; b = 0.25 nM; *l* = 16 pM.

using Model 5c. Curve A is responsible for linear complex formation and since Curve A does not fit the ascending limb of the "hook" this suggests that the circular complexes are responsible for the observed behavior. The experimental and theoretical values of affinities are almost the same for Curve A. No inhibition was observed for the experimental data for GHC 101. This is attributed to lack of competition between labeled and unlabeled hGH for the fixed amount of GHC 101 at low antigen concentration. GHC 072 data simply show an inhibition. Theoretical binding curves were also generated for the mixture and individual antibodies using Models 3 and 4. Theoretical curves for the single antibodies do fit with the experimental data. The curves for the individual antibodies in Figure 2.10 are symbolized as C and D. Curve E represents the theory for the 1:1 mixture assuming that these two antibodies do not interact each other. Theoretical values of affinities for GHC 101 and GHC 072 are assumed approximately 4 and 1.5 fold less than the reported experimental values. Theoretical curve E does not fit for the experimental data of 1:1 mixture again concluding that GHC 101 and GHC 072 bind to two different epitopes. As we predicted earlier the formation of linear complexes does not describe the low dose "hook" in the competitive binding assay. The competitive binding assay for a 1:1 mixture of antibodies was compared with individual monoclonal antibodies in the 0.1 - 9.0 nM (high) concentration range of hGH. The data are shown in Figure 2.11. The mixture of antibodies does show a "hook" at very low concentrations of hGH (< 0.3 nM) but because B_o is not included in the plot ($B_o\% = 34.0$) the dose-response curve appears normal. The data for the 1:1 mixture of antibodies should theoretically result in lower bound ^{125}I-hGH since the total antibody concentration used for the mixture is less than that of the individual antibodies. However, the reverse is observed for the data in this concentration range of hGH, suggesting higher affinity for the 1:1 mixture.

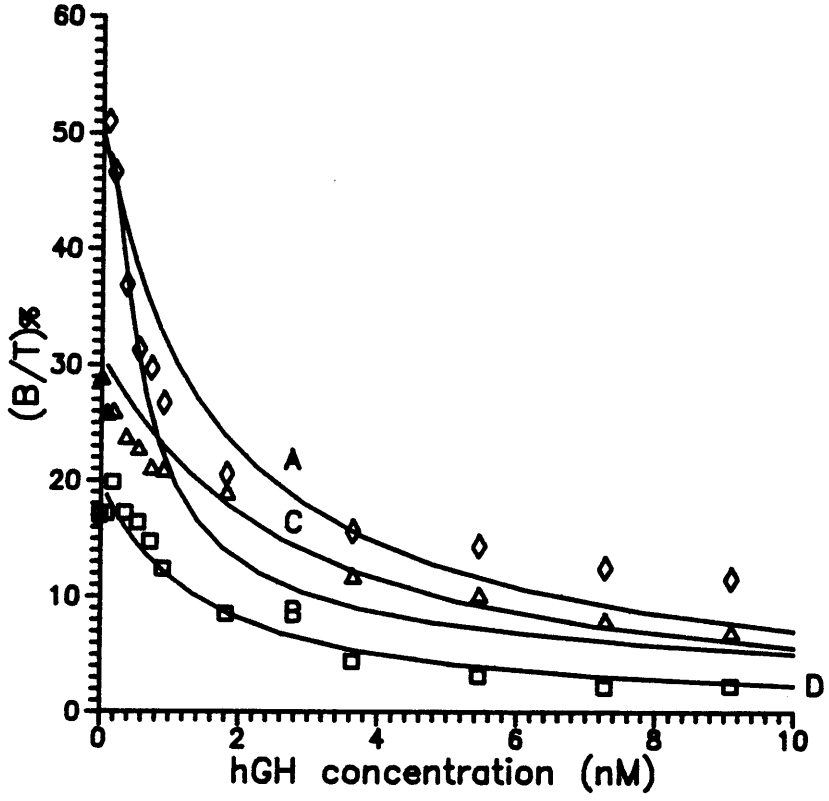

Figure 2.11 Comparison of theoretical and experimental results of competitive binding assay for hGH: **high** analyte concentration range. Antibody concentrations: Individual antibodies, GHC 101 (□) - 0.25 nM, GHC 072 (△) - 0.64 nM; and 1:1 mixture (◊) - 0.20 nM, total antibody. hGH concentration range: 0.09 - 9.0 nM. (B_o/T) % = 34.0 for the mixture of antibodies is not included.

Theoretical Curves A and B correspond to the experimental data for the mixture of antibodies. Theoretical Curves C and D correspond to the experimental data for GHC 072 and GHC 101 respectively. Theoretical binding curves: *Curve A -- Model 5c:* K_1 = 3.5 nM^{-1}; K_2 = 1.0 nM^{-1}; a = 0.1 nM; b = 0.1 nM, *l* = 16 pM. *Curve B -- Model 5d:* K_1 = 1 nM^{-1}; K_2 = 0.9 nM; K_r = 300 nM; a = 0.1 nM; b = 0.1 nM; *l* = 16 pM. *Curve C -- Model 3:* K_2 = 0.7 nM^{-1}; b = 0.64 nM; *l* = 16 pM. *Curve D -- Model 3:* K_1 = 1 nM^{-1}; a = 0.25 nM; *l* = 16 pM.

This is consistent with the titration data for 1:1 mixture (shown in Figure 2.4). The competitive binding assay data for the individual monoclonal antibodies in Figure 2.11 exhibit the expected inhibition behavior (cf: Figure 2.10 and Case I, Figure 2.1A). Theoretical curves for competitive binding assay consisting of single monoclonal antibodies were generated using Model 3. These curves are designated as C and D (Figure 2.11). Affinities of GHC 101 and GHC 072 are approximately 4 (GHC 101) and 1.5 (GHC 072) less than the experimental values. In this concentration range only the descending limb of the biphasic dose-response curve is considered. It is apparent that Model 5c fits the data for the mixture of antibodies (Curve A) in which a mixture of linear complexes is formed. Model 5d does deviate from the experimental data for the mixture of the antibodies indicating that the response decrease is due to the dissolution of circular complexes at high analyte concentrations. The low dose "hook" for a mixture of antibodies conforms to Model 5d suggesting that concomitant interactions of antibodies should result in highly stable circular complexes.

Competitive binding assays were further performed for different ratios of monoclonal antibodies, while keeping the total antibody concentration constant at 0.20 nM. All of these data were compared with a competitive binding assay at 0.40 nM antibody concentration. The unlabeled hGH concentration was 0.10 - 9.0 nM in the experiments. These results are shown in Figure 2.12 (B_o values for mixtures with 0.20 nM total antibody concentration are not shown). There is no significant difference in the shape of the curves for mixtures with ratios of 1:1 or 1:3 (GHC 101 : GHC 072), however, the curve for the 3:1 mixture reflects the greater contribution from the higher affinity antibody (GHC 101) and the curve accordingly exhibits increased slope (Figure 2.12). As the total concentration of antibodies is increased (0.40 nM), the response maximum corresponding to the

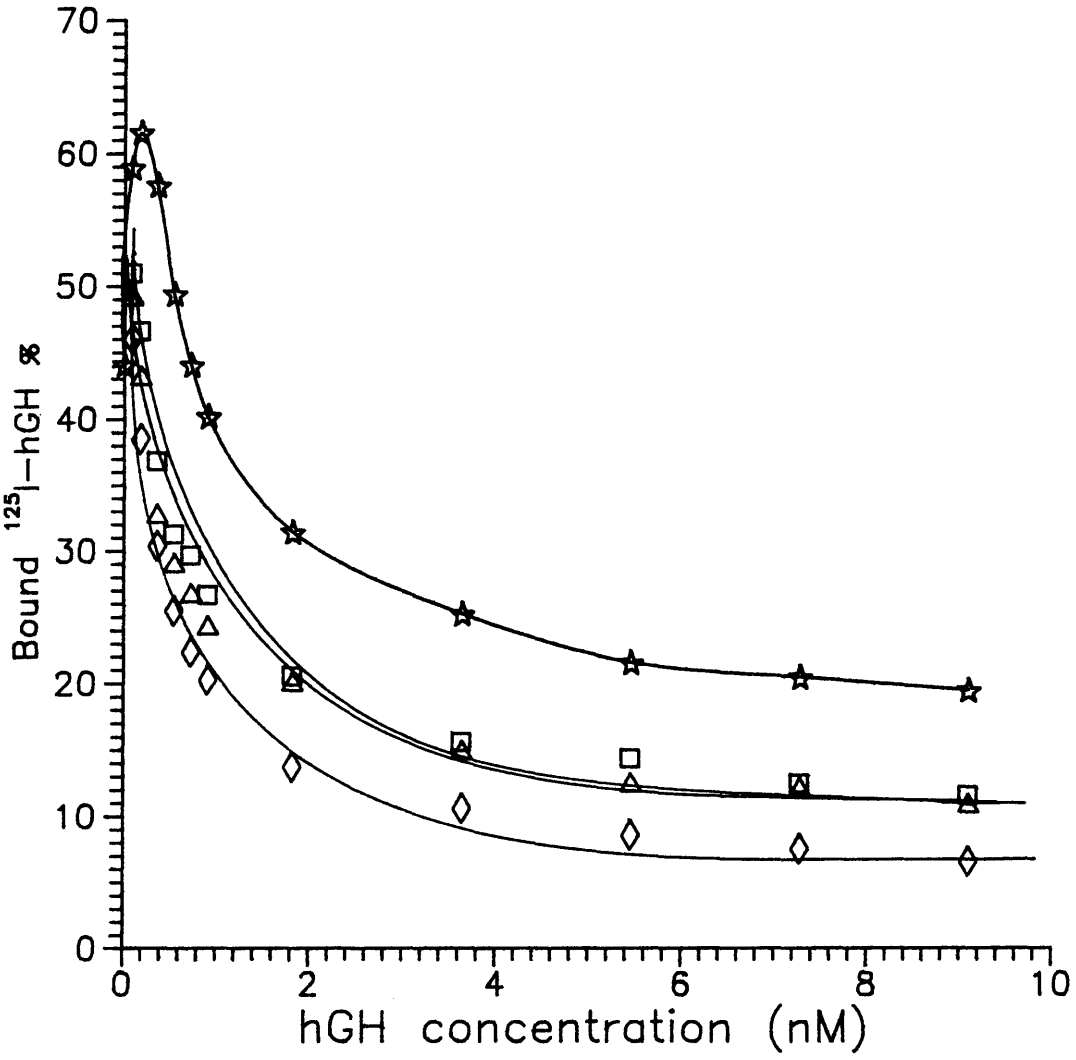

Figure 2.12 Competitive binding assay : Effect of antibody ratios on binding response. Total antibody concentrations: 1:1 mixture (*) - 0.40 nM. Total antibody concentration for the 1:1 mixture (□), 1:3 mixture (△) and 3:1 mixture (◊) is 0.2 nM. Ratio = GHC 101:GHC 072; % B_o/T values for mixtures with 0.20 nM total antibody concentration are as follows: 1:1 mixture -- 32.0; 1:3 mixture -- 32.7 and 3:1 mixture -- 29.4.

"hook" shifts to higher concentrations of unlabeled hGH (Figure 2.12). The total antibody concentration is in excess for each assay. The total hGH (labeled and unlabeled) concentration is limiting, therefore, the "hook" is predicted to appear at higher concentrations of hGH (Figure 2.12).

If the same experiment is performed in the 10 - 300 pM hGH concentration range, the "hook" is expected to disappear. In order to test this prediction, the competitive binding assay experiment was carried out with the following. Total antibody concentrations: 0.15, 0.10, 0.05 nM shown in Figure 2.13 and 0.40, 0.80, 1.2, 1.6 nM shown in Figure 2.14. The total hGH concentration is limiting as the antibody concentration is increased. Lower total concentration of antibody results in no significant change in the curves for the competitive binding assay. No "hook" was observed for 1.2 and 1.6 nM total antibody concentrations. The increased bound ^{125}I-hGH (for curves with 1.2 and 1.6 nM total antibody) is due to the presence of excess antibodies in the sample.

The competitive binding assay with higher ^{125}I-hGH concentrations (excess labeled hGH), should form higher molecular complexes with ^{125}I-hGH. Therefore the response for B_o should be considerably higher than that with lower ^{125}I-hGH. To avoid the difficulties of measuring and handling higher concentrations of ^{125}I-hGH, a known mixture of ^{125}I-hGH and unlabeled hGH was used. After the mixture of ^{125}I-hGH and hGH is added the maximum response is achieved. Thus the addition of hGH will simply replace ^{125}I-hGH in complexes, to generate a curve without showing a "hook". The competitive binding assay is carried out under the same conditions as the experiment in Figure 2.11, with an antibody ratio of 1:1 in the 10 - 300 pM range, except that the radiolabeled tracer is mixed with unlabeled antigen. The data are shown in Figure 2.15. It is clear that the peak maximum occurs at lower unlabeled hGH concentrations when higher total hGH

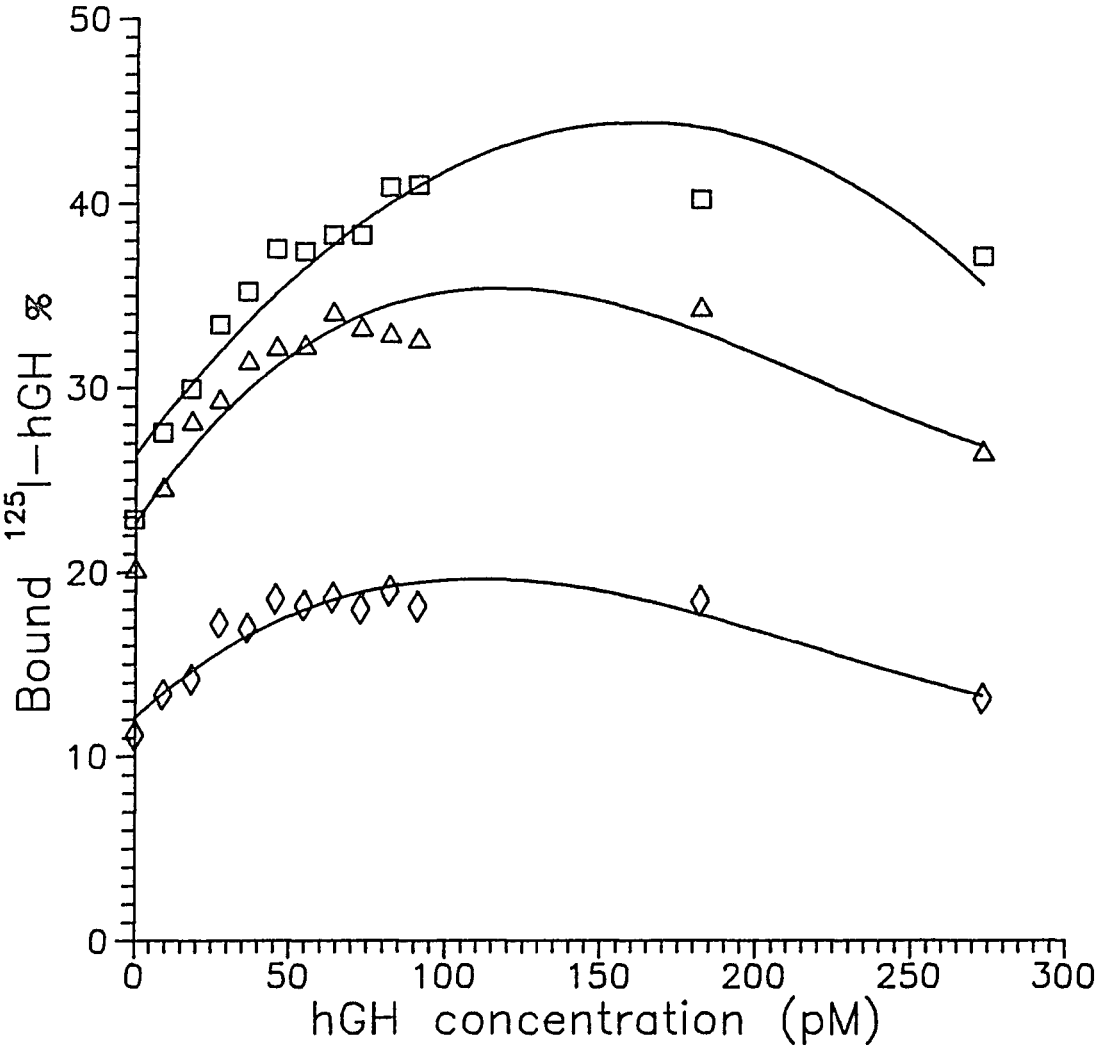

Figure 2.13 Competitive binding assay -- Effect of total antibody concentration on binding response: low antibody concentration range. Ratio of mixtures is 1:1. Total antibody concentrations: 0.15 nM (□), 0.10 nM (△) and 0.05 nM (◇).

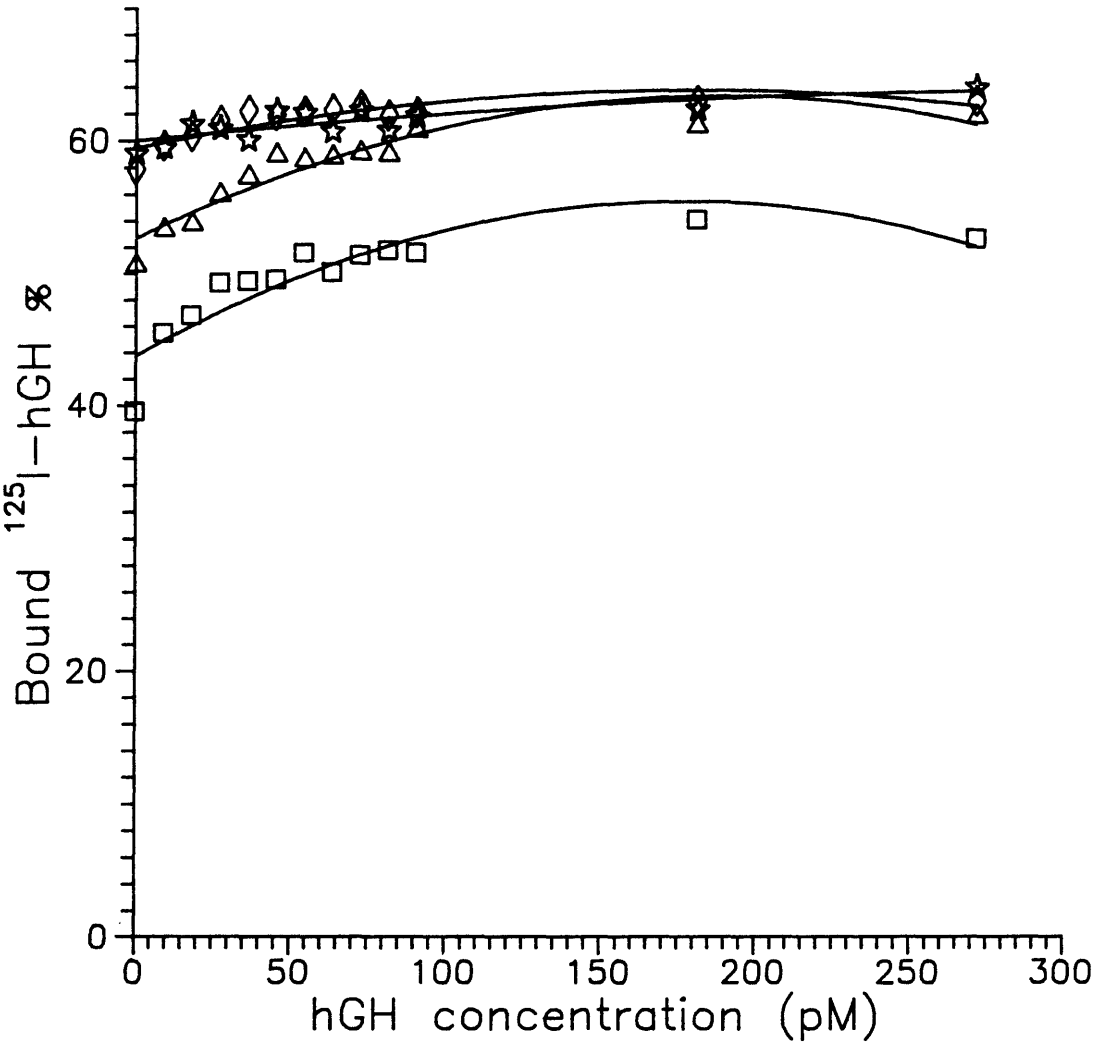

Figure 2.14 Competitive binding assay -- Effect of total antibody concentration on binding response: high antibody concentration range. Ratio of mixtures is 1:1. Total antibody concentrations: 0.40 nM (□), 0.80 nM (△), 1.20 nM (◊) and 1.60 nM (*) respectively.

concentrations (labeled and unlabeled) are used (cf: Figures 2.11, 2.13 and 2.15). These data suggest that higher amounts of labeled antigen will saturate the antibody, forming all possible complexes, and achieving the maximum measurable response at B_0 prior to the addition of hGH. Therefore the response curve generated by adding hGH should yield a normal inhibition curve rather than a low dose "hook".

2.3.14 Elucidation of antigenic determinants of hGH

A requirement for circular complex formation is the ability of GHC 101 and GHC 072 to bind two different epitopes on hGH simultaneously. Based on the available sequence information, hGH is known to have no repeating epitopes or identical sequences, indicating that the binding of the each single antibody would not increase the avidity (Riggin et al., 1988; Li and Graf, 1974).

Binding sites of GHC 101 and GHC 072 have been investigated (Surowy et al., 1984). Detailed results for GHC 072 were not reported, but GHC 072 was shown to have similar characteristics to antibody GHE 033. GHE 033 and GHC 101 were further evaluated by Cunningham et al. (1990). GHE 033 (referred to as Mab 2) was shown to recognize a discontinuous antigenic determinant involving helix 1 and the polypeptide segment (amino acids 96 -106) connecting helices 2 and 3. GHC 072 (referred to as Mab 4) was also shown be specific for a discontinuous determinant, but the determinant involved helices 1 and 3. An asparagine residue at position 12 of helix 1 was shown to be critical for binding of GHC 072 to hGH but was not important for GHE 033 binding. It was hypothesized that GHE 033 and GHC 072 bound to different faces of the hGH molecular surface. Overall, the data of Cunningham et al. (1990) supported the earlier predictions of Surowy et al. (1984). Further, it is possible to argue that the antibody mixture binds to spatially different epitopes of hGH, forming a

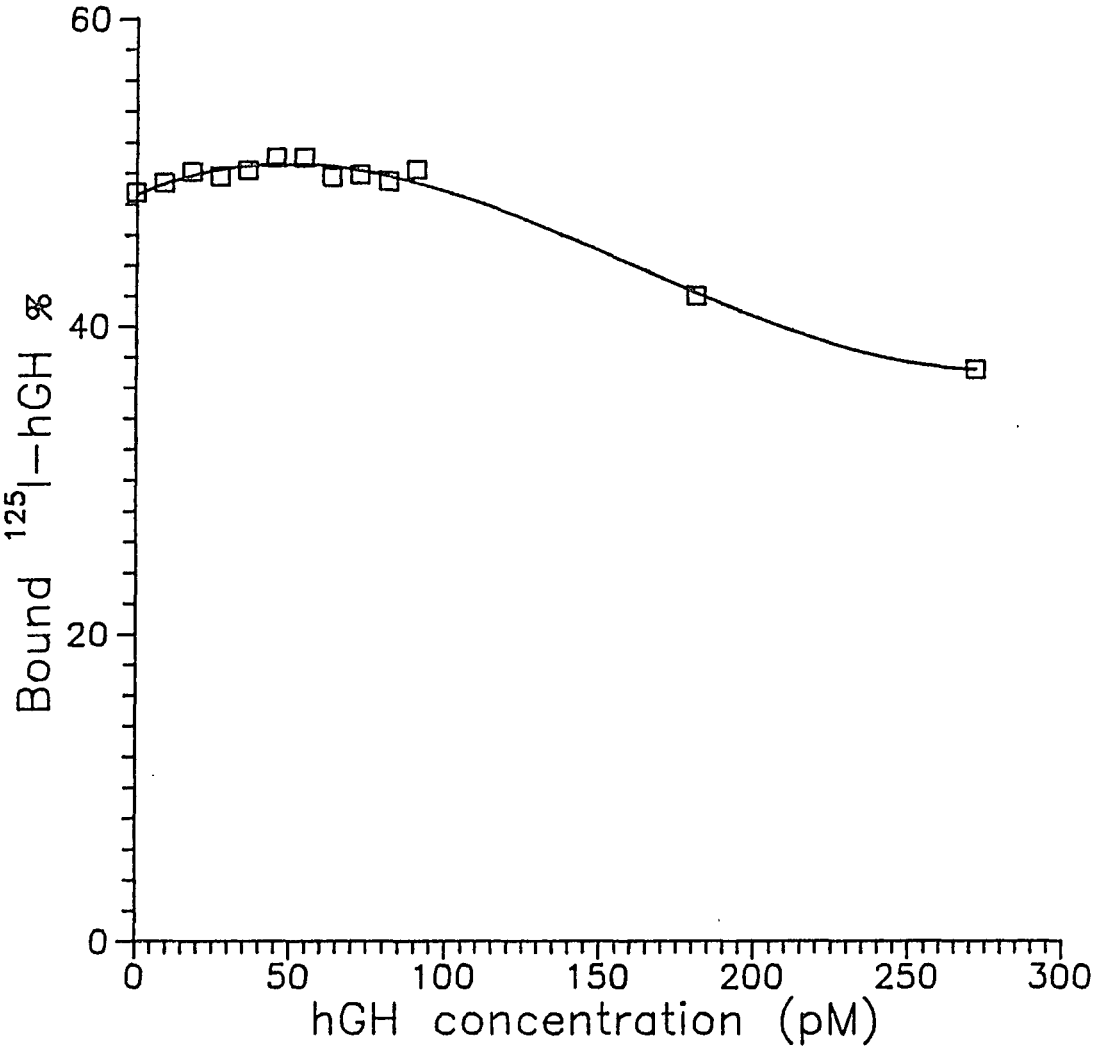

Figure 2.15 Competitive binding assay: Effect of total antigen concentration on binding response for the mixture of antibodies. Total antibody cocnentration -- 0.2 nM. Ratio -- 1:1. Concentration of antigen -- a mixture of ^{125}I-hGH (14.5 pM) and hGH (0.09 nM).

multicomponent complex resulting in binding enhancement.

2.3.15 Size exclusion chromatography for hGH

Size exclusion separations of antigen-antibody complexes were performed to further investigate the interaction of hGH with the mixture of monoclonal antibodies. Unlabeled hGH was mixed with GHC 101 and GHC 072 monoclonal antibodies (160 kDa), keeping the antibody ratio 1:1. The molar ratio of the antibody or antibody mixture to hGH was 0.87. All species were separated and their molecular weights compared with those of both individual antibodies and with that of hGH. Size exclusion separations of GHC 101 and GHC 072 monoclonal antibodies yielded a single peak (Figure 2.16 c,d Peak IV), demonstrating homogeneity of antibodies. hGH exhibited a single peak at a longer retention time (Figure 2.16f Peak II).

The two mixtures of single antibodies and hGH were chromatographed under the same conditions and the two components were resolved as shown in Figure 2.16a and e (Peaks II and III) respectively. The components having longer retention times are identical to hGH (Figure 2.16f Peak II). The interaction of single antibodies and hGH probably results in the formation of complexes with one or two hGH molecules and antibodies. All of these complexes eluted as a separate peak in the chromatographic separations (Figure 2.16a and e Peak III). Also, unbound monoclonal antibodies in the mixture probably coelute with the complexes under these conditions because of the similarity in molecular weight. The elution profile of the antigen and 1:1 mixture of the two antibodies suggests the formation of a higher molecular weight species, likely a complex (Figure 2.16b Peak I). The peak that would be caused by unreacted antibodies does not appear in the chromatographic profile, indicating their nearly complete reaction to form a

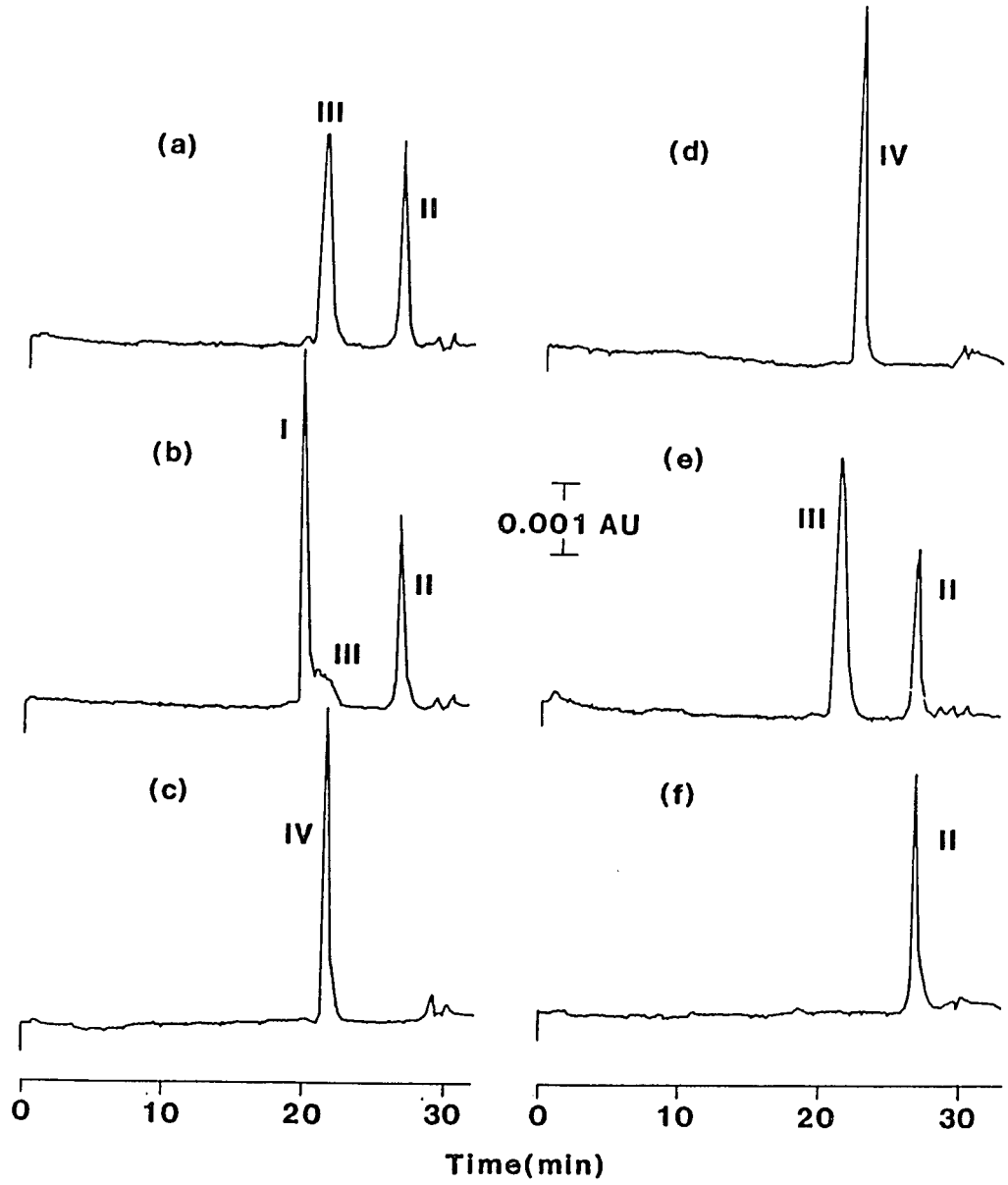

Figure 2.16 The chromatographic behavior of antigen-antibody complexes:
(a) GHC 101 and unlabeled hGH (b) 1:1 mixture of monoclonal antibodies and unlabeled hGH
(c) GHC 101 monoclonal antibody (d) GHC 072 monoclonal antibody (e) GHC 072 and unlabeled hGH
(f) unlabeled hGH. Concentrations of all reagents are reported in section 2.2.5.1.

complex. hGH was in excess in the mixture and the peak due to unbound hGH appears (Figure 2.16b Peak II) in the chromatographic profile. The cascade of reactions involving individual monoclonal antibodies and hGH can be represented by Figure 2.2. The maximum number of hGH molecules which can bind to a monoclonal antibodies is two. Furthermore, the hGH molecule cannot bind with two molecules of the same monoclonal antibody simultaneously. Chromatographic data for the monoclonal antibodies (Figure 2.16) show no complexes attributed to hGH-2(GHC 101) or hGH-2(GHC 072) (molecular weight = 342 kDa). Therefore, 1:1 and 1:2 complexes, HA, HB, HAH and HBH are the only permitted complexes that can exist for each monoclonal antibody under these conditions. Assuming that the two binding sites of the antibody molecule are identical and independent, there is the possibility of cooperativity (Mazza et al., 1989). In this hypothesis, binding of one antigen to the antibody leads to a conformational change in the antigen molecule which enhances the interaction with the second antibody. There is no experimental evidence to support this hypothesis in the present situation. The reactions resulting in the formation of all possible multicomponent complexes are summarized in Figure 2.3. As Figures 2.2 and 2.3 show the stoichiometry of complexes are given up to molecular weight 364 KDa.

Standard proteins were applied to the column under similar conditions to calculate the molecular weights of these complexes. The plot of log(molecular weight) vs retention time is shown in Figure 2.17 The retention times for catalase (232 kDa) and aldolase (158 kDa) are almost the same (Figure 2.17). The molecular weight of the complex corresponding to Peak I in Figure 2.16 is approximately 600 kDa. To account for the stoichiometry of the 600 kDa complex, the formation of higher molecular weight complexes have to be

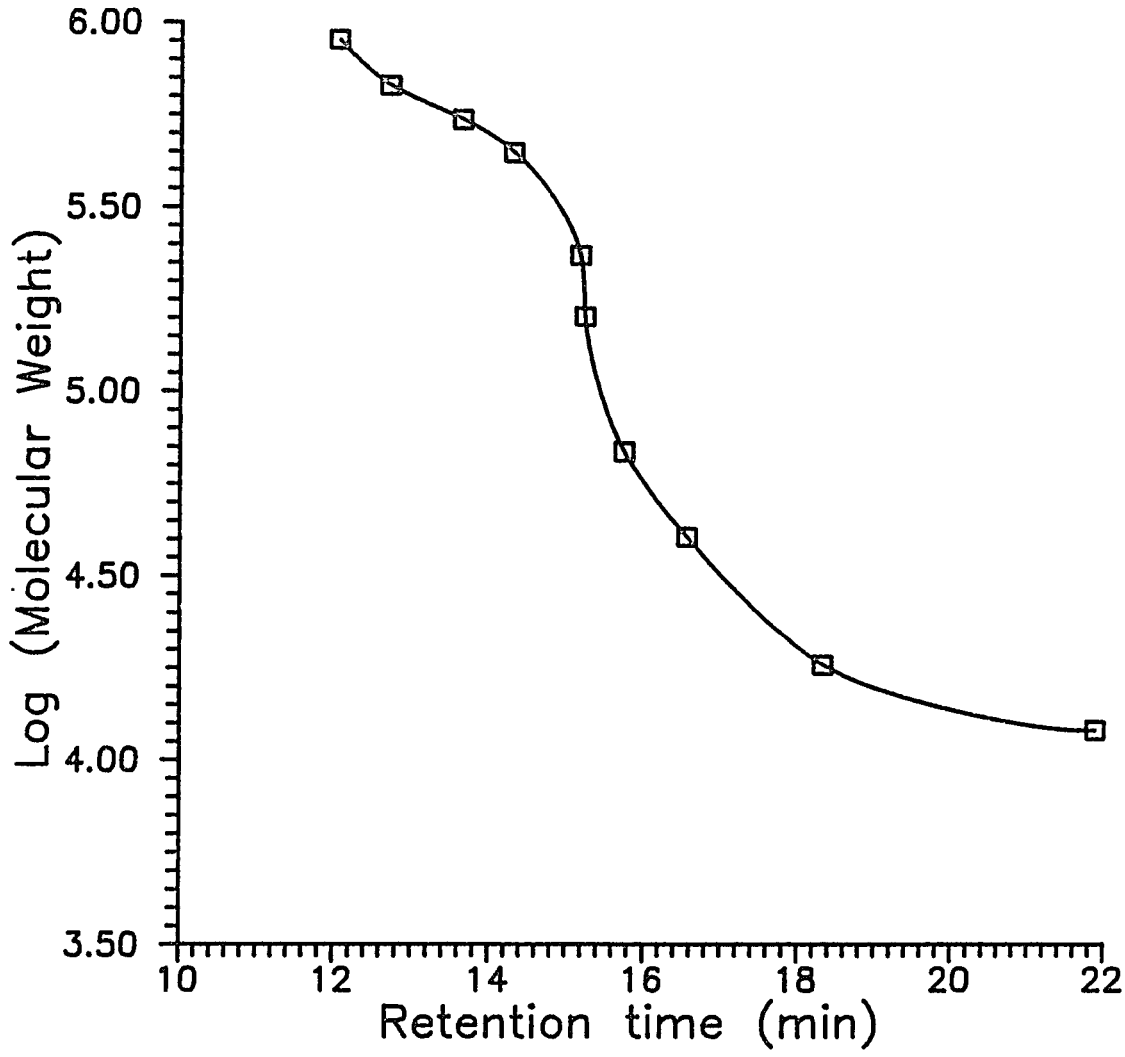

Figure 2.17 The calibration curve for molecular weight standards: IgM (900 kDa), thyroglobulin (669 kDa), beta-Galactosidase (540 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), BSA (68 kDa), HRP (40 kDa), myoglobin (18 kDa), cytochrome C (12 kDa).

considered. The possible stoichiometries for higher molecular weight linear and circular complexes are shown in Figure 2.18. Stoichiometries, XI and XII, linear complexes are the almost same except for the type of antibody present in the complex. The molecular weight of either complex (XI or XII) is 588 KDa. The stoichiometry of 600 kDa complex (Peak I, Figure 2.16) could probably be XI or XII.

