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Preparation and characterization of immunochemical reagents for bioanalytical applications

Wimalasena, Rohan Lalith, Ph.D.

The University of Arizona, 1991

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Preparation and Characterization of Immunochemical Reagents for Bioanalytical Applications

by

Rohan Lalith Wimalasena

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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 1991
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Rohan L. Wimalasena entitled Preparation and Characterization of Immunochemical Reagents for Bionalytical Applications and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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Dr. Michael Burke  
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January 31, 1991  
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1/31/91

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

George S. Wilson  
Dissertation Director  
March 29, 1991  
Date
STATEMENT BY AUTHOR

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SIGNED: [Signature]
Dedication

To Naomi, Nirosha and my Parents

To the memory of my mother-in-law who passed away while this work was in progress
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I would like to express my gratitude to Professor George S. Wilson who has served as a competent and faithful research advisor over the years. He has been a source of encouragement and guidance from whom I have learnt much, even beyond the realms of Science. I would also like to thank Professor Quintus Fernando and Professor Michael Burke for their helpful discussions and perspective suggestions. I wish also to extend my sincere thanks to Professors James M. Byers III and Walter B. Miller for their constructive criticism and valuable suggestions. I am greatly indebted to Dr. Uditha de Alwis for his sacrifice of time in educating me in many areas of my research projects. I am very grateful to Dr. Bruce I Meiklejohn for his extensive advise on how to prepare monoclonal antibodies. I wish to thank the staff of the Animal Care Unit at the University of Kansas for their support. I am especially grateful to Dr. Nanda Thilakarathne who gave freely of his time and talents to critique and upgrade the early drafts up till the final manuscript.

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Abstract

Immunological reagents were prepared and characterized for the development of analytical methodology in bioanalytical research. Monoclonal antibodies to glucose oxidase (E.C. 1.1.3.4) from Aspergillus niger were prepared with apoenzyme as the antigen. Five of these antibodies, all of the IgG, subisotype, were further characterized. The carbohydrate moiety of the enzyme is not immunogenic. Binding of the five antibodies to the enzyme had no detectable effect on its catalytic properties. All the antibodies are shown to be directed towards segmental epitopes of the enzyme, not involving the carbohydrate moiety. Each enzyme subunit has more than one non-overlapping epitope. All five antibodies bound enzyme in a non-native conformation when coated on ELISA plates in preference to the native solution conformation. The importance of having a solution phase screening procedure for monoclonal antibodies is demonstrated.

Factors affecting the specific activity of immobilized antibodies and their biologically active fragments were studied with goat anti-mouse and goat anti-human IgG. Antibodies were immobilized on HW 65 polymeric support matrix activated with carbonyldiimidazole, hydrazide and iodoacetic acid. The most significant factors influencing the specific activity of stochastic coupling of antibodies are multisite attachment, multiple orientations, and steric hindrance imposed by crowding of antibody and the size of the antigen. With oriented immobilization the specific activity is affected only by steric hindrance. The specific activity of immunosorbents prepared by immobilization of F(ab') fragments can be improved to almost 100% by limiting the amount of protein immobilization and the size of the antigen. The present study shows the protocols for optimizing immobilized antibody performance.

Preparation of fragments of immunoglobulin were studied. Within the same species different antibodies showed different sensitivities to proteolytic cleavage by pepsin. A
rapid, simple, high performance size exclusion chromatographic method was developed to monitor the reaction progress. Conditions must be optimized for each antibody in the preparation of F(ab')2. Preparation of F(ab') from F(ab')2 shows that 10-15% of goat anti-mouse F(ab')2 was resistant to reduction. The procedure causes reduction of disulfide bonds other than the inter-heavy chain disulfide bonds.
Chapter 1

General Introduction

1.1 Introduction

Antibodies and their biologically active fragments, in their native form or after conjugation to solid supports, enzymes, drugs and other molecules are generally referred to as immunochemical reagents. These reagents have been used in diagnosis, immunotherapy, affinity chromatography and in the determination of minute quantities of substances in biological fluids. The properties of immunological reagents depend on intrinsic properties of the antibodies and on the perturbation of these properties resulting from derivatization. Therefore to improve the properties of these reagents, derivatization methods should be optimized to minimize undesirable effects. Furthermore, it is important to improve the methods of preparation of antibodies with desired intrinsic properties. This includes the preparation of suitable immunogens and effective screening methods for antibody selection. Thus the progress of analytical methodology in bioanalytical research requires a greater emphasis on the preparation and characterization of immunochemical reagents.

Antibodies belong to a structurally related family of glycoproteins called immunoglobulins. They are divided into five main classes based on the type of the heavy chain, as IgG, IgM, IgA, IgD and IgE. In many analytical applications the IgG class of antibodies has been used, because it is the predominant class of immunoglobulins in mammalian sera (80% of total immunoglobulins). The IgG has a molecular weight of 150,000 - 160,000 and is composed of two identical halves, each of which contains two polypeptide chains (Figure 1.1) (Porter, 1962). One is a heavy chain of molecular weight 55,000 - 60,000 and the other a light chain of molecular weight 20,000 - 25,000. The two chains are held together by disulfide bridges and non-covalent interactions. The two halves of the molecule are joined to each other by disulfide bonds (Figure 1.1). There are two
Figure 1.1. Schematic Diagram of Four-Chain Structure of IgG
identical antigen binding sites for each molecule of IgG. In IgG most often the two heavy chains are glycosylated in the Fc region (Figure 1.1) and the average carbohydrate content is three percent. A wide range of proteolytic enzymes cleave IgG molecule in the hinge region but the most extensively used enzymes have been papain and pepsin. The pepsin cleaves the heavy chain of the IgG on the C-terminal side of the inter-heavy chain disulfide bridges (Figure 1.1). This yields a divalent fragment called $F(ab')_2$ which essentially consists of two F(ab) regions held together by disulfide bridges.

Antibodies to enzymes have many analytical applications. They have been used to prepare enzyme reactors in the determination of substrate by flow injection analysis (De Alwis et al., 1987; De Alwis and Wilson, 1985; 1988; Gunaratna and Wilson, 1990). In general the enzyme reactors have been prepared by covalent immobilization of enzymes on solid supports. Since the covalent binding of an enzyme to a solid support is usually attained by random coupling with the various corresponding chemically reactive groups of the enzyme, the probability of retaining the full enzymic activity is low. It is well documented that the loss of catalytic activity should be anywhere from 20 - 100\% (Solomon et al., 1986). Furthermore, the covalent immobilization is usually irreversible and therefore, the reactors cannot be recharged. On the contrary, antibodies can be used to reversibly immobilize enzymes, through preselected well defined sites of the enzyme, without loss of enzymic activity. However, the antibodies should possess well defined properties to fulfill these requirements. Since, this method of immobilization is reversible, it is possible to recharge the reactors by removing the impaired enzyme.

Antibodies to enzymes are useful in clinical diagnosis (Piran et al., 1987), where conventional methods are not able to provide the necessary information. An example, is found in the determination of creatine kinase isoenzyme MB. Determination of isoenzyme MB for the diagnosis of acute myocardial infarction is well established (Galen and
Gambino, 1975). CK-MB is a more specific biochemical marker of myocardial infarction than are lactate dehydrogenase and aspartate aminotransferase, owing to the high ratio of CK-MB/total CK in cardiac muscle (Lott and Stang, 1980). The other two isoenzymes of creatine kinase are CK-MM and CK-BB which are present in the muscle and the brain respectively. It is now believed that the accurate interpretation of myocardial infarction requires the knowledge of both the activity and the mass of CK-MB. None of the current methods are capable of determining the above parameters. Determination of the mass and activity is difficult without the use of antibodies to isoenzymes. As shown in Figure 1.2, immobilized antibodies to the B-subunit of the creatine kinase can be used to predominantly extract the CK-MB from the sample matrix, because the concentration of CK-BB in serum is low. After separating the CK-MB, the activity of the enzyme can be determined on the support matrix using an appropriate method. Using a labeled antibody to the M-subunit of the enzyme (anti CK-MM or anti CK-MB antibody), it is then possible to determine the total mass of the enzyme. In order for the method to be feasible the antibodies used for the extraction of CK-MB should recognize both the active and inactive forms of the enzyme. Also it should bind the isoenzyme without loss of enzymic activity. Furthermore, the second antibody should also recognize both the active and inactive forms of the CK-MB for the determination of the total mass of the enzyme. This approach could be adopted to any enzyme of interest which has two or more subunits or two or more non-overlapping epitopes. It should be noted that the antibodies should possess special properties to be useful for these applications. The above example illustrates the options available when antibodies with special characteristics are produced.

Antibodies immobilized on solid supports have been widely used in bioanalytical research: for affinity purification of enzymes (van Faassen et al., 1990) and receptors (Stauber et al., 1988), extraction of substances from plasma for purposes of analysis
Figure 1.2. Determination of the Mass and Activity of Creatine Kinase Isoenzyme -MB
Ideally, when antibodies are immobilized on solid supports the resulting immunosorbents should possess the following properties: high specific activity for the antigen, constant immunological activity on repeated use and storage, good mechanical stability, minimum non-specific interactions, and stable linkage between the solid support and the antibody. Properties such as non-specific interactions and a stable linkage are related to physicochemical properties of the support matrix and the chemistry of derivatization. The specific activity of the immobilized antibody could be a function of coupling chemistry, the density of antibody on the support matrix or the intrinsic properties of the antibody. Therefore to improve the properties of immobilized antibodies it is necessary to improve the characteristics of the support matrix, the coupling chemistry, and the selection of antibodies with desired properties.

More than thirty methods have been reported for covalent immobilization of proteins on solid supports. Functional groups such as primary amine, carboxyl and thiol, on the protein are used for coupling to support matrices activated with different groups. The structure of the IgG class of antibodies suggests that there are four types of functional groups: amino, carboxyl, aldehyde or sulfhydryl, which can be used for immobilization (Figure 1.3). Amino groups and carboxyl groups are naturally available on the molecule whereas aldehyde groups have to be created by the oxidation of the carbohydrate moiety in the Fc portion of the antibody. Likewise, sulfhydryl groups can be obtained by the reduction of the whole molecule or the F(\text{ab}')\text{2} fragments of the antibody. There are sixty to eighty \(\varepsilon\)-amino lysine groups on the molecule along with the four N-terminal amino groups. Due to their high abundance, coupling through the amino groups is random and results in immobilized antibodies with low specific activity (Cuatrecasas, 1970). Since the most reactive amino groups are the N-terminal ones, the probability of coupling the
Figure 1.3. Functional Groups Available for Immobilization of IgG Class of Antibodies
antibody through these amino groups is higher. This could be another reason for the loss of specific activity. On the contrary, if coupling is carried out through the carbohydrate moiety the antibodies can be immobilized with a well defined orientation (Hoffman and O'Shannessy 1988). Since the carbohydrate moiety is distal from the antigen binding site, this method should give immobilized antibodies with high specific activity. Similarly, coupling through the sulfhydryl group also favors active orientation (Prisyazhnoy et al., 1988) and therefore, should give high specific activity immunosorbents. Carboxyl groups can also be used for coupling, however, very few applications have been reported. This could be attributed to the lower accessibility of the carboxyl groups on the molecule. Since there are many methods for immobilization of an antibody on a support matrix, it is necessary to carefully evaluate these methods to optimize the properties of the resulting immunosorbents.

Preparation of antibody-enzyme conjugates is crucial for the satisfactory performance of enzyme immunoassays. Enzyme immunoassays are widely used in the determination of minute quantities of analytes in biological fluids. The first enzyme immunoassays (EIA) were reported independently by Engvall and Perlmann (1971) and Van Weemen and Schuurs (1971). However, enzyme antibody conjugates were used in immunocytochemistry prior to that date (Nakane et al., 1966). Different chemical and biological procedures have been developed for conjugation of enzymes to antibodies, but very often chemical methods result in conjugates with suboptimal properties. The object of any labeling procedure is to produce a conjugate which retains the highest activities of the enzyme and the molecular recognition properties of the antibody. Chemical conjugation methods often require an activation step, which increases the complexity of the labeling procedure. The chemical reaction used could involve parts of the molecules that are important for the activity (i.e. the antibody binding site or the active site of the enzyme),
and, as a result, either component could lose the activity. Furthermore, due to the lack of control of the conjugation reaction higher molecular weight polymers are often formed during chemical conjugation. These polymers can be inactive and can cause higher backgrounds in enzyme immunoassays. Since they are polyvalent, they can have entirely different immunological characteristics compared to a single molecule of antibody. On the contrary, if proper antibodies to the enzyme of interest are used as a part of a bispecific antibody (Figure 1.4) it should be possible to make conjugates with high enzymic activity, well defined stoichiometry and low non-specific interactions (Tijssen, 1985).