2.3.16 Effect of different concentrations of antibodies

This experiment is analogous to the titration curves in Figure 2.4 in which hGH concentration was kept constant and the concentration of antibodies was varied while keeping the ratio 1:1. The purpose of this experiment was to demonstrate the other possible complexes which might form at lower concentrations of hGH. The molar ratio of the total concentration of antibodies to hGH was in the 3.4 - 14.0 range and 0.18 nM of hGH was used. The 600 kDa peak appeared in chromatograms for each of the mixtures in Figure 2.19 (a-d,f) as Peak I. At higher total antibody concentration, the unreacted antibodies, as well as the 1:1 and the 1:2 complexes (antibody:hGH) coeluted as an identical peak (Peak III) in the chromatographic profile (Figure 2.19b-d,f). Another chromatographic peak, corresponding to a higher molecular weight complex, was observed at two concentrations of antibodies (Figure 2.19b,c Peak V). The molecular weight of this complex is about 720 kDa and was present at a low concentration compared to the major complex (600 kDa). Also, the mixture of pure antibodies coeluted as an identical peak (Figure 2.19e Peak VI), similar to the individual antibodies (Figure 2.16c,d Peak IV). The stoichiometry of the 720 kDa complex (Peak V, Figure 2.19) which supports the experimental data is shown in Figure 2.18 (IX) (molecular weight of 728 kDa). This complex could probably be cyclic, but it should also be noted that the complexes VIII and X have same molecular weight

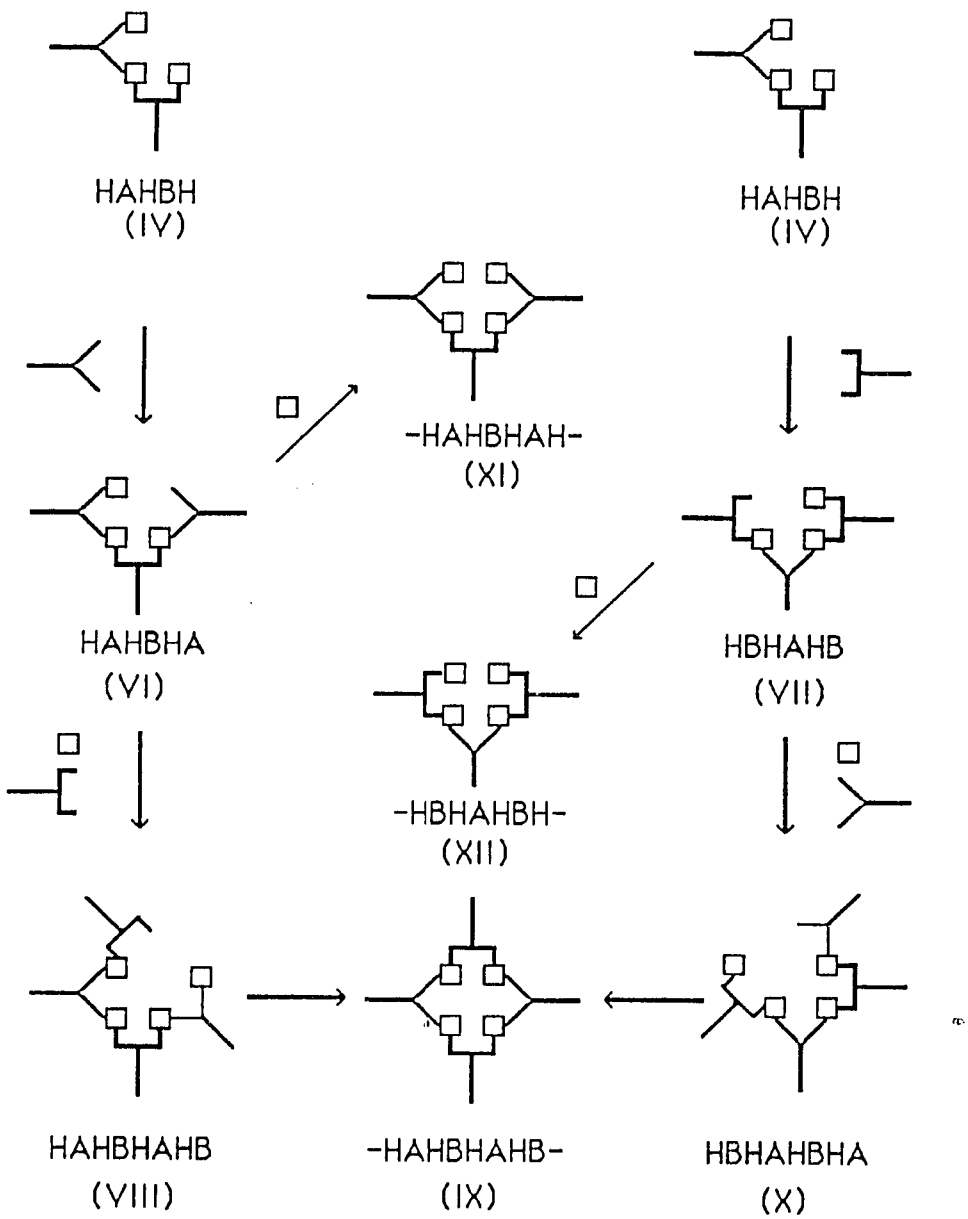

Figure 2.18 Schematic representation of octameric complexes with hGH and mixture of antibodies.

A:GHC 101, B:GHC 072 and H:hGH.

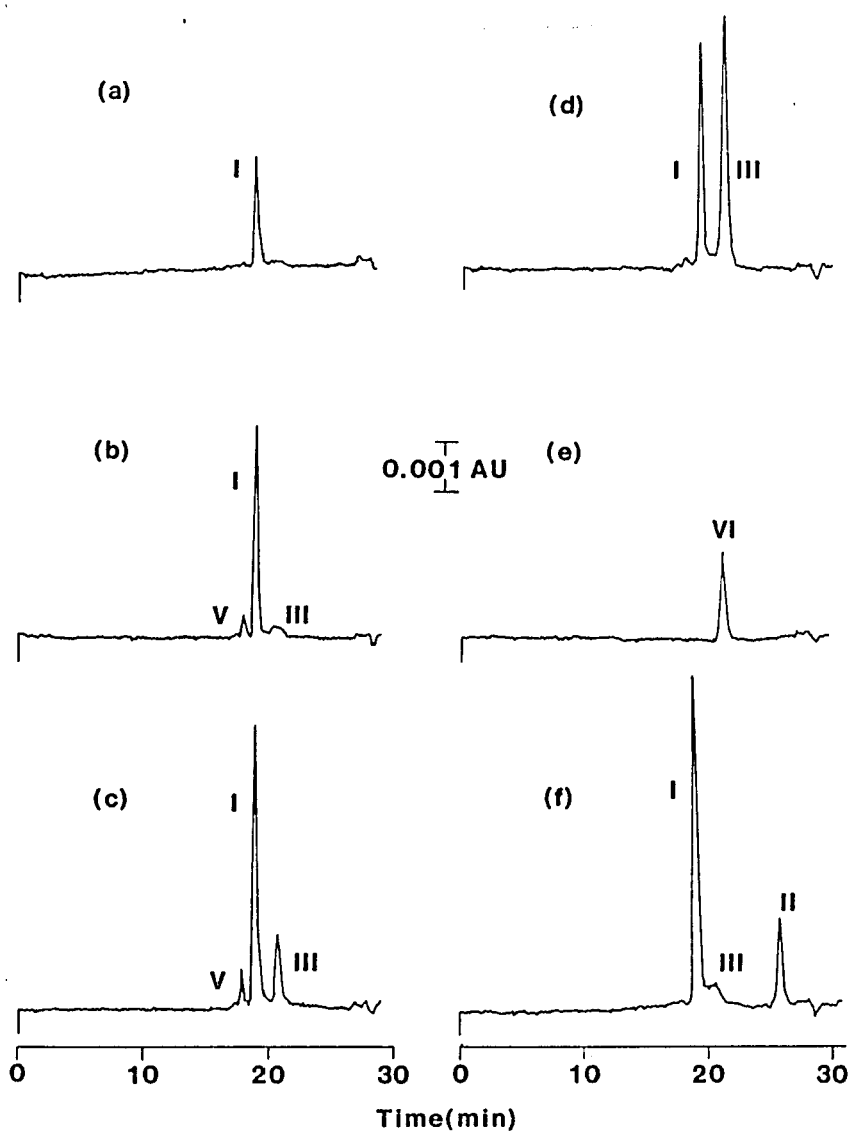

Figure 2.19 Chromatograms showing the effect of antibody concentration on complex formation: Concentration of hGH (a) - (d): 0.18 nM and (f) 0.90 nM and (e) no hGH.

Antibody ratio is 1:1. Total antibody concentration:

(a) 0.62 nM (b) 1.25 nM (c) 1.87 nM (d) 2.50 nM (e) 1.56 nM (f) 0.62 nM.

Experimental details are given in section 2.2.5.2.

as complex IX. However complexes VIII and X are not circular.

2.3.17 Effect of different concentrations of hGH

This experiment is analogous to the saturation experiment of Figure 2.8. A fixed amount of antibody mixture was reacted with different concentrations of hGH. Figure 2.20 shows the results for the 1:1 mixture. The major peak in the 1:1 mixture of antibodies is the 600 kDa complex (Figure 2.20a-d, peak I). A minute amount of unreacted antibodies is eluted as peak III (Figure 2.20). The unbound hGH is shown as the Peak II suggesting that hGH is in excess.

In order to show that monoclonal antibodies do not form higher molecular weight complexes with hGH, a parallel experiment was carried out with the monoclonal antibody, GHC 101. Figure 2.21 shows the results for GHC 101. As the concentration of hGH was increased, the unreacted antibodies, probably 1:1 and 1:2 complexes (antibody:hGH) eluted as an unresolved peak (Figure 2.21b-e, Peak III). The unbound hGH is shown as Peak II in Figure 2.21. The complexes formed for GHC 101 with a 1:1 mixture of antibodies support the experimental data for the saturation curve.

2.3.18 Stability of higher molecular weight complexes

The chromatographic profile for the mixture of antibodies can also be used to qualitatively identify and verify the stability of the higher molecular weight complexes. The hGH and antibodies react rapidly when these experiments are carried out at room temperature. A chromatographic profile was obtained just after reagent mixing (Figure 2.22a) that is similar to the profile after complete incubation (Figure 2.16a). The major components are the 600 kDa and 720 kDa complexes, as well as traces of unreacted antibodies. To identify the peak for unreacted antibodies, the sample was spiked with a known amount of GHC 101. This mixture, with added excess monoclonal antibody confirms the retention

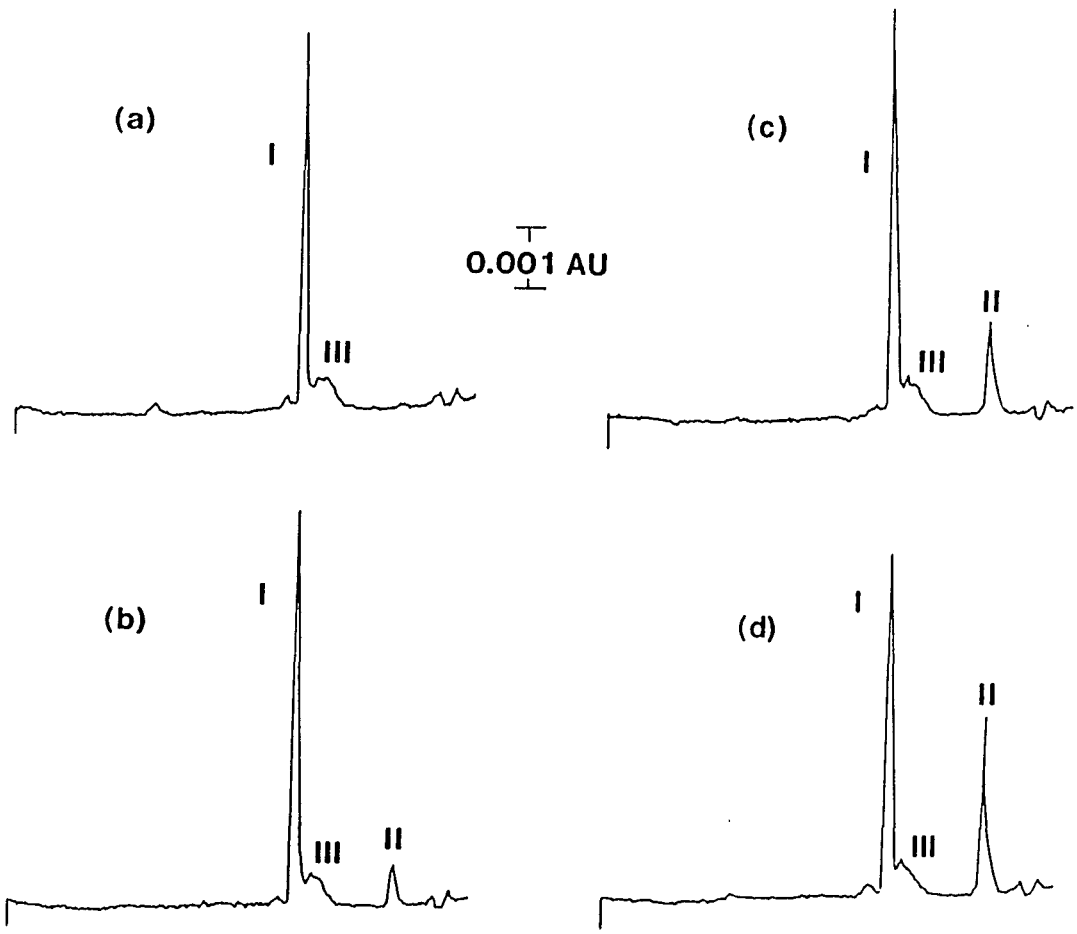

Figure 2.20 Chromatograms showing effect of hGH concentration on complex formation for a mixture of antibodies. Total antibody concentration - 0.5 nM. Ratio -- 1:1. hGH concentrations (a) 0.18 nM (b) 0.36 nM (c) 0.53 nM (d) 0.71 nM. Experimental details are given in section 2.2.5.4.

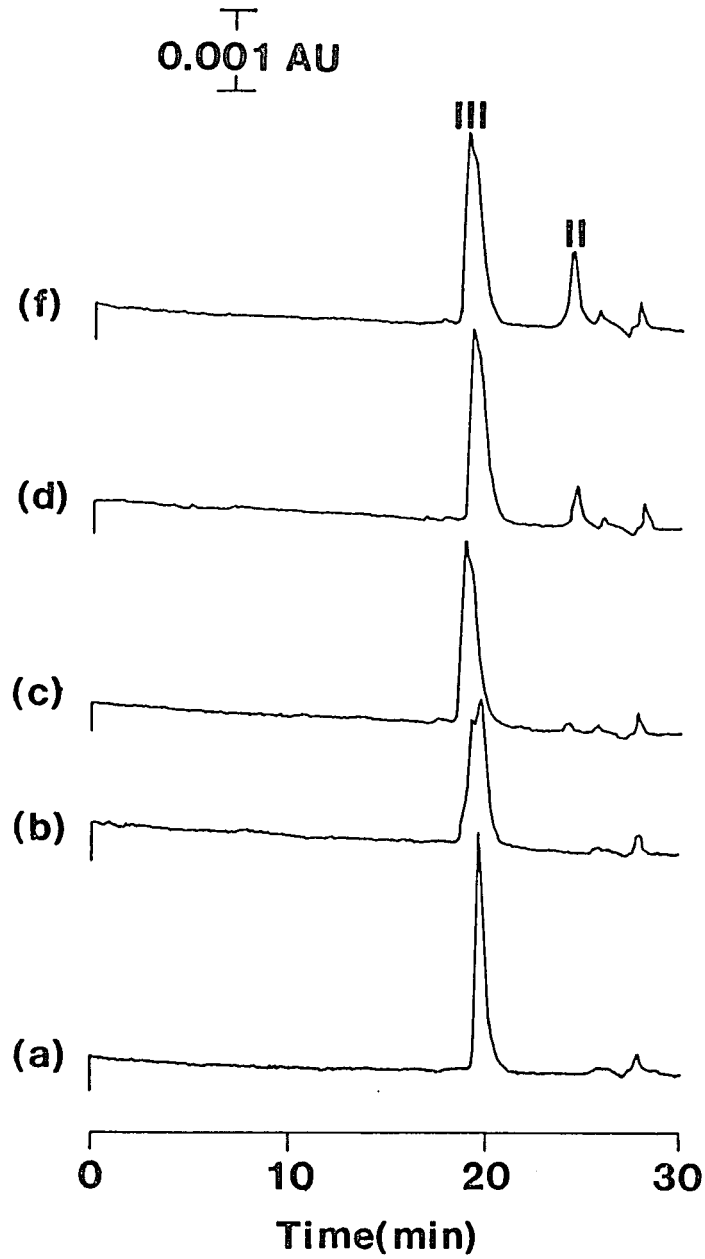

Figure 2.21 Chromatograms showing hGH-GHC 101 complexes. GHC 101 concentration -- 1.87 nM.

(a) GHC 101 (b) GHC 101 and 0.35 nM of hGH (c) GHC 101 and 0.71 nM of hGH (d) GHC 101 and 1.0 nM of hGH (e) GHC 101 and 1.4 nM of hGH. Experimental details are given in section 2.2.5.3.

profile of the unreacted antibodies (Figure 2.22b Peak III). When excess hGH is added to the same mixture, a chromatographic profile showing excess hGH and a unresolved peak caused by complexes with monoclonal antibodies and unreacted antibodies was observed (Figure 2.22c Peaks II and III). Finally, the addition of more GHC 101 caused formation of higher concentrations of the 1:1 and 1:2 antibody-hGH complexes (Figure 2.22d Peak III). The amount of the multicomponent complex (600 kDa) is not affected by addition of any other reagents such as excess hGH and GHC 101 after it is formed as shown in Figure 2.22a-d Peak I.

A similar experiment was carried out to show that both antibodies are required for concomitant interactions. This experiment was carried out at room temperature. The 1:1 mixture of antibodies shows (Figure 2.23a) the chromatographic profile seen previously (cf: Figure 2.22a). Increasing amounts of GHC 101 and GHC 072 were added to the same mixture and rechromatographed. These profiles are shown in Figure 2.23b-c. As the addition of both antibodies raises the concentration of unreacted antibodies, the excess causes the height at Peak III to increase. After addition of hGH to the above mixture, the peak caused by unreacted antibodies disappeared and the amount of 600 kDa complex increased as shown in Figure 2.23d.

The interaction of the three components to form the complexes was further demonstrated by varying the amounts of hGH for the fixed amount of antibodies. The experiments started with the mixture of antibodies yielding a single peak (Figure 2.24a). As increasing amounts of hGH were spiked into this mixture, the 600 kDa complex formed, as shown in Figure 2.24b-c. These chromatograms were compared with standard proteins as shown in Figure 2.24d-e. The formation of additional amounts of the 600 kDa complex was thereby shown to result from

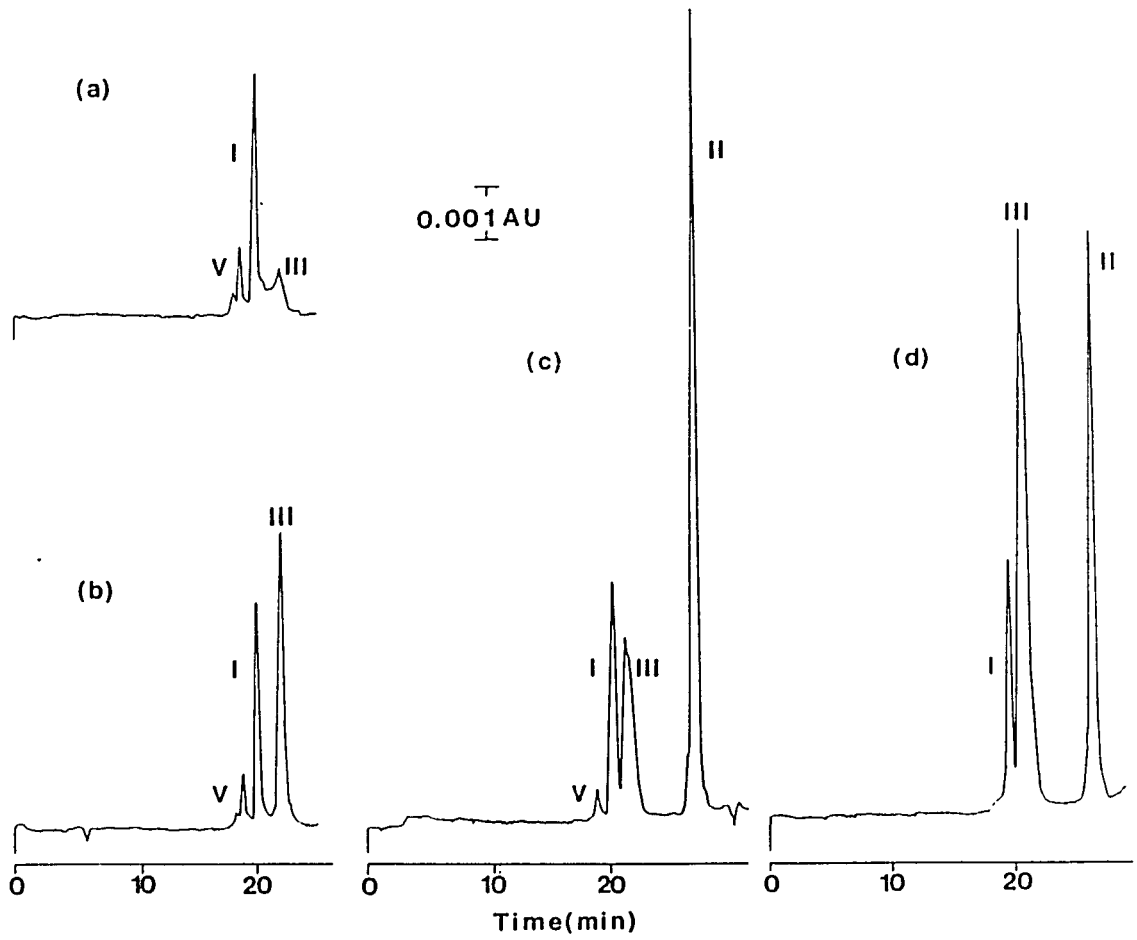

Figure 2.22 Identification of components in the mixture using size exclusion chromatography:

(a) 1:1 mixture of antibodies and hGH

(b) When GHC 101 is added to (a)

(c) When hGH is added to (b)

(d) When GHC 101 is added to (c). Experimental details are given in section 2.2.5.5.

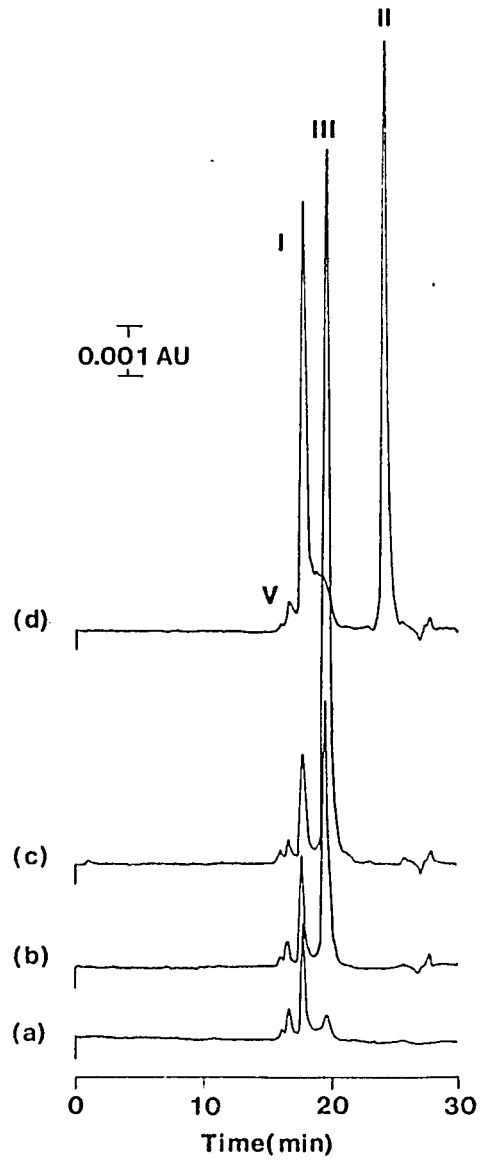

Figure 2.23 Identification of components in a mixture using size exclusion chromatography:

(a) 1:1 mixture of antibodies and hGH (b) When GHC 101 and GHC 072 are added to (a); (c) When GHC 101 and GHC 072 are added to (b); (d) When hGH is added to (c). Experimental details are given in section 2.2.5.6.

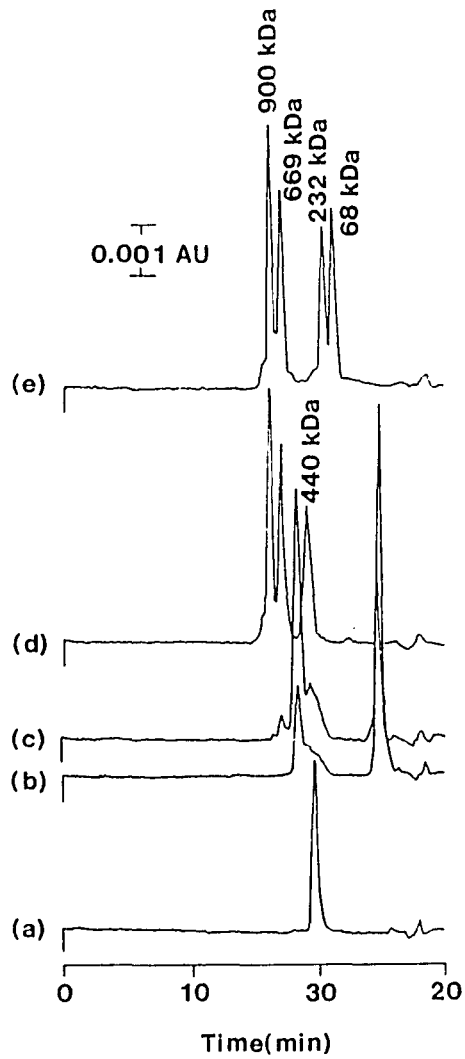

Figure 2.24 Identification of components in a mixture using size exclusion chromatography: (a) 1:1 mixture of antibodies

(b) When hGH is added to (a)

(c) When hGH is added to (b)

(d) Standard protein mixture; IgM, Thyroglobulin and ferritin

(e) Standard protein mixture; IgM, thyroglobulin, catalase and aldolase.

Experimental details are given in section 2.2.5.7.

either excess antibodies or excess hGH (cf: 2.22-2.24). These data again verify the higher stability of the complex and the requirement of participation of both antibodies for complex formation. The size exclusion experimental data show that the mixture of antibodies form a higher molecular weight species under these conditions.

2.3.19 Size exclusion chromatography for D-hGH

hGH can also exist in a dimeric form (Becker et al., 1987). The D-hGH can be used as a model molecule which has two repeating epitopes for each monoclonal antibody. No studies have previously shown the accessibility of all epitopes of D-hGH for simultaneous interaction with GHC 101, GHC 072 and a mixture of antibodies. Chromatographic separations of D-hGH complexes to elucidate the binding behavior of D-hGH. GHC 101 and GHC 072 were used to design a experiment analogous to the saturation curve in Figure 2.8. ^{125}I-hGH from the previous design was here replaced by D-hGH. The chromatographic profiles of GHC 101 and D-hGH complexes are shown in Figure 2.25. As the concentration of D-hGH is increased, the higher molecular weight complexes are eluted at low retention times (Figure 2.25a-c). A known amount of GHC 101 was added to the same sample and rechromatographed in order to identify the unbound antibody peak (Figure 2.25d). The same experiment was carried out using GHC 072 and these data are shown in Figure 2.26. The chromatographic profile is almost the same as in Figure 2.25. The molecular weight of the major complex is approximately 600 kDa.

To compare the ability of D-hGH to interact with antibodies in solution, the 1:1 mixture of antibodies was reacted with D-hGH. The elution profile for the 1:1 mixture of antibodies in Figure 2.27a is the peak previously seen in Figure 2.19e Peak VI. By just adding a small amount of D-hGH to the 1:1 mixture a variety of

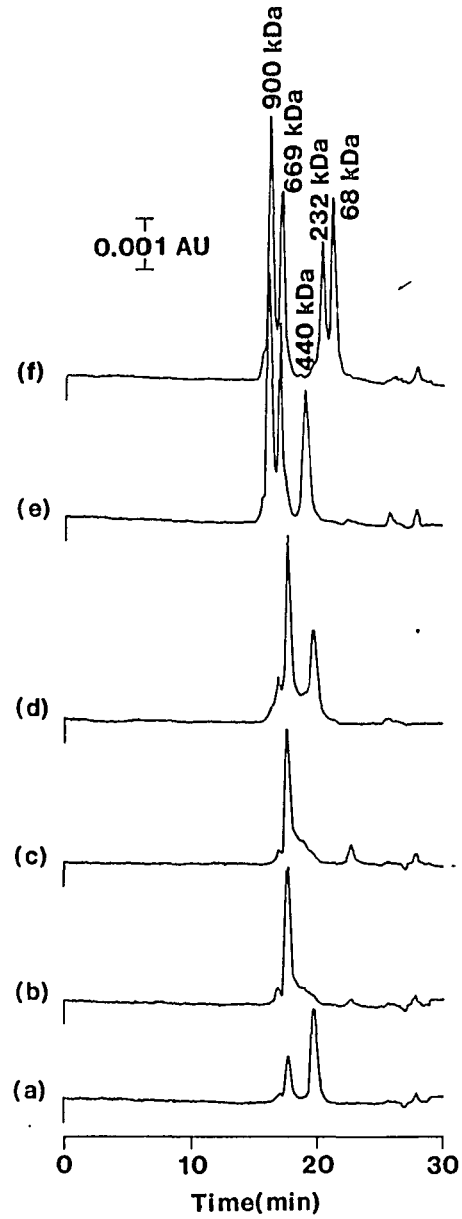

Figure 2.25 Separation of complexes with D-hGH and GHC 101: GHC 101 concentration - 1.87 nM.

D-hGH concentration (nM); (a) 2.24 (b) 6.72 (c) 8.96.

(d) addition of GHC 101 to (c).

(e) and (f) are standard protein mixtures. Experimental details are given in section 2.2.6.1.

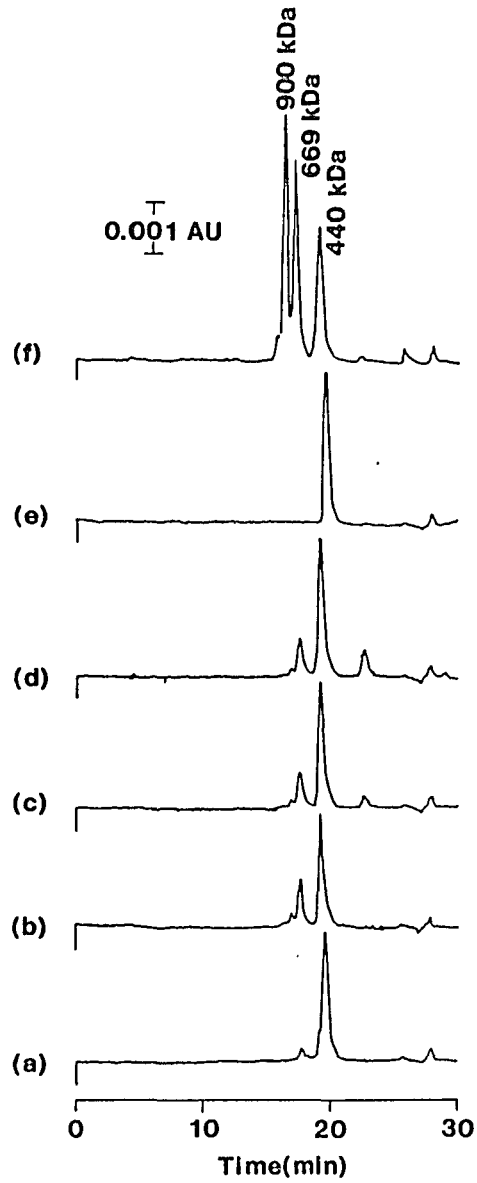

Figure 2.26 Separation of complexes with D-hGH and GHC 072:
GHC 072 concentration - 1.87 nM. D-hGH concentration (nM); (a) 2.24 (b) 4.48 (c) 6.72 (d) 8.96 (e) no D-hGH. Experimental details are given in section 2.2.6.2.

higher molecular weight complexes are formed (Figure 2.27b). As the concentration of D-hGH is increased, it is possible to generate a chromatographic profile consisting of several major, well resolved components (Figure 2.27c). The peak which represents higher retention time is correlated with unreacted D-hGH. The same sample was spiked with a 1:1 mixture of antibodies and subjected to chromatographic separation (Figure 2.27d). Similar complexes are formed to those seen previously with hGH. These two complexes exhibit approximately the same molecular weights when compared with standards. This data confirms the possibility of D-hGH binding concurrently with both antibodies.

2.3.20 Stoichiometry of complexes

Cyclic multicomponent complex IX as well as possible linear complexes are shown by comparing with the chromatographic data (Figure 2.18). The chromatographic data show that two major multicomponent complexes (600 and 720 kDa) do form. The -HABH- (Complex II) has a molecular weight of 364 kDa and this stoichiometry known to be the most stable form (Moyle et al., 1983; Holmes et al., 1983). Theoretically tetrameric and octameric complexes cannot be distinguished, however the chromatographic data show a reasonable indication of these complexes. The possibilities for the formation of circular complexes have been reported experimentally (Moyle et al., 1983; Holmes et al., 1983) and theoretically (Moyle et al., 1983; Schumaker et al., 1973) for different model analytes. Furthermore, the different sizes of complexes have been observed for the interaction of rabbit antibodies with chemically synthesized dimer of dinitrophenyl (DNP) hapten (Valentine and Green, 1967). The shortest carbon chain which allowed both DNP hapten groups to be combined simultaneously with an antibody was that of the octamethylenediamine (number of carbon atoms = 8). Electron micrographs show the formation of cyclic dimers, trimers, tetramers and

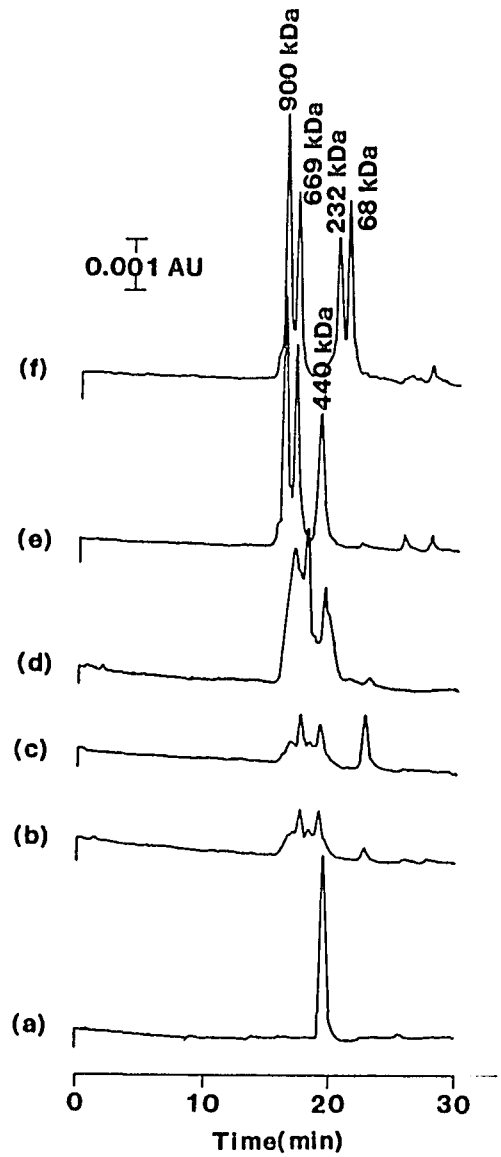

Figure 2.27 Separation of complexes with D-hGH and mixture of antibodies:

(a) 1:1 mixture of antibodies

(b) When D-hGH is added to (a)

(c) When more D-hGH is added to (b)

(d) When GHC 101 and GHC 072 are added to (c)

(e) and (f) are standard protein mixtures. Experimental details are given in section 2.2.6.3.

pentamers. Moreover, electron microscopic studies on another bivalent hapten, bis-dansyl cadaverine (Phillips et al., 1990) and monoclonal antibodies demonstrated ring-shaped complexes predominantly the dimeric form. Comparing with previous studies on theoretical and practical grounds, the low dose "hook" in competitive binding assay can be exhibited if a mixture of antibodies interact cooperatively.

2.3.21 Conclusions

Two general models for the low dose "hook" effect had been previously proposed (Thompson and Jackson, 1984). The first model suggested that the binding of one antibody produces an allosteric change in the antigen; altering the epitope recognized by the second antibody, so that the binding of this second antibody is greatly increased. The second model proposed that no allosteric interactions are involved but that the increased binding is produced by the formation of multimolecular cyclic complexes. The ultimate result is the formation of the multicomponent antigen-antibody complexes. This is entirely plausible for some type of antisera, particularly where the size of the antigen permits interaction with multiple epitopes and the formation of higher order complexes. It is clear that each antibody molecule is able to bind with two hGH molecules, providing a bridge between them. As the second antibody binds to the remaining epitope of one of the hGH-antibody-hGH oligomers, a large lattice of antigen and antibody results. This leads to formation of multicomponent complexes. The ultimate size of the multicomponent complex for a given system is determined by the concentrations, the affinities and the ratios of the components in the mixture. Moreover, it is possible to conclude that the three dimensional structure of a molecule and its epitope distribution play a major role in forming multicomponent complexes. Therefore, one can predict cooperative interactions for a given

molecule by understanding the three-dimensional structure and the binding behavior with antibodies for several model analytes. This study confirms the importance of careful development of methodologies to design sensitive immunoassays and predict the behavior of polyclonal antisera or monoclonal mixtures in advance.

The "hook" effect in competitive binding assay is due to cooperative interactions i.e., results a non-linear calibration curve. The "hook" effect for the standard curve gives ambiguous results for the unknown sample. But the "hook" in competitive binding assay is not a concentration effect of the analyte unlike in the sandwich immunoassay. One can also make use of the ascending limb of the calibration curve for the determination of lower analyte concentration in an unknown sample.

Chapter 3

Fundamental studies of the "hook" effect in the one-step sandwich immunoassay

3.1 Introduction

There are two types of two-site immunometric assay modes known as one-step and two-step. One-step sandwich immunoassays are currently enjoying an increase in popularity in clinical laboratories due to the assay speed. The practical advantages of these assays has been limited by the high dose "hook" effect i.e., a decrease in assay response at high analyte concentration or the biphasic nature of the dose-response curve. The "hook" effect can be avoided by using the conventional two-step sandwich immunoassay pioneered by Miles and Hales (1968). However, for some analytes such as ferritin, which possesses multiple epitopes the "hook" effect complicates even the two-step mode.

The one-step sandwich immunoassay often replaces the traditional two-step mode. The feature most significant in the performance of these two assays is the mode of addition of the necessary reagents (Sevier et al., 1981). The one-step immunoassay is carried out by simultaneous mixing of the solid-phase (capture) antibody, analyte, and the signal producing labeled antibody, followed by separation of the solid-phase for signal measurements. In contrast, in the two-step assay mode the analyte is first permitted to bind only with the immobilized antibody. After the reaction is completed, the excess analyte is washed away. The immobilized capture antibody-antigen complex is then incubated with the excess labeled antibody at the second step. If all of the steps in the formation of the "sandwich" consisting of the immobilized capture antibody, the analyte, and labeled antibody were reversible then the order or sequence of reagent addition would make no difference. However, this is not uniformly the case thus making a

detailed understanding of non-reversible behavior essential.

The generally accepted cause of the "hook" effect in one-step immunometric assays involving monoclonal antibodies is an excess of analyte which prevents simultaneous binding of solid-phase and liquid-phase monoclonal antibodies. This is assumed to be a reaction which reaches equilibrium. The three reactants -- analyte, solid-phase antibody, and liquid-phase antibody react simultaneously. If any one of the reactants is present in insufficient or excess amounts, the equilibrium may lead in either direction and formation of the response generating complex can cause significant deviation from expected behavior. The "hook" effect in the one-step immunoassay is primarily a concentration effect, however, the characteristics of the antibodies and their epitopes do play a significant role in producing the "hook".

The biphasic nature of the response is extremely critical when the assay is applied to the determination of analytes which may exist at very high levels. In some pathological situations, for example, tumors can secrete much higher levels of peptide markers than normally found. Accordingly it is possible to obtain, sometimes without warning, curve distortion. Such a potential analytical problem has been reported for the determination of prostate-specific antigen (PSA) in serum which is a marker for adenocarcinoma of the prostate (Alfthan and Stenman, 1988; Boder et al., 1989; Vaidya et al., 1988; Wolf et al., 1989). All these reports suggest that this artifact can be prevented by employing the two-step assay procedure (Vaidya et al., 1988; Wolf et al., 1989). However, the two-step assay demonstrated a "hook" effect identical to that observed in the one-step assay for PSA (Alfthan and Stenman, 1988). It is also reported (Alfthan and Stenman, 1988) that 9% of the serum PSA (30 kDa) in one "hook" sample is present as a 16 kDa PSA fragment. This brief report shows a "hook" effect in both two-site assays.

The cause is believed due to an affinity difference between the 16 kDa fragment and PSA. Boder et al. (1989) did not see the "hook" effect the two-step assay mode, suggesting that the Alfthan and Stenman observation resulted from increased incubation time.

Recent reports describe low results for lutropin (LH) and follitropin (FSH) in a one-step immunoassay which was developed for both hormones using two monoclonal antibodies (Duhlmann et al., 1990). This report also indicates that the modified two-step procedure should overcome this artifact. However, no supporting data are presented. Similarly, Gershagen et al. (1986) developed a one-step immunoradiometric assay (monoclonal antibody based) with no practical limitations (i.e., the dose-response curve does not "hook" at very high concentration of the analyte). It was suggested that the absence of a "hook" could be attributed to coupling of the antibody to high capacity polyacrylamide beads.

A time resolved immunofluorometric assay for hCG has been performed as a model to determine the effect of the concentration of the labeled antibody in a one-step assay. In this assay, the increase in the labeled antibody concentration proportionately increases the assay's tolerance to the "hook" effect. Also, the two-step modification did not exhibit the "hook" effect for the hCG assay (Khosravi, 1990; Khosravi et al., 1987). It is clear that inadequate concentrations of labeled antibody also appear to be a major cause of the "hook" effect in one-step assays.

Sandwich enzyme immunoassays for hCG have been developed using monoclonal antibodies (Gupta et al., 1985). Different enzyme tags have been utilized to design a one-step assay procedure. The "hook" effect for the one-step assay is shifted to higher concentrations of hCG as the amount of anti-alpha-hCG antibody-HRP conjugate is increased. However, the assay response for the two-

step modification shows no "hook" effect. According to this report, at high analyte concentrations, solid-phase antibodies interact monogamously with the analyte which is much less stable than bigamous binding. Therefore analyte molecules may be released from the solid-phase during the washing steps. A reasonable account of the analytical aspects of sandwich immunoassays have been established in this report (Gupta et al., 1985). Comitti et al. (1987) developed a monoclonal based, one-step sandwich enzyme immunoassay for insulin. This work suggests that the one-step immunometric assay gives an enhanced sensitivity over the two-step modification for the insulin system and can be performed without a "hook" effect in a clinically useful range. According to this report, the higher sensitivity of the one-step mode is attributed an "enhancement" effect, as described by Moyle et al. (1983). Another study shows (Rogier et al., 1989) the importance of optimizing the amount of solid- or liquid-phase antibody in order to avoid the "hook" effect in the two-step sandwich immunoassay.

Methodological problems with respect to sensitivity, precision and the "hook" effect in one-step commercial immunoassays for ferritin (Anido, 1984; Ng et al., 1983; Revenant et al., 1982) have been evaluated. Anido (1984) has estimated the maximum limits for the determination of ferritin and emphasized the importance of the sample dilution to avoid the possibility of misdiagnosis. Garcia-Webb et al. (1986) have also observed a high dose "hook" effect in the measurement of somatotropin.

Hoffman et al. (1984) have reported a novel method called "kinetic hook screening" which is capable of monitoring the "hook" effect in two-site immunometric assays. This technique has been designed to eliminate interference and reduce the incubation time resulting in immunometric assays of higher sensitivity. Achieving these attractive advantages, however, requires special

instrumentation (Parsons et al., 1983).

To be useful as an analytical technique, ambiguous immunoassay results for test samples should be minimized or eliminated. Examination of the literature reveals that by changing the mode of assay performance lower results for the sample can be eliminated. This approach, however, may not necessarily be appropriate for all analytes, as suggested by Alfthan and Stenman (1988) and no report has appeared concerning detailed investigations on the "hook" effect.

In this report we present a careful evaluation of the "hook" effect in the one-step sandwich immunoassay. Three model systems have been employed: human growth hormone (hGH, 22 kDa), non-covalent dimer of human growth hormone (D-hGH, 44 kDa), and ferritin (450 kDa). For all these systems well-characterized monoclonal antibodies are available. In the hGH system, two monoclonal antibodies were selected which bind two distant but different epitopes. These studies were extended with D-hGH as the simplest case of an analyte having two sets of repeating epitopes. The influence of repeating epitopes was further examined using ferritin. All the experimental data are supported by theoretical models.

3.2 Experimental section

3.2.1 Materials

The antigens, hGH and purified dimeric hGH, were donated by Eli Lilly (Indianapolis, IN). The following reagents were donated by Hybritech Inc. (San Diego, CA); Monoclonal antibodies for hGH, GHC 072 and GHC 101; Anti-ferritin antibodies, FEF021 and QCI054 ($F(ab)'_2$). The ^{125}I-FEF021 and ^{125}I-GHC 101 (approximately 10 μCi/mL) were prepared in the radioiodination laboratory of Hybritech, Inc. Chemical immobilization of GHC 072, GHC 101, FEF021 and QCI054 on plastic beads was carried out at Hybritech, Inc. Human Spleen ferritin (hs-ferritin) was purchased from Scripps Laboratories (San Diego, CA). "Maxisrop" (11 X 70 mm) and Immunostar (12 X 75 mm) polystyrene tubes were purchased from Thomas Scientific (Swedesboro, NJ). Bovine serum albumin (BSA, Cohn Fraction V) and polystyrene tubes (12 X 75 mm) (for assays with plastic beads) were purchased from Fisher Scientific Co. (St. Louis, MO).

3.2.2 Reagents

The sample buffer used throughout all experiments was phosphate buffered saline (PBS), pH = 7.4, 20 mM, containing 0.15 M sodium chloride with 5.0% BSA added. The washing buffer was the same except that 0.05% BSA was added. The stock solution of the coating buffer was 1.0 M carbonate buffer, pH = 9.6. All other reagents were reagent grade and all solutions were prepared weekly in water obtained from a Barnstead Nanopure II system and stored at 4 °C.

3.2.3 Apparatus

A computer controlled gamma counter (Compugamma 1882-003, Pharmacia LKB Biotech. Inc., Gaithersburg, MD) was used for all radioactivity measurements.

3.2.4 Methods/One-step sandwich immunoassay

3.2.4.1 Chemically immobilized antibodies

Each plastic bead (5/12 inch) was removed from the container and the residual drops were blotted without allowing the beads to dry. One plastic bead was introduced into each tube. GHC 072 immobilized plastic beads were supplied in dry condition and were used directly. Different amounts of the analyte (hGH, D-hGH or ferritin), and 100 μL of ^{125}I-labeled liquid-phase antibody were then added and diluted to 200 μL. Reagents were mixed and then incubated for 4 hours at room temperature. After incubation of the beads, 1.0 mL of washing buffer was dispensed to each tube and liquid was aspirated. All plastic beads were washed five times, aspirating the liquid after each washing step. After carefully removing all of the washing buffer, each bead was counted in the gamma counter. Assays for both analytes were performed with shaking to ensure mixing. All measurements were made in triplicate. Any deviations from this procedure are specified in the appropriate figure captions. The one-step sandwich immunoassay for hGH was performed at 37°C. Assays for hs-ferritin were carried out at room temperature.

3.2.4.2 Physically adsorbed antibodies

Antibodies (FEF021 or QCI054) were physically adsorbed on the polystyrene surface of plastic tubes by directly adding 200 μL of antibody in carbonate buffer (0.01 M, pH = 9.6) followed by incubation for three hours at room temperature. The antibody concentrations are reported in the appropriate figure captions. After coating with antibodies, all tubes were blocked for non-specific binding by incubation with 300 μL of sample buffer for one hour at room temperature followed by five washings with 250 μL of washing buffer. After washing, variable amounts of hs-ferritin and a fixed amount of ^{125}I-labeled antibody were added and diluted to 200 μL. Tubes were incubated for three hours.

These tubes were washed as before, then counted in the gamma counter. All steps were carried out at 37°C. Measurements were made in triplicate.

3.3 Results and discussion

3.3.1 General discussion

In the two-site immunometric assay the amount of analyte is measured by the binding of a labeled antibody to form the sandwich complex. As the concentration of analyte increases, so does the amount of bound labeled antibody. Ideally, the one-step sandwich immunoassay method should be preferred because it is simpler and faster. As the dynamic range of the assay is extended to high concentrations there is a substantial possibility that a "hook" will be observed. The appearance of the "hook" depends on the characteristics of the analyte. This is the first report which gives a concise picture of the problems related to one-step sandwich immunoassay.

3.3.2 Fundamental description of one-step sandwich immunoassay

As the simplest case, an analyte having two different epitopes can be chosen to illustrate the principles of the one-step sandwich immunoassay. The fundamental reaction resulting from the reaction of the analyte, solid- and liquid-phase antibodies in a one-step sandwich immunoassay can be represented by the following equation:

$$P + Q_1 + Q_2^* \rightleftharpoons Q_2^*PQ_1 + PQ_1 + PQ_2^* \qquad \text{----- (1)}$$

where Q_1 and Q_2^* represent the capture and labeled monoclonal antibodies, respectively. The concentration of Q_1 is defined as the total number of moles of immunologically active Q_1 immobilized on the surface divided by the solution volume. The capacity is defined as moles of Q_1 per unit surface area. The analyte P interacts with Q_1 and Q_2^* to form the sandwich complex, $Q_2^*PQ_1$ which generates the binding response for this assay. PQ_1 (solid-phase) and PQ_2^* (liquid-phase) are the only other forms of bound analyte except the sandwich complex. The resulting

linear hypothetical standard curve is shown in Figure 3.1. All antibodies used in this study are monoclonal and are assumed to be individually homogeneous. The solid-phase and the labeled antibodies are in excess with respect to the analyte concentration so that the calibration curve is linear under optimized conditions. When the assay is performed for the test sample it may contain unexpectedly high concentration of the analyte which results a lower response giving a errorneous sample concentration. Figure 3.2B shows the hypothetical calibration curve which demonstrate the effect of the higher analyte concentration in one-step sandwich immunoassay. For the sake of simplicity it is assumed that a large excess of solid-phase antibody is used for the reaction. According to Figure 3.2B, as the analyte concentration increases the concentration of labeled antibody is insufficient, therefore, a biphasic calibration curve is observed. X_0, X_1, X_2 and X_3 correspond to various analyte concentrations as shown in Figures 3.1 and 3.2. For example the analyte concentration, X_2 can be encountered from an unknown sample. The calibration curve in Figure 3.1 is used as the practical curve for the immunoassay. Generally, the ascending response of the calibration curve results from the labeled antibody reacting in excess with the analyte (i.e., concentrations X_0, X_1 and X_2). The descending response begins and continues when the analyte is in excess (Curve B, Figure 3.2, concentration range X_2 - X_3). The ideal curve, however, should demonstrate the high-dose plateau at infinite analyte concentration (Curve A, Figure 3.2). X_2 is the analyte concentration at which the maximum response is demonstrated in the dose-response curve.

To extend the characteristics of the analytes, the behavior of the dimeric form of P (symbolized as H) in a one-step sandwich immunoassay can be evaluated. H has a total of four epitopes for the interaction with antibodies Q_1 and Q_2^*, but each antibody can interact independently with the two equivalent

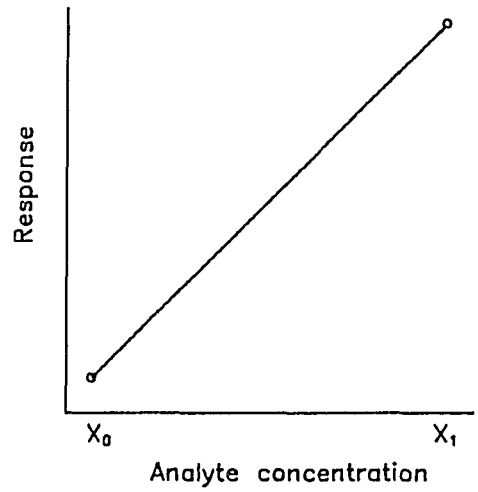

Figure 3.1

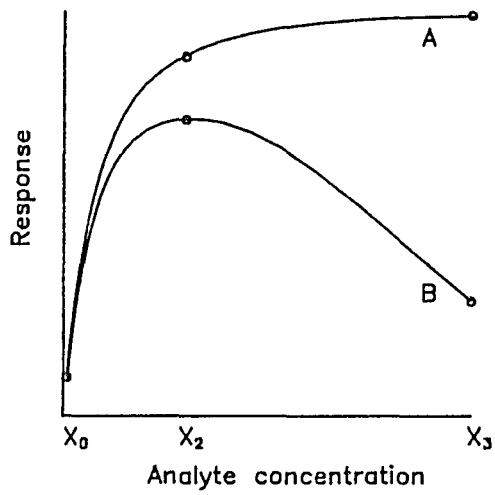

Figure 3.2

Figure 3.1 A hypothetical standard curve for the monoclonal based one-step sandwich immunoassay represented by an analyte having two different epitopes. The analyte concentration range is X_0 - X_1.

Figure 3.2 Hypothetical standard curves for the monoclonal based one-step sandwich immunoassay represented by an analyte having two different epitopes. The analyte concentration range is X_0 - X_3. The Curve A represents a non-hooked calibration curve. The Curve B exhibits the "hook" effect and the response is maximum at analyte concentration, X_2. Figure 3.1 shows a minute analyte concentration range. The analyte concentration, X_2 is greater than X_1.

epitopes. Thus, two options facilitate the one-step sandwich immunoassay for H. Analyte H has two-repeating epitopes which interact with one monoclonal antibody concurrently and effectively yield a one-step sandwich immunoassay. The nature of the interactions are, however, somewhat different from the analyte P (Equation 1). H has two equivalent epitopes for each monoclonal antibody. For such an analyte a one-step immunoassay can be established by using either Q_1 or Q_2^* alone. To illustrate the response generating reaction, the Q_1 monoclonal antibody is used both as the solid- and liquid-phase antibody. The similar results could be obtained by using Q_2^*. The reaction can be represented by Equation 2.

$$H + Q_1 + Q_1^* = Q_1^*HQ_1 + HQ_1 + HQ_1^* \qquad \text{----- (2)}$$

where Q_1 and Q_1^* are the capture and labeled monoclonal antibodies respectively. As Equation 2 shows, $Q_1^*HQ_1$ is the sandwich complex while HQ_1 and HQ_1^* are the other possible solid- and liquid-phase complexes. This assay will demonstrate the "hook" effect as shown in Figure 3.2.

H can be assayed in the one-step mode utilizing a similar principle adopted for the analyte P (Equation 1). The hypothetical binding reaction generates a response similar to Equation 1, except that the complexes formed are somewhat different. Equation 3 describes the possible complexes formed in such an interaction.

$$H + Q_1 + Q_2^* = Q_2^*HQ_1 + HQ_1 + HQ_2^* \qquad \text{----- (3)}$$

Q_1 and Q_2^* are the solid- and liquid-phase antibodies, respectively. H interacts with antibodies in a similar manner as P as shown in Equation 1. In the presence of excess H a "hook" is observed. To simplify the case, the solid-phase antibodies are assumed to react with a single analyte. Also, the response generating complex, $Q_2^*HQ_1$ is assumed to consist of one labeled antibody. All possible species formed will be considered in relevant models.

An analyte, such as ferritin, which consists of many repeating epitopes, is an example of an extended model for analyte H. Different epitopes of ferritin can also be used for the same purpose. The binding interactions for ferritin can be demonstrated using a model similar to that developed for analyte H (Equations 2 and 3), and the resulting standard curves should be similar to the those shown in Figures 3.1 and 3.2.

In order to explain the theoretical binding response, simple models can be adopted, guided by the following general assumptions. Specific appropriate assumptions are stated in connection with specific descriptions of models: (1) The antibody-antigen reaction on a solid-phase is governed by the law of mass action (2) All reactions reach equilibrium (3) The immunoreactivity of the solid-phase antibody is independent of immobilization by adsorption or covalent attachment (4) Solid-phase antibodies are assumed to be far apart so that the analyte having multiple epitopes cannot interact with two capture antibodies (5) Kinetics of the antigen-antibody reaction at solid-phase are similar to the corresponding reaction in solution (6) Physically adsorbed antibodies do not leach out of the solid-phase during the reaction (7) All liquid-phase antibodies are uniformly labeled (8) Bound and free fractions can be separated without disturbing the equilibrium (9) No cooperative interactions occur between two identical binding sites on the antibody.

Numerous models are suggested by the characteristics of the analyte. These models are useful for simulating and explaining the binding behavior for one-step sandwich immunoassay. In one-step sandwich immunoassay capture antibody is attached to the solid-phase. To reduce the problem of determining the concentration of solid-phase antibodies, all of these theoretical models assume that

all reagents are in solution in the initial stage of the reaction. At equilibrium, unbound solid-phase antibodies and the response generating bound solid-phase antibodies are separated from appropriate liquid-phase antibody species (Ehrlich et al., 1983).

In the one-step sandwich immunoassay, the analyte and the solid-phase and liquid-phase antibodies are added simultaneously so that the amount of these reagents, affinity of antibodies, and the nature of the analyte should control the assay response. For example, if the other parameters are kept constant, a change in the amount of analyte, or solid- and liquid-phase antibodies should drive the equilibrium in either direction as shown by Reactions 1, 2 or 3. These reagents therefore have to be well characterized to demonstrate the performance of this assay. The epitope characteristics of an analyte determine the assay response. An analyte may have equivalent, different or both types of epitopes. Only non-overlapping epitopes can be effectively used to design the immunoassay. Thus the effect of solid- and liquid-phase antibodies in the one-step sandwich immunoassay can be studied for each characterized antigen. Two models are needed to explain the assay response.

3.3.3 Model 1 -- Antigen with no repeating epitopes/two antibodies

Model 1 represents an antigen having two different epitopes. If the analyte has at least two different epitopes which are far apart, two antibodies, one binding at each epitope are sufficient to design a one-step sandwich immunoassay. Assuming the analyte has a reasonable size, one or two antigen molecules could interact with each antibody. Depending on the number of antigens binding to the antibodies, Model 1 can be divided into Model 1a binding one antigen, and Model 1b binding two antigen molecules.

3.3.3.1 Model 1a -- One Antigen interacting with one antibody

This is the simplest model used to describe the nature of the interaction of an analyte consisting two different epitopes. The fundamental reaction for this assay is shown above in Equation 1. The stoichiometric association constant for the interaction of P and Q_1 is symbolized as K_1. K_2 represents the stoichiometric association constant for the interaction of P and Q_2^*. The following relationships will describe the possible concentrations of the species formed in this model.

$$[PQ_1] = K_1[P][Q_1]$$
$$[PQ_2^*] = K_2[P][Q_2^*]$$
$$[Q_2^*PQ_1] = 2K_1K_2[P][Q_1][Q_2^*]$$

Under equilibrium conditions, the following mass balance equations can be written:

$$p = [P] + [PQ_1] + [PQ_2^*] + [Q_2^*PQ_1]$$
$$q_1 = [Q_1] + [PQ_1] + [Q_2^*PQ_1]$$
$$q_2^* = [Q_2^*] + [PQ_2^*] + [Q_2^*PQ_1]$$

where p is the total antigen concentration and q_1 and q_2^* define the total concentrations for solid-phase antibody (Q_1) and liquid-phase antibody (Q_2^*), respectively. [P] is the free antigen concentration, $[Q_1]$ is the unbound solid-phase antibody concentration, and $[Q_2^*]$ is the unreacted liquid-phase antibody concentration.

3.3.3.2 Model 1b -- Two antigens interacting with one antibody

Model 1b in which two antigens are assumed to interact with one antibody is an extension of the Model 1a. The response generating complex will still be $Q_2^*PQ_1$. Different liquid-phase complexes are formed in comparison with Model 1a. The concentrations of the resulting species are described below using the same set of symbols:

$$[PQ_1] = 2K_1[P][Q_1]$$
$$[PQ_2^*] = 2K_2[P][Q_2^*]$$
$$[PQ_1P] = K_1^2[P]^2[Q_1]$$
$$[PQ_2^*P] = K_2^2[P]^2[Q_2^*]$$
$$[Q_2^*PQ_1] = 4K_1K_2[P][Q_1][Q_2^*]$$

In addition, under equilibrium conditions the appropriate mass balance equations can be written:

$$p = [P] + [PQ_1] + [PQ_2^*] + 2[PQ_1P] + 2[PQ_2^*P] + [Q_2^*PQ_1]$$
$$q_1 = [Q_1] + [PQ_1] + [PQ_1P] + [Q_2^*PQ_1]$$
$$q_2^* = [Q_2^*] + [PQ_2^*] + [PQ_2^*P] + [Q_2^*PQ_1]$$

The bound species of Q_1 are given as PQ_1, $Q_2^*PQ_1$ and PQ_1P. Similarly PQ_2^*, $Q_2^*PQ_1$ and PQ_2^*P are the all possible complexes formed with Q_2^*, the labeled antibody.

3.3.4 Model 2 -- Analyte with repeating epitopes

Model 2 represents an antigen having non-overlapping repeating epitopes. For an analyte having two or more repeating epitopes, a single antibody can serve as both the capture and labeled antibody, but the same assay can also be done using two different antibodies against different epitopes. In Model 2a two different antibodies are used. In Model 2b a single antibody is used. This model deals with an analyte (H) having non-overlapping repeating epitopes. H is considered as the simplest model antigen and the resulting interactions for two different cases are described in Equations 2 and 3. Model 2 is compatible with the experimental data for D-hGH and ferritin. Model 2 has been subdivided (Model 2a and 2b) according to the type of epitopes of the analyte involved in the binding reaction. The fundamental treatment of theoretical concepts for the interaction of a

macromolecular analyte with monoclonal antibodies was developed according to the literature (Klotz and Hunston, 1979). As the antigen has multiple binding sites, K_1 and K_2 are defined as microscopic binding constants for Model 2.

3.3.4.1 Model 2a -- Analyte having two sets of repeating epitopes/assay with two different antibodies

This model deviates from Model 1 by allowing the antigen to exhibit multiple binding with the liquid-phase antibody. For simplicity, the solid-phase antibody is assumed to bind only one antigen. Also the signal generating complex is assumed to be composed of one each of capture and labeled antibody. Two epitopes of the antigen could interact with appropriate paratopes of the liquid-phase antibody as shown below:

$$[HQ_1] = 2K_1[H][Q_1]$$
$$[HQ_2^*] = 2K_2[H][Q_2^*]$$
$$[Q_2^*HQ_2^*] = K_2^2[H][Q_2^*]^2$$
$$[Q_2^*HQ_1] = 4K_1K_2[H][Q_1][Q_2^*]$$

The following mass balance equations can be written for the above model. The total concentrations of H, Q_1 and Q_2^* are h, q_1 and q_2^*.

$$h = [H] + [HQ_1] + [HQ_2^*] + [Q_2^*HQ_1] + [Q_2^*HQ_2^*]$$
$$q_1 = [Q_1] + [HQ_1] + [Q_2^*HQ_1]$$
$$q_2^* = [Q_2^*] + [HQ_2^*] + [Q_2^*HQ_1] + 2[Q_2^*HQ_2^*]$$

3.3.4.2 Model 2b -- Analyte having more than two equivalent epitopes/assay with a single antibody

Model 2b deviates from Model 2a by selecting one antibody (Q_1) against the equivalent epitopes of the analyte (H). H is assumed to have two or more repeating epitopes. This model assumes the binding sites of the solid- (Q_1) and liquid-phase (Q_2^*) antibodies (paratopes) are equivalent. Only one antigen is

assumed to interact with the solid-phase antibody. The other two epitopes are assumed to be accessible for the interaction with liquid-phase antibody (Q_1^*). Model 2b is compatible with the analyte, ferritin. The following are the concentrations of each of the species for Model 2b:

$$[HQ_1] = 2K_1[H][Q_1]$$
$$[HQ_1^*] = 2K_1[H][Q_1^*]$$
$$[Q_1^*HQ_1^*] = K_1^2[H][Q_1^*]^2$$
$$[Q_1^*HQ_1] = 4K_1^2[H][Q_1][Q_1^*]$$

The mass balance equations for the total concentration are written as follows:

$$h = [H] + [HQ_1] + [HQ_1^*] + [Q_1^*HQ_1^*] + [Q_1^*HQ_1]$$
$$q_1 = [Q_1] + [HQ_1] + [Q_1^*HQ_1]$$
$$q_1^* = [Q_1^*] + [HQ_1^*] + 2[Q_1^*HQ_1^*] + [Q_1^*HQ_1]$$

The total concentrations of H, Q_1 and Q_1^* are h, q_1 and q_1^* respectively.

3.3.5 Computer simulated data/avoidance of the "hook" effect

3.3.5.1 Model 1 -- The effect of capture antibodies

In one-step sandwich immunoassays, systems consisting of high capacity solid-phase antibodies are expected to shift the "hook" away from the analytically significant concentration range, preventing misleading results for test samples. Simulated dose-response curves using Model 1b are shown in Figure 3.3. Curve A represents an example of the "hook" effect in one-step sandwich immunoassay. The response progressively increases with increasing analyte concentration, passing through a maximum, but the sensitivity is very low. The response (B/T)% is defined as the ratio of the concentration of the bound labeled antibody in response generating complex divided by the total amount of labeled antibody (q_2^*). Sensitivity is here defined as the positive slope of the curve. Ideally, the response

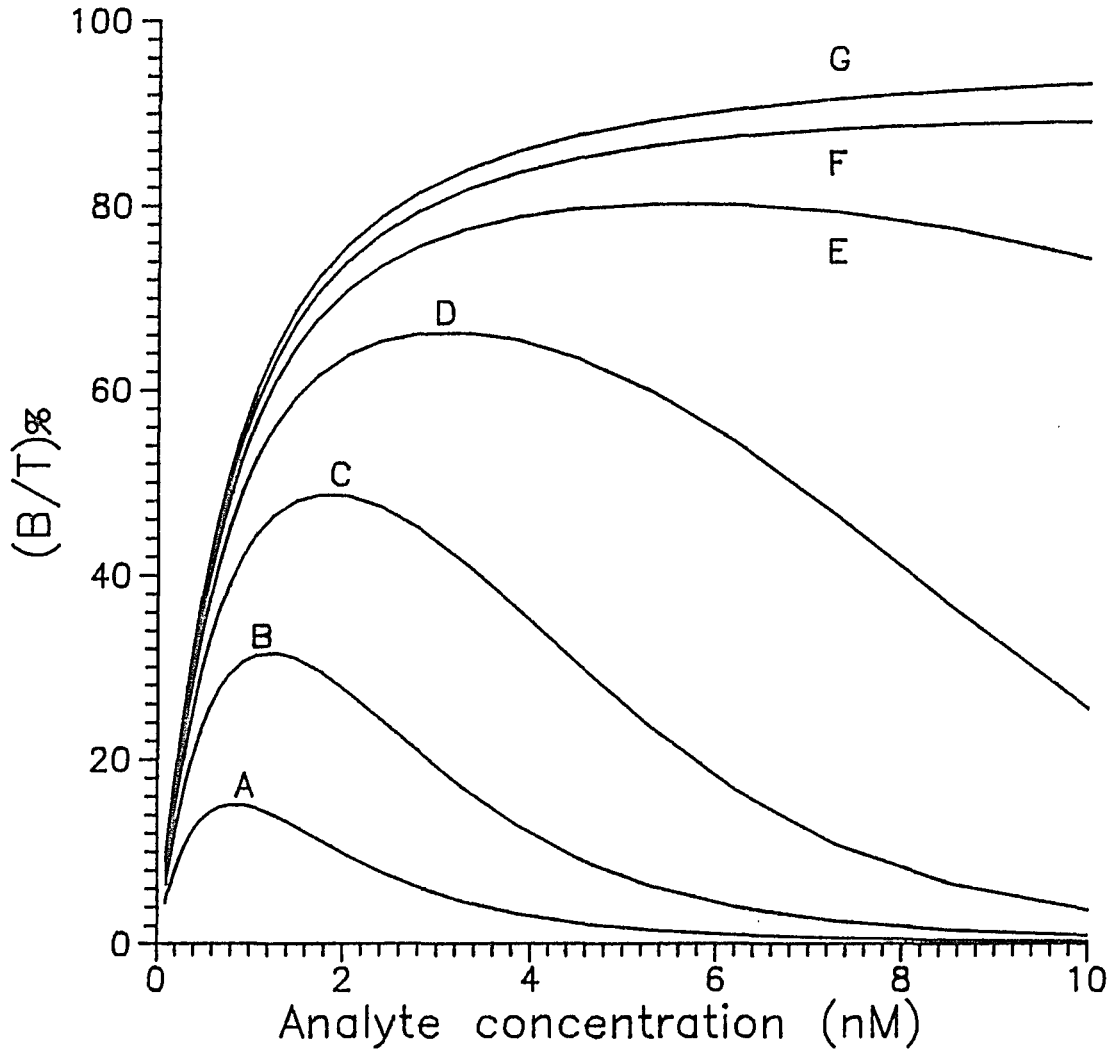

Figure 3.3 Theoretical: Effect of the capacity of solid-phase antibody (q_1) on "hook" effect in one-step sandwich immunoassay for Model 1b -- **low** analyte concentration range: K_1 = 1 nM^{-1}, K_2 = 1 nM^{-1}, q_1 (nM); 0.5 (A), 1.25 (B), 2.5 (C), 5 (D), 10 (E), 20 (F) and 50 (G), q_2^* = 0.5 nM. Analyte concentration (p) range is 0.1 - 10 nM.

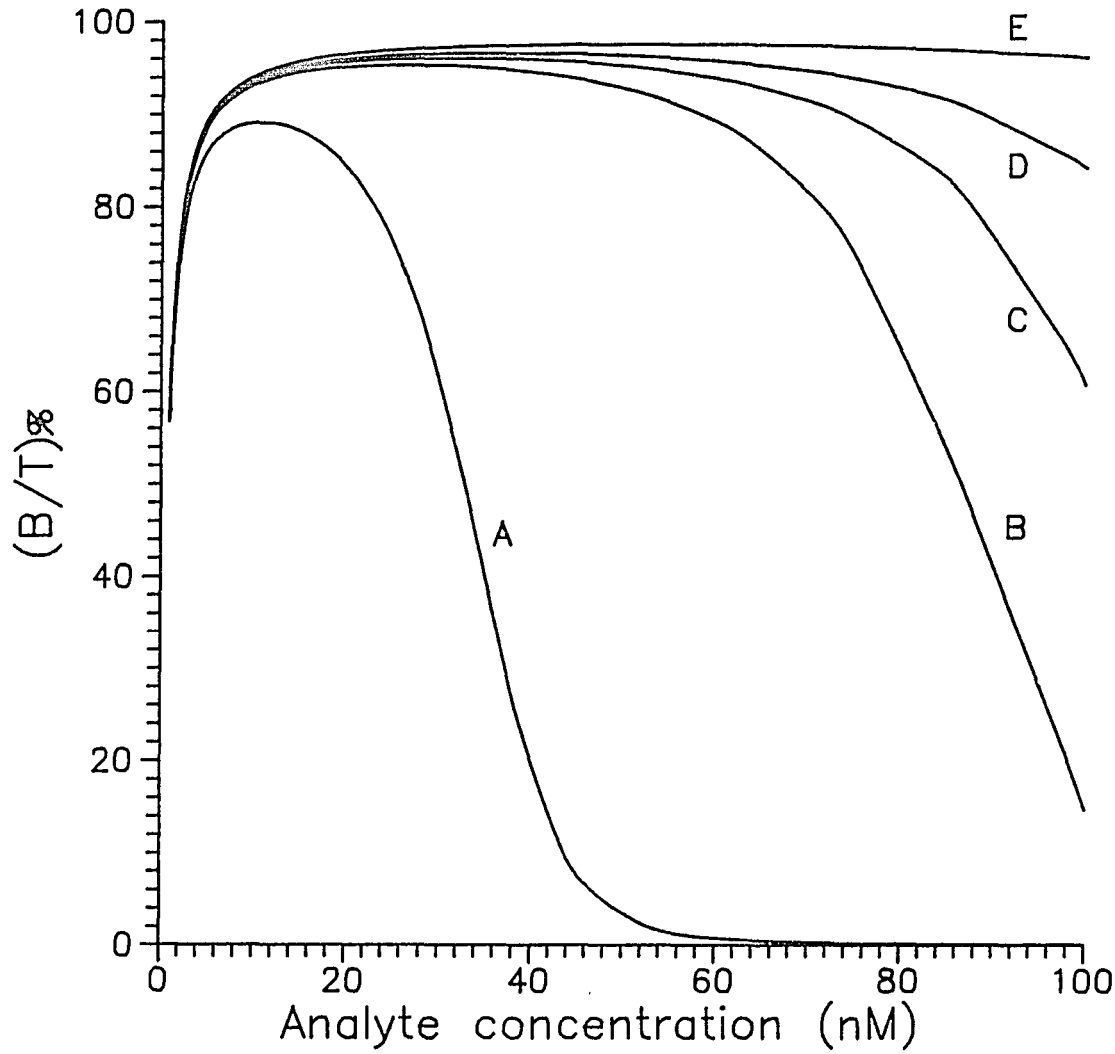

Figure 3.4 Theoretical: Effect of the capacity of solid-phase antibody (q_1) on "hook" effect in one-step immunoassay for Model 1b -- **high** analyte concentration range: K_1 = 1 nM^{-1}, K_2 = 1 nM^{-1}, q_1 (nM); 20 (A), 50 (B), 60 (C), 70 (D), 100 (E). q_2^* = 0.5 nM. Analyte concentration (p) range is 1 - 100 nM.

should show a plateau at high analyte concentration. The response in Curve A, however, decreases after the optimum response is achieved, because the large excess of analyte drives the equilibrium in the reverse direction, favoring formation of solution phase analyte-antibody complexes. The resulting array of dose-response curves generated from an increase in the solid-phase antibody concentration is shown in Figure 3.3 (Curves B-G). The relative sensitivities and linearity of these curves have increased, however Curves B - E also suffer from the "hook" effect. Note that the "hook" maximum shifts to higher analyte concentrations. For example, if the concentration of the capture antibody is raised by a factor of 10 (0.5 nM - Curve A, 5 nM - Curve D), the "hook" maximum is shifted along the x-axis (analyte concentration) from 0.80 nM to 2.0 nM. Curves F and G apparently show the ideal high-dose plateau. The apparent absence of a "hook" is misleading. If the analyte concentration is increased to still higher levels (up to 100 nM) (Figure 3.4) instead of 10 nM (Figure 3.3) "hook" is again observed in Curves F and G. Curves F and G in Figure 3.3 are analogous to Curves A and B in Figure 3.4. This suggests that optimization of the reagent concentrations is required if the "hook" is to be avoided. Again the "hooked" nature of Curves A and B can be eliminated by raising the concentration of the solid-phase antibody (Curves C-E, Figure 3.4). Note that these calculations are made assuming equal affinities for solid-and liquid-phase antibodies. These results indicate that use of high capacity capture antibody will effectively avoid or minimize ambiguous results for the test sample in a practical assay.