Both polyclonal and monoclonal antibodies can be used in analytical applications. Since polyclonal antibodies are heterogeneous it is difficult to make reproducible reagents with defined properties against any antigen. However, with the introduction of monoclonal antibodies (Kohler and Milstein, 1975) it has become possible to prepare homogeneous antibody populations with well defined properties. Since antibodies to enzymes that are useful for analytical applications should have special properties, preparation and characterization of monoclonal antibodies to enzymes are of great interest. In the present study monoclonal antibodies to glucose oxidase (GOx) were prepared and characterized to study their properties and their epitope distribution. GOx is of interest, because it has been used with antibodies in glucose determinations (De Alwis and Wilson, 1989; De Alwis et al., 1987) and as a label in immunoassays (De Alwis and Wilson, 1985; 1987). Despite the rigidity of the GOx molecule, it was found that the screening process has an influence on the properties of the monoclonal antibodies selected. Therefore, the investigation was extended to study this phenomenon.

Since most of the analytical applications of antibodies require immobilized antibodies on solid supports, it is important to optimize the conditions to prepare immunosorbents of high quality. A number of studies of covalent coupling reactions have
Figure 1.4. Schematic Representation of Antibody-Enzyme Conjugate with Bispecific Antibody
been previously reported (Nakamura et al., 1990; Cress and Ngo, 1989; Hoffman and O'Shannessy, 1988; Matson and Little, 1988; Prisyazhnny et al., 1988; Pfeiffer et al., 1987) but the data are difficult to compare. The conditions for individual coupling reactions were not optimized and different supports and different antibodies have been employed. Therefore, it was considered essential to do a systematic comparative study of different coupling methods in order to evaluate the relative merits of each. In this regard it was decided to use the same antibody or its biologically active fragment and the same support derivatized with different reagents. The objective of this research was to study the effects of multisite attachment, control orientation and antibody loading on biological activity of the resulting immunosorbent.

As described above, bispecific antibodies are useful for preparation antibody-enzyme conjugates with optimal properties. Bispecific antibodies can be produced by biological or chemical methods. Biological production involves the fusion of two monoclonal antibody producing hybridomas or of an immunized spleen cell and a hybridoma. This method is tedious and time consuming. In chemical production, F(ab') fragment of the parental antibodies are recombined using heterobifunctional reagents or by oxidation of the sulfhydryl groups (for review see Norlan and O'Kennedy, 1990). Chemical production of bispecific antibodies is faster and less complicated. Despite the relative simplicity, we and others (Runge et al., 1990) have been unable to increase the yield beyond 25% of total potential yield. By contrast Brennan et al. (1987) have reported a 50-70% yield. However, the chromatographic data presented in their paper did not unequivocally support this observation. Therefore, it was decided to reinvestigate the reaction conditions necessary to optimize the yield of bispecific antibody production. In previous reports more or less the same reducing conditions have been used to prepare the F(ab') fragments without considering the reactivity of the many disulfide bonds in the
parent molecule. It has been widely assumed that only the inter heavy chain disulfide bonds are reduced. However, this may not be the case and thus the number of sulfhydryl groups generated in the reduction step may have a significant effect on the yield of bispecific antibody. The purpose of the present study is to investigate the disulfide bond reduction conditions including the kinetics, the number of sulfhydryl groups formed and the biological activity of the resulting F(ab') fragment.
Chapter 2
Preparation and Characterization of Monoclonal Antibodies To Glucose Oxidase

2.1 Introduction

Antibodies to enzymes have three basic applications that are useful for analytical purposes. The antibodies can be used to reversibly immobilize enzymes to prepare enzyme reactors (De Alwis and Wilson, 1985; De Alwis et al., 1987), to link an enzyme to an antibody fragment (bifunctional antibody) for enzyme immunoassays (Gorog et al., 1989; Karawajew et al., 1988), or to immobilize an enzyme so that its mass and activity can be determined. Antibodies so employed should bind the enzyme with high affinity and with essentially no loss of activity in order to be useful (De Alwis and Wilson, 1985; De Alwis et al., 1987). Both polyclonal and monoclonal antibodies can be used for this purpose, however, there is some advantage in using essentially homogeneous preparations of monoclonal antibodies (MAbs) with well defined characteristics (Kohler and Milstein, 1975). It has been reported in the literature that some MAbs raised against native enzymes bind preferentially, if not exclusively to the denatured form of the enzyme (Wilson and Smith, 1985; Finney et al., 1984; Djavadi-Ohaniance et al., 1984; Dunn et al., 1985). Both inhibition and activation of enzyme activity by MAb binding have been observed (Dunn et al., 1985; Wolf, 1983; Suh et al., 1988, Rockwell et al., 1985; Makino et al., 1985; Hessova et al., 1985). Preparation of MAbs against native β-D galactosidase has yielded at least four types of functionally different antibodies on binding to the enzyme: those which increase the enzymatic activity of the denatured enzyme, those which inhibit the enzymatic activity of the enzyme, those which protect the heat denaturation of the enzyme and those which have no effect on the activity of the enzyme (Frackelton and Rotman, 1980). Therefore, it is important to prepare and characterize MAbs to enzymes to obtain antibodies of analytical interest.
Glucose Oxidase (GOx) is of interest because it has been used as an analytical reagent for glucose analysis (De Alwis and Wilson, 1989; De Alwis et al., 1987; Lomen et al., 1986) and in enzyme immunoassays (De Alwis and Wilson, 1985; 1987). GOx isolated from aspergillus niger has a molecular weight of 160,000 and contains approximately 16 percent carbohydrate (Tsuge et al., 1975). It consists of two identical subunits which are covalently linked by disulfide bonds and each mole of subunit has one mole of flavin adenine dinucleotide (Tsuge et al., 1975; O'Malley and Weaver, 1972).

Rabbit polyclonal antibodies prepared against GOx did not show immunological reactivity with the isolated subunits, but recognized the periodate oxidized derivative of the enzyme. These observations suggest that the epitope on the enzyme did not reside on the carbohydrate moiety, but rather in a domain bridging the polypeptide chains of the two subunits (Pazur et al., 1984; Nakamura et al., 1976). However, there was no information about the effect of antibody binding on the catalytic activity of the enzyme. Also it has been reported that native GOx was very toxic to mice as an immunogen, and therefore, it was very difficult to prepare MAbs with the native enzyme as the immunogen (Steinmetz and Pfreundschuh, 1987). However, it is known that MAbs raised against denatured antigen react with the native antigen (Vaishnav and Antony, 1989). Therefore, the apoenzyme can be used as the immunogen to raise MAbs to native GOx, thus avoiding the toxicity problem. The apoenzyme of GOx, devoid of FAD groups, has essentially the same molecular weight as the holoenzyme, but a different sedimentation coefficient, intrinsic viscosity and sensitivity to proteolysis (Swoboda, 1969). It exists mainly in the conformation of a loose flexible coil, whereas the native enzyme has a compact, nearly spherical form (Swoboda, 1969). The apoenzyme possesses no enzyme activity.

Very often preparation of antibodies to enzymes yields antibodies that inhibit the activity of the enzyme. Despite numerous efforts, we have been unable to produce such
antibodies to GOx. This prompted a detailed investigation of the immunological properties of antibodies to this enzyme. The objectives of the present study were to investigate: the properties of the GOx enzyme as an immunogen, the immunological characteristics of the enzyme and the epitope distribution of the antibodies. However, during the characterization of the antibodies to GOx, it was found that the screening process has an influence on the properties of the antibodies selected. Even though this has been observed for antibodies to peptides, it was not anticipated for GOx, because it has a very rigid structure. Therefore, the investigation was extended to study the influence of the screening process on the properties of the antibodies to GOx.

2.2 Materials and Methods

2.2.1 Preparation of Apoenzyme

The apoenzyme was prepared according to the method of Swoboda (1969) with the following modifications: Glucose oxidase (EC 1.1.3.4) from Aspergillus niger (Type GO3A, Biozyme Laboratories International Ltd., San Diego, CA), 20 mg in 1 ml of PBS (0.1 M phosphate buffer, 0.15 M NaCl pH 7.4) was used as the starting material. The enzyme was subjected to two cycles of acidified salt treatment (Swoboda, 1969) to obtain the apoenzyme completely free of FAD groups as confirmed by absence of absorbance at 450 nm. The final product was dissolved in PBS, dialyzed against the same buffer and then concentrated to a 4 mg/ml solution. This solution was sterilized by filtering through a 0.22 μm filter (Gelman Sciences, Ann Arbor, MI) and was used as the stock solution of the apoenzyme.
2.2.2 Immunization

Three different protocols were used to immunize the mice, with one of the objectives being to shorten the immunization period.

Protocol 1

Female, 4-6 weeks old, BALB/c mice were immunized by directly injecting 20 \( \mu \)g of sterile ApoGOx (0.4 mg/ml) into the spleen according to the method of Gearing et al., (1985). Three days after the injection mice were sacrificed for the fusion.

Protocol 2

This procedure is the same as Protocol 1 except that it involved two intrasplenic injections of 20 \( \mu \)g of ApoGOx on day 1 and day 12, followed by fusion 3 days after the final injection.

Protocol 3

Female, 4-6 weeks old, BALB/c mice were immunized with the same antigen according to the immunization schedule described by Cianfriglia et al., (1983) as summarized in Table 2.1.

2.2.3 Fusion

Both the fusion and the cell culture were carried out as described by Lane (1985). Splenocytes from the immunized mice were fused with mouse myeloma cells, P3-NS1/1-Ag4-1 (NS1) (American Type Culture Collection, Rockville, MD), (spleen:myeloma = 10:1) in the presence of polyethylene glycol, MW 3350 (Sigma, St. Louis, MO) and the fused cells were grown in Cell Wells 96 well plates (Corning, NY). After 2 weeks, wells
Table 2.1

Immunization Protocol 3

<table>
<thead>
<tr>
<th>Day</th>
<th>Dose (μg), condition, mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>50, CFA&lt;sup&gt;a&lt;/sup&gt;, ip&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>7</td>
<td>50, CFA, ip</td>
</tr>
<tr>
<td>12</td>
<td>400, S&lt;sup&gt;c&lt;/sup&gt;, ip</td>
</tr>
<tr>
<td>13</td>
<td>200, S, ip + 200, S, iv&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>14</td>
<td>200, S, ip + 200, S, iv</td>
</tr>
<tr>
<td>15</td>
<td>fusion</td>
</tr>
</tbody>
</table>

<sup>a</sup> Complete Freund's Adjuvant.  
<sup>b</sup> Intraperitoneal.  
<sup>c</sup> Saline.  
<sup>d</sup> Intravenous.
with a positive growth of cells were screened for specific antibody production using an Enzyme Linked Immunosorbent Assay (ELISA).

### 2.2.4 Screening Procedure

The Enzyme Linked Immunosorbent Assay (ELISA) was used for screening of hybridoma supernatants. The assay was performed according to the method of Bahr et al. (1980) with the following modifications: The ELISA plates (Corning, NY) were coated with 100 µl/well of 10 µg/ml solution of antigen in carbonate buffer 0.1 M, pH 9.4 for 2 hrs. at 37°C. Plates were washed four times with wash buffer, phosphate buffered saline (PBS), pH 7.4, containing 0.05% Tween 20. Culture supernatants were added, 50 µl/well with 50 µl of ELISA diluent buffer (PBS pH 7.4, 0.05% Tween 20, 0.2% BSA). The subsequent incubations were performed at 37°C for 1 hr., except for the last step. Goat anti-mouse IgG-HRP conjugate (American Qualex, La Mirada, CA) was used at a 1/5000 dilution in ELISA diluent buffer. In the final step plates were washed four times with nanopure water (Barnstead, Nanopure II, SYBRON/Barnstead, Boston, MA), and 100 µl of substrate solution of HRP (citrate-phosphate, 0.1 M, pH 4.5, 0.01% urea peroxide, 1 mg/ml O-phenylenediamine) was added to each well. After 20 min. of incubation the color reaction was stopped by adding 50 µl/well of 1 M HCl and the absorbances were read at 490 nm using a microplate reader (Vmax, Molecular Devices Corp., Palo Alto, CA). The supernatants were screened against both Glucose oxidase and apoglucose oxidase using this method.

The isotype of the antibodies from positive hybridoma supernatants was determined by an ELISA technique using specific rabbit anti-mouse subclass sera of a subisotype kit (American Qualex International, Inc., La Mirada, CA). Assays were performed according to the manufacturer's instructions.
2.2.5 Cloning and Production of Ascites

Hybridomas secreting specific antibodies were selected, expanded and cloned twice by the limiting dilution method (Goding, 1985). Monoclonals thus obtained were cultured and injected (10^6 cells/mouse) into pristane primed 3-5 months old BALB/c mice in order to induce ascites. The ascitic fluids were collected and centrifuged at 1000 g to remove the cells. The cell free ascitic fluid was stored at -20°C until use.

2.2.6 Affinity Purification of Antibodies

Immunoaffinity chromatography was used to purify the MAbs. The affinity column was prepared by covalently coupling periodate oxidized GOx to carbonyldiimidazole (CDI) (Fluka Chemical Corp., Ronkonkoma, NY) activated Sepharose 6B (Pharmacia, Piscataway, NJ) using triethylene tetraamine (TETA) as a spacer. CDI activated Sepharose 6B was prepared as previously described (Bethell et al., 1981). TETA tetrahydrochloride (Aldrich, Milwaukee, WI) (1 mmol/ml of gel in carbonate buffer, pH adjusted to 9.0) was added to the activated gel equilibrated with carbonate buffer 0.1 M, pH 9.0 and tumbled end over end for 18 hrs. at 4°C. The gel was washed alternatively with 5 x gel volume each of borate buffer 0.1 M, pH 8.0 and acetate buffer 0.1 M, pH 4.5. GOx (75 mg) was oxidized by sodium metaperiodate (Sigma, St. Louis, MO) (final concentration: NaIO₄, 2 mM; GOx, 2 mg/ml) according to the method of Zaborsky et al., (1976). The oxidized GOx solution, free of excess periodate, was then added to the gel and the pH of the mixture was adjusted to 7.0 by adding a few drops of 5 M NaOH. This mixture was tumbled end-over-end for 4 hrs. at 4°C and the uncoupled GOx was removed according to the wash procedure described above. The amount of GOx
coupled to the gel was determined as 66.8 mg. The gel was again equilibrated with PB 0.1 M, pH 7.0 and resuspended in twice the gel volume of the same buffer. This solution was then made 10 mM with respect to sodium cyanoborohydride (Sigma, St. Louis) and allowed to react for 48 hrs at 4°C. The gel was finally washed alternatively with 5 x gel volume each of PB 0.1 M, pH 7.4 and PB 0.1 M, pH 2.2. After washing with PB 0.1 M, pH 2.2, the immobilized GOx was converted to ApoGOx due to the loss of the prosthetic groups (FAD). This gel was then packed into a (2.3 cm x 5 cm) laboratory-made column and used for affinity purification of MAbs to GOx.

Thawed ascites fluid was filtered through glass wool to remove any aggregated lipids and applied to the immunoaffinity column equilibrated with wash buffer (PB 0.1 M, pH 7.4). The column was washed with buffer until the absorbance of the column eluent at 280 nm reached baseline. The specific antibody was eluted with PB 0.1 M, pH 2.2. The eluent containing the specific antibody was collected in one third of the column volume of PB 0.4 M, pH 7.4. Affinity purified antibodies were later dialyzed against PBS pH 7.4 and stored at 4°C for shorter periods and -20°C for longer periods. When the ascites fluid was bloody the IgG fraction was separated by salt fractionation with ammonium sulfate precipitation (33% of saturation; saturated solution at room temperature is 4.1 M) (Tijssen, 1985). The precipitate was dissolved in PB 0.1 M, pH 7.4 and dialyzed against the same buffer for 48 hrs with three changes of buffer. This IgG fraction was then subjected to affinity purification as described above.

2.2.7 Preparation of GOx-HRP Conjugate

GOx was conjugated to HRP (Type 4, Biozyme Laboratories International Ltd., San Diego, CA) according to the method of Tijssen (1985) and separated into two fractions on a Sephacryl HR-200 (Pharmacia, Piscataway, NJ) column (2.3 x 60 cm) at a flow rate
of 0.5 ml/min. The absorbances of the two fractions were read at 280 nm and 403 nm to determine the GOx to HRP ratio and the fraction with the 1:1 ratio was used for further studies.

2.2.8 Preparation of Biotin conjugates of MAbs

These conjugates were prepared according to the method of Yolken et al (1983) with the following modification: Sulfo N-hydroxysuccinimidobiotin (Pierce, Rockford, IL) was dissolved in nanopure water at a concentration of 0.05 M, and a 100 μl aliquot of this solution was added to 1 ml of 1 mg/ml protein solution in carbonate buffer 0.1 M, pH 8.5. The mixture was incubated for 3 hrs at room temperature and the conjugate was separated from the excess reagents by passing through a G-25 column. The resulting conjugates were then dialyzed against PBS, pH 7.4 for 24 hrs with two changes of buffer. Protein concentrations of the conjugate were determined by the absorbance at 280 nm.

2.2.9 Preparation of Avidin-HRP conjugate

Avidin was conjugated to HRP according to the method of Staros et al. (1986), with the following modifications: A solution of 5 mg of Avidin (Sigma, St. Louis, MO) in 0.5 ml of PB, 0.1 M pH 7.0 was prepared and 100 μl of 50 mM N-hydroxysulfosuccinimide (Pierce, Rockford, IL) and 19.17 mg of 1-Ethyl-3-(3 dimethylaminopropyl) carbodiimide were added. Then 13.3 mg of HRP in 0.3 ml PB was added and the final volume of the mixture was adjusted to 1.0 ml with the same buffer and stirred at room temperature for 24 hrs. The protein fraction was then separated from low molecular weight products and excess reagents by passing over a G-25 column, and extensively dialyzed against PBS pH 7.4. All the buffers used in the experiment were free of sodium azide.
2.2.10 Characterization of MAbs

Five monoclonals, obtained by cloning three hybridomas, resulting from three independent fusions were selected for further characterization. The designations and the properties are given in Table 2.2.

2.2.11 Determination of the Affinity of MAbs

2.2.11.1 Method I, Antigen in Solution

The affinity of the MAbs was determined by an ELISA technique. In this method, ELISA plates were coated with 10 \( \mu \text{g/ml} \) solution of affinity purified MAbs as described above. After 2 hrs of incubation at 37°C the plates were washed. Two fold serial dilutions of the GOx conjugated to HRP were prepared in ELISA diluent buffer from a solution of 42 \( \mu \text{g/ml} \). Then the dilutions of the conjugate were titrated in quadruplicate against the antibodies coated on the plate by adding 100 \( \mu \text{l/well} \) of each dilution. Subsequent steps of the assay were same as the steps following the addition of conjugate in the screening ELISA described above. Bovine Serum Albumin coated on the plate served as the blank in all the experiments.

The apparent affinity of the antibodies can be obtained from the reciprocal of the concentration of the antigen (GOx), where 50% of the maximum binding occurs (Tijssen et al., 1985). The titration curves for the five MAbs are shown in figure 2.1. The apparent affinities calculated from these curves are tabulated in Table 2.2.
Table 2.2

Designation and Properties of MAbs

<table>
<thead>
<tr>
<th>MAb</th>
<th>Sub-isotype</th>
<th>Formation Constant Method I $10^8 \text{M}^{-1}$</th>
<th>Formation Constant Method II $10^8 \text{M}^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1F10C12G5</td>
<td>IgG1</td>
<td>0.79</td>
<td>5.12</td>
</tr>
<tr>
<td>1F10C12C9</td>
<td>IgG1</td>
<td>0.49</td>
<td>5.12</td>
</tr>
<tr>
<td>2F12E12</td>
<td>IgG1</td>
<td>0.28</td>
<td>40.90</td>
</tr>
<tr>
<td>2F12D12</td>
<td>IgG1</td>
<td>0.42</td>
<td>40.90</td>
</tr>
<tr>
<td>3A2C8</td>
<td>IgG1</td>
<td>0.53</td>
<td>10.20</td>
</tr>
</tbody>
</table>
Figure 2.1. Titration Curves of MAbs (Method I)

Experimental details in section 2.2.11.1

- 1F10C12G5
- 1F10C12C9
- 3A2C8

- 2F12E12
- 2F12D12
2.2.11.2 Method II, Antigen Coated on the Plate

This method was essentially the same as Method I except for the following modifications: In Method II, ELISA plates were coated with 10 μg/ml solution of GOx and the affinity purified MAbs were titrated against the enzyme coated on the plates. Antibody dilutions were prepared from 40 μg/ml solutions. Apparent antibody bound was determined using goat anti-mouse HRP conjugate at a dilution of 1:2000. The working dilution of the conjugate was selected such that the conjugate concentration was not limiting under the experimental conditions. The apparent affinity of the antibodies was determined from the plot of response vs. antibody concentration as shown in Figure 2.2. The apparent affinities of the MAbs are tabulated in Table 2.2.

2.2.12 Preparation of GOx derivatives

2.2.12.1 GOx Subunits

Subunits were prepared and alkylated essentially as described by O'Malley et al., (O'Malley et al., 1972). The protein fraction was separated from the excess reagents and free FAD groups by passage through a Sephadex G-25 (Pharmacia, Piscataway, NJ) column (1.2 cm x 30 cm) equilibrated with PBS, pH 7.4.

2.2.12.2 Periodate Oxidized GOx

The derivative was prepared as described under the affinity purification of antibodies and the resulting aldehyde groups were blocked by reacting with Tris-HCl and reducing with sodium cyanoborohydride.
Figure 2.2. Titration Curves of MAbs (Method II).

Experimental details in section 2.2.11.2.

- 1F10C12G5
- 1F10C12C9
- 3A2C8
- 2F12E12
- 2F12D12
2.2.12.3 Heat Inactivated GOx

This was prepared by heating 5 mg of the enzyme in 1.0 ml of PBS pH 7.4 at 75°C for 30 min. The protein fraction was separated from the free FAD groups by passing through a Sephadex G-25 column equilibrated with the same buffer.

2.2.12.4 Apo Enzyme

This was prepared as described above.

2.2.13 Determination of the Immunoreactivity of GOx Derivatives

Immunoreactivity of the MAbs towards different derivatives of GOx was studied using an ELISA method, essentially the same as Method II described for the apparent affinity determination of the MAbs. The MAbs were titrated against different derivatives of GOx coated on plates. Apparent affinity of the MAbs towards the different derivatives can be determined as described above.

2.2.14 Inhibition ELISA

Inhibition ELISAs were carried out with each derivative of GOx in solution as an inhibitor. Antibody dilutions were prepared from affinity purified antibodies to give 50% of the maximum binding to GOx coated on ELISA plates (from titration ELISA, Method II). Each antibody was preincubated with various concentrations of each derivative for 30 min. to give 1:10, 1:100 and 1:500, antibody: derivative ratios by mixing 0.25 ml each of the antibody and the derivatives. Each of these mixtures was then added in triplicate, 100 µl/well to plates coated with GOx and incubated for 1 hr. at 37°C. To determine the percent inhibition, controls were run by preincubating 0.25 ml of the antibody dilution with 0.25 ml of PBS, pH 7.4, in place of the inhibitor. The controls were treated the same way
as the samples. The apparent MAb bound to the plate was determined using goat anti-mouse HRP conjugate as described above. The conjugate concentration was not limiting under the experimental conditions.

2.2.15 Sandwich ELISA

In order to study the epitopes of the MAbs on the enzyme a sandwich ELISA assay was developed. An ELISA plate was coated with one of the MAbs (primary coating antibody) as described above. After washing the plate, two fold serial dilutions of each GOx derivative, prepared from 100 μg/ml solutions, were added in duplicate (100 μl/well) and incubated for 1 hr at 37°C. Then the plate was washed and 100 μl/well of biotin-labeled secondary antibody was added. One experiment was carried out, with the same antibody as both the primary and the secondary antibody. Another experiment was performed by using different primaries and the same secondary antibody labeled with biotin to give every combination of antibody pairs. Avidin labeled with HRP was used to monitor the binding of the secondary antibody. Preliminary studies were made to optimize the concentrations of the avidin HRP conjugate and the secondary antibody labeled with biotin.