The conditions defined to generate Curve C in Figure 3.3 are used as an example to explain all possible complexes formed in the one-step sandwich immunoassay for Model 1b. Concentration profiles of all the species are shown in Figure 3.5. The solid-phase antibody (Q_1) concentration is 10 times greater than

the liquid-phase antibody concentration (Q_2^*) so that higher amounts of Q_1 species are formed. Initially PQ_1 concentration is rapidly increased. As the analyte concentration is increased, PQ_1 concentration is progressively increased, passing through a maximum, and declining at high analyte concentrations. A similar response is shown for PQ_2^*, but the concentration is lower. Although the amounts of PQ_1P and PQ_2^*P are initially formed at considerably lower levels, these complexes are the major species formed up to the analyte concentration 10 nM, and even at higher concentrations. Note that the response generating species, $Q_2^*PQ_1$ demonstrates a "hook". Model 1a assumes the simplest case of binding only one antigen with the antibody, forming fewer species. Using Model 1a, under similar conditions, theoretical curves are constructed assuming that antigen and antibody bind in a 1:1 ratio. The results are shown in Figure 3.6 and are similar to Model 1b. Comparing Figure 3.3 with Figure 3.6, we see that at higher analyte concentration the curves for Model 1a are less steep than the curves generated for Model 1b. Figure 3.7 shows the concentrations of possible species, PQ_1, PQ_2^* and $Q_2^*PQ_1$ formed as the analyte concentration increased for Model 1a. PQ_1 and PQ_2^* are formed similar to Model 1b (Figure 3.5) at low analyte concentrations. In contrast to Model 1b both of these species do not show any "hook" at high analyte concentrations. Deviation in the slopes in response generating curves is due to the formation of different solid- and liquid-phase complexes in Model 1b. In Model 1b, PQ_2^* and PQ_2^*P are both formed and responsible for the decline in the assay response. PQ_2^* is the only possible liquid-phase complex for Model 1a. For both Models 1a and 1b, the selected analyte concentration range produce the hypothetical "hooked" curves presented in Figures 3.1 and 3.2.

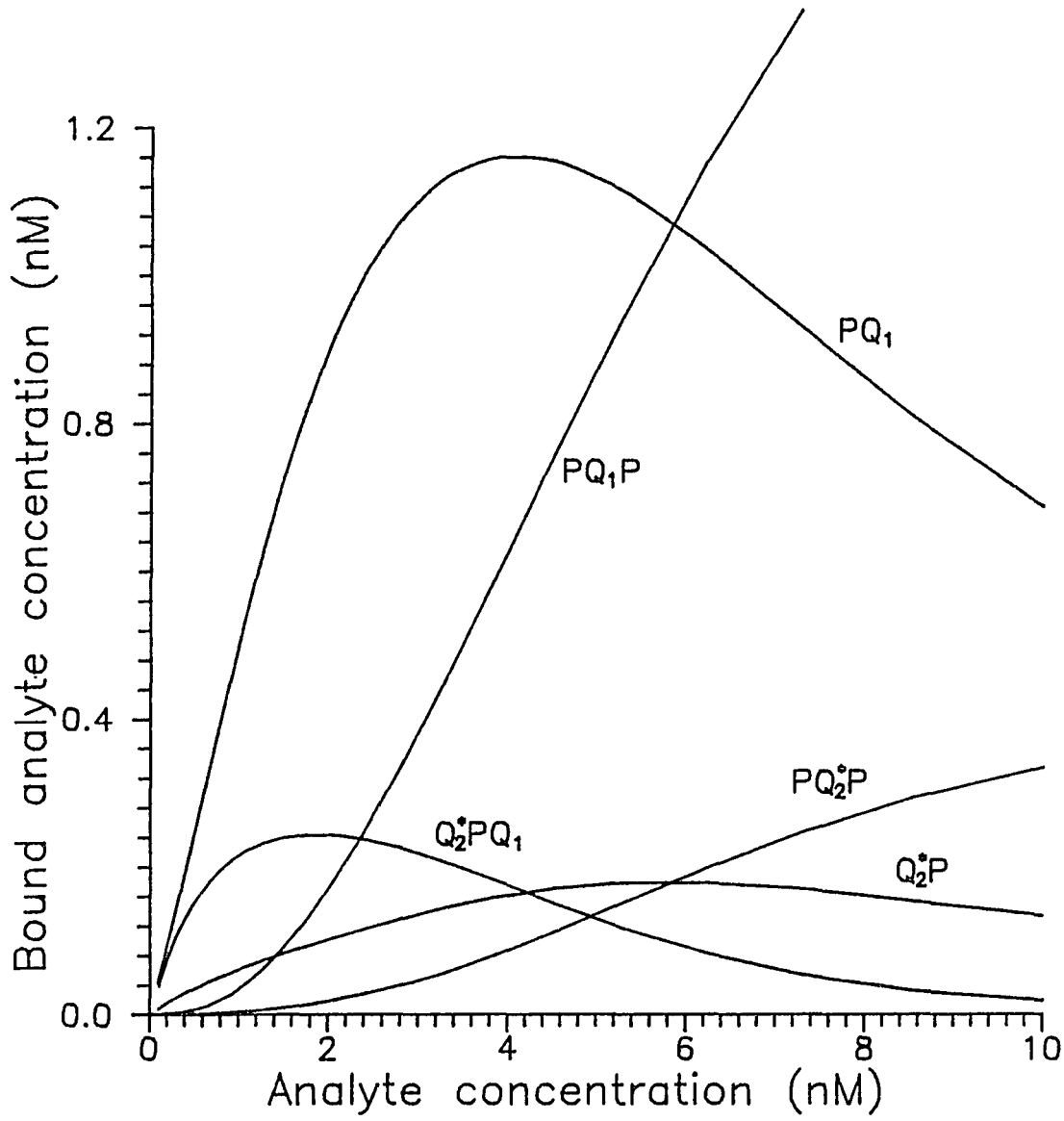

Figure 3.5 Possible complexes formed in the reaction of two monoclonal antibodies with an analyte in Model 1b. K_1 = 1 nM^{-1}, K_2 = 1 nM^{-1}, q_1 = 5 nM, q_2^* = 0.5 nM. Analyte concentration range (p) is 0.1 - 10 nM.

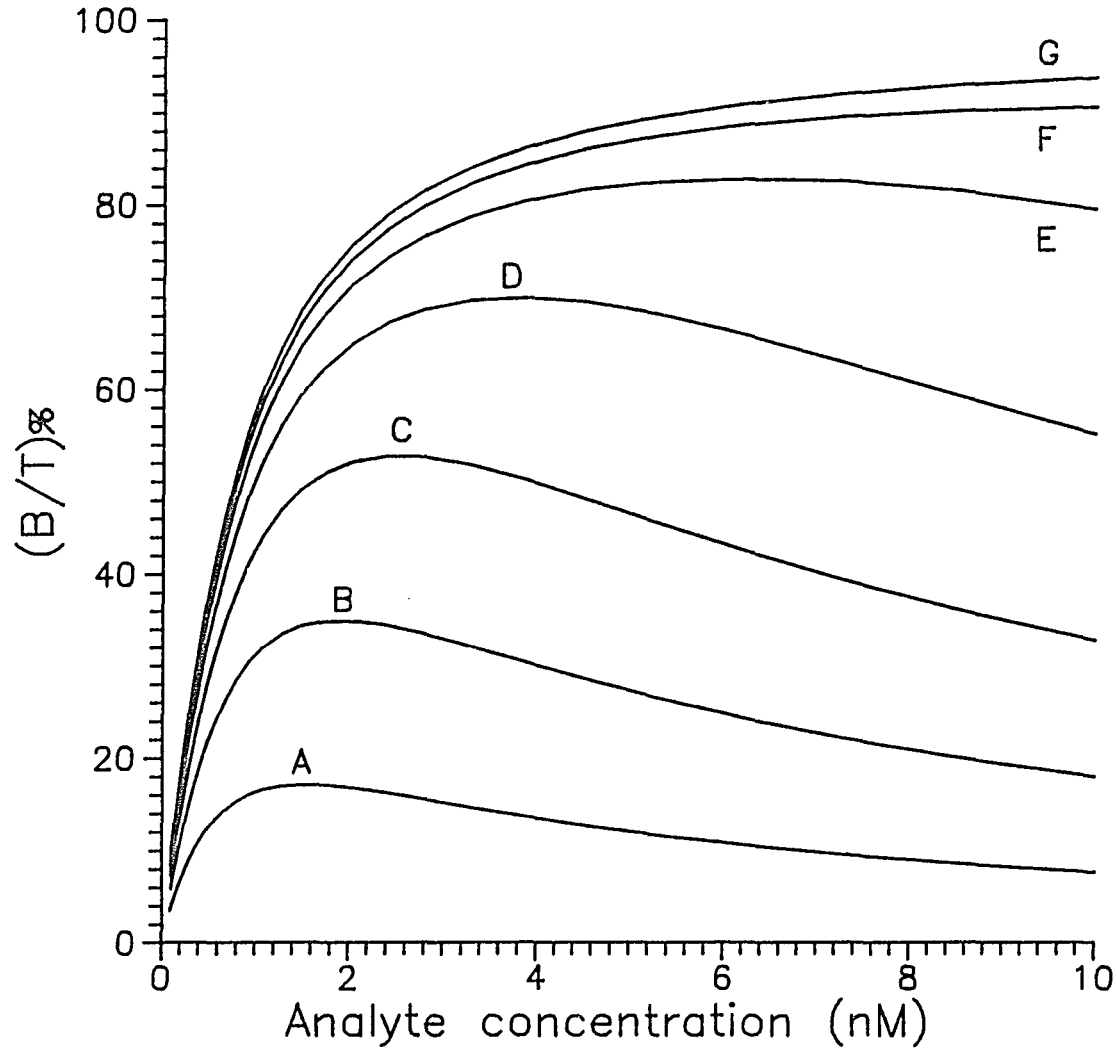

Figure 3.6 Theoretical: Effect of the capacity of the solid-phase antibody (q_1) on "hook" effect in one-step sandwich immunoassay for Model 1a: K_1 = 1 nM^{-1}, K_2 = 1 nM^{-1}, q_1 (nM); 0.5 (A), 1.25 (B), 2.5 (C), 5 (D), 10 (E), 20 (F) and 50 (G). q_2^* = 0.5 nM. Analyte concentration (p) range is 0.1 - 10 nM.

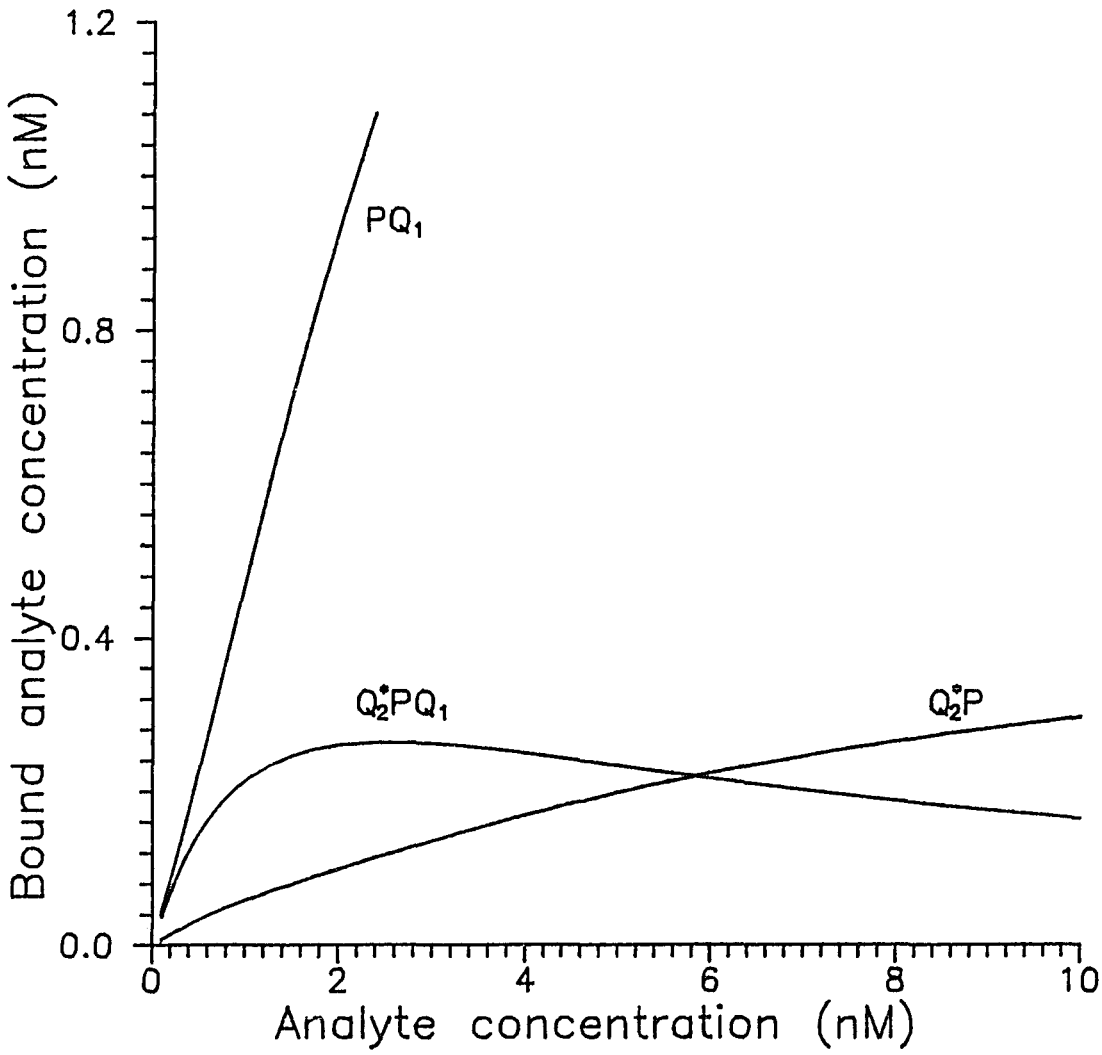

Figure 3.7 Possible complexes formed in the reaction of two monoclonal antibodies with an analyte in Model 1a. K_1 = 1 nM^{-1}, K_2 = 1 nM^{-1}, q_1 = 5 nM, q_2^* = 0.5 nM. Analyte concentration range (p) is 0.1 - 10 nM.

Model 2 -- The effect of capture antibody

Theoretical values for Model 2 show similar effects to those just detailed in Model 1. A high capacity solid-phase antibody would eliminate the "hook" effect. These results are shown in Figures 3.8 and 3.9. Assay responses are similar to each Model because the affinity of solid- and liquid-phase antibodies are assumed to be equal. But different complexes are formed in Model 2a and 2b.

The studies on Model 1 and Model 2 show that the characteristics of the analyte specifically control the shape of the dose-response curve even if the affinities of antibodies are equal. Higher concentrations of solid-phase antibodies could theoretically be used to increase the sensitivity and eliminate the biphasic nature in the one-step sandwich immunoassay format.

3.3.5.3 Model 1b -- Effect of labeled antibody

The performance of one-step sandwich immunoassays can be controlled by selecting appropriate concentrations of labeled antibody. The labeled antibody is responsible for the formation of the response generating complex and the liquid-phase complexes. High concentrations in the liquid-phase would theoretically suppress the "hooked" response and could increase the assay sensitivity. Practical limitations, however, prohibit using high concentrations of radiolabeled antibody. A study of Model 1b is sufficient to explain the effect of the labeled antibody concentration. This model was detailed for three different concentrations of the solid-phase antibodies selected from Figure 3.3, 0.5 nM (low), 5 nM (moderate), and 20 nM (high) (Curves A, D and F respectively).

For the low solid-phase concentration (0.5 nM), Curve F in Figure 3.10 is analogous to Curve A from Figure 3.3. Curve F suffers from the "hook" effect. If the concentration of the labeled antibody is raised while keeping both the solid-phase and analyte concentration range the same, resulting computer simulated

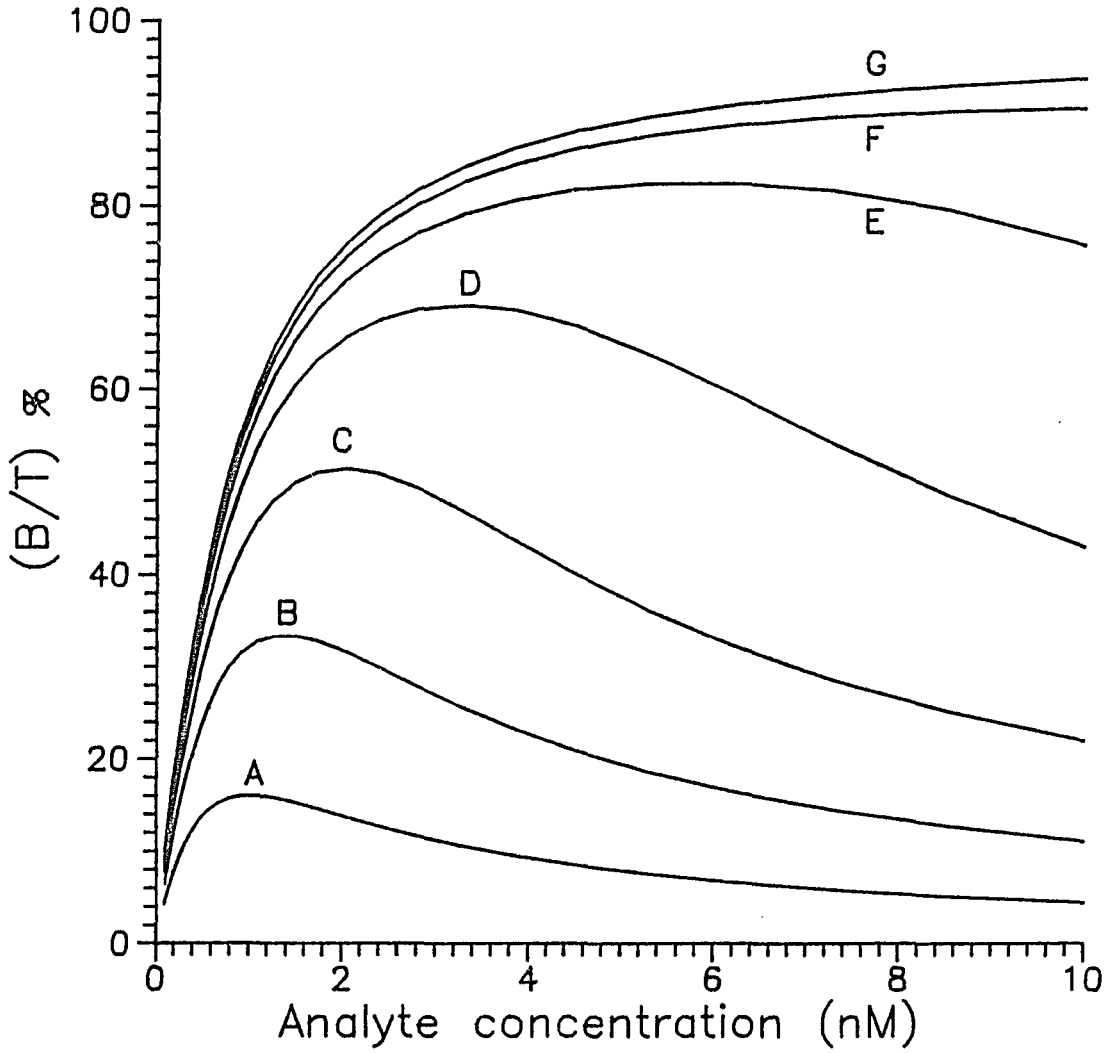

Figure 3.8 Theoretical: Effect of the capacity of the solid-phase antibody (q_1) on "hook" effect in one-step sandwich immunoassay for Model 2a: K_1 = 1 nM^{-1}, K_2 = 1 nM^{-1}, q_1 (nM); 0.5 (A), 1.25 (B), 2.5 (C), 5 (D), 10 (E), 20 (F) and 50 (G). q_2^* = 0.5 nM. Analyte concentration (p) range is 0.1 - 10 nM.

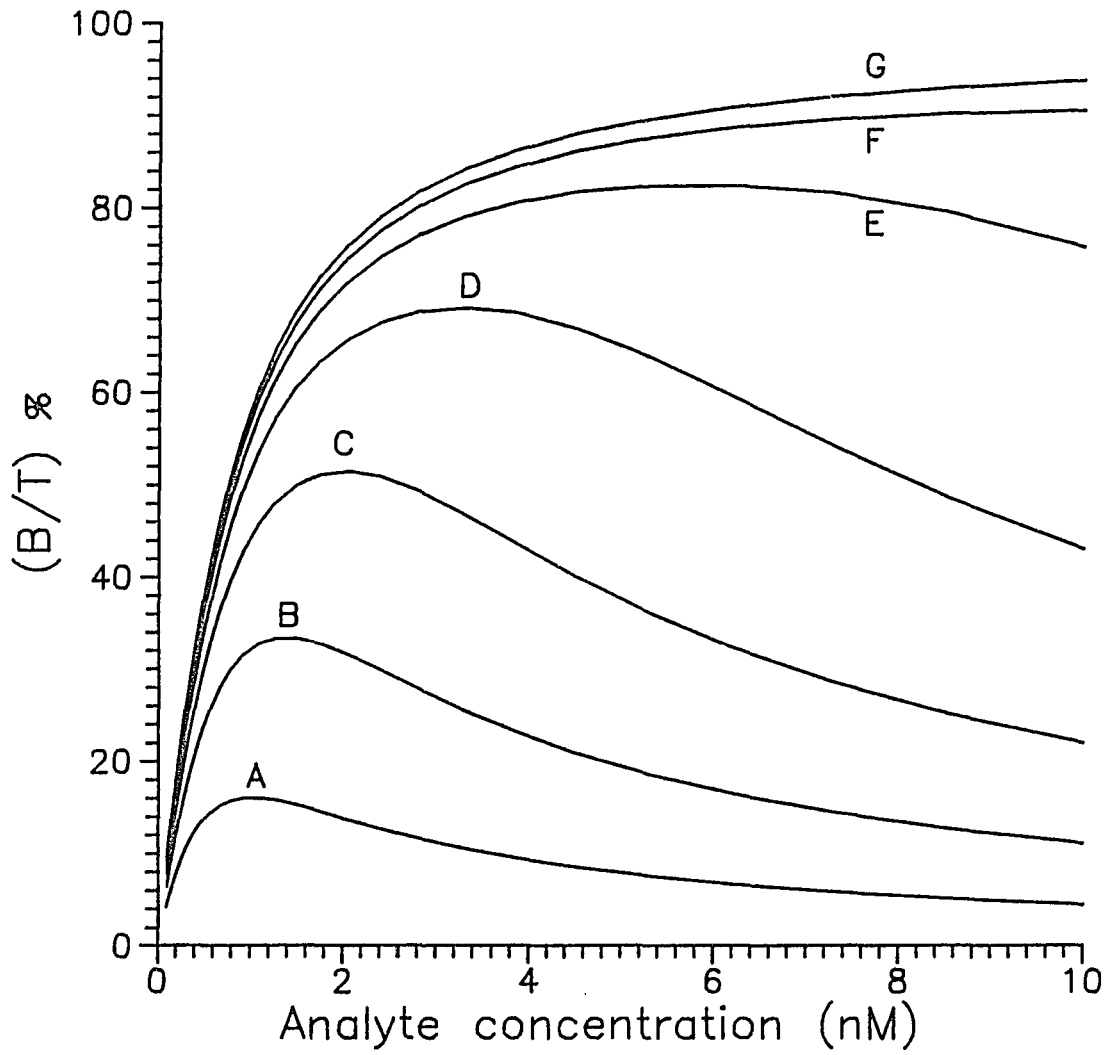

Figure 3.9 Theoretical: Effect of the capacity of the solid-phase antibody (q_1) on "hook" effect in one-step sandwich immunoassay for Model 2b: K_1 = 1 nM^{-1}, q_1 (nM); 0.5 (A), 1.25 (B), 2.5 (C), 5 (D), 10 (E), 20 (F) and 50 (G). q_2^* = 0.5 nM. Analyte concentration (p) range is 0.1 - 10 nM.

data show that the "hook" shifts to higher analyte concentrations (Figure 3.10, Curves C-E). The sensitivity, however, is decreased. Very high concentrations of labeled antibody show no "hook" (Curves A and B), but it is impractical to use radiolabeled antibody at these high concentrations. Very low concentrations of labeled antibody (Curves G and H) give high sensitivity but a sharp "hook".

If the concentration of solid-phase antibody is increased to moderate levels (5 nM), the "hook" becomes lower and broader as the concentration of labeled antibody increases (Figure 3.11, Curves A-D). Curve E is analogous to Curve D from Figure 3.3, and shows that the sensitivity for each of the Curves A-D is lowered from E. These theoretical values also suggest that some of the assays have sufficient sensitivity to permit measurements in the low analyte concentration range thus avoiding the "hook" effect (Figure 3.11, Curves E-G).

Assays with high concentrations of solid-phase antibody (20 nM) show no "hook" effect (Figure 3.3, Curves F and G), and consequently, those assays with variable amounts of liquid-phase antibody show no "hook" as well (Figure 3.12, Curves A-E). The theoretical study underlines the importance of selecting appropriate concentrations of labeled antibody to control the performance of one-step sandwich immunoassay. In a one-step sandwich immunoassay all three reagents are required to react together. As the theoretical study shows excessive concentrations of any of the reagents (q_1, q_2^* and p) should control the effective species formation. Across all concentrations of the solid-phase, there is an upper limit in the improvement in sensitivity that can be obtained by restricting the concentration of labeled antibody in the assay. According to theoretical studies on Model 1b the following general guidelines can be adopted:

(1) The "hook" appears when $p > q_1, q_2^*$

(2) No "hook" appears when $q_1 > p$ (if $q_1 > q_2^*$) or $q_2^* > p$ (if $q_2^* > q_1$).

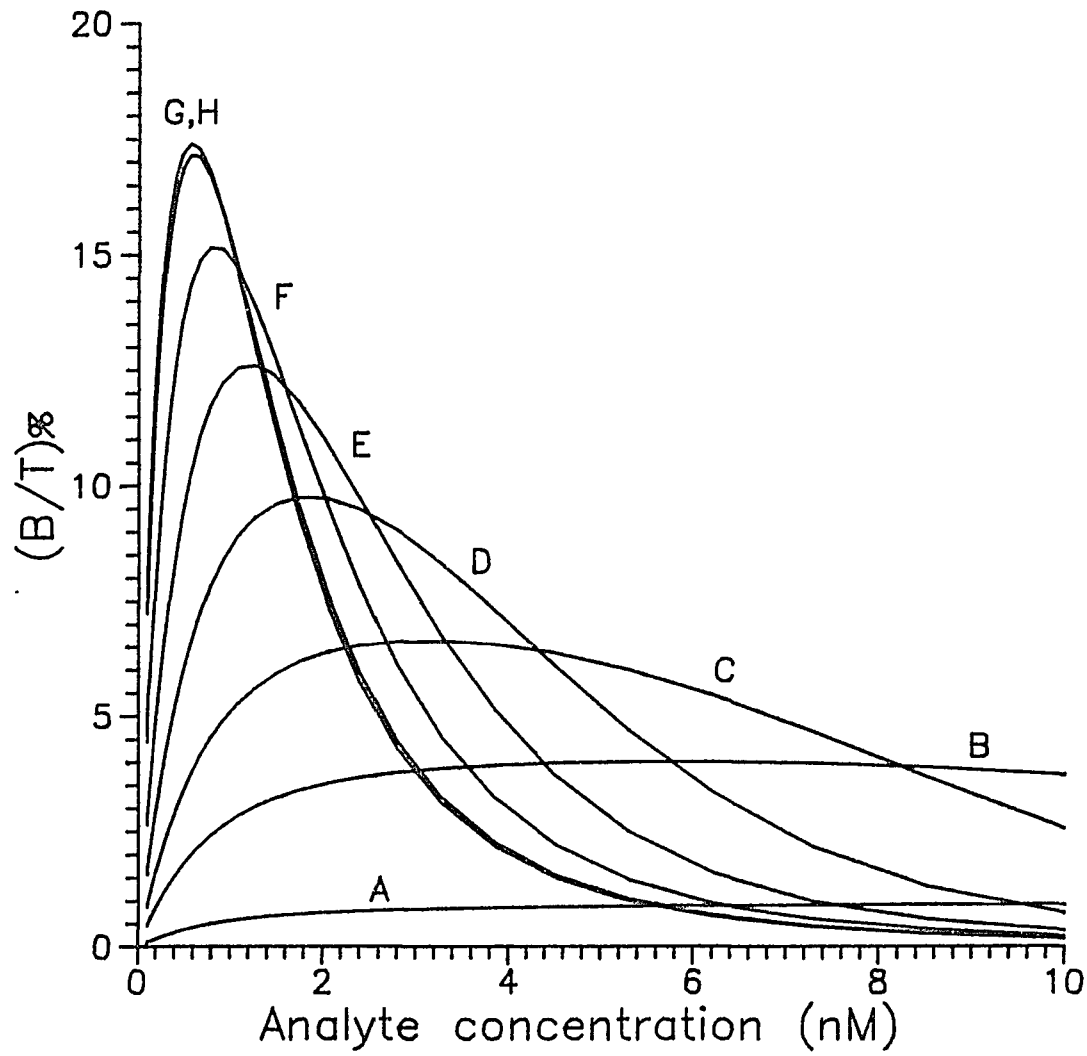

Figure 3.10 Theoretical: Effect of the labeled antibody concentration (q_2^*) on "hook" effect in one-step sandwich immunoassay for Model 1b: K_1 = 1 nM^{-1}, K_2 = 1 nM^{-1}, q_2^* (nM); 50 (A), 10 (B), 5 (C), 2.5 (D), 1.25 (E), 0.5 (F), 50 pM (G), 5 pM (H). q_1 = 0.5 nM. Analyte concentration range is 0.1 - 10 nM.

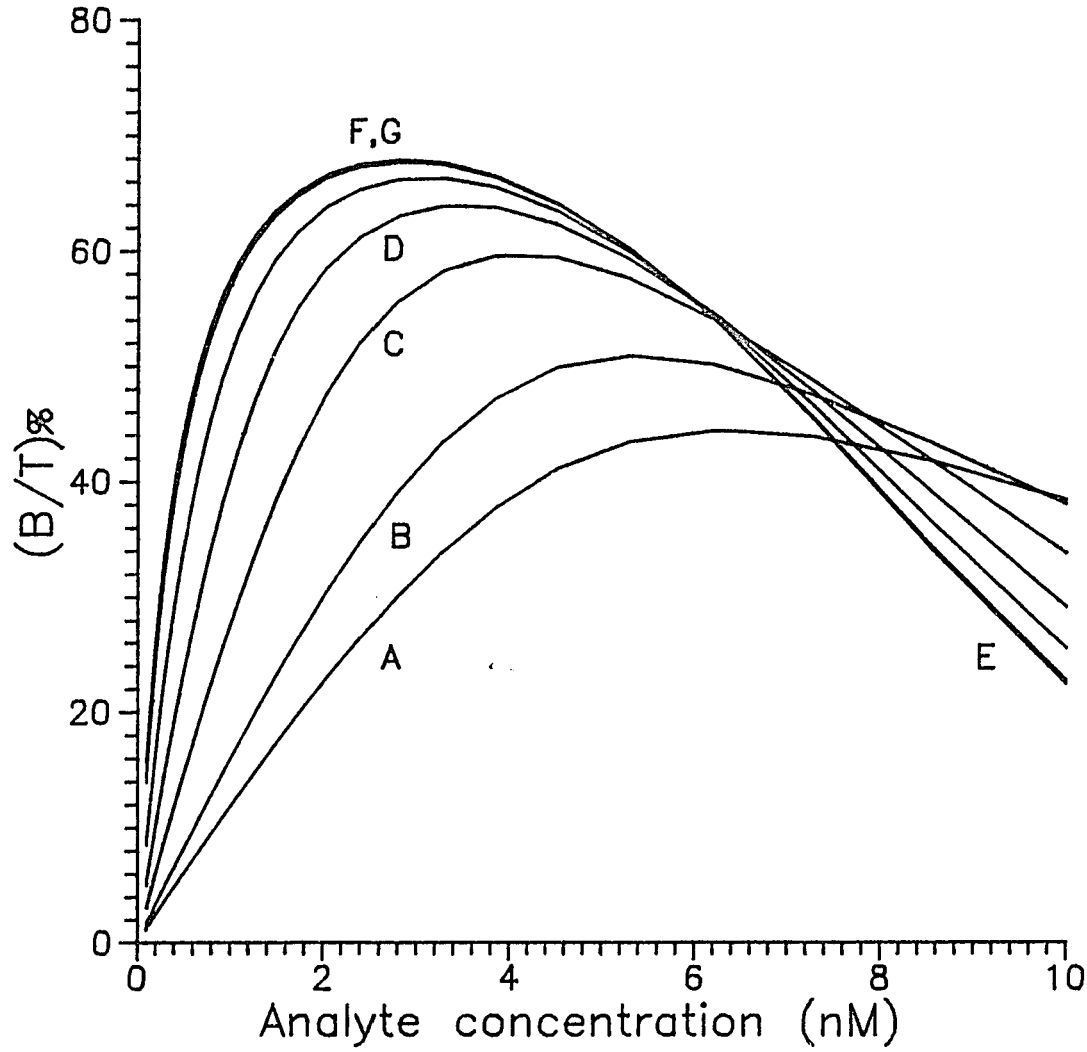

Figure 3.11 Theoretical: Effect of the labeled antibody concentration (q_2^*) on "hook" effect in one-step sandwich immunoassay for Model 1b: K_1 = 1 nM^{-1}, K_2 = 1 nM^{-1}, q_2^* (nM); 50 (A), 10 (B), 5 (C), 2.5 (D), 1.25 (E), 0.5 (F), 50 pM (G), 5 pM (H). q_1 = 5 nM. Analyte concentration range is 0.1 - 10 nM.

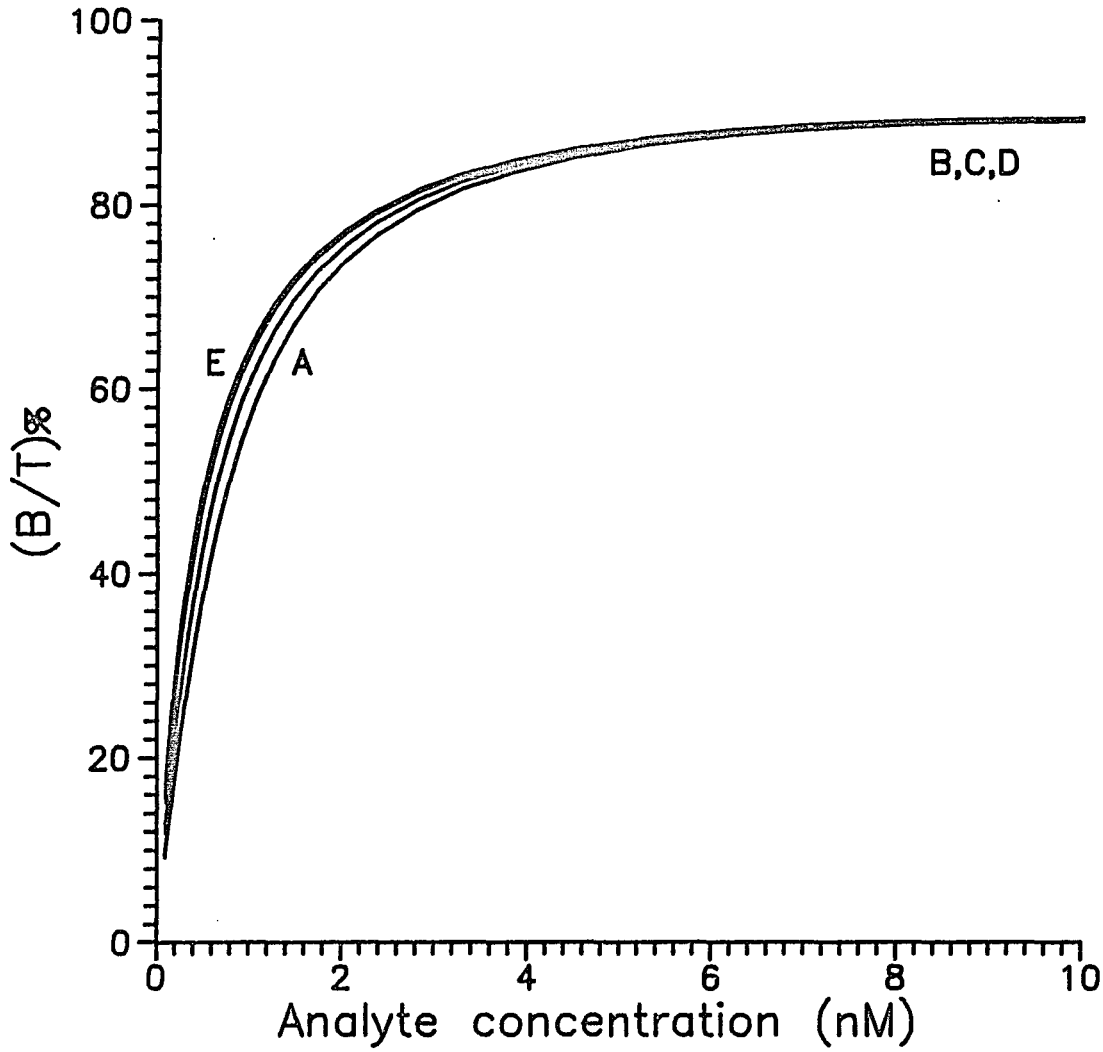

Figure 3.12 Theoretical: Effect of the labeled antibody concentration (q_2^*) on "hook" effect in one-step sandwich immunoassay for Model 1b: K_1 = 1 nM^{-1}, K_2 = 1 nM^{-1}, q_2^* (nM); 50 (A), 10 (B), 5 (C), 2.5 (D), 1.25 (E), 0.5 (F), 50 pM (G), 5 pM (H). q_1 = 20 nM. Analyte concentration range is 0.1 - 10 nM.