2.2.16 Determination of the Effect of Antibody Binding on Catalytic Activity of the Enzyme

The enzyme GOx was diluted with PBS, pH 7.4 to give 10 μg/ml of enzyme solution. The antibodies were then allowed to react with the enzyme by mixing 0.25 ml each of the enzyme solution and the antibody dilutions to give 100:1, 20:1, 1:1 and 1:5 Ab:Ag ratios and incubating for varying lengths of time at 37°C. Controls were treated essentially the same as the samples, with PBS, pH 7.4 in place of the antibodies. The
enzyme activity was determined according to the method described elsewhere (Decker, 1977). The results are shown in Table 2.3.

### 2.3 Results and Discussion

The results of the three fusions carried out with the mice immunized by the three different immunization protocols are summarized in Table 2.4. Almost all (97%) the antibody producing hybridomas obtained by Protocol 1 were of the IgM class. This is not very surprising because the fusion was done three days after the primary injection. Protocol 2 also gave more or less the same results (55% IgM and 36% mixture of IgG and IgM). This shows that the secondary response had not enough time to mature to a higher percentage of IgG class antibodies. Based on these observations we believe that a more extensive immunization protocol should be employed consisting of three biweekly immunizations. A primary intrasplenic injection is given, followed by two intraperitoneal booster injections. A final intravenous boost is then given 3-4 days prior to fusion. In contrast to these, Protocol 3 gave a substantial percentage (45%) of hybridomas producing antibodies of the IgG class in a relatively short time period (2 weeks). However, a potential drawback of this method is that it requires a large quantity of antigen and therefore is not suitable for both toxic and low abundance antigens.

GOx is known to lose its prosthetic group, resulting in apoenzyme under the low pH conditions (ca. pH 2.2) used to elute antibodies in immunoaffinity chromatography. Therefore, an affinity column prepared by covalent immobilization of GOx will convert to an immobilized apoenzyme column after conditioning for affinity chromatography as described in the procedure. Thus, one might imagine that this could be a problem for affinity purification of MAbs to GOx. Since both the apo- and the holo-enzyme are
Table 2.3

Effect of Antibody Binding on Catalytic Activity of GOx

<table>
<thead>
<tr>
<th>Ab Designation</th>
<th>Ab:Ag Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100:1</td>
</tr>
<tr>
<td>% activity of mixture&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>1F10C12G5</td>
<td>103</td>
</tr>
<tr>
<td>1F10C12C9</td>
<td>108</td>
</tr>
<tr>
<td>2F12E12</td>
<td>102</td>
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<td>2F12D12</td>
<td>106</td>
</tr>
<tr>
<td>3A2C8</td>
<td>110</td>
</tr>
</tbody>
</table>

<sup>a</sup> \[
\text{% activity of mixture} = \frac{\text{activity of mixture}}{\text{activity of control}} \times 100
\]
Table 2.4

Fusion Efficiencies

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Total no. of wells</th>
<th>No. of growing wells</th>
<th>Specific efficiency(^a) (%)</th>
<th>Class of specific IgM (%)</th>
<th>IgG (%)</th>
<th>IgG + IgM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>384</td>
<td>102</td>
<td>29.4</td>
<td>97</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>384</td>
<td>215</td>
<td>29.7</td>
<td>55</td>
<td>9</td>
<td>36</td>
</tr>
<tr>
<td>3</td>
<td>288</td>
<td>238</td>
<td>30.2</td>
<td>25</td>
<td>45</td>
<td>30</td>
</tr>
</tbody>
</table>

\(^a\) No. of specific wells/No. of growing wells x 100

\(^b\) (No. of wells with specific class / total No. of specific wells) x 100
Figure 2.3. Immunological Reactivity of 2F12E12

The ELISA plates were coated with 100 μl per well of GOx derivatives, (10 μg/ml). Antibody titrated against GOx derivatives coated on plate with two-fold serial dilutions. Initial concentration, 40μg/ml.

- Native
- Apoenzyme
- Subunits
- Heat Inactivated
- Oxidized GOx
recognized by these antibodies as shown by the screening assay, an affinity column prepared by covalent immobilization of GOx can be used for the affinity purification.

Because of its large molecular size, it is reasonable to assume that direct covalent immobilization of GOx on a solid support would have no significant effect on immunological reactivity. On the contrary, GOx lost its immunological reactivity towards the monoclonal antibodies as evidenced by non-recovery of the antibodies from the respective ascites fluids, using affinity columns prepared by this method. When a spacer arm was used to immobilize the GOx, such affinity columns then gave excellent yields of affinity purified antibodies confirming that the loss of immunological reactivity is due to direct covalent immobilization.

The observation that the apoenzyme is recognized by the MAbs is not sufficient to confirm that the binding of the antibodies would not affect the catalytic activity of the enzyme. Therefore the effect of antibody binding on the catalytic activity of the enzyme was studied. As depicted in Table 2.3 none of the MAbs have any effect on the catalytic activity of the enzyme (activity ca. 100%), under the assay conditions. These two observations together confirm that the epitopes of these MAbs are not at the active site of the enzyme, nor do they affect its conformation. Therefore, the MAbs can be used to immobilize GOx reversibly, without loss of catalytic activity to prepare enzyme reactors. The MAb 2F12E12 has been used to prepare such an enzyme reactor for sub femtomole levels of glucose determinations (De Alwis and Wilson, 1989). However, due to the moderate binding constant (ca. $10^7$ l mol$^{-1}$) of the antibodies with native GOx in solution such reactors lost their enzyme activity rapidly with time.

To our knowledge, the complete structure of GOx is not available. Therefore, the epitope mapping is a difficult task. Instead it was decided to localize the epitopes of the antibodies on specific segments of the enzyme. With this in mind the immunoreactivity of
the MAbs towards different derivatives of GOx was evaluated. Results of the ELISA assay are shown in Figure 2.3 for MAb 2F12E12. Because the antibody dilution for fifty percent binding is more or less the same for all the derivatives as observed from Figure 2.3, 2F12E12 has the same apparent affinity for all the proteins. Similar behavior is observed for the other MAbs as well. The observation that the MAbs recognized the subunits of the enzyme makes it evident that the epitopes of the MAbs are not in a polypeptide domain bridging the two subunits. This is in contrast to the observation of Pazur et al. (1984) for polyclonal rabbit-antiGOx antibodies. Because all the MAbs showed immunoreactivity with periodate oxidized GOx, it is reasonable to conclude that the epitopes are not residing on the carbohydrate moiety of the enzyme. This suggests that this part of the enzyme is not very immunogenic. This observation is also in agreement with those reported by others for GOx (Pazur et al., 1984; Nakamura et al., 1976). The MAbs also recognized the heat denatured GOx, and this makes it evident that the epitopes of these monoclonals are not susceptible to conformational changes caused by denaturation of the enzyme under the above experimental conditions. Since each MAb showed approximately the same apparent affinity towards all GOx derivatives, it is possible to conclude that the antibodies are directed towards segmental rather than topographical epitopes of GOx.

A sandwich assay was performed to understand the distribution and the distinction of the epitopes of the MAbs on the enzyme. The results of the assay appeared to be similar for all five MAbs using the same antibody as the primary and the secondary. The results of 2F12E12 are shown in Figure 2.4. As depicted in Figure 2.4 the response of the assay increases with the increasing concentration of GOx. This confirms that a sandwich assay
Figure 2.4. Response of Sandwich Assay.

Plates coated with primary antibody, 2F12E12 (10 μg/ml). Biotin-labelled 2F12E12 used as secondary antibody (0.46 μg/ml). Details in section 2.2.14.
can be carried out for GOx using the same primary and secondary antibody. Since a recent study of cloning and gene sequence of GOx has confirmed that the enzyme has two identical subunits (Frederick et al., 1990), this observation is not surprising. However, the observation that the subunits of GOx also gave a positive response under the same conditions suggests that each of the subunits has more than one non-overlapping epitope, spatially separated by at least 35 Å for 2F12E12 antibody (Tzartos et al., 1981). Since similar results have been observed for the other four MAbs, it is possible to draw the same conclusion for them as well. When the assay was carried out using different primary antibodies and the same secondary antibody, the results appeared to be the same. This further supports the idea of non-overlapping epitopes for each subunit, but it is not possible to say unequivocally whether the epitopes of different monoclonals are different or identical.

The apparent binding affinities of the MAbs, determined by Method I and Method II are different as shown in Table 2.2. In Method I, the antigen GOx was in solution whereas in Method II, it was adsorbed on the ELISA plates. Therefore, it is reasonable to assume that the MAbs preferentially recognized the antigen adsorbed on the plate over the antigen in solution. Similar behavior has been observed with many antigens including rat brain hexokinase, lactate dehydrogenase, the β2 subunit of Escherichia Coli tryptophan synthase and human growth hormone (Finney et al., 1984; Mazza and Retegui, 1989; Hollander and Katchalski-Katzir, 1986; Friguet et al., 1984; Miller et al., 1983). In order to confirm the idea of preferential recognition, we performed an inhibition ELISA experiment. The results of the experiment as shown in Figure 2.5 (for 1F10C12G5) reveal that the inhibition observed with a one hundred fold excess of the denatured derivatives of GOx was about 62-86% whereas that observed with GOx and periodate oxidized GOx was 6-14%. The other four MAbs also exhibit similar results. This clearly confirms the idea of preferential
Figure 2.5. Inhibition of 1F10C12G5.
Experimental details in section 2.2.15.
recognition. The results also provide additional support for the low response of the sandwich assay for GOx and periodate oxidized GOx as observed in Figure 2.4.

The apparent affinities of the MAbs for GOx in solution and GOx adsorbed on the ELISA plate together with the results of the inhibition assay (Figure 2.5) suggest that the enzyme GOx undergoes some denaturation when adsorbed on to the ELISA plates. Further they suggest that the derivatives of GOx tested, when adsorbed on the ELISA plates, revert to a common conformation which is recognized by all the antibodies under consideration. This, in turn, has introduced an artifact into the screening of hybridomas, and as a result, the prepared monoclonals preferentially recognized the enzyme adsorbed on to the plate over the enzyme in solution. Therefore, it is very important to have a solution phase screening procedure as suggested by Miller et al. (1983), in the preparation of MAbs to be used in solution phase immunochemistry.
Chapter 3
Evaluation of Coupling Methods for the Preparation of Immunosorbents by Immobilization of IgG and their Biologically Active Fragments

3.1 Introduction

The ability to elicit an immune response to an antigen, and more particularly the advent of monoclonal antibody technology has led to the widespread application of immobilized antibodies for analytical purposes. Antibodies covalently immobilized on a solid support matrix have been found to be useful for the immunoaffinity purification of enzymes (Abouaki et al., 1988; Yang et al., 1988; Aarsman et al., 1989; Thompson et al., 1990; van Faassen et al., 1990), receptors (Moncharmont et al., 1982; Phillips and Frantz 1988; Stauber et al., 1988; ) and extraction of substances from plasma (Davis et al., 1986; Wojchowski et al., 1987; van Ginkel et al., 1989). Immobilized antibodies have been used to prepare enzyme reactors in the determination of substrates (De Alwis et al., 1987; De Alwis and Wilson, 1989; Gunaratna and Wilson, 1990) and to prepare immunoreactors in flow injection immunoassays (De Alwis and Wilson 1985, 1987; Lee and Meyerhoff, 1990).

There are several methods available for immobilization of an antibody on a support matrix. The most popular coupling method has been the cyanogen bromide (CNBr) activation of agarose beads, followed by the direct attachment of antibody molecules or the attachment of antibody via an N-hydroxysuccinimide ester (Cuatrecasas, 1970). Other methods used include the following reagents: periodate (Ferrua et al., 1979), 1,1'-carbonyldiimidazole (CDI) (Bethel et al., 1979), tresyl chloride (Nilsson et al., 1981), 2-fluoro-1-methylpyridinium toluene-4-sulfonate (FMP) (Ngo, 1986) or epichlorohydrin (Matsumoto et al., 1979). The reagents activate the support matrix, and this step is followed by direct or indirect attachment of the antibody.
Generally antibodies are covalently coupled to the solid supports through ε amino groups. This method of coupling has several inherent problems associated with it. Since the antibody molecule has many lysine groups present on the surface, multisite attachment is unavoidable. For the same reason multiple orientations frequently occur during immobilization and as a consequence of both these processes the antibody loses its activity (Cuatrecasas, 1970). Some orientations of the antibody molecule may result in an active antibody that physically cannot bind an antigen, while other orientations of the antibody molecule may result in an inactive antibody due to a covalent bond at or near the antigen binding site. Random coupling results in an immunosorbent with low specific activity, generally in the order of 1 to 30% of the theoretical binding efficiency (Cress and Ngo, 1989; Brizgys et al., 1988). Multisite attachment is further enhanced due to the availability of excess active groups on most of the commercially available support matrices.