3.3.6 Experimental results/One-step sandwich immunoassay

3.3.6.1 An Analyte with two different epitopes/assay for hGH: GHC 072/hGH/GHC 101 system

Theoretical studies have shown that one-step sandwich immunoassay can be performed at high analyte concentrations if the solid-phase antibody has high capacity. This holds irrespective of the nature of the analyte. As Equation 1 shows, the analyte combines with two different monoclonal antibodies to form a sandwich complex. hGH was used as an model analyte, to study the effect of the analyte concentration on the response and each set of data was fitted with a theoretical curve. Figure 3.13 shows the experimental data for each concentration range of hGH. According to these data the response is progressively increased reflecting low hGH concentrations. Curve A represents the theoretical binding curve using Model 1a in which antibody is permitted to interact with hGH at 1:1 ratio. Plastic beads provides high capacity solid-phase antibody, and the labeled antibody is in excess at low hGH concentrations. Therefore Model 1a is assumed to be the most reasonable to obtain Curve A (Figure 3.13). The experimentally determined affinities of GHC 072 and GHC 101 are 1.1 nM^{-1} and 3.8 nM^{-1} (Sportsman et al., 1990). These affinities were determined while the antibody was in solution. Curve A was generated using affinities of GHC 072 (K_1) and GHC 101 (K_2) of 0.05 nM^{-1} and 0.6 nM^{-1} respectively. The solid-phase antibody concentration is 8 nM (q_1). The affinity for the solid-phase antibody is about 22 fold lower than the experimental value. The difference in affinity is often attributed to the modification of GHC 072 in the immobilization process. The affinity value for the liquid-phase antibody only differs by 6 fold. If the assay in Figure 3.13 is reproduced at high analyte concentrations the amount of labeled antibody is certainly limited. But GHC 072 and GHC 101 are known to have synergistic

interactions with hGH which could enhance the binding response. A detailed investigation of the synergistic interactions was documented in Chapter 2.

The developed dose-response data and the theoretical curves at higher analyte concentration ranges are shown in Figures 3.14 and 3.15. The analyte concentration is the only deviation from the data obtained for Figure 3.13. The response is progressively increased with increasing hGH concentration and a plateau results (Figure 3.14). The response is reduced gradually compared to the increase in analyte concentration as presented in Figure 3.15. Therefore by changing the concentration of hGH, theoretical curves can be drawn similar to Curve A in Figure 3.13. However, the parameters have to be changed considerably to fit the data for Curve A in Figures 3.14 and 3.15. The theoretical parameters for Curve A in Figure 3.14 are K_1 (0.1 nM^{-1}), K_2 (0.2 nM^{-1}) and q_1 (50 nM) respectively. Curve A in Figure 3.15 was generated using K_1 (0.1 nM^{-1}), K_2 (0.2 nM^{-1}) and q_1 (160 nM). The estimated values of K_1 and K_2 lower than 10 and 19 fold from the experimental data. Note that q_1 has changed by 6 fold and 20 fold compared to q_1 in Figures 3.13. hGH forms a series of complexes when it coexists with GHC 101 and GHC 072 monoclonal antibodies in solution. The formation of these complexes depends both on the concentration of hGH and antibodies. These deviations are inferred to be the result of concomitant interactions of both antibodies with the antigen, forming higher molecular weight linear and circular complexes.

3.3.6.2 An analyte having two repeating epitopes/assay for D-hGH: GHC 101/D-hGH/GHC 101 system

D-hGH is well characterized (Becker et al., 1987). It can provide a model antigen having two known repeating epitopes. The selection of the solid- or liquid-phase antibody will determine the binding characteristics of D-hGH in a one-step

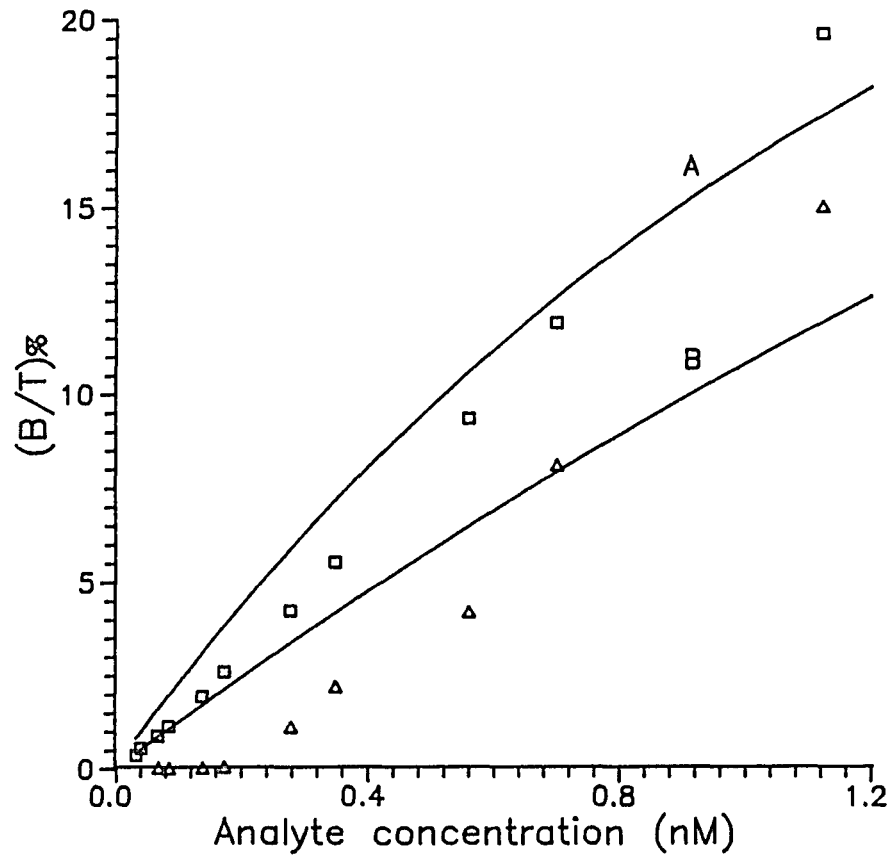

Figure 3.13 Dose-response curves for hGH and D-hGH in one-step sandwich immunoassay: **low** analyte concentration range. The concentration range of analytes, hGH (□) and D-hGH (△) is 30 pM - 1.2 nM. The concentrations of labeled antibody, ^{125}I-GHC 101 are 0.59 nM (□) and 0.8 (△) nM. Solid-phase antibodies, GHC 072 (□) and GHC 101 (△) are covalently attached to plastic beads.

Theoretical Curves A and B correspond to the experimental results for GHC 072 (□) and GHC 101 (△) systems. Theoretical binding curves: Curve A -- Model 1a: K_1 = 0.05 nM^{-1} , K_2 = 0.6 nM^{-1}, q_1 = 8 nM, q_2^* = 0.6 nM. Curve B -- Model 2b: K_1 = 0.1 nM^{-1}, q_1 = 14 nM and q_2^* = 0.8 nM.

sandwich immunoassay. As discussed in Model 2b, a single antibody (GHC 101 or GHC 072) can be used to design a one- step sandwich immunoassay for D-hGH. D-hGH has two epitopes to interact with the antibody, and the assay design should be similar to that of hGH. The experimental data for the one-step immunoassay for D-hGH were obtained at three different concentrations. The response deviate slightly from linearity at low concentration range as shown in Figures 3.14 and 3.15. The deviation, at low analyte D-hGH concentrations, may be mainly due to fact that the labeled GHC 101 can effectively mask both epitopes of D-hGH and inhibit the binding to the solid-phase. These are shown in Figures 3.13 - 3.15. The theoretical discussion from Figures 3.1 and 3.2 shows that the response increases linearly at low analyte concentrations, and a plateau results. The response decreases at high analyte concentrations, owing to the formation of soluble liquid-phase complexes (Figure 3.15). The possible liquid-phase complexes are described in Model 2b. The Curve B represents the simulated values in each figure. These theoretical curves diverge somewhat from the experimental data, especially in low D-hGH concentrations (data show a sigmodial pattern). In Model 2b, both epitopes are assumed to have similar affinities, however, the possible distortions of the epitopes in D-hGH (when the dimer is formed or after the reaction with a single antibody) may cause a deviation of the experimental data from theoretical model. The theoretical value of K_1 used to generate Curve B is about 11 fold (Figures 3.13 and 3.15) lower than the experimental value. It is approximately 15 fold lower for Curve B in Figure 3.14. The concentration of solid-phase antibody (q_1) is 14 nM (Figures 3.13), 22.7 nM (Figure 3.14) and 27 nM (Figure 3.15) respectively.

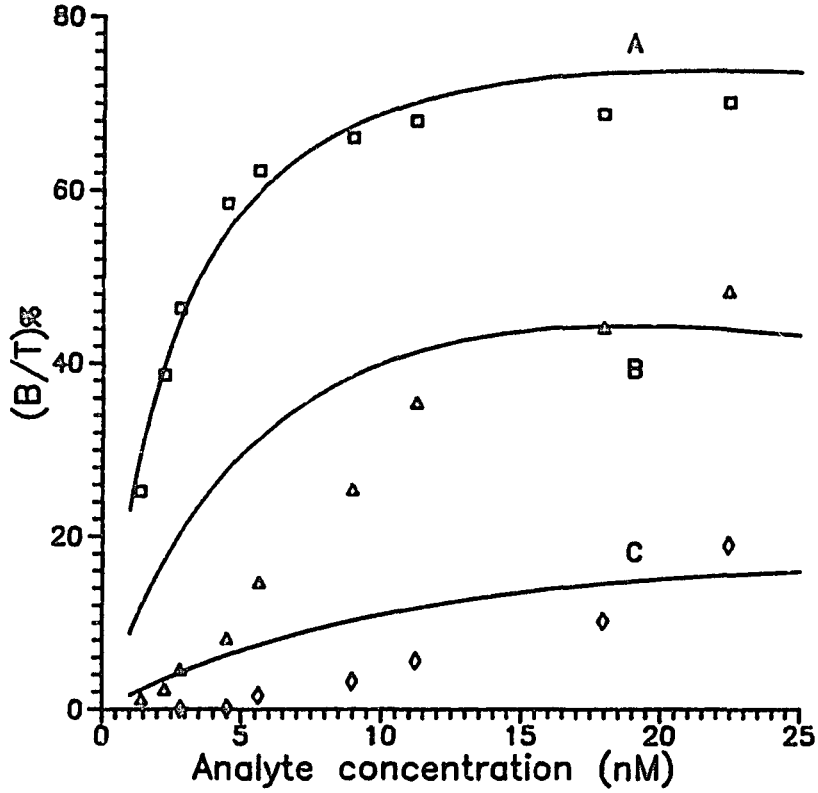

Figure 3.14 Dose-response curves for hGH and D-hGH in one-step sandwich immunoassay: **moderate** analyte concentration range. The concentration range of analytes, hGH (□) and D-hGH (△ , ◊) is 1.4 - 22 nM. The concentrations of labeled antibody, ^{125}I-GHC 101 are 0.59 nM (□) and 0.8 nM (△ , ◊) respectively. Solid-phase antibodies GHC 072 (□ , ◊) and GHC 101 (△) are chemically attached to plastic beads.

Theoretical Curves A and C correspond to the experimental results of GHC 072 (□) and (◊) systems respectively. Theoretical Curve B corresponds to the experimental data of GHC 101 (△) system. Theoretical binding curves: Curve A -- Model 1b: K_1 = 0.1 nM^{-1}, K_2 = 0.2 nM^{-1}, q_1 = 50 nM, q_2^* = 0.56 nM. Curve B -- Model 2b: K_1 = 0.072 nM^{-1}, q_1 = 22.7 nM, q_2^* = 0.8 nM. Curve C -- Model 2a: K_1 = 20 μM^{-1}, K_2 = 20 μM^{-1}, q_1 = 20 nM, q_2^* = 0.8 nM.

3.3.6.3 An analyte having two repeating epitopes/assay for D-hGH: GHC 072/D-hGH/GHC 101

Alternatively a similar one-step sandwich immunoassay can be developed using two different epitopes of D-hGH. This sandwich immunoassay is analogous to the assay developed for hGH by selecting GHC 072 and GHC 101 as the solid- and liquid-phase antibodies. However, some of the epitopes in D-hGH may be perturbed. The possible complexes formed are discussed in Model 2a and the dose-response should show results similar to the assay with only one antibody as previously discussed for D-hGH. However, D-hGH can interact with two liquid-phase antibodies in this assay mode. At very low concentrations of D-hGH, the response is very poor and the experimental data are not shown (Figure 3.13). In this assay D-hGH can effectively interact with two GHC 101 molecules, depending on the concentration of the labeled antibody present in the reaction. The poor response might be due to inhibition of binding of D-hGH to the solid-phase antibody at higher concentrations of labeled antibody (GHC 101). As the D-hGH concentration is increased, the response is gradually increased and eventually generates a "hook" (Figure 3.14 and 3.15). Curve C represents the computer simulated curve for this assay. The theoretical curve shows a reasonable fit, however, experimental data in Figure 3.14 demonstrate a quite low response especially at very low analyte concentrations. Curve C in Figure 3.14 has been obtained using approximately 55 and 190 fold smaller values for GHC 072 (K_1) and GHC 101 (K_2). The solid-phase antibody concentration, q_1 was 20 nM. Similarly, K_1 and K_2 differ about 15 and 48 fold from the experimental values (Curve C, Figure 3.15). The deviated data for affinities could be related to the distorted nature of the D-hGH compared to hGH. The poor response of this assay, at very low concentrations, should be attributed to the following: The interaction

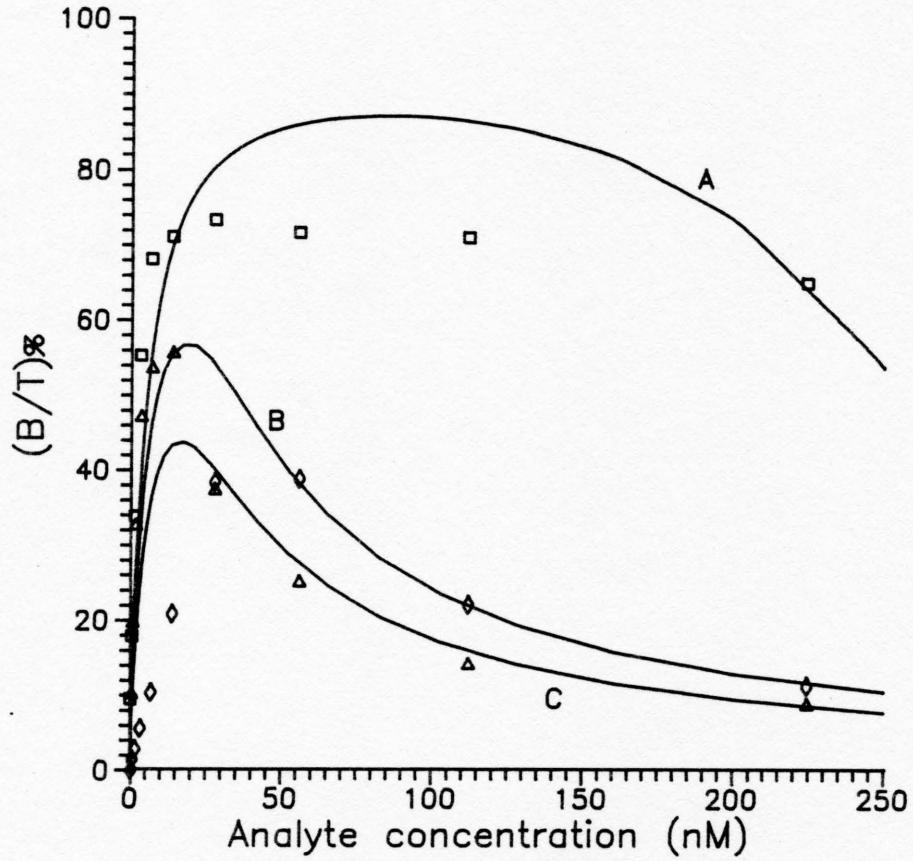

Figure 3.15 Dose-response curves for hGH and D-hGH in one-step sandwich immunoassay: **high** analyte concentration range. The concentration range of analytes, hGH (□) and D-hGH (△ ,◊) is 0.40 nM - 0.25 μM. The concentration of labeled antibody, ^{125}I-GHC 101 is 0.59 nM (□) and 0.8 nM (△ ,◊). Solid-phase antibodies, GHC 072 (□) and GHC 101 (△ ,◊) are chemically attached to plastic beads. The concentration range of hGH is 0.40 nM - 0.25 μM.

Theoretical Curves A corresponds to the experimental data for GHC 072 (□). Theoretical Curves B and Correspond to the experimental data for GHC 101 (△) and GHC 101 (◊) systems. Theoretical binding curves: Curve A -- Model 1b: K_1 = 0.1 nM^{-1}, K_2 = 0.1 nM^{-1}, q_1 = 0.16 μM, q_2^* = 0.56 nM. Curve B -- Model 2b: K_1 = 0.1 nM^{-1}, q_1 = 27 nM, and q_2^* = 0.8 nM. Curve C -- Model 2a: K_1 = 80 μM^{-1}, K_2 = 80 μM^{-1}, q_1 = 20 nM and q_2^* = 0.8 nM.

of two labeled antibodies (GHC 101) in the liquid-phase may prohibit the formation of the sandwich complex with the solid-phase, as GHC 101 is in excess at very low analyte concentrations. At high D-hGH concentrations, perhaps each analyte molecule can react with an individual labeled antibody. Thus GHC 072 would be accessible to interact with D-hGH to form the signal generating sandwich complex. Additional studies of D-hGH in two-step assay mode (detailed in Chapter 4), indicate consistent data which specifically permit multiple epitope interactions with liquid-phase antibody (GHC 101) to form liquid-phase complexes. The solid-phase antibody is prohibited from interacting with D-hGH if the analyte reacts with two molecules of the liquid-phase antibody. All the analytes exhibit the "hook" effect at high analyte concentrations. Moreover, the selection of the solid- and liquid-phase antibody also contributes to the specificity of the one-step assay mode.

3.3.6.4 An analyte having multiple epitopes/assay for ferritin:

c,p-FEF021/hs-ferritin/FEF021 and c,p-QCI054/hs-ferritin/FEF021 systems

The theoretical studies demonstrate that high capacity solid-phase matrices shift the "hooked" maximum of the curve to higher analyte concentrations. Experimental results support this theoretical prediction. As a practical matter, the effect of the capacity of the solid-phase capture antibody can only be demonstrated by studying the dose-response curves at different concentration ranges of the analyte while maintaining the liquid-phase labeled antibody constant. As described in Model 2b, an individual antibody can be selected as the solid- and liquid-phase antibody to design a one-step sandwich immunoassay. In such an assay the solid-phase antibody is covalently attached to the plastic beads (c-FEF021) or physically adsorbed on the plastic tubes (p-FEF021). This assay can also be redesigned by replacing the capture antibody c-FEF021 with c-QCI054

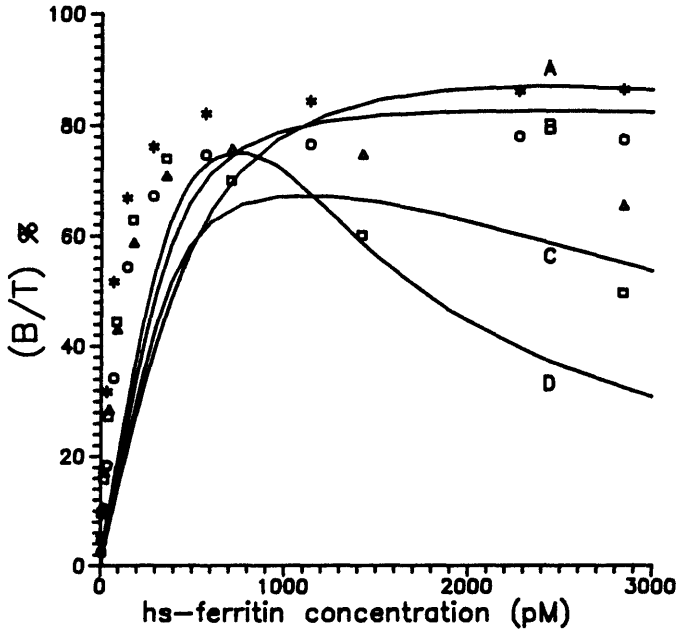

Figure 3.16 Effect of **low** hs-ferritin concentrations on "hook" effect in one-step sandwich immunoassay for chemically and physically immobilized antibodies. Capture antibodies: physically adsorbed (p) to plastic tubes FEF021 (□), QCI054 (△) and chemically attached (c) plastic beads: QCI054 (○) and FEF021 (*). Concentrations of the coating solutions for physically adsorbed antibodies, FEF021 and QCI054 are (50 μg/mL) and (9.0 μg/mL) respectively. ^{125}I-FEF021 concentrations are 0.375 nM (□, ○ , △) and 0.5 nM (*) respectively. hs-ferritin concentration range is 4.0 pM - 3.0 nM.

Theoretical Curves A and D correspond to the experimental results for c-FEF021 (*) and p-FEF021 (□) systems respectively. Theoretical Curves B and C correspond to the experimental results for c-QCI054 (○) and p-QCI054 (△) systems respectively. Theoretical binding curves: Curve A -- Model 2b: K_1 = 4 nM^{-1}, q_1 = 4 nM, q_2^* = 0.5 nM. Curve B -- Model 2a: K_1 = 0.5 nM^{-1}, K_2 = 10 nM^{-1}, q_1 = 8 nM, q_2^* = 0.375 nM. Curve C -- Model 2a: K_1 = 1 nM^{-1}, K_2 = 12 nM^{-1}, q_1 = 2.3 nM, q_2^* = 0.375 nM. Curve D -- Model 2b: K_1 = 10 nM^{-1}, q_1 = 1.0 nM, q_2^* = 0.375 nM.

(chemically attached QCI054 on the plastic beads) or p-QCI054 (physically adsorbed QCI054 to the plastic tubes). The labeled antibody for all assays was FEF021. Assays for all systems were performed in three different concentration ranges and the resulting data are shown in Figures 3.16-3.18. To learn if these observations could be explained by the theory, reasonable values were assigned for each relevant model. The experimentally determined affinity constant for FEF021 antibody is 56 nM^{-1}. The affinity has been determined in solution. Simulated dose-response curves for Model 2a and 2b are shown in each figure. Computer simulated Curves represent as Curve A (c-FEF021), Curve B (c-QCI054), Curve C (p-QCI054) and Curve D (p-FEF021) respectively. At very low analyte concentrations, all ferritin assays show a linear dose-response similar to the linear hypothetical response in Figure 3.1. The response is progressively increased as the analyte concentration rises and a plateau results, but all one-step sandwich immunoassays demonstrate the "hook" at high analyte concentrations. The "hook" maximum is at low analyte concentrations for physically adsorbed solid-phase systems. The data for p-FEF021 and p-QCI054 systems exhibit poor assay response at very high analyte concentrations and are not shown. The "hook" maximum is moved to higher analyte concentrations for the chemically immobilized solid-phase antibody systems. Plastic beads permit higher surface area than the plastic tubes.As shown by Cantarero et al. (1980) one can estimate the surface saturation capacity for an antibody. As an example, the surface saturation capacity of bovine IgG is approximately 250 ng/cm^2. The surface area of a plastic bead is greater than the plastic tube. Therefore it is apparent that plastic beads can accommodate more antibodies than plastic tubes. These differences are discussed with the experimental data. It is also known that the immobilization process alters the binding affinity of the solid-phase antibody (Olson et al., 1989). The

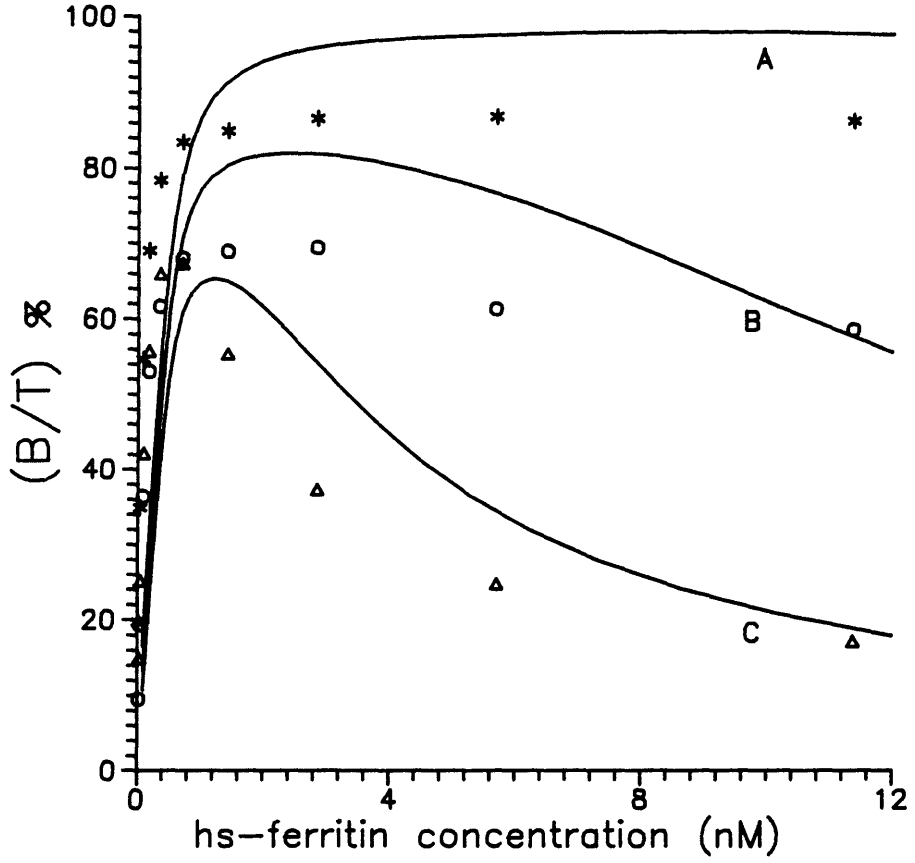

Figure 3.17 Effect of **moderate** hs-ferritin concentrations on "hook" effect in one-step sandwich immunoassay for chemically and physically immobilized antibodies. Capture antibodies: physically adsorbed plastic tubes, p-QCI054 was coated with is 5 μg/mL solution; chemically immobilized, QCI054 (o) and FEF021 (*). ^{125}I-FEF021 concentration is 0.5 nM. hs-ferritin concentration range is 20 pM - 11.3 nM.

Theoretical Curves A corresponds to the experimental data for c-FEF021 (*) system. Theoretical Curves B and Correspond to the experimental data for c-QCI054 (o) and p-QCI054 (□) systems respectively. Theoretical binding curves: Curve A -- Model 2b: K_1 = 6 nM^{-1}, q_1 = 18 nM, q_2^* = 0.5 nM. Curve B -- Model 2a: K_1 = 0.5 nM^{-1}, K_2 = 10 nM^{-1}, q_1 = 8 nM, q_2^* = 0.5 nM. Curve C -- Model 2a: K_1 = 1 nM^{-1}, K_2 = 12 nM^{-1}, q_1 = 2.3 nM, q_2^* = 0.5 nM.

experimental data for covalently immobilized plastic beads suggest that the altered binding affinity can be compensated by increasing the concentration of the solid-phase antibody. The binding capacity of physically adsorbed antibodies is somewhat lower, which specifically affects the dynamic range of the dose-response curve. The affinity of physically adsorbed antibodies should be similar or greater than the chemically immobilized antibodies. The Curve A in Figures 3.16-3.18 represents a general view of the theoretical binding response for Model 2b in which FEF021 is used as both the solid- and liquid-phase antibodies. It will be noted that the theoretical curves do not correlate well with the experimental data. At high ferritin concentrations there is a substantial deviation of theory and experiments. Model 2a and 2b can be used to quantify the experimental data. With reagents mixed together, therefore, ferritin molecules can exhibit multiple binding, especially with the liquid-phase antibody even before it interacts with the solid-phase antibody. In both models $Q_2^*PQ_1$ is assumed to be the response generating complex, however, the response generating species can have multiple binding with Q_2^* antibody. Therefore the overall binding response is magnified. Moreover, affinities of the solid- and liquid-phase are assumed to be the same in establishing Curve A. Although the same antibody is used for both solid- and liquid-phase in the experimental procedure, the affinity of the solid-phase antibody appears to be significantly different from the liquid-phase antibody. As a result, the experimental data is shifted from the theoretical curves. The affinity of antibody (K_1) used to generate Curve A is about 14 fold (Figure 3.16) and 9 fold (Figures 3.17 and 3.18) lower from the experimental value. Note that the Curve D is also generated using the same model, but K_1 is only about 5 fold lower from the experimental value. The concentrations of the solid-phase antibody (q_1) for theoretical Curve A are 4 nM (Figure 3.16), 18 nM (Figure 3.17) and 16 nM

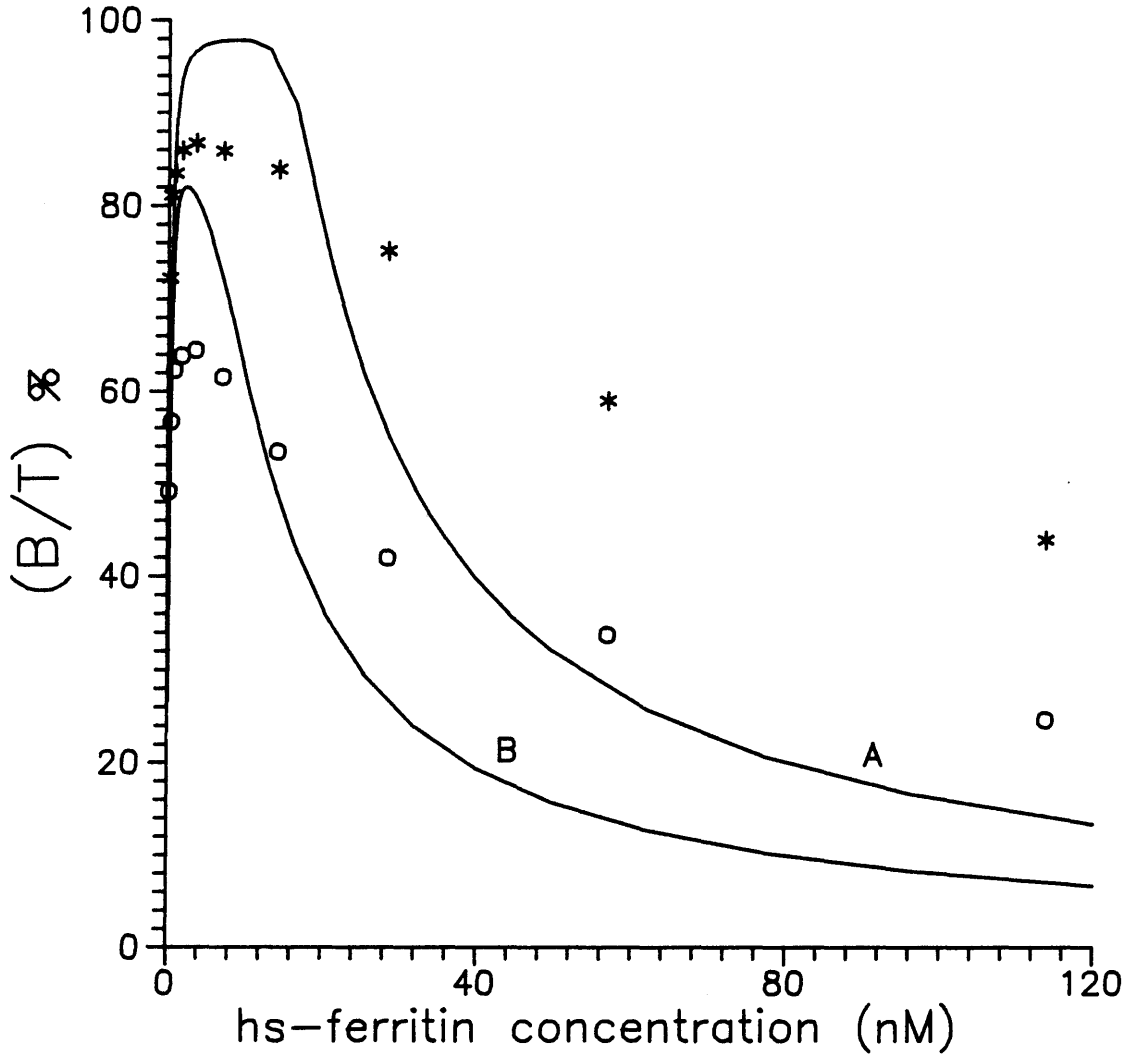

Figure 3.18 Effect of **high** hs-ferritin concentrations on "hook" effect in one-step sandwich immunoassay for chemically immobilized antibodies. Capture antibody is chemically immobilized (c): c-QCI054 (o) and c-FEF021 (*). ^{125}I-FEF021 concentration is 0.5 nM. The concentration range of hs-ferritin is 0.22 - 60 nM. Theoretical Curves A and B correspond to the experimental data for c-FEF021 (*) and c-QCI054 (o) systems respectively. Theoretical binding curves: Curve A -- Model 2b: K_1 = 6 nM^{-1}, q_1 = 16 nM, q_2^* = 0.5 nM. Curve B -- Model 2a: K_1 = 0.5 nM^{-1}, K_2 = 10 nM^{-1}, q_1 = 8 nM, q_2^* = 0.5 nM.

(Figure 3.18), respectively. The value of q_1 is quite low for Figure 3.18, but an increase in q_1 would not effectively approximate the experimental data. For contrast, Curve D was generated using a 4 fold lower value of q_1 compared with Curve A, attributed to the low capacity of the physically adsorbed solid-phase antibodies (Figure 3.16). The value of the affinity for the solid-phase (K_1, QCI054) antibody used to generate Curve B in each figure (3.16-3.18) was 0.5 nM^{-1}. The experimental value of K_1 has not been determined for QCI054, but it is assumed to be similar to that of FEF021. Curve C was generated using Model 2a (similar to Curve B) the difference being the solid-phase antibody. The theoretical value of K_1 is 1 nM^{-1}. The values for affinity of labeled antibody (K_2, FEF021) are acceptable which approximately 5 (Figure 3.16), 6 fold (Figures 3.17 and 3.18) lower than the experimentally determined value. The theoretical estimated value of K_1 for Curve C (Figure 3.16 and 3.17) is also about 5 fold lower than the experimental value. Curve C was generated using a 4 fold lower solid-phase antibody concentration compared to Curve B suggesting that the one-step sandwich immunoassay uses plastic tubes which have less capacity than the plastic beads. The experimental concentration of the labeled antibody is same as the theoretical value for all of these calculations. These studies underline the importance of increasing the capacity of the capture antibody to forestall the "hook" effect. On the other hand the number of epitopes of the analyte can also contribute to the sensitivity of the assay, postponing the "hooked" nature of dose-response curve. The replacement of the capture antibody seems to show no adverse effects on the dose-response curve for analytes such as ferritin.

Assayist is interested to predict the "hook" effect in advance before the assay is applied to the samples. To illustrate this Model 2 can be used. Protein concentration on a plastic surface can be 160 - 320 ng/cm^2. Assume the antibody

concentration is 160 ng/cm^2 and the total volume used for the reaction is 0.2 mL. The liquid volume, 0.2 mL, approximately corresponds to 1.55 cm^2 surface area. Therefore the theoretical total solid-phase antibody concentration is 7.75 nM, however, practically it is impossible to get 100% surface coverage. If only 50% of the total antibody is immunoreactive and covers the plastic surface then the solid-phase antibody concentration should be 4 nM. For the sake of simplicity it is assumed that the binding constants of the solid- and liquid-phase antibodies are equal. Also the labeled antibody concentration assumed to be 0.5 nM. Thus, it can be predicted that the test samples exceeding an analyte concentration of 2.5 nM would give a lower result due to the "hook" effect in Model 2.

3.3.7 Conclusions

This study underlines the various circumstances in which one can obtain analytically descriptive results in one-step sandwich immunoassay. The assayist has an advantage in using one-step mode to speed up the assay process. However, our study indicates that the optimization of the assay could avoid unexpectedly lower values for the unknown sample using this technique. Generally, all the one-step sandwich immunoassays exhibit the "hook" effect, irrespective of the characteristics of the analyte. Accordingly the biphasic nature of the one-step sandwich immunoassay is a result of a much higher level of the analyte. This is the general disadvantage of this assay mode. In principle, the concentration effect of the analyte is not necessarily applicable to the two-step mode as the necessary reagents are added sequentially. In a one-step assay, it is apparent that the use of excess solid- and liquid-phase antibody is necessary to shift the "hook" to higher analyte concentrations. Theoretical and experimental studies show that the use of high capacity solid-phase antibodies should prevent the "hook" at analytically significant

concentrations.

In addition, analyte characteristics must be considered as the interactions of the solid- and liquid-phase antibodies with the analyte are multifold. The careful selection of the reagents is necessary to avoid or minimize the ambiguous results in the one-step sandwich immunoassay. One step sandwich immunoassay can be designed by properly selecting two spatially different epitopes of an analyte. Macromolecular analytes are presumed to have equivalent as well as different classes of independent epitopes. Thus, the multiple epitope interactions can specifically improve the sensitivity of the assay using either identical or different epitopes. For macromolecules, the assay can use either one antibody reacting with identical epitopes or two antibodies binding with different epitopes. However, assays for analytes having a discrete number of identical or different epitopes may lead to considerable changes in the assay response unless the two different antibodies for non-repeating epitopes are selected. The one-step sandwich immunoassays could not be used in the analytically significant concentration range if the liquid-phase antibody can permit multiple interactions, especially at low analyte concentrations. However, this effect may not be serious in a two-step assay mode. The effect of analyte concentration on the "hook" effect may be mitigated with analytes having multiple epitopes. In such an assay more labeled antibody binds to the analyte, the sensitivity of the assay is improved, and the "hook" shifts to higher analyte concentrations.

Chapter 4

Multiple epitope interactions in the two-step sandwich immunoassay

4.1 Introduction

The immunometric assay is increasingly recognized as a potentially important immunoassay technique (Miles and Hales, 1968). This assay offers several advantages over limited reagent labeled assays (e.g., competitive binding assay, radioimmunoassay (RIA)) for analytes, with respect to lower detection limits, higher specificity, wider working range and shorter incubation time.

In a two-site immunometric assay, the entity to be measured is "sandwiched" between two antibodies which recognize either equivalent epitopes (symmetrical) or different epitopes (asymmetrical). One of the antibodies (capture antibody) is either covalently bound, or physically adsorbed on a solid-phase. In conventional, two-site immunometric assays, the antigen and the labeled antibody react sequentially with the capture antibody (Sevier et al., 1981). Excess reagent is removed before the addition of the next. The response is generated by the labeled antibody, and it is directly proportional to the analyte concentration. One such "sandwich type" immunometric assay (Woodhead et al., 1974) is the immunoradiometric assay (IRMA). Since IRMA was developed, the use of sandwich immunoassays has rapidly expanded, particularly in the area of clinical diagnosis for biologically active analytes (Gosling, 1990). One of the most serious analytical problems associated with the two-step sandwich immunoassay is the high dose "hook" effect -- a paradoxical decrease in response at high analyte concentrations.

Polyclonal antibodies have been commonly used as the solid-phase antibody in the two-step immunometric assay (Miles et al., 1974). With the advent of

monoclonal antibodies (Kohler and Milstein, 1975), an additional, simplified procedure was adopted in involving two monoclonal antibodies directed against spatially distant antigenic sites (epitopes) on the same molecule (David et al., 1981). Analytes consisting of non-overlapping but repeating epitopes, however, permit a single monoclonal antibody to act concurrently and effectively as both capture and tracer antibody for the same assay (Chi et al., 1987).

Rodbard and Feldman (1978) developed a theoretical model to optimize conditions for the two-step sandwich immunoassay which permits performance evaluation of the ideal assay system. Their model predicts the effects on the dose-response curve of random errors in the concentration of reagents, the rate constants for antigen-antibody complex formation or the reaction time. It fails, however, to provide an explanation for the "hook" effect, because the solid- and liquid-phase antibody populations were assumed to be homogeneous. Other reports have also suggested improvements in models for IRMA (Rodbard and Lewald, 1970; Rodbard and Weiss, 1973). Studies were undertaken to evaluate the possible factors (Miles et al., 1974) which affect performance in sandwich immunoassays. According to the literature, analytes such as ferritin (Casey et al., 1979) and human growth hormone (Miles, 1977) are initially captured by the polyclonal solid-phase antibody but are released into solution during the second step of the assay. The immunological properties of ferritin and human growth hormone differ widely yet behave similarly in these assays. Furthermore, their reports suggest that the "hook" may be due to low affinity solid-phase antibodies, inadequate washing, insufficient amounts of labeled antibody at the second step, or excessive incubation times.

Rodbard et al. (1978) soon established a theoretical basis for the "hook" effect using extended mathematical models based on two mechanisms. Their

model suggests that the solid-phase antibody is heterogeneous and exhibits binding sites with differing affinity constants. Rapid dissociation of the analyte from the low affinity sites occurs during the second "wash" period. This accentuates the "hook" effect. Systems consisting of homogeneous solid-phase antibodies may still demonstrate a "hook" effect if washing is incomplete after the first incubation with the antigen. The results obtained from theoretical studies on two-step immunometric assays indicate (Ryall et al., 1982) that a "hooked" response could be obtained under conditions which do not involve low affinity antibodies. This simulation of the "hook" effect involved homogeneous antibodies of high binding affinity, but the concentration of labeled antibody added in the second assay step was insufficient. This model assumed that ferritin was lost from the surface and might have formed soluble complexes with labeled antibody during the second-step of the assay. The proposed model is specially designed for large molecules such as ferritin. Some of the assumptions may not be applicable for analytes which do not possess multiple identical epitopes. According to their theoretical calculations (Ryall et al., 1982) an additional population of low affinity antibodies on the solid-phase will demonstrate a "hook" in the two-step immunoassay as suggested by other reports.

Ferritin has been used as the model antigen in experimental studies to investigate in detail the fundamental analytical problems in the two-step sandwich immunoassay (Perera and Worwood, 1982). Dual labeling provided a more detailed understanding of the probable reasons for the "hook" in a sandwich assay involving a polyclonal solid-phase antibody. By monitoring the second-step of the assay, it was reported that a significant amount of ferritin bound at the first-step was lost during the second incubation. Another experiment, without the labeled

antibody, demonstrated that 10% of the bound ferritin was lost during the second-step of the assay. These data clearly show that the hook effect in a polyclonal system results in part from heterogeneity of the capture antibody. The "hook" was not eliminated in an assay developed by using monoclonal capture antibodies. Moreover, the studies by Perera and Worwood (1982) indicated that non-specific interactions should be eliminated to provide assays without "hook" effects. However no studies have investigated the influence of the characteristics of the analyte on "hook" effects in two-step sandwich immunoassays. It is evident, therefore, that there are a number of possible causes of the "hook" effect. As this phenomenon is clearly detrimental to reliable immunoassays, the conditions under which the effect can be observed must be delineated and its occurrence predicted.

This study integrates the various theoretical approaches with experimental tests of analyte characteristics. To gain insight into the variables affecting the "hook" effect, monoclonal antibodies have been used throughout all experiments. The effect of antigenic determinant properties of the antigen on the "hook" effect has been examined to demonstrate the nature of the interactions. The performance of the two-step sandwich immunoassay has been compared for different analytes to model the "hook" effect in terms of the characteristics of the analytes. Biosynthetic human growth hormone (hGH) has been selected as the simplest model to illustrate the binding behavior for an antigen having two different non-overlapping epitopes. This protein is known to form an extremely stable non-covalent dimer (D-hGH) which will serve as the simplest case of an analyte with two repeating epitopes. Ferritin was employed as a model for an antigen having multiple epitopes. The influence of varied analyte and antibody concentration, and non-specific interactions of the analyte with the solid-phase were all investigated to identify conditions under which the "hook" effect may be

observed. Kinetic studies were performed to explain the interactions that can release analyte from the solid-phase.

All the possible reasons previously reported for the "hook" effects in two-step sandwich immunoassay can be summarized: Low affinity of the solid-phase antibodies, inadequate washing, insufficient amounts of labeled antibody, excessive incubation times and non-specific interactions. Our study centers on the high dose "hook" effect which can be exhibited under excess and inadequate amounts of solid- and liquid-phase antibodies. The other factors were held to a minimum in this study.

4.2 Experimental section

4.2.1 Materials

Biosynthetic human growth hormone (hGH) and purified dimeric hGH (D-hGH) were donated by Eli Lilly (Indianapolis, IN). The following reagents were donated by Hybritech Inc. (San Diego, CA); Monoclonal antibodies for hGH, GHC 072 and GHC 101, Anti-ferritin antibodies, FEF021 and QCI054 ($F(ab)'_2$). The ^{125}I-FEF021 and ^{125}I-GHC 101 (approximately 10 μCi/mL) were prepared in the radioiodination laboratory of Hybritech, Inc. Chemical immobilization of GHC 072, GHC 101, FEF021 and QCI054 on plastic beads was carried out at Hybritech, Inc. Human spleen ferritin (hs-ferritin) and Human liver ferritin (hl-ferritin) were purchased from Scripps Laboratories (San Diego, CA). "Maxisorp" (11 X 70 mm) and Immunostar (12 X 75 mm) polystyrene tubes were purchased from Thomas Scientific (Swedesboro, NJ). Bovine serum albumin (BSA, Cohn Fraction V) and polystyrene tubes (12 X 75 mm) (for assays with plastic beads) were purchased from Fisher Scientific Co. (St. Louis, MO).

4.2.2 Reagents

The sample buffer throughout all experiments was phosphate buffered saline (PBS), pH = 7.4, 20 mM, containing 0.15 M sodium chloride with 5% BSA added. The washing buffer was the same except that 0.05% BSA was added. The stock solution of the coating buffer is 1.0 M carbonate, pH = 9.6. All other reagents were Reagent Grade. All solutions were prepared weekly in water obtained from a Barnstead Nanopure II system and were stored at 4 °C.

4.2.3 Apparatus

A computer controlled gamma counter (Compugamma 1882-003, Pharmacia LKB Biotech. Inc., Gaithersburg, MD) was used for all radioactivity

measurements.

4.2.4 Methods/Two-step sandwich immunoassay

4.2.4.1 Chemically immobilized capture antibodies

Each plastic bead (5/16 inch) was removed from the container and the residual solvent was blotted but the bead was not permitted to dry. One plastic bead was introduced into each tube. GHC 072 immobilized beads were supplied in dry form and were used directly. Different amounts of the analyte (hs-ferritin, D-hGH or hGH) were added to each bead and diluted to 200 μL, followed by incubation for 2 hours at room temperature. Assays for all analytes were performed with shaking to ensure mixing. After incubation of the beads, 1.0 mL of washing buffer was dispensed to each tube and the liquid was aspirated. All plastic beads were washed five times, aspirating the liquid after each washing step. After carefully removing all of the washing buffer in the tube, a fixed volume of the ^{125}I-antibody was added and diluted to 200 μL, then incubated for 3 hours. After incubation, the above washing procedure was repeated five times, the buffer was removed, and each bead was counted in the gamma counter. All measurements were made in triplicate. Any deviations from this procedure are indicated in the figure captions. Two-site immunoassays for hGH were performed at 37 °C.

4.2.4.2 Physically adsorbed capture antibodies

Antibodies (FEF021 or QCI054) were physically adsorbed on the polystyrene surface of plastic tubes by directly adding 200 μL of antibody in carbonate buffer (0.01 M, pH = 9.6) and incubating 3 hours at room temperature. The antibody concentrations are indicated in the appropriate figure captions. Once coated with antibodies, all tubes were blocked for nonspecific binding by incubation with 300 μL of sample buffer for 1 hour at room temperature, and followed by washings with 250 μL of washing buffer. After washing, variable amounts

of hs-ferritin were added and diluted to 200 μL. The standard incubation time was 3 hours. After washing as before, labeled antibody was added to the tubes and incubated for 4 hours. These tubes were washed as before and counted in the gamma counter. All measurements were made in triplicate.

4.2.5 Ferritin assay kinetics with chemically immobilized capture antibodies

4.2.5.1 First assay step:

Plastic beads were introduced into the plastic tubes as indicated in the sandwich immunoassay procedure. A fixed amount of hs-ferritin in 200 μL was added to each bead and incubated for different time intervals. After incubation, the beads were washed as described above, 200 μL of ^{125}I-FEF021 antibody was added, then incubated for 3 hours. The concentrations of ferritin and ^{125}I-FEF021 are given in the figure captions. Once the unreacted labeled antibody was separated, beads were counted in the gamma counter.

4.2.5.2 Second assay step:

Plastic beads were introduced into plastic tubes as explained in the two step sandwich immunoassay procedure. A fixed amount of hs-ferritin in 200 μL was added to each bead and incubated for 2 hours. Then the unreacted hs-ferritin was separated. 200 μL of ^{125}I-FEF021 were added and incubated, then bound, labeled material was separated at different time intervals. Beads were counted in the gamma counter. The concentrations of ferritin and ^{125}I-FEF021 are reported in the figure captions. All kinetic experiments were carried out in triplicate.

4.3 Results and discussion

4.3.1 General discussion

Ideally, the two-step sandwich immunoassay should be highly specific for the analyte(s) to be measured, have a sensitivity sufficient to detect the analyte(s) and a broad enough measurement range to cover all the concentrations occurring in the sample. To fulfill all these requirements it is necessary to know in detail the immunological characteristics of the analyte(s) and to obtain antibodies with high affinity and specificity. The essential aspect of this immunoassay method is the use of constant and excess amounts of capture and labeled antibodies so that the resulting protocol would be analytically useful for the assayist. In order to determine the factors which characterize the dose-response curve, this study combines theory and experimental results of two-step sandwich immunoassay systems consisting of different analytes. Practically, antigen concentration range and the amounts of antibodies can both be manipulated, however the working range of the analyte should be selected before optimizing the assay. If the working range for the analyte is established, the performance can be controlled by choosing the optimal amounts of solid- and liquid-phase antibodies. Therefore, special considerations are necessary to manipulate the binding characteristics of these analytes and to elucidate the fundamental analytical problems in two-step sandwich immunoassays.

4.3.2 Fundamental description of two-step sandwich immunoassay

If a two-step sandwich immunoassay is designed for an analyte using two monoclonal antibodies, the first and second steps can be represented by the following equations: $P + Q_1 = PQ_1$ ----- (1)

$$Q_2^* + PQ_1 = Q_2^*PQ_1 \qquad \text{----- (2)}$$

where P is the analyte, Q_1 and Q_2^* (labeled) are monoclonal antibodies which bind to two different sites on P. PQ_1 and $Q_2^*PQ_1$ are formed at the first and second steps of this assay and the measured response is generated by the $Q_2^*PQ_1$ complex. The concentration of Q_1 is defined as the total number of moles of immunologically active Q_1 immobilized on the surface divided by the solution volume. The capacity is defined as the moles of solid-phase antibody per unit area. If the conditions such as the amount of solid- and liquid-phase antibodies and the working range are optimized, the resulting dose-response curve shown in Figure 4.1 will be obtained. X_1, X_2 and X_3 are the increasing concentrations of the analyte. The calibration curve is linear up to concentration X_2. At higher analyte concentrations, X_2 and X_3, the inadequate amounts of capture and labeled antibody cause a plateau. A similar dose-response curve can result for systems under conditions with an excess of capture antibody and insufficient concentration of labeled antibody.

For an analyte consisting of two or more equivalent epitopes (e.g., ferritin) a single antibody is sufficient to develop the sandwich immunoassay. To extend the discussion about the epitopes of the analyte, a dimeric molecule of P (denoted as H) can be considered, which has two different types of epitopes, with each epitope repeated, for a total of four. This study assumes that all epitopes are accessible for binding interaction with appropriate sites in antibody. To describe the two-site sandwich immunoassay for an analyte having two repeating epitopes, the antibody Q_1 has been chosen, but the fundamental reaction is similar for antibody Q_2. The capture antibody Q_1 is again in the solid-phase. The labeled antibody Q_1^* is introduced in the liquid-phase. The reactions for each step of this assay are shown in Equations 3 and 4:

$$H + Q_1 = HQ_1 \qquad \text{----- (3)}$$

$$Q_1^* + HQ_1 = Q_1^*HQ_1 \qquad \text{----- (4)}$$

HQ_1 and $Q_1^*HQ_1$ are the solid-phase products generated at each step respectively. The response is monitored by measuring the radioactivity from the $Q_1^*HQ_1$ complex. The other possible liquid-phase complexes which might form are not discussed here for the sake of simplicity. The results obtained for the immunoassay for analyte H could be similar to analyte P (Figure 4.1). For an analyte such as ferritin, having multiple repeating epitopes (Luzzago et al., 1986), the shape of the dose-response curve would be expected to be similar to Curve A, Figure 4.2. Y_1, Y_2 and Y_3 denote various analyte concentrations as shown in Figure 4.2. On the contrary, the above assay gives experimental results corresponding to Curve B in Figure 4.2. The maximum response for the "hooked" dose-response curve is at analyte concentration Y_2. The linear range of the calibration curve is only useful for the assayist (i.e., concentration range, X_1 - X_2, Fig 4.1). Therefore the assay designer normally does not concern about the non-linear calibration curves when the assay is established (Figure 4.2). The "hooked" region of the calibration curve is considered when the assay is applied to real samples. For example, If the test sample contain an unexpected higher amount of the analyte a lower response is observed resulting misdiagnosis. The same assay can be performed if the unknown sample is diluted. The "hooked" calibration is studied in order to predict and avoid such ambiguous results for the test sample in advance.

Besides the two-site immunoassay already explained for the analyte possessing repeating epitopes, it is also possible to design an assay using one antibody for capture and another antibody for labeling. To develop a sandwich

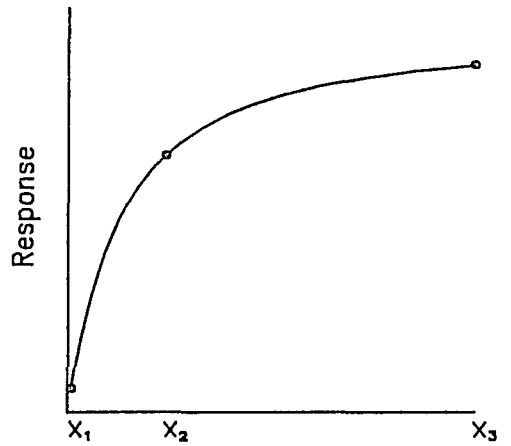

Figure 4.1

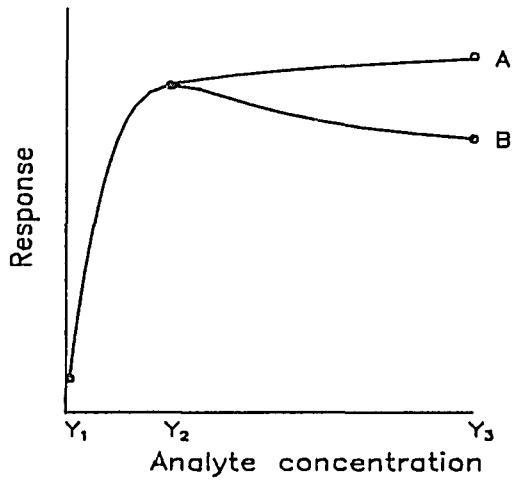

Figure 4.2

Figure 4.1 A hypothetical dose-response curve for two-step sandwich immunoassay using two antibodies directed at sterically distinct determinants of an analyte. The labeled antibody is in excess to analyte concentration, X_1. As the analyte concentration increases (range X_2 - X_3) a plateau results.

Figure 4.2 Hypothetical dose-response curves for two-step sandwich immunoassay using an analyte having non-overlapping repeating epitopes. Curve A represents the non-hooked dose-response curve. Curve B shows a "hook" effect. The analyte concentration range is Y_1-Y_3. The maximum response is at analyte concentration, Y_2.

immunoassay for the analyte H, two antibodies can be chosen which bind to two spatially different epitopes. This immunoassay is analogous to the experimental design discussed for the analyte P (Equations 1 and 2). Each step can be described using Q_1 and Q_2^* as the capture and labeled antibody respectively:

$$H + Q_1 = HQ_1 \qquad \text{----- (5)}$$

$$Q_2^* + HQ_1 = Q_2^*HQ_1 \qquad \text{----- (6)}$$

The basic response directed reactions are shown in Equations 5 and 6. In this case the liquid-phase antibody, Q_2^* can interact with two epitopes which is quite different from the assay defined by Equations 3 and 4. Theoretically, the standard curve is expected to be similar to the hypothetical curve of Figure 4.1, but no experimental evidence has previously been published to show that the response curve deviates from the hypothetical situation. Note that the analyte H can interact with two molecules of Q_2^* simultaneously, as a result of the multiple interactions a "hook" may be introduced into the standard curve.

Again this concept can be extended for an analyte such as ferritin and the fundamental reactions involved are basically similar to Equations 5 and 6. The response generating curve for ferritin is similar to Curve B, Figure 4.2. The "hooked" Curve B is attributed to possible multiple binding of ferritin with the liquid-phase antibody.

In order to resolve the complexity associated with the two-step sandwich immunoassays, model experiments have been designed with the support of theoretical data. Monoclonal antibodies reacted against these analytes permit the study of the effects of the nature of the analyte, with the goal of predicting the binding interactions so that two-step sandwich immunoassays can be properly designed and optimized. As denoted above, symbol P represents hGH, H represents D-hGH and ferritin in the description of models.

To simplify the interactions discussed in each model, the following general assumptions are made: (1) All reactions reach equilibrium (2) The immunoreactivity of solid-phase antibody is unaffected by immobilization by adsorption or covalent attachment (3) Capture antibodies are assumed to be far apart so that the analyte having multiple epitopes can not interact with more than a single antibody (4) Kinetics of the antigen-antibody reaction at a solid-phase are similar to the corresponding reaction in solution (5) Physically adsorbed antibodies do not leach from the solid-phase during the binding reaction (6) All liquid-phase antibodies are uniformly labeled (7) Bound and free fractions can be separated without disturbing the equilibrium (8) All labeled antibodies are uniformly labeled (9) No cooperative interactions occur for the binding of the antigen to the antibody. Other relevant assumptions are clearly mentioned in the description of each model.

4.3.3 Model 1 - Two-step sandwich immunoassay -- "hook" effect:

Analyte having two different epitopes/each epitope is repeated/two antibodies

This model assumes the interaction of a antigen with two different antibodies. The analyte is assumed to possess at least two different and repeating epitopes for the interaction with capture and labeled antibody respectively.

4.3.3.1 First assay step -- reaction of the analyte and the solid-phase antibody

The first assay step corresponds to the reaction of the analyte with an antibody-coated tube or bead as indicated in Equation 1. The stoichiometric equilibrium constant for the interaction is given by K_1:

$$K_1 = 2K_a = [HQ_1]/[H][Q_1]$$

where K_a is the microscopic binding constant. [H] and $[Q_1]$ refer to the free analyte and antibody concentrations, while $[HQ_1]$ is the bound analyte

concentration. The total concentrations of analyte and antibody are symbolized by h and q_1, respectively, and the following mass balance equations can be written:

$$h = [H] + [HQ_1]$$

$$q_1 = [Q_1] + [HQ_1]$$

Assuming $R = [HQ_1]/[H]$, a relationship can be developed which would then allow simulation of the binding curves for the first step of the two-site immunometric assay (Ekins et al., 1968).

$$R^2 + AR + B = 0$$

where $A = K_1(h - q_1) - 1$ and $B = -q_1K_1$.

It is possible to write $[H] = h/(1 + R)$, $[Q] = R/K_1$ and $[HQ_1] = hR/(1 + R)$.

4.3.3.2 Second assay step -- reaction with the labeled antibody

If the analyte has more than two equivalent epitopes then reaction scheme can be extended. Some of the complexed analyte, HQ_1 dissociates to form uncomplexed analyte, H, in the presence of inadequate amounts of labeled antibody. As the assay is optimized for a given analyte concentration range, the amount of labeled antibody should be insufficient at high analyte concentration range assuming that a sufficient amount of capture antibody is used for the assay. The dissociation reaction can be symbolized as follows:

$$HQ_1 \rightleftharpoons H + Q_1$$

The analyte is assumed to migrate from the solid-phase to react with the liquid-phase antibody primarily resulting in the formation of soluble complex HQ_2^*:

$$H + Q_2^* \rightleftharpoons HQ_2^*$$

Moreover several labeled antibodies could react with HQ_2^* to form soluble complexes and $Q_2^*HQ_2^*$ is the simplest case. The reaction is shown below:

$$Q_2^* + HQ_2^* \rightleftharpoons Q_2^*HQ_2^*$$

$$K_2 = 2K_b = [HQ_2^*]/[H]_0[Q_2^*]$$

$$K_2 = K_b/2 = [Q_2^*HQ_2^*]/[Q_2^*][HQ_2^*]$$

The stoichiometric equilibrium constant for the second step is K_2. The microscopic binding constant is K_b. Therefore the concentration of each species will be calculated by taking the above reactions into account. The total labeled antibody concentration is q_2^*. There is assumed to be no reaction between antibody Q_2^* and the solid-phase. The following mass balance equations can be written:

$$[HQ_1] = [H]_0 + [HQ_2^*] + [Q_2^*HQ_2^*] + [Q_2^*HQ_1] + [HQ_1]_0$$

$$q_1 = [Q_1] + [HQ_1]_0 + [H]_0 + [Q_2^*HQ_1]$$

$$q_2^* = [Q_2^*] + [HQ_2^*] + [Q_2^*HQ_1] + 2[Q_2^*HQ_2^*]$$

$[H]_0$ is the concentration of the analyte which migrate from the solid-phase. The remained bound analyte concentration is symbolized as $[HQ_1]_0$.

4.3.4 Model 2 -- Analyte having two or more equivalent epitopes/one antibody

This model assumes all conditions are the same as Model 1 except the following:

$k_a = k_b$; $K_2 = k_b/2$

$q_1^* = q_2^*$ ($Q_1^* = Q_2^*$)

This model differs from Model 1 in that a single antibody is employed. The analyte is assumed to possess at least two equivalent epitopes for the interaction with the solid- and liquid-phase antibodies. The fundamental reactions are given by Equations 3 and 4 in which Q_1 is used as the capture and labeled antibody. The Model 2 is compatible with the theoretical calculations reported by Ryall et al. (1982).

4.3.5 Model 3 -- Analyte having two sets of repeating epitopes/one antibody

These conditions deviate from Model 1: $k_a = k_b$; $K_2 = k_b/2$

$q_1^* = q_2^* (Q_1^* = Q_2^*)$

$[H] = 0$

$[HQ_2^*] = 0$

$[Q_2^*HQ_2^*] = 0$

This model differs from Model 2 in that no antigen molecules are leached out of the solid-phase at the second step of the assay ([H] = 0). Both epitopes of the antigen are assumed to allow simultaneous binding with either capture or labeled antibody. The binding reactions are shown in Equations 3 and 4.

4.3.6 Model 4 -- Analyte having two spatially different epitopes/two antibodies

The following are changed from Model 1 and the analyte is symbolized as P (H = P). The reactions for the first and second steps are given in Equations 1 and 2:

$K_1 = K_a$

$K_2 = K_b$

$[H] = 0$

$[HQ_2^*] = 0$

$[Q_2^*HQ_2^*] = 0$

In this model the interaction of two different epitopes of an antigen with appropriate antibodies is considered. This model is similar to Model 1 in that two different epitopes of an antigen is considered, however, this model differs from Model 1 by assuming that each epitope is not repeated and no desorption of the antigen ([P] = 0) occurs at the second assay step.

4.3.7 Model 4 -- Theoretical model for analyte having spatially different epitopes

As indicated in the beginning of the discussion, a two-step sandwich immunoassay can be developed if two antibodies are selected to bind different epitopes of an analyte (Equations 1 and 2). Such an assay has been described in

Model 4. This hypothetical model exhibits typical saturation curves at fixed amounts of labeled antibody (Figure 4.3). Response, (B/T)% is defined as the ratio of the concentration of the response generating complex divided by the total amount of liquid-phase labeled antibody (q_2^*). Consistent with Rodbard and Feldman (1978), this analysis shows the increase in the slope of these curves as the amount of solid-phase antibody is raised and no "hook" is observed (Curves A-G, Figure 4.3).

4.3.8 Model 2 -- Theoretical model for analytes possessing repeating epitopes

4.3.8.1 The effect of capture antibodies

Macromolecular antigens have repeating epitopes allowing multiple interactions with antibodies, consequently, theoretical two-step sandwich immunoassays demonstrate a high dose "hook" effect. The behavior of modeled monoclonal based two-step sandwich immunoassays for such macromolecular analytes, were predicted to behave in this way (Ryall et al., 1982) under inadequate concentrations of liquid-phase antibody, but the effect of the capacity of the solid-phase antibody on the high dose "hook" effect has not been discussed. The theoretical results presented here show that a high capacity solid-phase should prevent the "hook" effect. Practically, however, there is also an upper limit to the concentration of the solid-phase antibody which can be achieved. In addition, one standard curve might show no "hook" in a given concentration range, but if the assay is performed at increased analyte concentration the "hook" will result again, unless the amount of labeled antibody is increased in such a way that the conditions are optimized. To better predict the effect of the amount of solid-phase antibody on dose-response curves, parametric studies were undertaken at fixed concentrations of liquid-phase antibody based on Model 2 in which the analyte is permitted to have multiple interactions with the liquid-phase antibody. In most

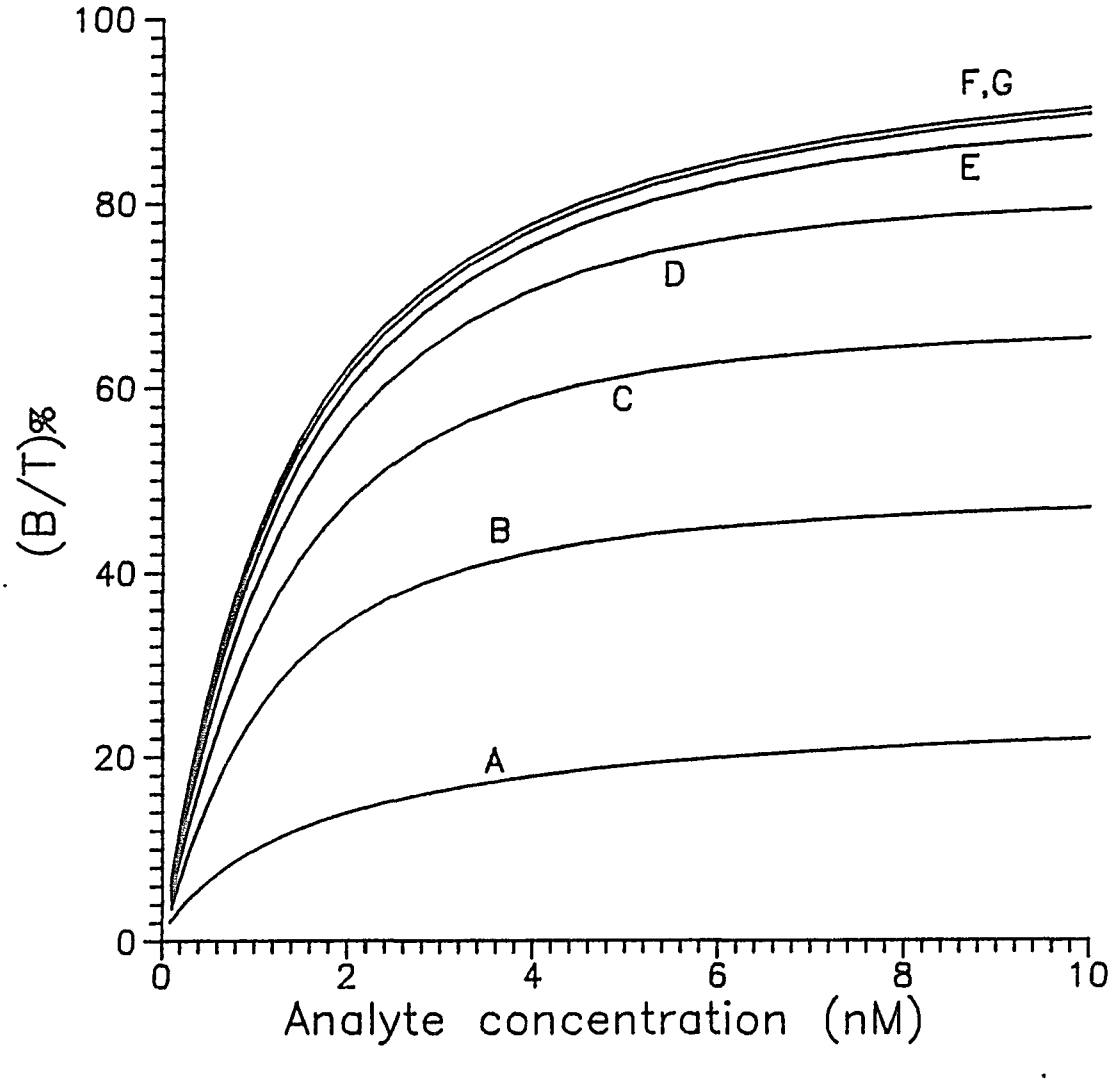

Figure 4.3 Simulated dose-response curves showing the effect of the capacity of solid-phase antibody in two-step sandwich immunoassay consisting of monoclonal antibodies against two different epitopes of an analyte. Theoretical parameters for Model - 4: K_1 = 1 nM^{-1}; K_2 = 1 nM^{-1}; q_1 (nM) = 0.5 (A), 1.25 (B), 2.5 (C), 5 (D), 10 (E), 20 (F), 50 (G); q_2^* = 0.5 nM.

instances, low capacity solid-phase antibodies demonstrate the "hook" effect as shown in Figure 4.4 (Curves B-E). Curve A shows almost no binding of analyte. As explained in Model 2, the equilibrium is reestablished after addition of Q_2^* in the second assay step. Several reactions can occur with bound HQ_1: it can react with Q_2^* to form the response generating complex, $Q_2^*HQ_1$; the analyte, H, can leach into the liquid-phase and react with Q_2^* to generate liquid-phase complexes such as HQ_2^* and $Q_2^*HQ_2^*$. Significant amounts of H and Q_2^* can be consumed in these reactions. As a result the $Q_2^*HQ_1$ concentration decreases at high analyte concentrations resulting in a "hook". As the capacity of solid-phase antibody is raised, larger amounts of the analyte bind in the solid-phase in the first step of the assay. Then the resulting dose-response curves show no "hook" (Curves F & G, Figure 4.4) and those necessarily follow hypothetical behavior (Curve A, Figure 4.2) in this concentration range. Under these conditions, however, insufficient Q_2^* is available. As the solid-phase has a sufficient amount of bound analyte, most of the labeled antibody molecules are consumed to form the signal generating complex. However, a greater proportion of the bound and desorbed analytes in the assay remains without forming a complex. A insignificant concentration of soluble complexes are formed in the analyte concentration range studied for the system containing high capacity solid-phases. As shown in Figure 4.4, if the capacity of solid-phase is greater than the highest analyte concentration ($q_1 > h$) the response generating complex is predominant. According to Figure 4.4, the highest analyte concentration is 10 nM and the solid-phase antibody concentrations for Curves F and G are 20 nM and 50 nM respectively. Calculated results show that an infinitesimal amount of analyte is released from the solid-phase under the conditions used to generate Curves F and G. As earlier predicted Curves F & G could demonstrate a "hook" at high analyte concentration range if the labeled

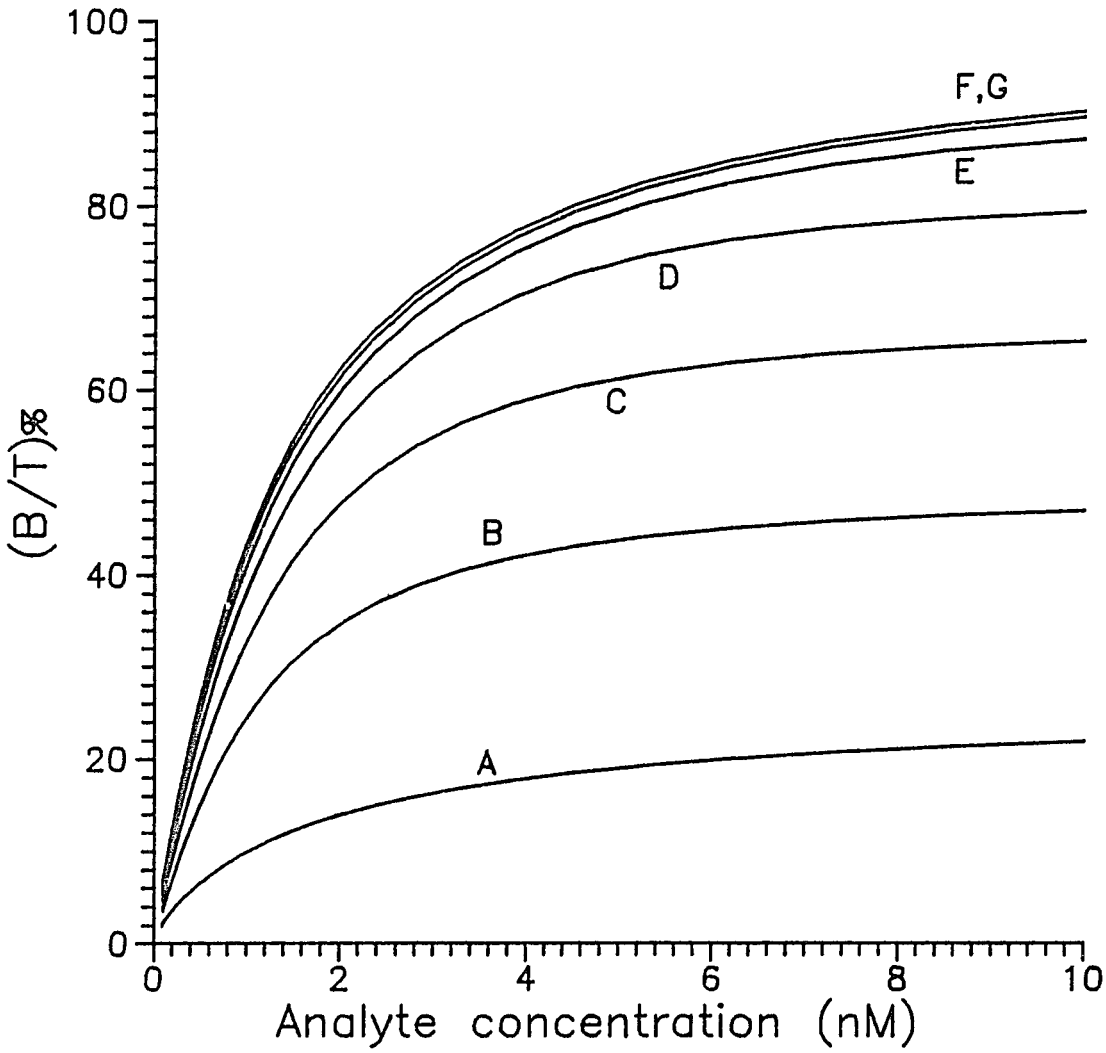

Figure 4.4 Simulated dose-response curves showing the effect of the capacity of solid-phase antibody (q_1) on "hook" effect in two-step sandwich immunoassay for an analyte having non-overlapping repeating epitopes. Theoretical parameters for Model 2: K_1 = 1 nM^{-1}; q_1 (nM) = 0.5 (A), 1.25 (B), 2.5 (C), 5 (D), 10 (E), 20 (F), 50 (G); q_2^* = 0.5 nM.

antibody concentration is not increased selectively. According to Model 2, high capacity solid-phase antibodies should demonstrate a plateau at high analyte concentrations preventing ambiguous results for the test sample.

4.3.8.2 The effect of labeled antibodies

The performance of two-step sandwich immunoassays can be controlled by selecting appropriate concentrations of labeled antibody. Theoretically, sufficient high concentrations of labeled antibody would predominate to suppress the "hook" effect. However the amounts of solid- and liquid-phase antibodies should carefully adjusted to be compatible in these assays. This study is limited to Model 2 in which the effect of labeled antibody concentration was investigated for a 2.5 nM concentration of solid-phase antibody selected from Figure 4.4 (Curve C). Curve E in Figure 4.5 is analogous to Curve C from Figure 4.4. Curve E suffers from the "hook" effect in which the selected capture antibody concentration (q_1) is greater than the labeled antibody concentration (q_2^*). If the concentration of labeled antibody is decreased while keeping both solid-phase and analyte concentrations the same, resulting calculated data show that the "hook" shifts to slightly lower analyte concentrations. These data are shown in Curves F-I, Figure 4.5. Note that the sharpness of the "hook" increase as the amount of labeled antibody is decreased (Curves F-I). Also the positive slope (sensitivity) of these curves are increased. Insufficient concentrations of labeled antibody in the mixture form higher amounts of soluble complexes. Consequently a smaller amount of signal generating sandwich complex is formed at high analyte concentrations. Increased concentrations of labeled antibody favor the formation of soluble analyte complexes in the second step of the assay and consequently diminish the assay response. Again note that the Curve D in Figure 4.4 shows a "hook" effect because $q_1 > q_2^*$. The concentrations q_1 and q_2^* are 2.5 nM and 1.0 nM respectively.