The support matrix of an immunosorbent is one of the most important components, and therefore, careful consideration must be given to the nature of the matrix which should have a number of desirable characteristics. The matrix should be: open and have a loose porous network; composed of rigid spherical beads of uniform porosity and size; easily derivatized; and mechanically and chemically stable (Cuatrecasas et al., 1968; Cuatrecasas and Anfinisen, 1971; Lowe and Dean, 1974). The linkage between the support matrix and the immobilized antibody has a significant effect on the properties of the resulting immunosorbent. The linkage depends on the method of matrix activation and the functional group of the antibody used for coupling. Despite its popularity, the CNBr method of activation has several drawbacks. For example, coupling of amines to activated polysaccharides introduces N-substituted isourea bonds that are not completely stable, particularly in the presence of nucleophiles (Wilchek et al., 1975). The isoureas are positively charged at physiological pH (pK_a = 9.4), and as a result such immunosorbents
exhibit ion exchange properties that can give rise to non-specific interactions. In contrast, the reaction of CDI and N-hydroxysuccinimide activated support matrices with the amino groups give stable N-alkylcarbamate (urethane) linkage, devoid of additional charge groups over the wide pH range (ca. pH 2-10), normally used in affinity chromatography (Bethell et al., 1979). Similarly the tresyl and FMP activated supports give stable non-ionic linkages with the coupling proteins.

The two most important parameters used to characterize an immunosorbent are the coupling efficiency and the specific activity. The coupling efficiency is defined as the percentage of the ratio of the amount of antibody coupled to the amount of antibody offered. The specific activity is defined as, the number of moles of antigen bound per mole of antibody coupled expressed as a percentage. From either an analytical or a commercial standpoint, the usefulness of an immunosorbent will be determined by the values of these two parameters.

Studies have reported that the amount of antibody immobilized on a support matrix will affect the specific activity of the resulting immunosorbent. Immobilization of polyclonal antibodies to estradiol on CNBr activated Sepharose 4B at different protein:matrix mass ratios resulted in a loss of immunoreactivity from 10 to 95 percent as the mass ratio was increased (Comoglio et al., 1976). Similar experiments using constant amounts of goat anti-human albumin antibodies and a fixed volume of Sepharose 4B, activated to different levels by CNBr have shown immunosorbents with 21 to 54 percent specific activity with decreasing levels of activation (Eveleigh and Levy, 1977). When the level of activation was constant and the amount of protein available for coupling was decreased, the specific activity increased from 30 to 42 % (Eveleigh and Levy, 1977). In a comparative study of Sepharose and Cellulose as supports for immobilization of antibody and antigen, it was found that above 3 to 4 mg of antibody per gram of Sepharose,
additional bound IgG was inactive (Weston and Scorer, 1977). A similar effect was observed for polyclonal antibodies to bovine serum albumin (Sada et al. 1986). In contrast, the steric hindrance due to high antibody density did not appear to be a significant problem when monoclonal antibodies to asparagine synthetase were immobilized on Affi Gel 10. The observed difference in antigen binding efficiency between the highest (13.5 mg antibody per ml of gel) and the lowest (0.6 mg antibody per ml of gel) concentrations was less than 5% (Pfeiffer et al., 1987). According to these authors the most important factor influencing the antigen binding capacity is the extent to which each antibody molecule is coupled to the resin. Cuatrecasas (1970) and Goding (1983) have also suggested that the extent of coupling (multipoint attachment) is an important factor influencing the antigen binding capacity of immunosorbents.

The pH of the coupling reaction is an important factor to be considered in covalent immobilization of antibodies to activated support matrices. Many reported procedures recommend the coupling of antibody to activated support matrix at pH 8 to 9.5 (Bethell et al., 1981; Dean et al., 1985). However, it has been found that sheep anti-porcine insulin antibodies retained the insulin binding activity when coupled at pH 6.5, but lost almost all the binding capacity when coupled at pH 9.5 to CNBr activated agarose (Cuatrecasas, 1970). The conclusion of this study was that at high pH, multipoint attachment was favored and as a result the antibody lost the tertiary structure necessary for optimum antigen-antibody interactions. In contrast to these findings, Pfeiffer et al. (1987) have reported that the antigen binding capacity of monoclonal antibodies to asparagine synthetase has been partially retained when immobilized at high pH (ca. 8.7).

In order to eliminate some of the problems associated with random (stochastic) coupling of antibodies, two approaches have been used to immobilize the antibody or its fragment with well defined orientations. In one approach F(ab') fragments of antibodies...
have been immobilized on support matrices. The monovalent F(ab') fragments are obtained by the reduction of the inter heavy chain disulfide bonds, in the hinge region of the F(ab')_2 fragment of the antibody (Nissonoff et al., 1960; 1961). It is known that the F(ab') fragments retain immunoreactivity (Nissonoff et al., 1960; 1961). The reduction of the disulfide bonds introduces one or more thiol groups in the C-terminal region of the F(ab') fragment, depending on the subisotype and the host species of the antibody. Therefore the F(ab') fragments can be immobilized on an appropriately activated support matrix through these thiol groups. Since the number of thiol groups is limited and they are located distal to the antigen binding site, the immunosorbents prepared by this method of immobilization would be expected to exhibit high specific activity. This approach has been used to immobilize F(ab') fragments on liposomes (Martin et al., 1981), small monolayer vesicles (Laserman et al., 1980), and thiolated cellulose (Skvortsov and Gurvich, 1984). Immobilization in these cases was accomplished through the formation of disulfide bridge between the support and the F(ab') fragment. Even though the coupling reaction is highly efficient, the immunosorbents prepared by this method are not stable under reducing conditions. However, stable immunosorbents could be prepared by using activated support matrices possessing maleimide or iodoacetamide functionalities. Both these groups react specifically with thiol groups forming a stable covalent thioether bond. This approach has been used to prepare immunosorbents with moderately high specific activity (ca. 57%) by immobilizing rabbit anti-mouse F(ab') fragments (Prisyazhnoy et al., 1988).

The other method of oriented coupling is based on the immobilization of the antibody through the carbohydrate moiety in the Fc region. The carbohydrate moiety is in a domain of the antibody spatially separated from the antigen binding sites and is not involved in antigen binding. Therefore this method of coupling would be expected to give immunosorbents with high specific activity. Immobilization is carried out by the oxidation
of the antibody using periodate to generate aldehydes which can then be condensed with support matrices containing amino groups to produce a Schiff base. The Schiff base is subsequently stabilized by reduction with cyanoborohydride. An alternative to the use of amines is to use hydrazide containing supports which on reaction with aldehydes produce stable hydrazone bonds. Studies have shown that the immunosorbents prepared by oriented coupling using hydrazide supports have higher specific activity (ca. 54 %) compared to those prepared by random coupling using Affi Gel 10 (ca. 18 %) (Hoffman and O'Shannessy, 1988). Another study has reported that rabbit anti-human IgG antibodies immobilized on hydrazide gel have a specific activity of 29 % compared to 6 % for the same antibodies immobilized on activated carboxyl gel (Cress and Ngo, 1989).

The ideal immunosorbent would possess very high specific activity, stability and minimum non-specific interactions. Since there are many factors which influence the properties of the immunosorbent, careful consideration must be given to each of them to optimize the conditions to obtain an immunosorbent with ideal properties.

The purpose of the present study is to make a systematic comparison of the covalent coupling methods taking into account the support matrix, coupling chemistry and the nature of the immobilized protein. Previous reports have dealt with specific systems under specific reaction conditions, but it is extremely difficult to make comparisons between coupling modes developed from widely divergent protocols. In the present study the same antibody or its biologically active fragments are coupled to the same support derivatized with different reagents. The purpose of this approach is to examine the questions of controlled orientation, multisite attachment and antibody loading on the biological activity of the resulting immunosorbents.
3.2 Materials and Methods

3.2.1 Effect of the Coupling Reaction pH on Immobilization of IgG on CDI Activated HW 65 Gel (Reacti-Gel HW 65)

Immobilization of IgG was carried out in one of the following four coupling buffers: 0.1 M phosphate buffer (PB) pH 6.0, 7.0 and 8.0 or 0.1 M carbonate buffer pH 9.0. In each case, the activated support matrix Reacti-Gel HW 65 (Pierce, Rockford, IL) was washed and equilibrated with the buffer according to the manufacturer's instructions. Approximately 0.5-1.0 ml aliquots of the gel equilibrated with the coupling buffer were placed in glass test tubes (Fisher Scientific, Pittsburgh, PA) and centrifuged at 500 g for 5 min. Then the residual supernatants were carefully removed by aspiration. A 2.0 ml solution of affinity purified goat anti-mouse IgG (anti-serum prepared by immunizing goats with mouse IgG) (ca. 5 mg/ml) or goat anti-human IgG (anti-serum from Pel-Freeze Biologicals, Rogers, AR; purified in laboratory) (ca. 5 mg/ml) dialyzed against the appropriate coupling buffer was added to each tube containing the respective gel. The resulting gel slurry was tumbled end over end for 18 hrs at 4°C, and was transferred to a disposable chromatography column (Pierce, Rockford, IL). The columns were washed alternatively with 5 x 1 ml each of acetate buffer 0.1M, pH 4.5 and borate buffer 0.1M, pH 8.5, then with 1.0 ml of PB 0.1M, pH 2.2 and finally with 2.0 ml of PB 0.1 M, pH 7.4 to remove the unbound proteins. The washings were collected, pooled and assayed for the protein content using the BCA Protein Assay method (Pierce, Rockford, IL). The amount of protein coupled to the gel was determined by taking the difference between the amount of protein added and the amount of protein in the washings after the coupling reaction. For convenience, this method will be referred to as the method of difference. The columns were treated with 0.2 M Tris HCl, pH 9.0 for an additional 4 hrs at room temperature to block any unreacted active groups. Finally, the columns were washed with PB 0.1 M, pH 7.4 and stored in the same buffer until use.
3.2.2 Determination of the Volume of Immunosorbent

The volume of each immunosorbent was measured by carefully transferring the gel into a calibrated 10 ml disposable pipette (Fisher Scientific, Pittsburgh, PA). The end of the pipette was plugged with glass wool to retain the gel. Residual buffer in the gel slurry was removed by applying 20 psi nitrogen pressure on the top of the pipette. The volume was read directly from the calibrated scale. Alternatively the volume of the immunosorbent was determined from the geometric dimensions of the column.

3.2.3 Study of the Kinetics of Immobilization of IgG

Immobilization was carried out either in 0.1 M PB pH 6.0 or in 0.1 M carbonate buffer pH 9.0. Coupling of the antibody (affinity purified goat anti-mouse or goat anti-human IgG) to the support matrix (Reacti-Gel HW 65) was carried out essentially as described in the section 3.2.1. After a specified coupling time, 50 μl of supernatant was withdrawn from each tube by centrifuging the tubes at 500 g for 5 min. This 50 μl sample was then added to 450 μl of 0.1 M PB pH 7.4 and assayed for the protein content by the BCA Protein Assay method. Samples were withdrawn at times 0.5, 1, 2, 9, 12, and 18 hrs from the start of the coupling reaction. The amount of protein coupled after a specified coupling time was determined by the method of difference.

3.2.4 Effect of Coupling Time on the Specific Activity of the Immunosorbent

Coupling of each antibody (affinity purified, goat anti-mouse IgG or goat anti-human IgG) to Reacti-Gel HW 65 was carried out in two different coupling buffers (0.1 M PB, pH 6.0 and 0.1 M carbonate, pH 9.0) as described in section 3.2.1. The coupling
reaction was terminated after 0.5, 2.0 and 18 hrs by the addition of 0.5 ml of 2 M ethylenediamine (Fisher Scientific, Pittsburgh, PA) (pH adjusted to 9.75) to each reaction mixture and mixing for an additional 6 hrs. The slurries were then transferred to disposable chromatography columns and washed as described above. The volume of the immunosorbents and the amount of antibody coupled were determined as described in section 3.2.1.

3.2.4.1 Determination of the Specific Activity of the Immunosorbents

In order to determine the specific activity of the immunosorbents the following procedure was used: First, immunosorbents packed in disposable chromatography columns were washed with 1.0 ml of 0.1 M PB, pH 2.2 and reequilibrated with 0.1 M PB pH 7.4. Then 1.0 ml aliquots of either affinity purified mouse IgG (mouse IgG from American Qualex Inc., La Mirada, CA; purified in laboratory) (10 mg/ml) or human IgG (Gammastan, Cutter Biologicals, Division of Miles Inc., West Haven, CT) (10 mg/ml) γ globulin fraction free of glycine was applied to the respective immunosorbent and allowed to pass through the columns until the immunosorbent was completely covered with antigen solution. Then the columns were capped and allowed to equilibrate for 1 hr at room temperature. Next, the columns were washed with 15 ml of 0.1 M PB, pH 7.4 to remove all the unbound proteins as confirmed by the absence of absorbance at 280 nm. The bound antigens were eluted with 1.0 ml of 0.1 M PB, pH 2.2 followed by 4.0 ml of 0.1 M PB, pH 7.4 and the final volumes of the eluates were adjusted to a known volume with the latter buffer. The amount of antigen bound to each immunosorbent was determined by measuring the absorbance of the respective eluates at 280 nm. The volumes of the immunosorbents were determined as described in section 3.2.1. In order to establish that the antibody columns were saturated, pilot experiments were carried out by varying the
concentration of the antigen solution and the incubation time. The antigen concentration of 10 mg/ml and the incubation time of 1 hr were found to be the optimum conditions.

3.2.5 Preparation of Iodoacetamide Gel

A 10 ml sample of Reacti-Gel HW 65 was transferred to a sintered glass funnel and washed according to the manufacturer’s instructions to remove the acetone. Immediately prior to use, the gel was washed with 0.1 M carbonate buffer, pH 9.0, drained and rapidly transferred to 20 ml of 0.5 M ethylenediamine the pH of which was adjusted to 9.75 with 5 M HCl. The resulting gel slurry was mixed end over end for 8 hrs at room temperature and then transferred to a sintered glass funnel to wash off the unreacted material. The gel was washed with 0.1 M PB, pH 7.4 until the washings were free from amines as confirmed by the absence of reaction with 2,4,6 trinitrophenylsulfonic acid (Sigma, St. Louis, MO). Then the gel was drained and transferred to 20 ml of a solution, composed of 0.1 M iodoacetic acid (Sigma, St. Louis, MO), 0.1 M 1-Ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride (Sigma, St. Louis, MO) and 50 mM N-hydroxysulfosuccinimide (Pierce, Rockford, IL). This mixture was tumbled end over end for 24 hrs at room temperature. The gel was then washed as described above with 200 ml of 0.1 M PB, pH 7.4 to remove all the unbound materials and stored in the same buffer at 4°C until use.

3.2.5.1 Determination of the activity of Iodoacetamide Gel

A 2 ml solution of 2-mercaptoethylamine (MEA) in Tris-buffer (50 mM Tris aminomethane, 1 mM EDTA, 0.01% NaN₃, pH adjusted to 8.5 with 5 M HCl) was coupled to approximately 0.5 ml of iodoacetamide gel as described in section 3.2.1. After 6 hrs of coupling at room temperature the gel slurry was carefully transferred to a
disposable column and washed with 15 ml of the coupling buffer to remove the unreacted material. The amount of MEA coupled was determined by the method of difference. Quantitation of MEA was carried out as described elsewhere, using 4,4'-dithiopyridine (Aldrich, Milwaukee, WI) as the assay reagent (Grassetti and Murray, 1967). Standard solutions of MEA for the calibration curve were prepared in the Tris-buffer. The volume of the gel was determined by the two methods described above.

3.2.6 Preparation of F(ab')

Affinity purified goat anti-mouse and goat anti-human F(ab')2 (ca. 8 mg/ml) were dialyzed against 0.1 M PB (pH 6.0, 1 mM EDTA) for 16 hrs. The F(ab')2 was then reduced by the addition of 50 μl of 0.2 M MEA to 1 ml of each F(ab')2 solution. The reaction mixtures were incubated at 37°C for 90 min. Resulting F(ab') was then separated from excess reducing agent by passing the reaction mixture through a Sephadex G-25 column (1 cm x 30 cm) equilibrated with Tris buffer (50 mM, Tris-aminomethane, 1 mM EDTA, pH adjusted to 8.5 with 5 M HCl) at 1.0 ml/min. After separation, the concentration of F(ab') was determined from the absorbance at 280 nm, assuming a value of 14.2 for ε1%280.

3.2.7 Immobilization of F(ab') on Activated Iodoacetamide Gel

The method of immobilization of F(ab') on iodoacetamide gel was essentially the same as that of IgG as described in section 3.2.1 with the following modifications. Coupling of F(ab') was carried out in the Tris buffer for 6 hrs at 4°C. Both goat anti mouse and goat anti human F(ab') were used to prepare the immunosorbents. A 4.0 ml aliquot of F(ab') solution was used with approximately 0.5 ml of the gel. In order to obtain various antibody F(ab') densities on the support matrix, different amounts of F(ab')
were reacted with a fixed volume of the gel. The procedure described above was used to
determine the specific activity of the resulting immunosorbents. The amount of F(ab')
coupled and the volume of the resulting immunosorbents were determined as described
above.

3.2.7.1 Kinetic Studies of Immobilization of F(ab')

This procedure was the same as that in section 3.2.3 with the following
modifications. A solution of F(ab') was used instead of IgG and the coupling was carried
out for 6 hrs at 4°C. Samples were withdrawn at times 0.5, 1.0, 2.0, 4.0 and 6 hrs after
initiating the coupling reaction.

3.2.8 Preparation of Hydrazide Gel

Reacti-Gel HW 65 (10 ml) was equilibrated with 0.1 M PB, pH 8.0 as described in
section 3.2.1. Then a 20 ml solution of 0.5 M adipic dihydrazide (Aldrich, Milwaukee,
WI), pH 7.0 (prepared in 0.1 M PB, pH 7.4) was added to the gel and tumbled end over
end for 6 hrs at room temperature. The gel was then transferred to a sintered glass funnel
and washed with 0.1 M PB, pH 7.4 until the eluent was free of adipic dihydrazide as
confirmed by the absence of reaction with 2,4,6 trinitrophenylsulfonic acid. The gel was
then stored in the same buffer at 4°C until use.

3.2.8.1 Oxidation of IgG

Affinity purified goat anti-mouse IgG and goat anti-human IgG were dialyzed
overnight at 4°C against acetate buffer (0.1 M sodium acetate / acetic acid, 0.15 M NaCl,
0.01% NaN₃, pH5.5). The antibodies were then oxidized by the addition of 50 μl and
82.5 μl of 0.2 M sodium metaperiodate to 1 ml of goat anti-human IgG (13.1 mg/ml) and
1.65 ml of goat anti-mouse IgG (15.7 mg/ml) respectively. Oxidation was carried out at room temperature in the dark for 30 min. The oxidized antibodies were then passed through a Sephadex G-25 column (1 cm x 30 cm) equilibrated with the acetate buffer at 1 ml/min to remove the excess periodate and non-protein associated aldehydes. After separation, the concentration of oxidized antibodies was determined from the absorbance at 280 nm, assuming a value of 14.2 for \( \varepsilon_{1\%}^{280} \).

### 3.2.8.2 Immobilization of Oxidized IgG

Immobilization of oxidized IgG on hydrazide gel was carried out as described for IgG in section 3.2.1 with the following modifications: Coupling of oxidized IgG was carried out in the acetate buffer for 6 hrs at room temperature. A solution of 4.0 ml of oxidized IgG (affinity purified, goat anti-human IgG or goat anti-mouse IgG) was used with approximately 0.5 ml of the gel. In order to obtain different antibody densities on the support matrix, different amounts of oxidized IgG were reacted with a fixed volume of the gel. Amounts of oxidized IgG coupled, the specific activity and the volume of each immunosorbent were determined using the procedures described above.

### 3.3 Results and Discussion

In the preparation of immunosorbents the pH of the coupling reaction has been shown to be an important factor affecting the coupling efficiency (Cuatrecasas, 1970) and the specific activity of immunosorbents (Cuatrecasas, 1970; Matson and Little, 1988). In order to investigate the effect of the pH on immobilization of antibodies to Reacti-Gel HW 65, experiments were carried out with affinity purified goat anti-mouse IgG and goat anti-human IgG. The results are shown in Figure 3.1. As can be seen, the coupling efficiencies of the two antibodies after 18 hrs of reaction at 4 °C were around 85-95% over
Figure 3.1. Effect of pH on the Immobilization of Antibody on Reacti-Gel HW 65

Coupling Efficiency

<table>
<thead>
<tr>
<th>Coupling Efficiency</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat anti-mouse IgG</td>
<td>Goat anti-mouse IgG</td>
</tr>
<tr>
<td>Goat anti-human IgG</td>
<td>Goat anti-human IgG</td>
</tr>
</tbody>
</table>
the entire pH range (pH 6-9) under investigation. Coupling of antibody to Reacti-Gel HW 65 is mainly accomplished through the ε-amino groups (pKₐ = 10.3) of the antibody. Therefore, one would expect to see a low coupling efficiency at a lower pH (ca. 6-7) compared to that at a higher pH (ca. 8-9). But the overall coupling efficiency depends on two competing events: rate of coupling of the antibody and the rate of hydrolysis of the active groups on the support matrix. The results of the experiment suggest that the antibody can be coupled to Reacti-Gel HW 65 over a wide pH range (ca. 6-9) with a high coupling efficiency (> 85%). Similar observations have been made by Pfeiffer et al. (1987) and Matson and Little (1988) for random immobilization of monoclonal antibodies to CNBr activated agarose and Affi-Gel 10 (N-hydroxysuccinimide) respectively. The observed results could be due to the presence of 60-80 amino groups on the surface of the antibody molecule and the availability of a large excess of reactive groups on the support matrix. These would compete with one another to give a high overall coupling efficiency over a wide pH range.

As depicted in Figure 3.1, the specific activity of the immunosorbents did not change significantly with the pH of the coupling buffer. The specific activity of immobilized goat anti-mouse IgG varied from 30% at pH 6.0 to 27% at pH 9.0. The corresponding values for immobilized goat anti-human IgG were 22% to 20%. Pfeiffer et al. (1987) has also reported that the pH of the coupling buffer has no significant effect on the specific activity of the resulting immunosorbents prepared by random coupling of monoclonal antibodies. In contrast it has been reported that immunosorbents prepared by stochastic coupling of antibodies at high pH (ca. 8.5) have lost almost 100% of their specific activity (Cuatrecasas, 1970; Matson and Little, 1988). Even though the specific activity of immunosorbents was not sensitive to the pH of the coupling buffer the observed values were low (ca. 20-30%). This could be due to either multisite attachment
and multiple orientations or steric hindrance as a result of crowding of the antibody. The size of the antigen can also impose steric hindrance which, in turn, would give low specific activity.

In order to illustrate the hypothesis that the multisite attachment causes a decrease in the specific activity, the effect of the coupling time on the specific activity of the immunosorbents was investigated as described in section 3.2.4.1. The results of these experiments are shown in Table 3.1 and Table 3.2 for goat anti-mouse IgG and goat anti-human IgG respectively. As shown, the specific activity of the goat anti-mouse immunosorbents decreased from 37% to 31% as the time of coupling increased from 0.5 to 18 hrs at pH 6.0. When the coupling was carried out at pH 9.0, the specific activity decreased from 37% to 27% for the same coupling times. Similar results were observed for the goat anti-human immunosorbents as shown in Table 3.2. The specific activity varied from 29% to 22% at pH 6.0 and from 27% to 20% at pH 9.0 as the time increased from 0.5 to 18 hrs. It is evident from the results that the specific activity of the immunosorbents decreases as the extent of the coupling reaction increases. Hence, the results of the experiment support the above stated hypothesis, that the multisite attachment causes a decrease in the specific activity of the immunosorbents as the extent of the coupling reaction increases. However, the decrease in specific activity (ca. 7-10%) with the increase in coupling time (from 0.5 to 18 hrs) is very small, compared to the loss of activity (ca. 63-73%) during the first 0.5 hrs of the coupling reaction.