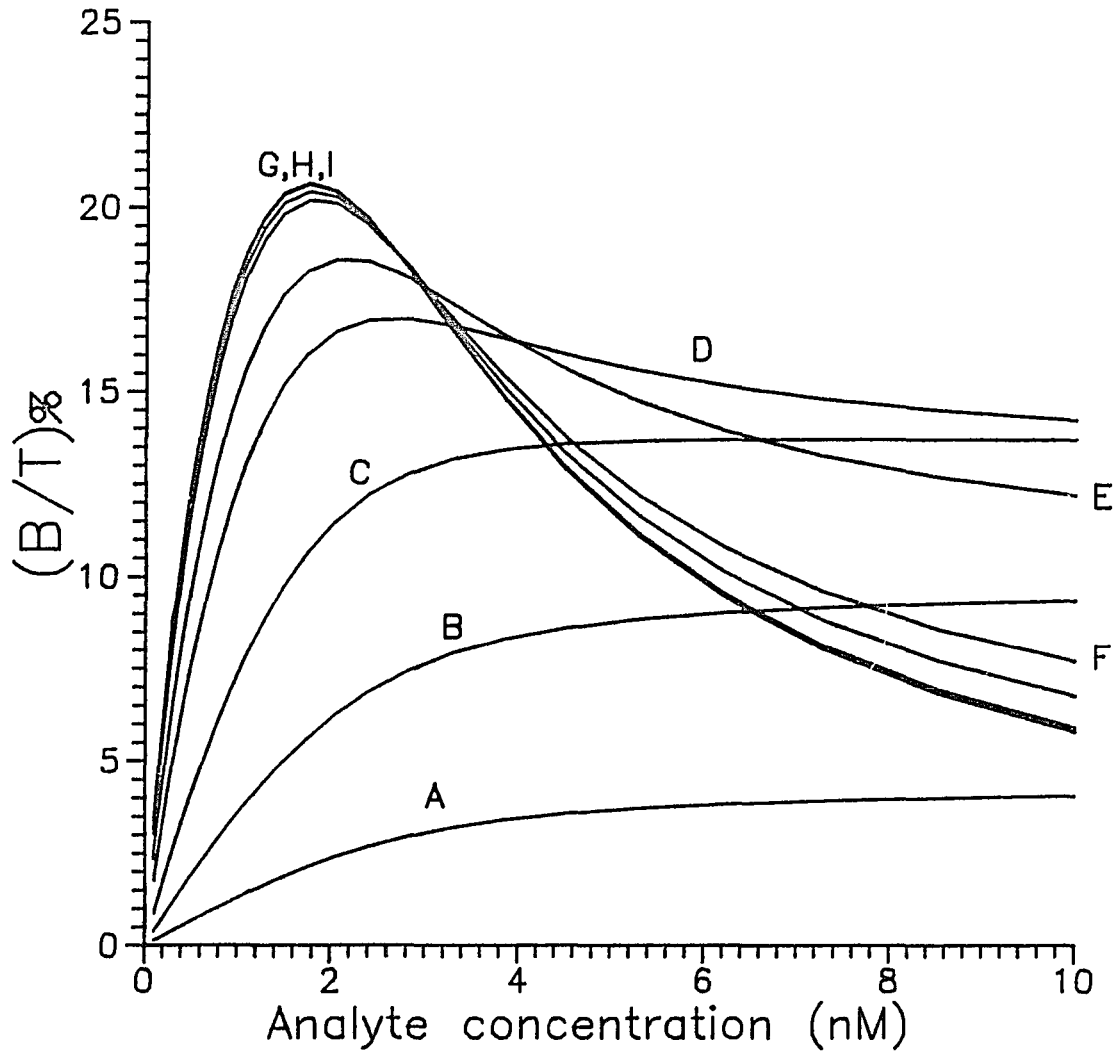

Figure 4.5 Simulated dose-response curves showing the effect of the concentration of the labeled antibody (q_2^*) on "hook" effect in two-step sandwich immunoassay. Theoretical parameters for Model 2: K_1 = 1 nM^{-1}, q_1 = 2.5 nM, q_2^* (nM) = 10.0 (A), 5.0 (B), 2.5 (C), 1.0 (D), 0.5 (E), 0.1 (F), 50 pM (G), 10 pM (H), 5 pM (I).

Furthermore Curves A-C in Figure 4.5 do not show a "hook" effect. Note that the concentration of labeled antibody is greater than or equal to the concentration of capture antibody ($q_2^* \geq q_1$) for Curves A-C. The parameters q_1 is 2.5 nM and q_2^* are 10 nM (Curve A), 5 nM (Curve B) and 2.5 nM (Curve C) respectively. However the assay sensitivity rapidly decreased as the concentration of the labeled antibody is raised. According to this study one can avoid the "hook" effect by selecting suitable concentrations of solid- or liquid-phase antibodies ($q_1 > q_2^*$) if the analyte concentration range is fixed. These conditions are compatible with Model 1 if the affinities of antibodies are assumed to be equal. A similar result has been reported (Ryall et al., 1982).

These theoretical data indicate that more selective experiments are needed to assess such changes. The above predictions, however, will also be determined by several parameters, including the affinities of solid- and liquid-phase antibodies and concentration of the labeled antibody.

4.3.9 Experimental results showing the effect of the analyte concentration: Assay for hGH -- GHC 072/hGH/GHC 101 system

Analytes such as hGH which contain no repeating epitopes are permitted to interact with two different antibodies so that the developed immunoassay for the analyte shows no "hook". Such an assay should generate a sandwich as defined in Equations 1 and 2. The resulting dose-response curve for hGH should be similar to Figure 4.1 (Model 4). Figure 4.6 shows experimental data points and the standard linear curve for hGH in which the binding behavior can be described using Equations 1 and 2. As the hGH concentration is increased, the response increases progressively as shown in Figure 4.7. Moreover, Figure 4.8 indicates the saturation of the response at high levels of hGH. The combination of data in Figures 4.6 - 4.8 comprise the hypothetical dose-response curve in Figure 4.1. The simulated curves

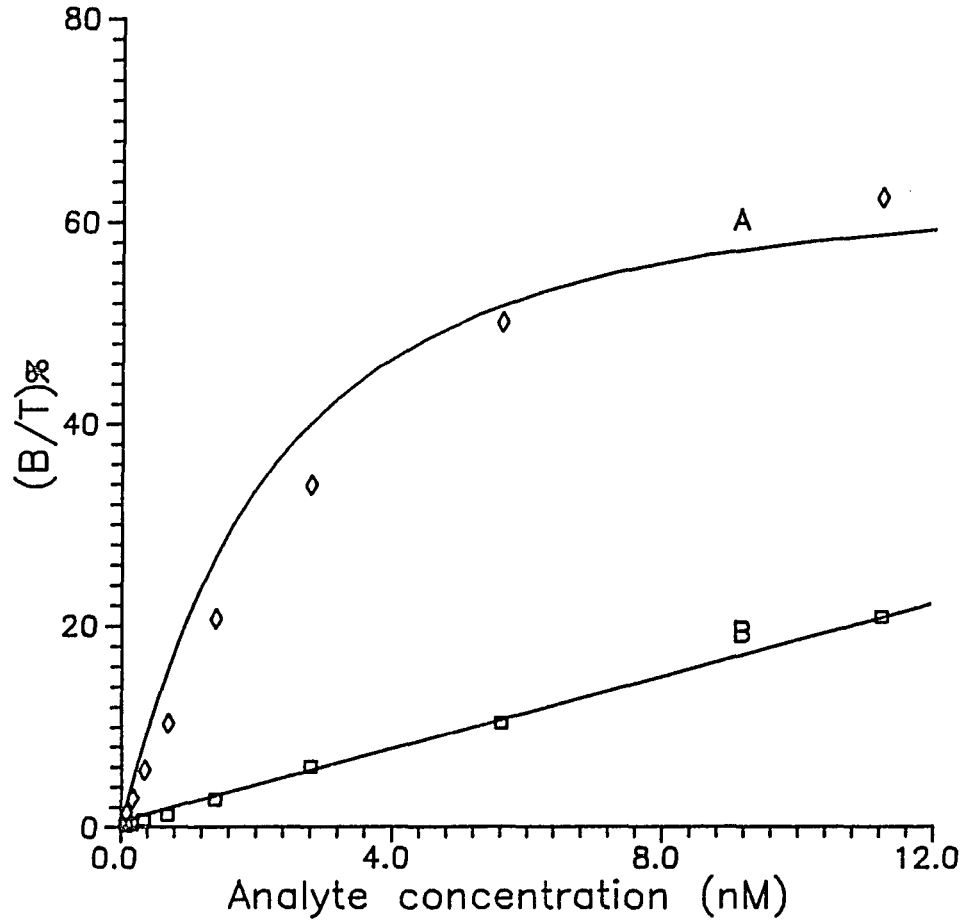

Figure 4.6 Two-step sandwich immunoassay for hGH and D-hGH in **low** analyte concentration range. Analyte: hGH (□) and D-hGH (◊). Solid-phase antibody is GHC 072 (◊, □). ^{125}I-GHC 101 is the labeled antibody for both analytes. ^{125}I-GHC 101 concentration: 1.22 nM (□), 0.81 nM (◊). Analyte concentration range is 0.08 - 12 nM. Incubation time for the first and second-steps of the assay are 4 and 3 hours respectively.

Theoretical Curves A and B correspond to the experimental data for D-hGH (◊) and hGH (□) respectively. Simulated dose-response curves: Curve A -- Model 1: $K_1 = 15\,\mu M^{-1}$; $K_2 = 0.48\ nM^{-1}$; $q_1 = 80$ nM; $q_2^* = 0.81$ nM. Curve B -- Model 4: $K_1 = 0.03\ nM^{-1}$; $K_2 = 1\ nM^{-1}$; $q_1 = 2$ nM; $q_2^* = 1.2$ nM.

for each figure are marked B. The liquid-phase interactions of individual antibodies with hGH permit only 1:1 or 1:2 complexes (Chapter 2), suggesting that Model 4 could explain this binding behavior. The experimental values for the affinities of GHC 072 (K_1) and GHC 101 (K_2) are 1.1 nM^{-1} and 3.8 nM^{-1} respectively (Sportsman et al., 1989). These affinities have been determined while the antibody is in solution. The theoretical values of K_1 and K_2 are 36 fold and 4 fold lower than the experimental values. The affinity of the solid-phase antibody used to fit the theoretical curve is considerably different from the experimental value. The low affinity of the solid-phase antibody may be attributed to chemical immobilization of the antibody.

4.3.10 Assay for D-hGH -- GHC 101/D-hGH/GHC 101 system

D-hGH is the non-covalent dimer of hGH which has been characterized for its chemical, physical and biological properties (Becker et al., 1987). It offers the obvious advantage of a model molecule having two known repeating epitopes. The antibody chosen for the solid- or liquid-phase should contribute some additional selectivity on the binding response in the sandwich immunoassay. The repeating epitopes of D-hGH allow a single antibody (either GHC 101 or 072) to form a sandwich complex. The sandwich immunoassay designed for D-hGH used GHC 101 as the solid- and liquid-phase antibodies at different concentration ranges. As D-hGH has only two epitopes to interact with GHC 101 the dose-response curve should be similar to hGH. The data are shown in Figure 4.7 and 4.8 for moderate and high D-hGH concentration ranges. The Curve C represents the simulated binding data for the each figure. Simulated Curves were generated using Model 3. The response for D-hGH at low concentration is weaker than that for hGH. The binding curves are similar to the data obtained for hGH in moderate (Figure 4.7) and high (Figure 4.8) analyte concentrations. Data for D-hGH correlate with the

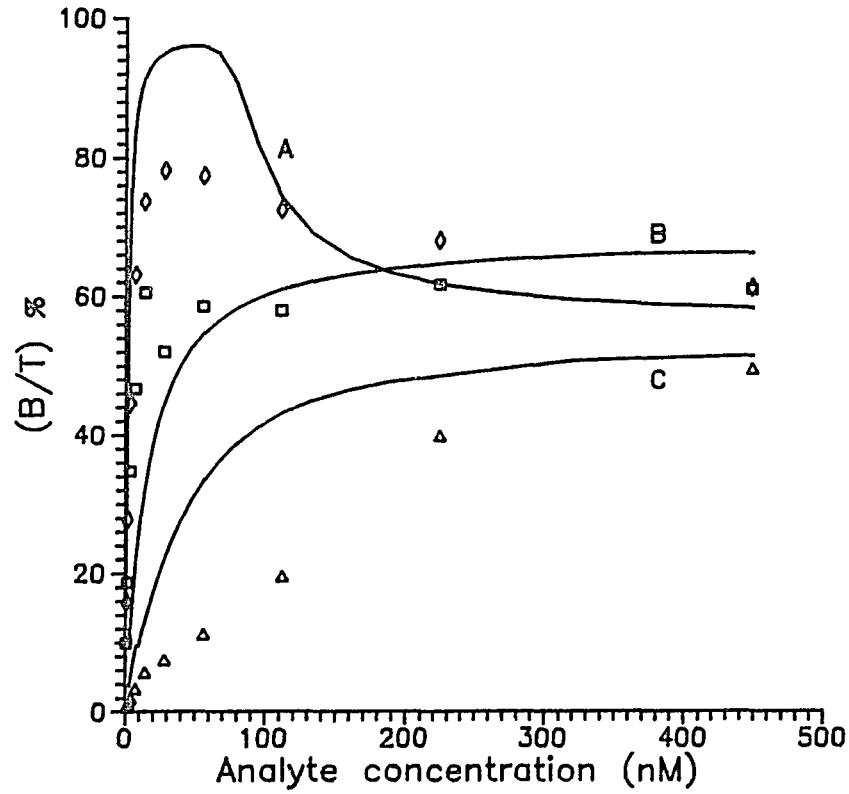

Figure 4.7 Two-step sandwich immunoassay for hGH and D-hGH in **moderate** analyte concentration range. Analyte: hGH (□) and D-hGH (◊, △). Solid-phase antibodies: GHC 101 (△) and GHC 072 (◊,□). ^{125}I-GHC 101 is the labeled antibody for both analytes. ^{125}I-GHC 101 concentration: 1.22 nM (□), 0.81(◊, △). Analyte concentration range is 3.0 - 0.45 μM. Incubation time for the first and second steps of the assay are 4 and 3 hours respectively.

Theoretical Curves A and C correspond to the experimental data for D-hGH (◊) and D-hGH (△) systems respectively. Curve B corresponds the experimental data for hGH (□). Simulated dose-response curves: Curve A -- Model 1: K_1 = 0.9 nM^{-1}; K_2 = 0.48 nM^{-1}; q_1 = 80 nM; q_2^* = 1.7 nM. Curve B -- Model 4: K_1 = 30 μM^{-1}; K_2 = 1 nM^{-1}; q_1 = 3 nM; q_2^* = 1.2 nM. Curve C -- Model 3: K_1 = 38 μM^{-1}; K_2 = 19 μM^{-1}; q_1 = 60 nM; q_2^* = 1.7 nM.

hypothetical curve in Figure 4.1. Model 3 does not fit to the experimental data in the moderate analyte concentration range which may be attributed to the difference in affinity of the solid- and liquid-phase antibodies (Figure 4.7). The affinity of GHC 101 for D-hGH is 19 μM^{-1} and 30 μM^{-1} to generate theoretical Curve C in Figures 4.7 and 4.8 respectively. The value of q_1 used generate Curve C in both figures are not experimentally known and are 60 nM (Figure 4.7) and 40 nM (Figure 4.8) respectively. This experiment suggest that the GHC 101 can interact with both epitopes of D-hGH to form the sandwich complex, however, the assay sensitivity is not practically useful.

4.3.11 Assay for D-hGH -- GHC 072/D-hGH/GHC 101 system

Alternatively a similar two-site sandwich immunoassay can be developed by selecting two antibodies for different epitopes of D-hGH. Theoretically, results should be similar to the case involving a single antibody, discussed previously. This sandwich immunoassay is analogous to the assay developed for hGH (Figure 4.6-4.8). The binding reactions can be explained using Model 1, in which two epitopes of D-hGH are accessible for the interaction with the liquid-phase antibody. The two-step sandwich immunoassay was constructed using GHC 072 and GHC 101 as the solid- and liquid-phase antibody, respectively. At very small concentrations of D-hGH (below 12 nM), the response is increased with the rise in concentration of D-hGH as shown in Figure 4.6. Curve A represents the theoretical binding curve which gives a good fit for the experimental data. The affinity of solid-phase antibody, GHC 072 was about 74 fold less for the theoretical Curve A. The affinity of the liquid-phase antibody, GHC 101 is about 8 fold less than the experimental value. Theoretical value of the solid-phase antibody concentration is 80 nM (experimental value is not known). The concentration of the labeled antibody, q_2^*, is same as the theoretical value. The response obtained at higher D-hGH

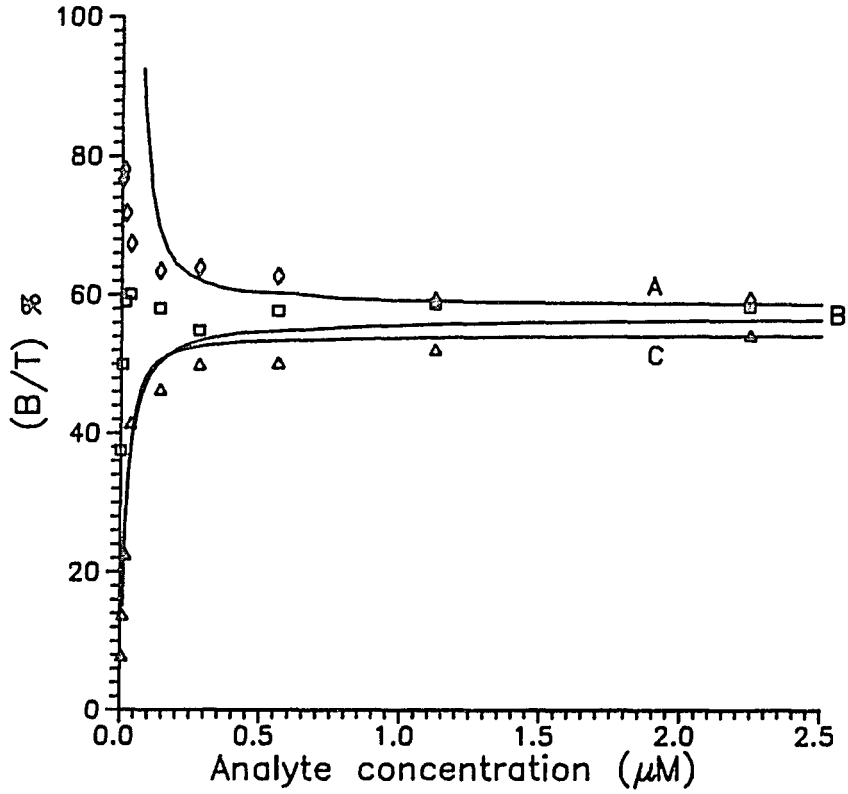

Figure 4.8 Two-step sandwich immunoassay for hGH and D-hGH in **high** analyte concentration range. Analyte: hGH (□) and D-hGH (◊, △). Solid-phase antibodies: GHC 101 (△) and GHC 072 (◊,□). ^{125}I-GHC 101 is the labeled antibody for both analytes. ^{125}I-GHC 101 concentration is 1.22 nM (□) and 0.81 (◊, △).Analyte concentration range is 4.0 nM - 2.2 μM. The incubation times for the first and second steps of the assay are 4 and 3 hours.

Theoretical Curves A and C correspond the experimental data for D-hGH (◊) and D-hGH (△) systems respectively. Theoretical Curve B corresponds the experimental data for hGH (□). Simulated dose-response curves: Curve A -- Model 1: K_1 = 0.9 nM^{-1}; K_2 = 0.48 nM^{-1}; q_1 = 80 nM; q_2^* = 2.0 nM. Curve B -- Model 4: K_1 = 30 μM^{-1}; K_2 = 1 nM^{-1}; q_1 = 2 nM; q_2^* = 1.2 nM. Curve C -- Model 3: K_1 = 60 μM^{-1}; K_2 = 30 μM^{-1}; q_1 = 40 nM; q_2^* = 0.81 nM.

concentrations declined, however, resulting in a "hook" (Figure 4.7) which further decreases and maintains a constant response at infinite D-hGH concentrations (Figure 4.8). Note that Curve A in Figure 4.7 also shows a sharp "hook" in the theoretical dose-response curve. However, the binding parameters, K_1 and q_2^* used to generate Curve A in Figure 4.6 have to be changed in order to fit the experimental data in Figures 4.7 and 4.8 respectively. The values of K_1 and q_2^* are approximately 60 fold and 2 fold higher than the values used to generate Curve A in Figure 4.6. The combination of Figures 4.6 - 4.8 should result in the hypothetical "hooked" curve from Figure 4.2 (Curve B). To generate the theoretical curves in Figures 4.7 and 4.8, binding parameters have been changed considerably comparing with the Curve A in Figure 4.6, suggesting that the descending limb of the "hook" is not directly explained by assuming that D-hGH just comes off from the solid-phase and form soluble liquid-phase complexes at high analyte concentrations.

Comparing the data for hGH with D-hGH allows the following conclusions to be drawn. For hGH, with no repeating epitopes, the "hook" effect can be avoided by using two different antibodies. For D-hGH, using a single antibody avoids the "hook" effect, but two different antibodies allows the "hook" at high concentrations of the analyte. If the analyte is capable of forming multiple adducts with several liquid-phase antibodies at high analyte concentrations, a plateau will not result in the dose-response curve (Figure 4.7). Moreover, at high analyte concentrations, more analyte will react with the solid-phase antibody at the first step of the sandwich immunoassay so that the labeled antibody might react in a random fashion in the second step, resulting in multiple interactions with some analytes. Multiple interactions of the solid-phase analyte and the liquid-phase antibody, may result in conformational changes of the analyte due to steric effects.

Thus, it could preferentially lead to weakening of the interaction of the analyte with the solid-phase antibody. Therefore, the bound antigen may easily be released from the solid-phase, resulting in a decrease in assay response. This effect may not be prominent if only a small amount of analyte is bound to the solid-phase.

4.3.12 Assay for ferritin -- Chemically immobilized antibodies: FEF021/hs-ferritin/FEF021 and QCI054/hs-ferritin/FEF021 systems

According to the above scheme, ferritin should also exhibit the "hooked" response due to multiple interactions with the liquid-phase antibody in the two-step sandwich immunoassay. In order to rule out the possibility that the capture antibody might desorb during reaction, beads with anti-ferritin antibody covalently attached were employed. To design a sandwich immunoassay in which the solid-phase antibody is covalently attached to the plastic beads (c-FEF021), as described in Model 2, an individual monoclonal antibody against ferritin was chosen for both the solid- and liquid-phase antibody. A second assay was designed by replacing c-FEF021 with c-QCI054 (covalently attached QCI054 to the plastic beads) which is comparable to Model 1. The labeled antibody for both assays was FEF021. Both assays were performed at three different concentration ranges and the resulting data are shown in Figures 4.9 - 4.11. Simulated dose-response curves from Model 1 and Model 2 are also shown in each figure. The affinity constant of FEF021 is 56 nM^{-1} and QCI054 has an affinity in the same range. To understand the effect of the amount of the labeled antibody, these assays were performed at different concentrations of the liquid-phase antibody. According to Figure 4.9, as the hs-ferritin concentration increases, the corresponding responses progressively increase and the maximum responses are achieved for each system. Curve A

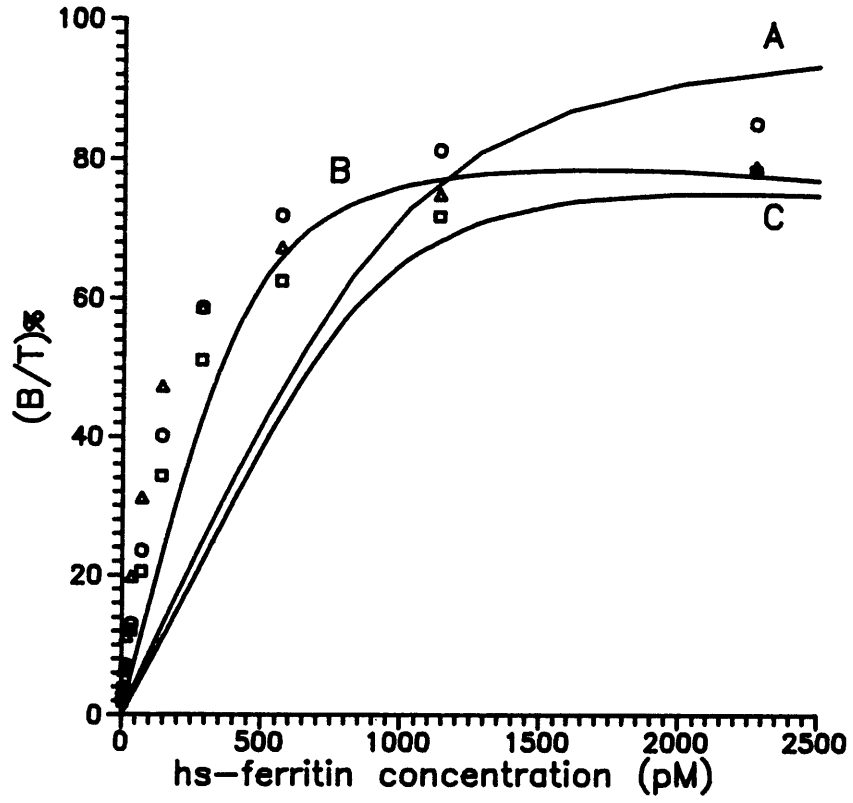

Figure 4.9 Two-step sandwich immunoassay for hs-ferritin in **low** analyte concentration range. Antibodies QCI054 (△, □) and FEF021 (○) are covalently attached to plastic beads. ^{125}I-FEF021 is the labeled antibody for all experiments and the concentrations are 0.75 nM (□), 0.375 nM (△) and 1.0 nM (○). hs-ferritin concentration range is 4.0 pM - 2.5 nM. Incubation time for first and second steps are 4 and 6 hours respectively.

Theoretical Curves A, B and C correspond to the experimental data for c-FEF021 (○) and c-QCI054 (△,□) systems respectively. Simulated dose-response curves: Curve A -- Model 2: K_1 = 20 nM^{-1}; q_1 = 25 nM; q_2^* = 1 nM. Curve B -- Model 1: K_1 = 1 nM^{-1}; K_2 = 10 nM^{-1}; q_1 = 4 nM; q_2^* = 0.375 nM. Curve C: Binding parameters are same as for Curve B except, q_2^* = 0.75 nM.

represents the theoretical curve for c-FEF021 system. The theoretical value of the affinity for FEF021 only differ about 6 fold from the experimental value. Note the close correlation between the experimentally obtained data and the simulated dose-response curves. All the binding parameters are described in the relevant legends. If this assay behaves ideally, since the curves in Figure 4.9 achieve the maximum response, the plateau should not be affected by increasing the analyte concentration, because the labeled antibody concentration is limiting and expected to follow the behavior of Curve A in Figure 4.2. However, the experimental data show a gradual decrease in response with increasing hs-ferritin concentration for the c-QCI054 system (Curves B and C, Figure 4.10). The c-FEF021 system does not show this effect in this analyte concentration range. Both systems demonstrate a "hook" in the assay. Note the high dose plateau for c-FEF021 and c-QCI054 systems (Figure 4.11).

These data are generally in agreement with the theory with the individual curves as shown in Figure 4.11. There is, however, no correlation between the theoretical and experimental data for assays developed at different labeled antibody concentrations. This finding is contradictory to theoretical predictions suggested by Ryall et al. (1982) using Model 2. Model 2 predicted the disappearance of the "hook" as the amount of the labeled antibody is increased. The computer simulated curves are shown in Figure 4.5. In contrast to the theoretical results in Figure 4.5 at much smaller concentrations of labeled antibody, assays for c-QCI054 and c-FEF021 systems do not show the decline in assay response at higher analyte concentrations. Note that the sharpness of the "hook" is diminished for the assay using c-FEF021 when the labeled antibody concentration is decreased from 1.0 nM to 0.25 nM (4 fold decrease in labeled antibody concentration). Again note the "hooked" dose-response data for c-

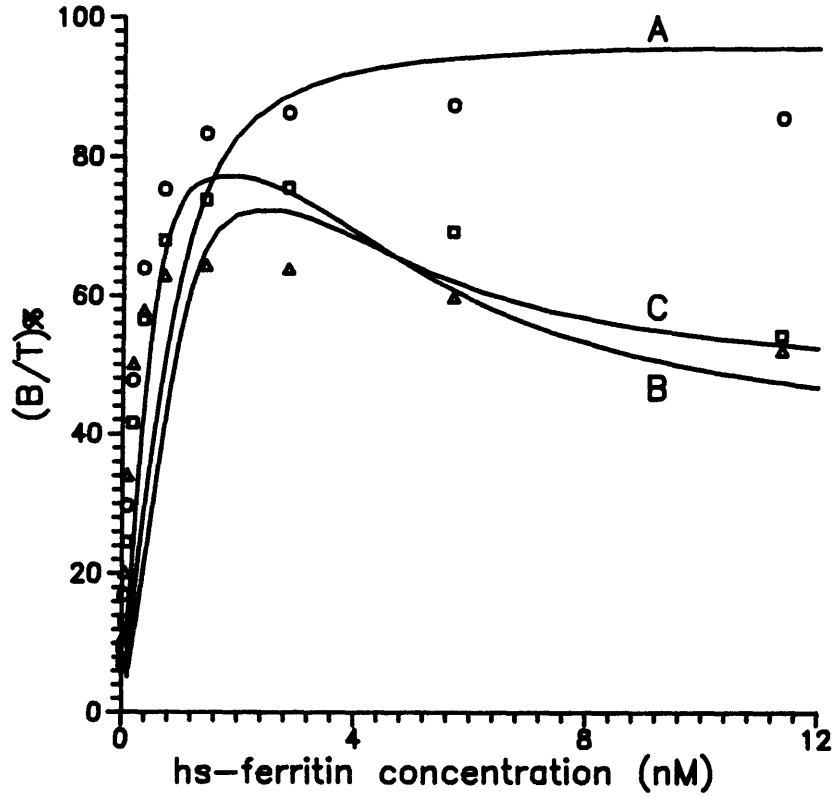

Figure 4.10 Two-step sandwich immunoassay for hs-ferritin in **moderate** analyte concentration range. QCI054 (△,□) and FEF021 (○) antibodies are covalently attached to plastic beads. ^{125}I-FEF021 is the labeled antibody and the concentrations are 1.0 nM (□, ○), 0.50 nM (△). hs-ferritin concentration range is 0.50 - 12 nM.

Theoretical Curves A, B and C correspond to the experimental data for c-FEF021 (○) and c-QCI054 (△,□) systems respectively. Simulated dose-response curves: Curve A -- Model 2: K_1 = 10 nM^{-1}; q_1 = 20 nM; q_2^* = 1 nM. Curve B -- Model 1: K_1 = 1 nM^{-1}; K_2 = 10 nM^{-1}; q_1 = 4 nM; q_2^* = 0.5 nM. Curve C: Binding parameters are same as for Curve B except, q_2^* = 1.0 nM.

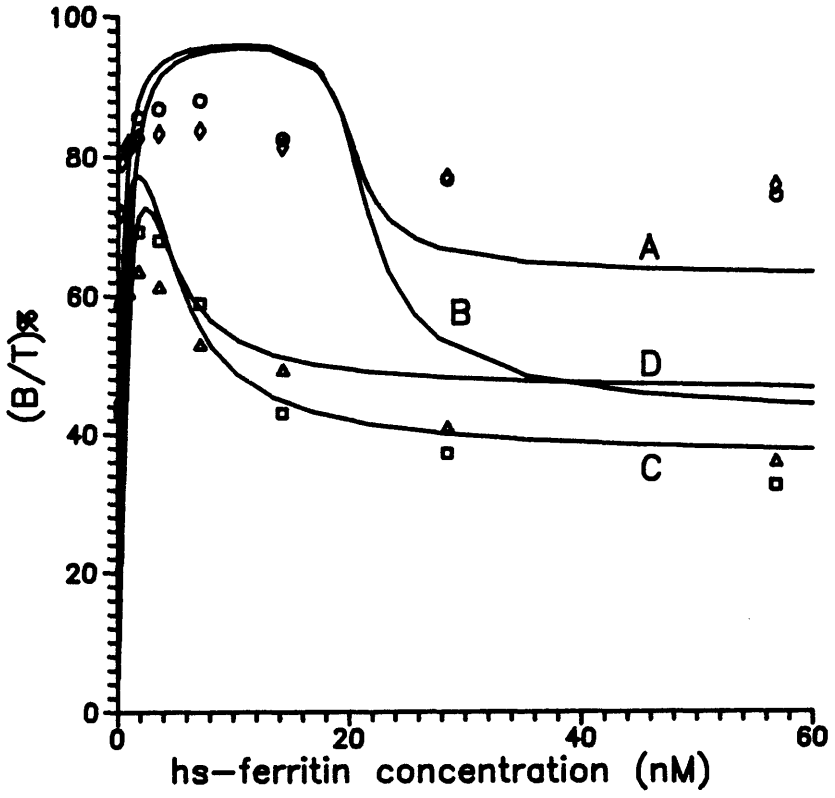

Figure 4.11 Two-step sandwich immunoassay for hs-ferritin in **high** analyte concentration range. Solid-phase antibodies QCI054 (△,□) and FEF021 (○,◊) are covalently attached to plastic beads. ^{125}I-FEF021 is the labeled antibody and concentrations are 1.0 nM (□,○), 0.50 nM (△) and 0.25 nM (◊). hs-ferritin concentration range is 0.9 nM - 0.06 μM.

Theoretical Curves A and B correspond to the experimental data for c-FEF021 (○) and c-FEF021 (◊) systems respectively. Theoretical Curves C and D correspond to the experimental data for c-QCI054 (△) and c-QCI054 (□) systems respectively. Simulated dose-response curves: Curve A -- Model 2: K_1 = 10 nM^{-1}; q_1 = 20 nM; q_2^* = 1 nM. Curve B: Binding parameters are same as Curve A except, q_2^* = 0.25 nM. Curve C -- Model 1: K_1 = 1 nM^{-1}; K_2 = 10 nM^{-1}; q_1 = 4 nM; q_2^* = 0.5 nM. Curve D:. Binding parameters are the same except, q_2^* = 1.0 nM.

QCI054 system at 1.0 nM and 0.5 nM labeled antibody concentration respectively. When the procedure was run with labeled antibody concentrations at 0.375 nM and 0.19 nM (an approximately 3 to 5 fold decrease in labeled antibody concentration from the highest concentration, 1.0 nM), the sharpness of the "hook" declined resulting essentially in a plateau (cf. Figure 4.11 and 4.12). But according to theoretical curves the "hook" becomes sharper as the amount of the labeled antibody becomes insufficient (Figure 4.5). Combinations of the theoretical Curves C, D (Figure 4.11) and Curves A, B (Figure 4.12) show the "hook" effect at different concentrations of labeled antibody. It is clear from the experimental data that the labeled antibody is inadequate. The concentration of the labeled antibody, however, is also critical in this assay. The "hook" appears at higher analyte concentrations as predicted previously. The "hook" disappears for assay systems consisting of insufficient labeled antibody concentrations in contrast to what is expected.

To test for optimization procedures, incubation time was increased in the second step of the assay in an experiment parallel to the design in Figure 4.11. The longer incubation did improve the assay response, but the "hook" reappeared as shown in Figure 4.13.