Since it was not possible to explain why the immunosorbents have lost nearly 63 - 73% of their specific activity even after 0.5 hrs of coupling, the kinetics of the coupling reaction were studied. Coupling of antibodies to Reacti-Gel HW 65 was carried out at 4°C in pH 6.0 and pH 9.0 coupling buffers. The results shown in Figure 3.2 illustrate that the efficiency of the coupling is independent of the pH of the coupling buffer. In the first
Table 3.1

Effect of Coupling Time on the Specific Activity of Immobilized Goat anti-mouse IgG

Experimental details in section 3.2.4

<table>
<thead>
<tr>
<th>Experiment number</th>
<th>Coupling time (hr)</th>
<th>Antibody Coupled (mg/ml of gel)</th>
<th>Volume of gel (ml)</th>
<th>Antigen bound (mg)</th>
<th>Specific activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.5</td>
<td>11.22</td>
<td>0.49</td>
<td>1.64</td>
<td>37</td>
</tr>
<tr>
<td>2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.0</td>
<td>12.20</td>
<td>0.35</td>
<td>1.57</td>
<td>37</td>
</tr>
<tr>
<td>3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.0</td>
<td>13.87</td>
<td>0.33</td>
<td>1.40</td>
<td>31</td>
</tr>
<tr>
<td>4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.5</td>
<td>12.72</td>
<td>0.31</td>
<td>1.46</td>
<td>37</td>
</tr>
<tr>
<td>5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.0</td>
<td>13.13</td>
<td>0.35</td>
<td>1.47</td>
<td>32</td>
</tr>
<tr>
<td>6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.0</td>
<td>14.51</td>
<td>0.29</td>
<td>1.13</td>
<td>27</td>
</tr>
</tbody>
</table>

<sup>a</sup> 0.1 M PB pH 6.04

<sup>b</sup> 0.1 M carbonate pH 9.05

<sup>c</sup> defined in text (average of 3 determinations)
Table 3.2

Effect of the Coupling Time on the Specific Activity of Immobilized Goat anti-Human IgG

Experimental details in section 3.2.4

<table>
<thead>
<tr>
<th>Experiment number</th>
<th>Coupling time (hr)</th>
<th>Antibody coupled (mg/ml of gel)</th>
<th>Volume of gel (ml)</th>
<th>Antigen bound (mg)</th>
<th>Specific activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.5</td>
<td>11.61</td>
<td>0.60</td>
<td>2.05</td>
<td>29</td>
</tr>
<tr>
<td>2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.0</td>
<td>12.98</td>
<td>0.60</td>
<td>2.18</td>
<td>28</td>
</tr>
<tr>
<td>3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.0</td>
<td>14.86</td>
<td>0.60</td>
<td>1.97</td>
<td>22</td>
</tr>
<tr>
<td>4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.5</td>
<td>12.76</td>
<td>0.55</td>
<td>1.90</td>
<td>27</td>
</tr>
<tr>
<td>5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.0</td>
<td>13.90</td>
<td>0.55</td>
<td>1.85</td>
<td>24</td>
</tr>
<tr>
<td>6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.0</td>
<td>15.27</td>
<td>0.55</td>
<td>1.70</td>
<td>20</td>
</tr>
</tbody>
</table>

<sup>a</sup> 0.1 M PB pH 6.04

<sup>b</sup> 0.1 M carbonate pH 9.05

<sup>c</sup> defined in text (average of 3 determinations)
Figure 3.2. Time course of Immobilization of Antibody to Reacti-Gel HW65

- Goat anti-mouse IgG pH 6.04
- Goat anti-mouse IgG pH 9.05
- Goat anti-human IgG pH 6.04
- Goat anti-human IgG pH 9.05
0.5 hr. of the reaction, about 70% of the antibody was coupled to the support matrix. During the rest of the time the coupling efficiency increased slowly and reached an upper value of 90-95% after 18 hrs. Immediately after an antibody molecule is coupled to the support matrix, it can undergo multisite attachment, because of the proximity of the molecule to the surface of the gel and the availability of excess reactive groups on the support matrix as well as on the antibody molecule. Further the rate of the multisite attachment would be highest in the beginning of the reaction because of the high concentrations of the reactive groups. Since about 70% of the antibody was coupled to the support matrix in the first 0.5 hrs of the reaction it is reasonable to assume that the multisite attachment would be critical and unavoidable even in this time frame. Thus the multisite attachment and multiple orientations is one of the main reasons for the observed low specific activity of the immunosorbents (ca. 37% for goat anti-mouse IgG - Table 3.1 and 27-29% for goat anti-human IgG - Table 3.2) prepared by coupling of antibodies for 0.5 hrs. Apart from this, crowding of the antibody can also be a significant factor affecting the specific activity.

Immobilization of monovalent F(ab') fragments of an antibody through the hinge region sulfhydryl groups theoretically should produce immunosorbents with high specific activity. The method of immobilization does not permit multisite attachment. It further results in defined orientation of the F(ab') fragment leaving the antigen binding site unobstructed. However, the immobilization of goat anti-mouse F(ab') fragments on iodoacetamide activated HW 65 gel has produced immunosorbents with low specific activity (ca. 26-30%). The F(ab') density (amount of F(ab') per ml of gel) on the support matrix of these immunosorbents were approximately 7-10 mg/ml of gel. This observation has led us to hypothesize that steric hindrance due to the crowding of the immobilized F(ab') fragment causes a loss of specific activity of the immunosorbents. In order to
illustrate this hypothesis the effect of the F(ab') density on the specific activity of the immunosorbents was investigated. The coupling efficiencies of the immunosorbents prepared by the immobilization of goat anti-mouse F(ab') are shown in Table 3.3. These results show that the coupling efficiency increases from 49% to 66% as the amount of F(ab') applied to the gel decreases from 4.50 mg to 1.91 mg. This suggests that the amounts of F(ab') initially offered to the gel exceeded the number of available iodoacetamide groups. Results for the immobilization of goat anti-human F(ab') shown in Table 3.4 were similar. The coupling efficiency increases from 50% to 67% as the concentration of F(ab') decreases from 5.0 mg per ml to 0.94 mg per ml. The coupling efficiencies observed for the coupling of F(ab') to iodoacetamide gel were lower than the values reported for the coupling of either half molecule of human IgG (prepared by reduction) or human F(ab') (ca. 90%) (Domen et al., 1990). The observed high efficiency could be due to the availability of more than one sulfhydryl groups for the coupling of the reduced human IgG or F(ab') as compared to only one in F(ab') of goat IgG. The same authors have done experiments with reduced rabbit anti-HSA IgG which has one sulfhydryl group, but the coupling efficiencies were not reported making a valid comparison impossible. The specific activity of the immunosorbents prepared with different F(ab') densities are shown in Table 3.5. As can be seen, the specific activity of the immunosorbents dramatically increase from 37% to 78% as the density of F(ab') decreases from 3.98 mg/ml of gel to 1.05 mg/ml of gel. Similar results were observed for the goat anti-human F(ab') immunosorbents. As shown in Table 3.6, the specific activity of the immunosorbents increases from 25% to 42% as the density decreases from 4.54 to 1.14 mg per ml of gel. These observations clearly support the above hypothesis that the steric hindrance due to the crowding of the F(ab') fragments causes a decrease in specific activity of the immunosorbents. A similar effect has been observed for the immobilization
### Table 3.3

**Coupling Efficiency of Goat anti-Mouse F(ab') on Iodoacetamide HW 65 Gel**

Experimental details in section 3.2.7

<table>
<thead>
<tr>
<th>Amount of F(ab') added (mg)</th>
<th>Amount coupled (mg)</th>
<th>Volume of gel (ml)</th>
<th>Coupling efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.50</td>
<td>2.19</td>
<td>0.55</td>
<td>49</td>
</tr>
<tr>
<td>3.83</td>
<td>1.99</td>
<td>0.55</td>
<td>52</td>
</tr>
<tr>
<td>1.91</td>
<td>1.27</td>
<td>0.55</td>
<td>66</td>
</tr>
<tr>
<td>0.96</td>
<td>0.58</td>
<td>0.55</td>
<td>61</td>
</tr>
</tbody>
</table>

*a* defined in the text (average of 2 determinations)
Table 3.4
Coupling Efficiency of Goat anti-Human F(ab') on Iodoacetamide HW 65 Gel
Experimental details in section 3.2.7

<table>
<thead>
<tr>
<th>Amount of F(ab') added (mg)</th>
<th>Amount coupled (mg)</th>
<th>Volume of gel (ml)</th>
<th>Coupling efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.00</td>
<td>2.50</td>
<td>0.55</td>
<td>50</td>
</tr>
<tr>
<td>3.75</td>
<td>1.89</td>
<td>0.55</td>
<td>50</td>
</tr>
<tr>
<td>1.25</td>
<td>0.78</td>
<td>0.55</td>
<td>62</td>
</tr>
<tr>
<td>0.94</td>
<td>0.63</td>
<td>0.55</td>
<td>67</td>
</tr>
</tbody>
</table>

a defined in the text (average of 2 determinations)
Table 3.5

Effect of F(ab') Density on the Specific Activity of Immobilized Goat anti-Mouse F(ab')

Experimental details in section 3.2.7

<table>
<thead>
<tr>
<th>Density of F(ab') (mg/ml of gel)</th>
<th>Volume of gel (ml)</th>
<th>Amount of antigen bound (mg)</th>
<th>Specific activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.98</td>
<td>0.55</td>
<td>2.82</td>
<td>37</td>
</tr>
<tr>
<td>3.61</td>
<td>0.55</td>
<td>2.44</td>
<td>35</td>
</tr>
<tr>
<td>2.30</td>
<td>0.55</td>
<td>2.00</td>
<td>45</td>
</tr>
<tr>
<td>1.05</td>
<td>0.55</td>
<td>1.57</td>
<td>78</td>
</tr>
</tbody>
</table>

*a* defined in the text (average of 3 determinations)

*b* M.W. of goat anti-mouse F(ab') 46,000 and mouse IgG 160,000
### Table 3.6

**Effect of F(ab') density on the specific activity of Immobilized Goat anti-Human F(ab')**

Experimental details described in section 3.2.7

<table>
<thead>
<tr>
<th>Density of F(ab') (mg/ml of gel)</th>
<th>Volume of gel (ml)</th>
<th>Amount of antigen bound (mg)</th>
<th>Specific activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.54</td>
<td>0.55</td>
<td>2.20</td>
<td>25</td>
</tr>
<tr>
<td>3.43</td>
<td>0.55</td>
<td>1.81</td>
<td>28</td>
</tr>
<tr>
<td>1.42</td>
<td>0.55</td>
<td>1.08</td>
<td>40</td>
</tr>
<tr>
<td>1.14</td>
<td>0.55</td>
<td>0.92</td>
<td>42</td>
</tr>
</tbody>
</table>

*a* defined in the text (average of 3 determinations)

*b* M.W. of goat anti-human F(ab') 46,000 and human IgG 160,000
of intact antibodies on different support matrices (Nakamura et al., 1990; Fleminger et al., 1990; Hadas et al., 1990; Comoglia et al., 1976; Eveleigh and Levy, 1977; Matson and Little, 1988). To the best of our knowledge, there have been no previous reports on the effect of the F(ab') density on the specific activity. Yet the specific activity of the F(ab') immunosorbents could not be improved to a value closer to 100%. This could be due to the large size of the antigen (mouse IgG or human IgG) which would impose steric hindrance even at very low F(ab') densities.