4.3.13 Non-specific interactions -- chemically immobilized antibodies: FEF021/hs-ferritin/FEF021 and QCI054/hl,hs-ferritin/FEF021 systems

Perera and Worwood (1984) have claimed that avoiding non-specific interactions of the analyte should overcome the "hook" effect. According to their report, the non-specific binding of ferritin has been demonstrated using physically adsorbed human serum albumin as the solid-phase. However, the data from the following experiment show a similar "hook" effect at minimum non-specific binding of ferritin (Figure 4.14). In this experiment, hs-ferritin was assayed using the c-

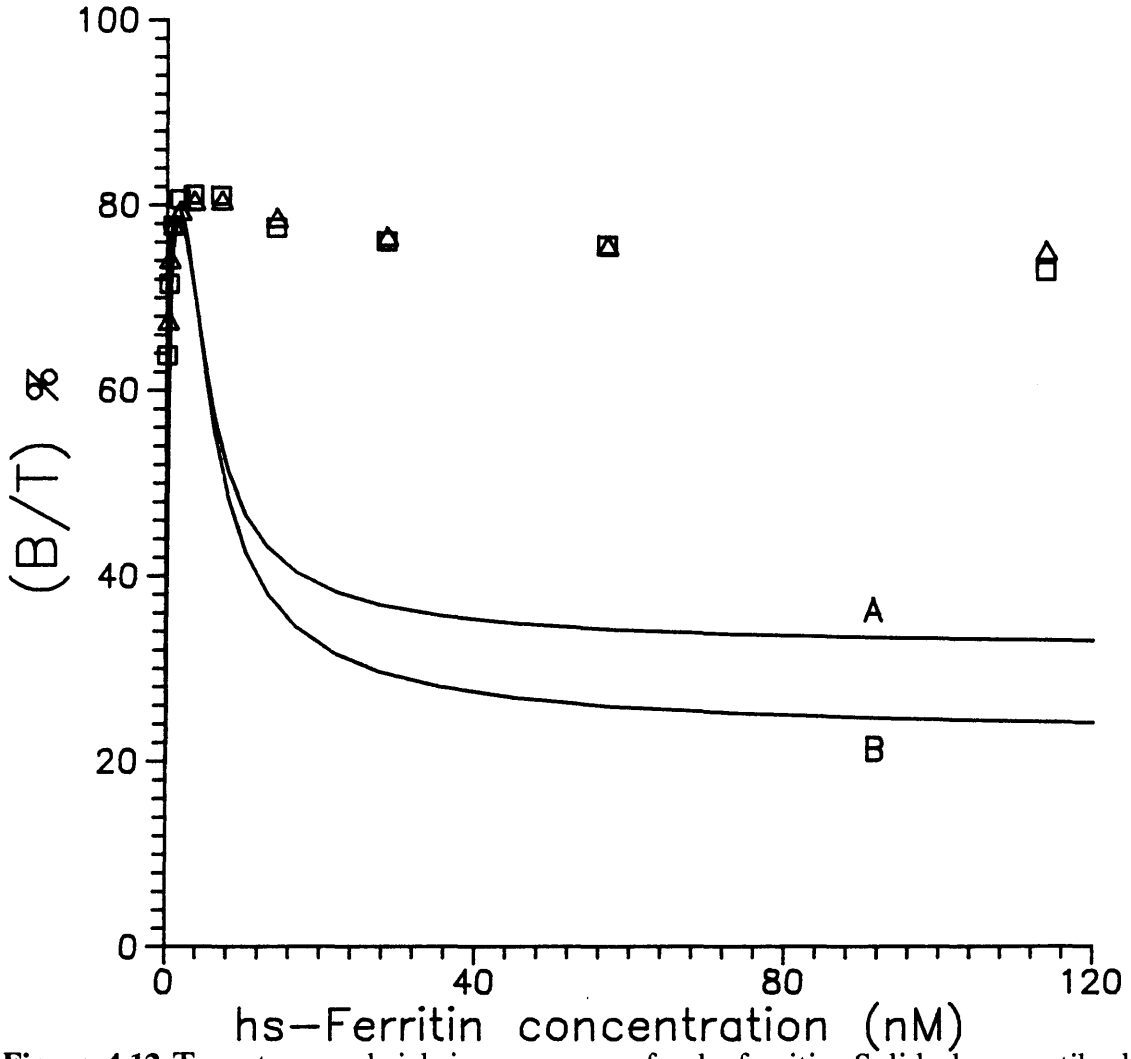

Figure 4.12 Two-step sandwich immunoassay for hs-ferritin. Solid-phase antibody, QCI054 is covalently attached to plastic beads. ^{125}I-FEF021 is the labeled antibody and the concentrations are 0.375 nM (□) and 0.19 nM (△) respectively. Theoretical Curves A and B correspond to the experimental data for c-QCI054 (□) and c-QCI054 (△) systems respectively. Simulated dose-response curves: Curve A -- Model 1: K_1 = 1 nM^{-1}; K_2 = 10 nM^{-1}; q_1 = 4 nM; q_2^* = 0.38 nM. Binding parameters for Curve B are the same as Curve A except, q_2^* = 0.2 nM.

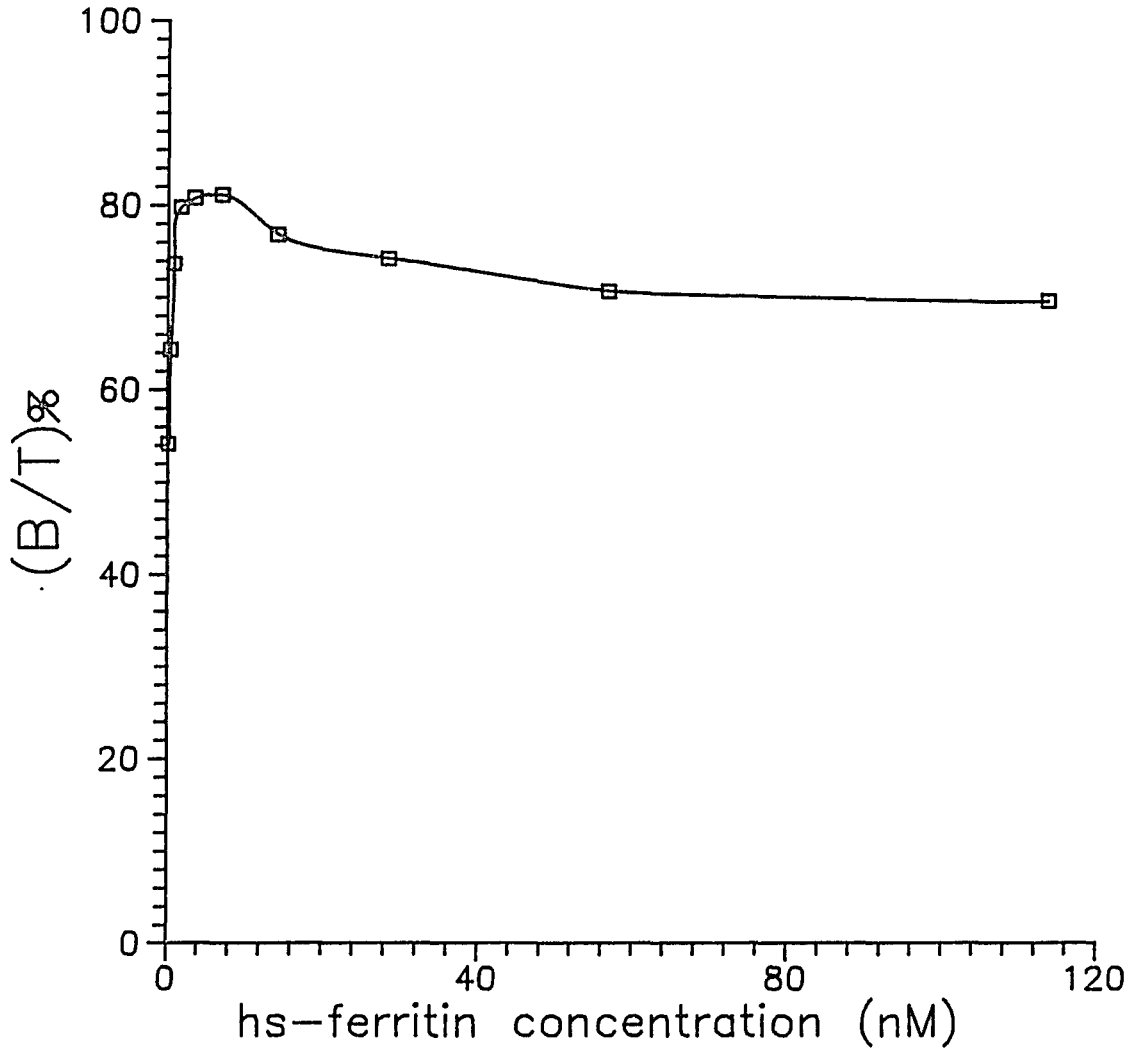

Figure 4.13 Two-step sandwich immunoassay for hs-ferritin. Solid-phase antibody, QCI054 is covalently attached to plastic beads. ^{125}I-FEF021 is the labeled antibody and concentration is 0.75 nM. The incubation time for first and second steps of the assay are 2 and 8 hours respectively.

QCI054 and c-FEF021 systems. These data suggest that the "hook" effect is not directly affected by non-specific interactions of the analyte. Moreover, the curve for hl-ferritin also shows a similar "hook" suggesting that both analytes have similar immunological properties.

Non-specific interactions (Perera and Worwood, 1984) cannot be eliminated at high analyte concentrations. In this assay two types of non-specific interaction have to be considered. These are the non-specific interactions of the analyte with the immunosorbent and also that of the labeled antibody. The contribution of the labeled antibody to the non-specific binding is less than the effect from the analyte. If the non-specific interactions of the analytes are not minimized, both the non-specifically and biospecifically bound analytes will contribute to the "hook" effect. Figure 4.14 compares the non-specific interactions of hs-ferritin using different solid-phase antibodies (covalently attached GHC 101 and GHC 072 plastic beads). The labeled antibody was FEF021 and the analyte was in PBS containing 0.5% BSA, 10% BSA and 10% horse serum. The contribution from the non-specific interaction of labeled antibody ("blank") has been subtracted in all calculations. These data show that the non-specific interactions are less than 4% in horse serum. However the "hook" effect constitutes an approximate 15% decrease in assay response (response difference between the maximum response and the response at 120 nM ferritin concentration), irrespective of the type of ferritin used under conditions of minimum non-specific interactions (Figure 4.14).

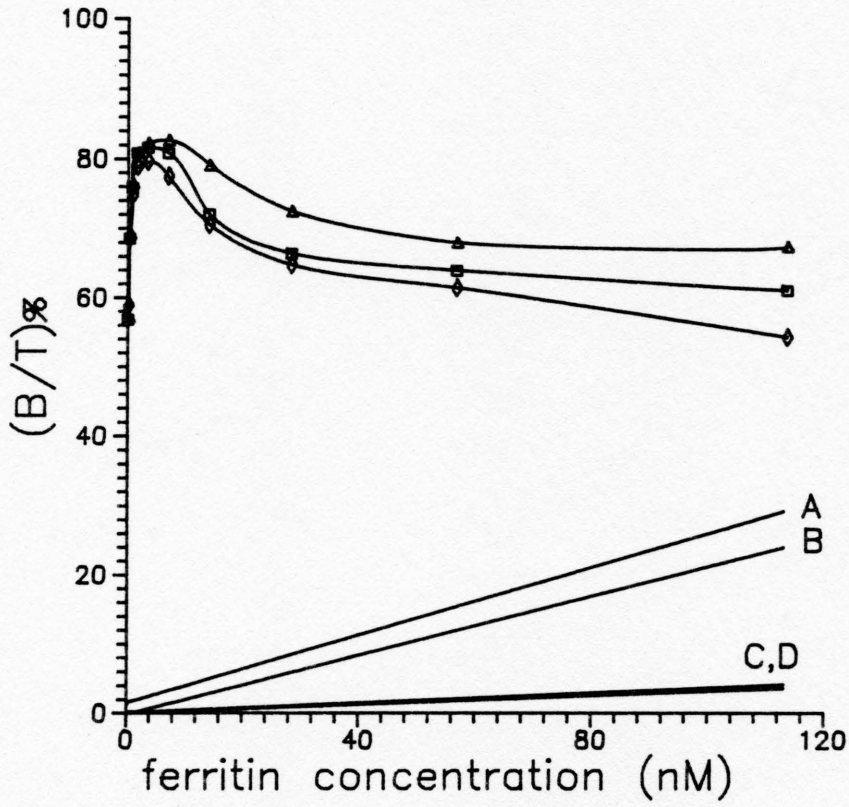

Figure 4.14 Two-site sandwich immunoassay for hs-ferritin and hl-ferritin and comparison with the non-specific interactions. Analyte: hs-ferritin (□, △) and hl-ferritin (◊). Both analytes were prepared in 20 mM PBS, 10% horse serum. Solid-phase antibodies are covalently attached to the solid-phase, QCI054 (□, ◊) and FEF021 (△). ^{125}I-FEF021 concentration is 1.0 nM. Non-specific binding of hs-ferritin: 20 mM PBS, 0.5% BSA: Curve A (solid-phase antibody, GHC 101), Curve B (solid-phase antibody, GHC 072); 20 mM PBS, 10% BSA: Curve C (solid-phase antibody, GHC 101); 20 mM PBS, 10% horse serum: Curve D (solid-phase antibody, GHC 101).

4.3.14 Assay for ferritin -- Physically adsorbed antibodies: FEF021/hs-ferritin/FEF021 and QCI054/hs-ferritin/FEF021 systems

The assay response could also be controlled by adjusting the amount of antibody in the solid-phase and in the liquid-phase. Plastic tubes permit small amount of solid-phase antibody because of the less surface area (approximately 160 - 320 ng/cm^2 of protein) (Cantarero, et al., 1980). The decreased amount of solid-phase antibody does suppress the "hook" effect for the ferritin system. However, It is not possible to compare the capacities of covalently attached and physically adsorbed solid-phases as there may be a apparent difference in affinities in these two systems. The following experiments illustrate the effect of the combination of amount of capture antibody and it's affinity on the dose response curves. Two physically adsorbed systems, p-QCI054 and p-FEF021 were studied at different concentrations of capture (using different antibody coating concentrations) and labeled antibody. The dose-response curve for p-QCI054 system is shown in Figure 4.15. The response is increased with increasing hs-ferritin concentration. After the maximum response is produced, the assay response is decreased approximately by 6% and demonstrates a constant upper limit at high dose (Figure 4.15). The theoretical dose-response Curve A was generated using Model 1. Theoretical results are in agreement with the experimental data. Note that the experimental design for p-QCI054 in Figure 4.15 is analogous to the c-QCI054 from Figure 4.10, the only difference is the solid-phase. However, it is apparent that the capacity of the capture antibody for p-QCI054 system much less than that of c-QCI054 system. Note that the estimated maximum responses for p-QCI054 and c-QCI054 systems are almost the same. The appearance of the small "hook" suggests that the affinity of physically adsorbed QCI054 must be higher than the covalently attached QCI054. The assay for p-

QCI054 was performed at higher analyte concentration (up to 120 nM, compared with 12 nM hs-Ferritin concentration in Figure 4.15) to further investigate on the dose-response curve. The labeled antibody concentration is increased by 2 fold. The experimental data demonstrate a constant upper response indicating that maximum response has been achieved. The experimental data is correlated with the theoretical results (Figure 4.16). In comparison, hs-ferritin was assayed using p-FEF021 solid-phase antibody. The dose-response curves have also been compared at two different concentrations of labeled antibody (0.1 and 0.3 nM). These data are shown in Figure 4.15. The experimental data for the c-FEF021 system (Figure 4.10) are also analogous to these data except for the method of immobilization. The p-FEF021 system demonstrates a significantly lower response than the p-QCI054 system. Dose-response data at both concentrations of labeled antibody appear to demonstrate a saturation curve similar to the hypothetical Curve A in Figure 4.2. Theoretical curves also show a similar response supporting the experimental data (Curves B and C, Figure 4.15). Moreover, a similar assay was extended by increasing hs-ferritin concentration up to 120 nM. The labeled antibody concentration was 0.75 nM. The experimental data were obtained using two coating concentrations of FEF021. These data are shown in Figure 4.16. Even under above conditions the p-FEF021 system shows no "hook", but increase in the capture antibody concentration resulted a comparable maximum response observed for p-QCI054 system. The dose-response curves demonstrate the hypothetical behavior as shown in Curve A at Figure 4.2. Data for p-QCI054 also demonstrate a constant response at infinite analyte concentration (Figure 4.16). Theoretical dose-response curves are also shown in Figure 4.16. The p-FEF021 system does not show the "hook" effect under these circumstances most probably

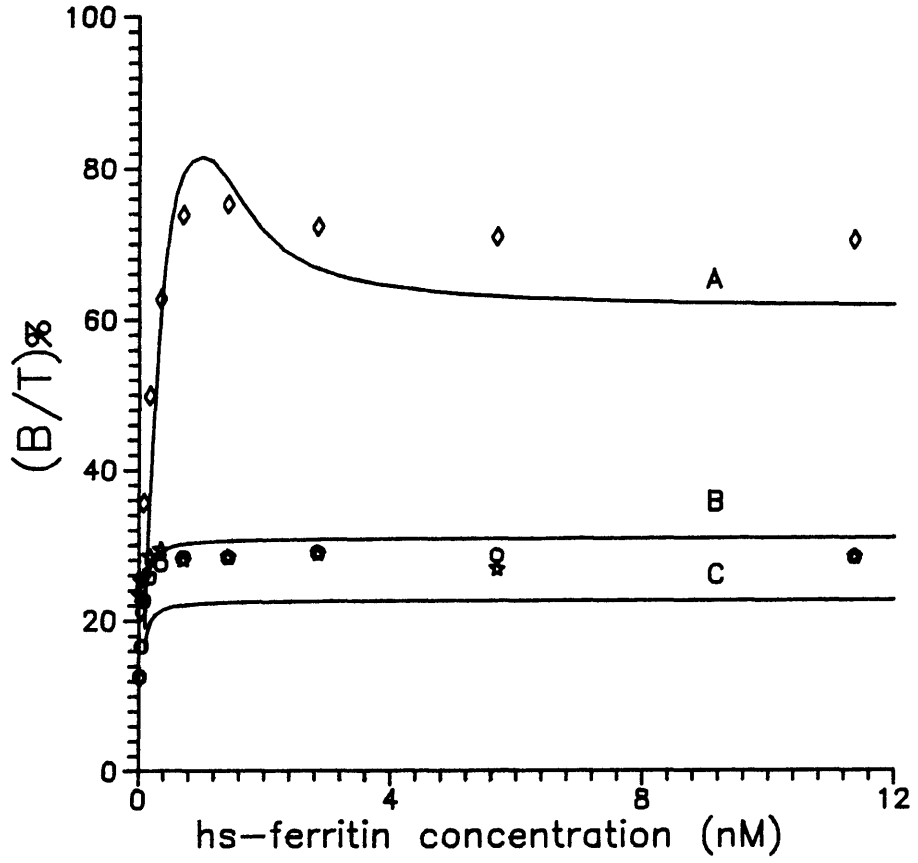

Figure 4.15 Two-step sandwich immunoassay for hs-ferritin in **moderate** analyte concentration -- physically adsorbed antibody. QCI054 (◊) and FEF021 (*, ○) antibodies are physically adsorbed to plastic tubes. ^{125}I-FEF021 is the labeled antibody and concentrations are 0.375 nM (◊), 0.3 (*), and 0.1 nM (○). All the experimental conditions are given in materials and methods. hs-Ferritin concentration range is 0.9 - 12 nM.

Theoretical Curves A correspond to the experimental data for p-QCI054 system (◊). Theoretical Curve B and C correspond to the experimental data for p-FEF021 system (*, ○). Simulated dose-response curves: Curve A -- Model 1: K_1 = 8 nM^{-1}; K_2 = 10 nM^{-1}; q_1 = 1.5 nM; q_2^* = 0.375 nM. Curve B -- Model 4: K_1 = 50 nM^{-1}; K_2 = 25 nM^{-1}; q_1 = 80 pM; q_2^* = 0.3 nM. Theoretical parameters for Curve C is same as Curve B except, q_2^* = 0.1.

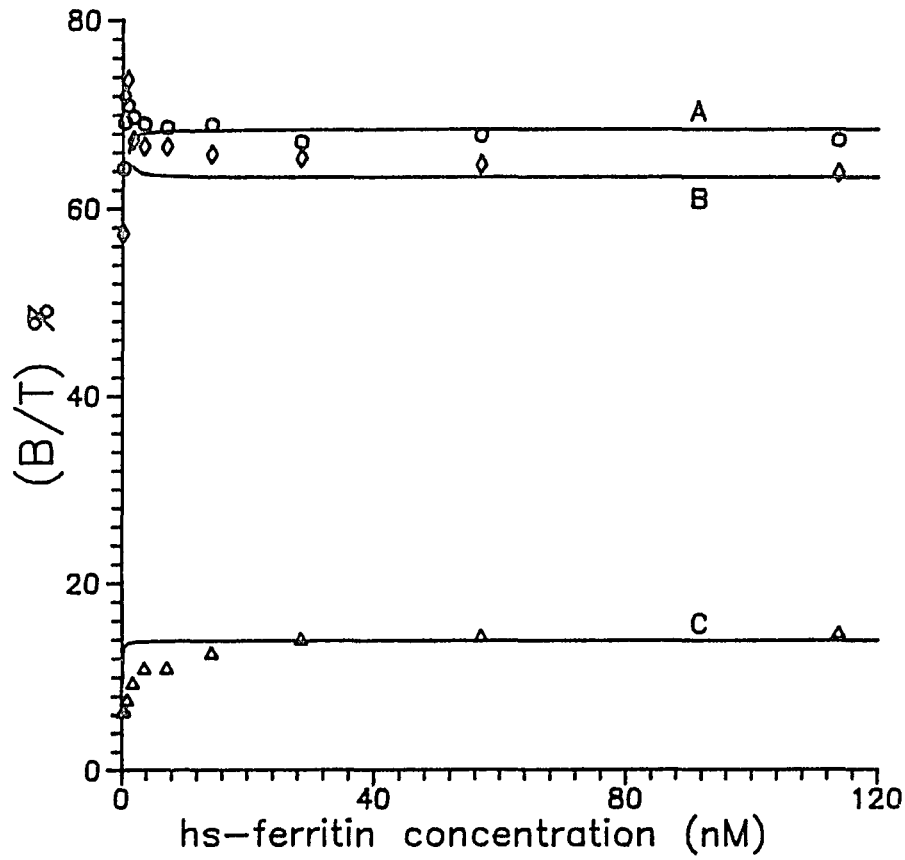

Figure 4.16 Two-site sandwich immunoassay for hs-ferritin in **high** concentration range of the analyte -- physically adsorbed antibody. All antibodies are physically adsorbed to plastic tubes: FEF021 (△, ○), QCI054 (◇). The concentrations of antibody coating solutions: 5 μg/mL (◇, △), 50 μg/mL (○). ^{125}I-FEF021 is the labeled antibody and the concentration is 0.75 nM.

Theoretical Curves A and C correspond to the experimental data for p-FEF021 (△) and p-FEF021 (○) systems respectively. Theoretical Curve B correspond the experimental data for p-QCI054 (◇). Theoretical parameters: Curve A -- Model 4: K_1 = 50 nM^{-1}; K_2 = 25 nM^{-1}; q_1 = 0.6 nM; q_2^* = 0.75 nM. Curve B -- Model 1: K_1 = 30 nM^{-1}; K_2 = 10 nM^{-1}; q_1 = 1.0 nM; q_2^* = 0.75 nM. Curve C -- Model 4: K_1 = 50 nM^{-1}; K_2 = 25 nM^{-1}; q_1 = 90 pM; q_2^* = 0.6 nM.

due to the low capacity of the solid-phase antibody. Experimental dose-response curves for p-FEF021 system might demonstrate a "hooked" response if the amount of solid- or liquid-phase is adjusted. However, the similar assay for c-FEF021 system demonstrated the "hook" effect (Figure 4.11). These experiments suggest that the amount of antibody in the solid- and liquid-phases have a close relationship with the "hook" effect. It is apparent from the above data that the "hooked" nature of the calibration curve could be compared for the capacity of the solid-phase if the amount of solid-phase antibody is controlled by using different size beads.

4.3.15 Kinetics of ferritin assay -- chemically immobilized antibodies: QCI054/hs-ferritin/FEF021 system

Kinetic studies further support that the "hook" effect in two-step sandwich immunoassay is attributed to multiple epitope interactions. Kinetic measurements were made for c-QCI054 and c-FEF021 systems at low and high analyte concentrations. Each step of the sandwich immunoassay for c-QCI054 system was studied. The amount of analyte bound in the first step of the two-step sandwich immunoassay depends on the concentrations of the solid-phase antibody and the analyte. Antibody immobilized on the beads has a fixed high capacity, and the two concentrations of hs-ferritin chosen for these kinetic studies were added in the first step of the immunoassay. If the capacity of the solid-phase antibody is high, the amount of bound antigen at the first step should be proportional to the hs-ferritin concentration. The overall response was monitored after the addition of the labeled antibody at the second step with incubation for a fixed time. Working with a hs-ferritin concentration of 4.44 nM (at the linear range of the calibration curves of Figure 4.9), longer incubation times at the first step proportionally increase analyte binding. The data for the low hs-ferritin concentration are shown in Curve

A (Figure 4.17). The reaction is 75% complete in 1 minute. The incubation time for the first step of the assay at 4.44 nM hs-ferritin was also extended for several hours and the data are shown in Curve A, Figure 4.18. According to these data, at low concentrations of the analyte, the maximum response is achieved from an increase in the incubation time.

The reaction between the solid-phase antibody and the analyte is rapid at high analyte concentrations. The data of Figure 4.17B,C show that the maximum response for c-QCI054 and c-FEF021 systems is achieved in less than one minute of the reaction time for the first step. As the incubation time for the first step is increased, the resulting bound radioactivity is decreased because the some of the bound ferritin molecules are desorbed from the solid-phase. Further decrease in assay response is shown at longer incubation times for the c-QCI054 system in Curve C, Figure 4.18. These data suggest that the amount of bound ferritin is raised by high analyte concentrations (cf: Curve A, B and C in Figure 4.17). The appearance of the "hook" in the ferritin assay is also supported by kinetic data. The labeled antibody is permitted to have random multiple interactions with ferritin. If the analyte interacts with labeled antibody at multiple epitopes, conformational changes of the analyte due to steric effects leach the analyte from the solid-phase. With longer incubation times, this migration of the analyte from the solid-phase could account for the response decrease shown by the experimental measurements.

Experimental data developed by varying the incubation times during the second step, with a fixed incubation time at the first step, further support this interpretation. The c-QCI054 system was studied with a two-hour incubation time at the first step, and variations up to 210 minutes during the second step. The data are shown in Curve B, Figure 4.18. These data suggest that if limiting amounts of

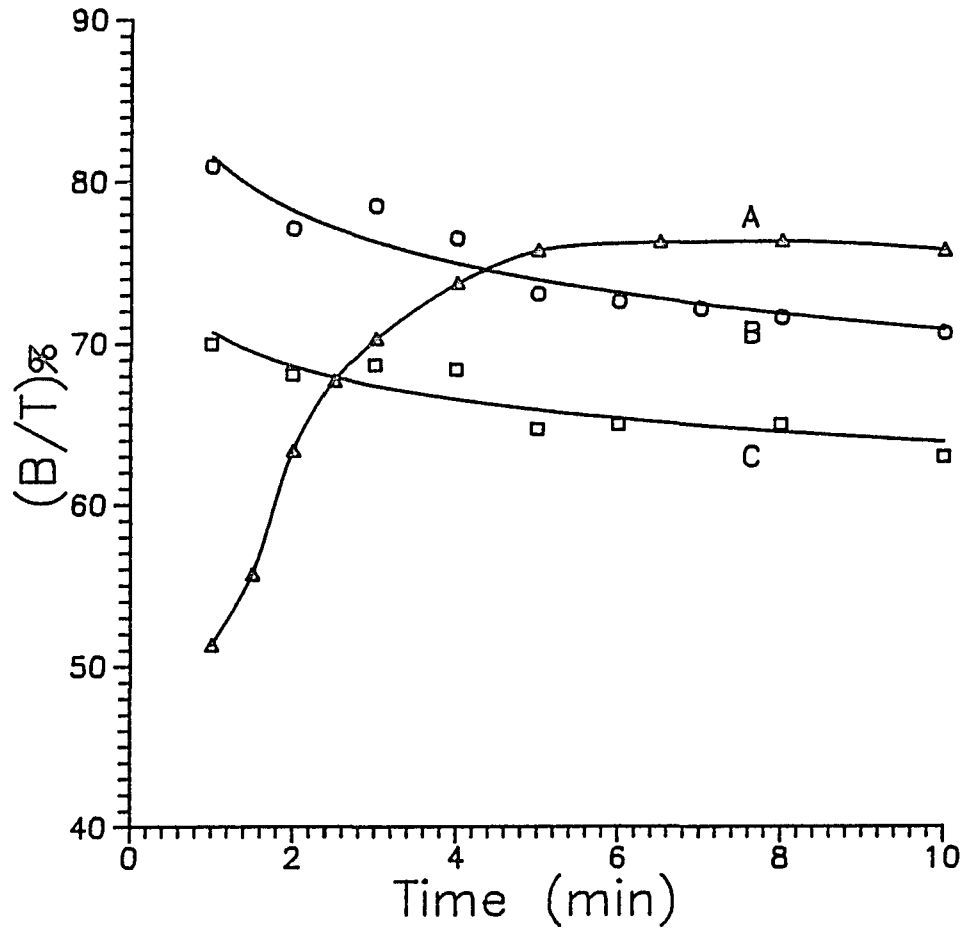

Figure 4.17 Effect of the response on time: kinetics of the first step of the two-step sandwich immunoassay for ferritin system. Solid-phase antibodies are c-QCI054 (□, △) and c-FEF021 (○) respectively. The concentrations of hs-ferritin are 4.44 nM (△ - Curve A), and 113 nM (○ - Curve B,□ - Curve C) respectively. ^{125}I-FEF021 concentrations are 0.75 nM (□, △) and 0.5 nM (○) respectively. Incubation time for the first step of the assay is 1 - 12 minutes. The incubation time for second assay step is 3 hours.

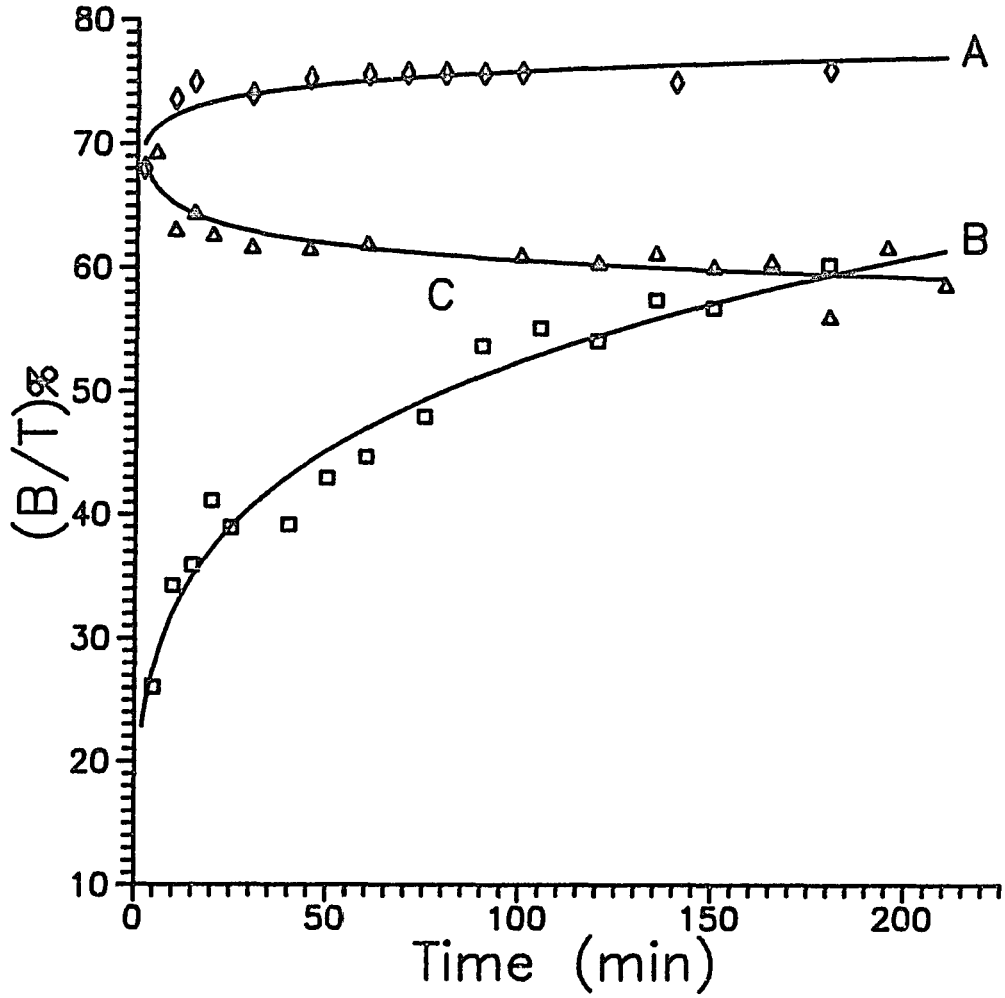

Figure 4.18 Effect of the response on time: comparison of the first and second steps of the two-step sandwich immunoassay for hs-ferritin. The solid-phase antibody is c-QCI054. The incubation time for the first step of the assay is 10 - 210 minutes. These results are shown in Curve A (◊) and Curve B (△) respectively. hs-ferritin concentrations are 113 nM (□) and 4.44 nM (□, ◊). ^{125}I-FEF021 concentration is 0.75 nM. Curve B (□) represents the effect of the response on second step of the two-site sandwich immunoassay for hs-ferritin. The incubation time for the first step of the assay is 2 hours. The incubation time for the second step of the assay is 5 - 210 minutes.

labeled antibody are employed longer incubation times are necessary to maximize the response. Theoretically Curve B, Figure 4.18 should exceed the response of Curve C, Figure 4.18 at the longest incubation times. The maximum response for Curve B, however, is at the minimum response for Curve C. This result is consistent with multiple antigen-antibody interactions causing release from the solid-phase and lowering the radioactivity measurements. This confirms that some ferritin molecules are leached from the solid-phase to the liquid-phase after the labeled antibody has reacted.

4.3.16 Conclusions

This study shows some of the fundamental analytical discrepancies of the monoclonal antibody based two-step sandwich immunoassay which result from the epitope characteristics of the analytes. The two-step sandwich immunoassay has an advantage over the one-step mode in that it is expected to show a "hook". However, our studies conclude that the monoclonal antibody based two-step sandwich immunoassay also demonstrates the "hook" effect if the analyte can undergo multiple epitope interactions with the labeled antibody in the second step of the assay. Due to these multiple interactions, distortion or conformational changes of the analyte may occur which could lead to desorption of the analyte from the solid-phase thus resulting in a "hook". Reaction is rapid at high analyte concentrations, and the increased multiple interactions increase the likelihood of a "hook". At low analyte concentrations, some analytes may also desorb from the solid-phase due to multiple epitope interactions. Extensive studies on analytes having different immunological properties suggest that the mode of performing the two-step sandwich immunoassay is critical for the "hook" effect. hGH cannot have multiple interactions, and establishes a normal dose-response plateau showing no "hook" at high analyte concentrations. Two approaches to designing the sandwich

immunoassay for D-hGH illustrate the conditions for obtaining the "hook" or plateau. If the assay for D-hGH is designed with a single antibody, no "hook" is observed as no multiple epitope interactions are permitted between the analyte and the liquid-phase antibody. If D-hGH is assayed with at least two epitopes accessible for binding with the liquid-phase antibody (two monoclonals) the "hook" effect is observed. Macromolecular analytes such as ferritin have many multiple epitopes and are capable of reacting with several antibody molecules while it is on the solid-phase. Apart from the analyte concentration, the concentrations of the capture and labeled antibodies can also effectively contribute to the "hook" effect. Higher concentrations of labeled antibody contribute more multiple epitope interactions to occur evidently the greater the extent of "hooking" of the dose-response curve. The effect of the concentration of labeled antibody provides the most direct argument for the incidence of multiple interactions causing the analyte to desorb from the solid-phase. A monoclonal based two-step sandwich immunoassay can effectively be designed for an analyte having two different epitopes if these epitopes are not repeated. For an analyte having multiple epitopes, either use of single antibody or different antibodies might not be possible to design the immunoassay without "hook" effect. Therefore, careful adjustment of capture and labeled antibody concentrations are needed to achieve a plateau and avoid the "hook" in the dose-response curve at higher analyte concentrations.

Chapter 5

Future directions

The conclusions of the preceding chapters indicate several possibilities for future research work. These include further understanding of fundamental analytical problems related to immunoassays. As noted in several instances, these assay systems have been primarily documented more than decade ago. However, no workable solutions for most of the analytical problems related to "hook" effect are provided in the literature. Therefore, further investigation of the characteristics of the analytes to understand problems related to immunoassays is utmost important. These studies would be helpful to design and implement new protocols for immunoassay systems.

All the studies thus for on "hook" effect are centered on analyte characteristics. For this purpose well characterized analytes have to be employed. Further investigations on epitope characterization of biosynthetic human growth hormone (hGH), dimeric form of hGH (D-hGH) and ferritin are feasible as the preliminary studies of these analytes have already been made.

5.1 Epitope mapping of hGH and D-hGH

Previously, a quite number of elegant reports have been published on epitope mapping related to hGH. However, these studies have not been integrated into immunoassay development. Our scope is to design immunoassays based on the characteristics of the analytes and recognize the analytical problems related to each assay at the fundamental level. A panel of monoclonal antibodies have to be employed for this purpose. The hGH molecule is feasible as the amino acid sequence is already known. Similarly antibody binding epitopes could be established for D-hGH.

5.2 Binding reactions of hGH and D-hGH

The purpose of this project is design and implement various protocols that enable us to predict the performance of any assay system. Solid- and liquid-phase competitive binding assays for individual antibodies and mixture of antibodies are established. Each appropriate pair of antibodies are essentially search for the accessibility of the formation of sandwich. Both assay protocols could lead to find evidences for conformational changes in binding interactions which leads to enhancement of inhibition.

5.3 Solid-phase/Liquid-phase/Cooperativity

The goal of this proposal is to elucidate the antigen-antibody interactions in solid- and liquid-phase to further investigate mechanism of conformational interactions. Solid- and liquid-phase binding reactions of hGH and D-hGH with individual antibodies and antibody mixtures are investigated. This study should give a clear picture of the antigenic topography of hGH and D-hGH. For example, some of the monoclonal antibodies could inhibit reacting with the D-hGH in liquid-phase as the dimer is formed by the non-covalent interaction of two identical hGH molecules (tentative region of interaction, amino acids 120 and 160). The liquid-phase reactions can be performed using the titration or the saturation procedures developed in Chapter 2. The solid-phase experiments are established with a fixed concentration of antibody or antibody mixtures. The concentration of antibody or antibody mixtures could not able to control in solid-phase experiments.

5.4 Competitive binding assay

Each couple of antibodies can be used to perform competitive binding assay to further illustrate epitope interactions. Ehrlich et al. (1983) has been carried out

some of these studies. However more work needs to be integrate to elucidate this fundamental aspect. This dissertation only covers one pair of antibodies. The binding behavior of hGH and D-hGH in liquid- and solid-phases should be carefully understood with a panel of antibodies to investigate the ability of producing cooperative interactions. solid- and liquid-phase competitive binding assays are designed. Both antibodies is each pair are used as the solid- and liquid-phase antibody.

5.5 Two-step/One-step sandwich immunoassay

Preliminary studies on one- and two-step sandwich immunoassays reveal that the performance deviate significantly from the assay mode. All non-interacting couples of antibodies form a sandwich complex which can be used to design one- or two -step sandwich immunoassays. Each antibody is used in the solid- and liquid-phase. These factors remains to be understood. This is a good starting experimental design. Further studies on the analyte characteristics can be performed for macromolecular analytes such as ferritin.

5.6 Binding reactions with fragmented antibodies

The fundamental studies on development of immunoassays with fragmented antibodies are in infancy. As described earlier titration and saturation curves, competitive binding assays and sandwich immunoassays can be designed parallel assays with intact antibodies to make the system simple and to investigate the possible analytical problems specially for macromolecular analytes.

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