Immobilization of oxidized IgG on hydrazide HW 65 gel also should give high specific activity because the method of coupling involves oriented immobilization. The immobilization is accomplished through the aldehyde groups created by oxidation of the carbohydrate moiety in the Fc region. Thus the site of immobilization is distal from the antigen binding site. On the contrary the immobilization of antibodies (goat anti-human and goat anti-mouse) by this method gave low specific activity (ca. 30%). The antibody density was approximately 6-7 mg/ml of gel. Cress and Ngo (1989) have also reported specific activity of 16-30% for similar antibody-antigen system at approximately the same antibody density. These observations have suggested that the steric hindrance due to crowding of the antibody would be the reason for the low specific activity. In order to investigate this phenomenon experiments were carried out with different antibody densities. Coupling efficiencies observed for oxidized goat anti-mouse IgG and oxidized goat anti-human IgG are shown in Table 3.7 and Table 3.8. As can be seen the coupling efficiency again increases (ca. 47-71%) with decreasing amounts of oxidized IgG (ca. 9-1 mg). This observation tends to suggest that the amounts of oxidized IgG offered to hydrazide gel were closer to the amount required to saturate the gel. However, the coupling efficiencies are in agreement with those reported by others (Cress and Ngo, 1989). The specific activity of the immunosorbents are shown in Table 3.9 and Table 3.10 for
Table 3.7

Coupling Efficiency of Oxidized Goat anti-mouse IgG on Hydrazide HW 65 Gel

Experimental details described in section 3.2.8.2

<table>
<thead>
<tr>
<th>Amount of antibody added (mg)</th>
<th>Amount coupled (mg)</th>
<th>Volume of gel (ml)</th>
<th>Coupling(^a) efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.2</td>
<td>4.80</td>
<td>0.70</td>
<td>52</td>
</tr>
<tr>
<td>4.6</td>
<td>2.92</td>
<td>0.70</td>
<td>63</td>
</tr>
<tr>
<td>2.3</td>
<td>1.58</td>
<td>0.70</td>
<td>69</td>
</tr>
</tbody>
</table>

\(^a\) defined in the text (average of 2 determinations)
### Table 3.8

**Coupling Efficiency of Oxidized Goat anti-human IgG on Hydrazide HW 65 Gel**

Experimental details described in section 3.2.8.2

<table>
<thead>
<tr>
<th>Amount of antibody added (mg)</th>
<th>Amount coupled (mg)</th>
<th>Volume of gel (ml)</th>
<th>Coupling(^a) efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.74</td>
<td>3.50</td>
<td>0.88</td>
<td>47</td>
</tr>
<tr>
<td>4.46</td>
<td>2.36</td>
<td>0.88</td>
<td>53</td>
</tr>
<tr>
<td>0.93</td>
<td>0.66</td>
<td>0.88</td>
<td>71</td>
</tr>
</tbody>
</table>

\(^a\) defined in the text (average of 2 determinations)
Table 3.9
Effect of Antibody density on the Specific Activity of Immobilized Goat anti-Mouse IgG
Experimental details described in section 3.2.8.2

<table>
<thead>
<tr>
<th>Antibody density (mg/ml)</th>
<th>Volume of gel (ml)</th>
<th>Amount of antigen bound (mg)</th>
<th>Specific activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.48</td>
<td>0.70</td>
<td>1.37</td>
<td>29</td>
</tr>
<tr>
<td>3.31</td>
<td>0.70</td>
<td>1.05</td>
<td>36</td>
</tr>
<tr>
<td>1.79</td>
<td>0.70</td>
<td>0.81</td>
<td>51</td>
</tr>
</tbody>
</table>

\( a \) defined in the text (average of 3 determinations)

\( b \) M.W. of oxidized goat anti-mouse IgG and mouse IgG = 160,000
Table 3.10

Effect of Antibody density on the Specific Activity of Immobilized Oxidized Goat anti-human IgG

Experimental details described in section 3.2.8.2

<table>
<thead>
<tr>
<th>Antibody density (mg/ml)</th>
<th>Volume of gel (ml)</th>
<th>Amount of antigen bound (mg)</th>
<th>Specifica,b activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.97</td>
<td>0.88</td>
<td>0.81</td>
<td>23</td>
</tr>
<tr>
<td>2.68</td>
<td>0.88</td>
<td>0.61</td>
<td>26</td>
</tr>
<tr>
<td>0.75</td>
<td>0.88</td>
<td>0.31</td>
<td>47</td>
</tr>
</tbody>
</table>

a defined in the text (average of 3 determinations)
b M.W. of oxidized goat anti-human IgG and human IgG = 160,000
immobilized goat anti-mouse IgG and goat anti-human IgG respectively. As depicted in Table 3.9 the specific activity has increased from 29% to 51% as the density decreases from 6.48 mg/ml to 1.79 mg/ml. Similarly for goat anti-human IgG the specific activity has increased from 23% to 47% when the antibody density decreased from 3.97 mg/ml to 0.75 ng/ml (Table 3.10). These observations conclude that the density of antibody on the support matrix is a dominant factor that determines the specific activity of the immunosorbent.

In order to illustrate the effect of steric hindrance imposed by the size of the antigen experiments were carried out with human F(ab') (M.W. 60,000) as the antigen for immunosorbents prepared from goat anti-human F(ab'). As shown in Table 3.11 the specific activity of the immunosorbents has increased from 39% to 100% when the density decreased from 4.54 to 1.14 mg/ml of gel. This clearly confirms that the site directed immobilization of F(ab') does not result in loss of immunological activity (specific activity 100% for 1.14 mg/ml F(ab') density). The observed loss of specific activity at high densities is either due to steric hindrance caused by crowding of the F(ab') itself or to the size of the antigen.

A study of the effect of IgG density on specific activity of immunosorbents prepared by random coupling of antibodies has shown that the specific activity has not increased as expected when the density of IgG decreased (Table 3.12, specific activity ca.20% for all densities studied). The observed behavior can be explained by taking into account, the effect of three factors that govern the specific activity: multisite attachment, multiple orientation, and steric hindrance. As the antibody density increases the effect of multisite attachment and multiple orientation decreases, but steric hindrance increases. Conversely, the effect of multiple orientations and multisite attachment is dominant at low antibody densities. Therefore, it is reasonable to assume that multiple orientation and
Table 3.11

Effect of Size of Antigen on the Specific Activity of Immobilized Goat anti-Human F(ab')

Experimental details described in section 3.2.7 with human F(ab') as the antigen.

<table>
<thead>
<tr>
<th>Density of F(ab') (mg/ml of gel)</th>
<th>Volume of gel (ml)</th>
<th>Amount of antigen bound (mg)</th>
<th>Specific activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.54</td>
<td>0.55</td>
<td>1.26</td>
<td>39</td>
</tr>
<tr>
<td>3.43</td>
<td>0.55</td>
<td>1.10</td>
<td>45</td>
</tr>
<tr>
<td>1.42</td>
<td>0.55</td>
<td>0.87</td>
<td>86</td>
</tr>
<tr>
<td>1.14</td>
<td>0.55</td>
<td>0.82</td>
<td>100</td>
</tr>
</tbody>
</table>

a defined in the text (average of 3 determinations)

b M.W. of goat anti-human F(ab') 46,000 and human F(ab') 60,000.
Table 3.12
Effect of IgG Density on the Specific Activity of Immobilized Goat anti-Human IgG

Experimental details described in section 3.2.1 with different amounts of IgG at pH 6.0.

<table>
<thead>
<tr>
<th>Density of IgG (mg/ml of gel)</th>
<th>Volume of gel (ml)</th>
<th>Amount of antigen bound (mg)</th>
<th>Specific activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.18</td>
<td>0.60</td>
<td>1.43</td>
<td>21</td>
</tr>
<tr>
<td>5.26</td>
<td>0.60</td>
<td>0.66</td>
<td>21</td>
</tr>
<tr>
<td>3.50</td>
<td>0.60</td>
<td>0.39</td>
<td>19</td>
</tr>
<tr>
<td>1.70</td>
<td>0.60</td>
<td>0.19</td>
<td>18</td>
</tr>
</tbody>
</table>

*a defined in the text (average of 3 determinations)*
multisite attachment are the dominant factors in random immobilization. This is in agreement with the work of others (Cuatrecasas, 1970; Matson and Little, 1988).

From the present study it is possible to conclude that three factors are of importance for the preparation of immunosorbents with high quality. They are multisite attachment, multiple orientations and steric hindrance as a result of antibody density and the size of the antigen. In the preparation of immunosorbents by random coupling of antibodies all three of these factors govern the specific activity of the resulting immunosorbent. Therefore, depending on the availability of reagents and the cost of the support matrix, care must be taken to achieve the highest specific activity by maneuvering the above three factors. In the site directed immobilization the main factor that governs the specific activity is the crowding of the antibody. Therefore, this method is less complicated and thus easier to optimize. Depending on the size of the antigen the density of F(\(ab'\)) one can achieve almost 100% specific activity by the site directed immobilization of antibodies.
Chapter 4
Preparation of F(ab') Fragments from Intact IgG

4.1 Introduction

Fragments of immunoglobulin molecule such as F(ab')$_2$, F(ab') and F(ab) have been used for many analytical and clinical applications including immunoassays (Inoue et al., 1985; Karawajew et al., 1988), preparation of immunosorbents with high specific activity (Prisyazhnoy et al., 1988), immunotherapy (Reading et al., 1989; 1990) and immunodiagnostics (Smith et al., 1977; Takahashi and Fuller, 1988). Preparation of immunoreactive fragments of polyclonal (Porter, 1959; Nisonoff et al., 1961) and monoclonal (Parham, 1982; 1983) antibodies has been described. The enzymes pepsin and papain have been most often used in the preparation of these fragments. The use of pepsin digestion for the preparation of F(ab')$_2$ is well documented (Nisonoff et al., 1961). However, it has been reported that antibodies from different species differ greatly in their sensitivity to proteolytic digestion (Parham, 1982; Nisonoff et al., 1975; Tijssen, 1985). The available information also reveals that large variations exist between and within mouse monoclonal subclasses with regard to the sensitivity to proteolytic digestion by pepsin (Lamoyi and Nisonoff, 1983; Parham, 1983). Furthermore, we have observed that different antibodies from the same species have varying degrees of sensitivity to proteolytic cleavage by pepsin. Therefore, it is necessary to develop rapid methods to monitor the progress of the proteolytic cleavage of antibodies by pepsin in order to optimize the conditions for the preparation of F(ab')$_2$.

The F(ab')$_2$ fragment is a dimer of two identical F(ab') units linked together by at least one disulfide bond between the heavy chains (Nisonoff et al., 1961). Depending on the host species and the subclass of the antibody the number of inter-heavy chain disulfide
bonds vary. It has been reported that goat and rabbit antibodies have one disulfide bond (De Preval et al., 1970; Grey et al., 1971) whereas mouse antibodies have two to three disulfide bonds depending on the subclass (Svasti and Milstein, 1972). Similarly, human IgG subclasses have two to six inter-heavy chain disulfide bonds (Pink et al., 1970).

Preparation of F(ab') from F(ab')₂ by reduction of inter-heavy chain disulfide bonds appears to be simple. The reduction has been carried out using disulfide reducing reagents such as dithiothreitol (DTT) (Ghetie et al., 1988) 2-mercaptoethanol (ME) (Stevenson et al., 1985) and 2-mercaptoethylamine hydrochloride (MEA) (Nisonoff et al., 1960). Even though the F(ab')₂ has many inter and intra chain disulfide bonds the inter heavy chain disulfide bonds are exceptionally labile (Nisonoff et al., 1961). Thus they can be selectively reduced under certain reaction conditions (Nisonoff et al., 1961) to produce F(ab') fragments. However, the F(ab') fragments prepared by the described procedures using MEA did not give high yields when used for preparation of bispecific antibodies. This prompted a more careful study of preparation of F(ab') fragments, in order to optimize the reaction conditions for a particular antibody. In the course of doing this analytical methods were developed to monitor the progress of reduction and to determine the number of sulfhydryl groups generated in the F(ab') fragment.

4.2 Materials and Methods

4.2.1 Purification of IgG from Anti-Serum

The IgG fraction was separated from anti-serum by salt fractionation with ammonium sulfate. First, the anti-serum was centrifuged at 2000 x g to remove any aggregated materials and then diluted with PBS (0.1 M PB, 0.15 M NaCl), pH 7.4 to a final protein concentration of 25 mg/ml prior to salting-out. Anhydrous ammonium sulfate was then added to the serum sample with stirring, up to 33% of saturation (0.2 g/ml of
serum) (saturated solution at room temperature is 4.1 M). Addition of ammonium sulfate was carefully carried out over a period of about 1 hr. to avoid local high concentrations. The solution was stirred for another 2 hrs at room temperature before centrifugation at 2000 g for 20 mins. The supernatant was then discarded and the precipitate was resuspended in 40% saturated ammonium sulfate solution. The precipitate was collected again by centrifugation. This process was repeated until the precipitate was almost white in color (2-3 times). Finally the precipitate was dissolved in PBS (approximately half the original volume of serum) and dialyzed against the same buffer for 48 hrs with three changes of buffer.

4.2.2 Preparation of F(ab')$_2$ by Pepsin Digestion

Preparation of F(ab')$_2$ from IgG was carried out according to the method of Nisonoff et al. (1960) with the following modification: the purified IgG fraction of goat anti peroxidase, goat anti-glucose oxidase (Jackson Immunoresearch Laboratories Inc., West Grove, PA) and goat anti-mouse (anti-serum prepared by immunizing goats with mouse IgG) (ca. 20 mg/ml) was dialyzed for 24 hrs against 0.1 M acetate buffer pH 4.5 at 4°C. A solution of pepsin (2 x crystallized; Sigma, St. Louis, MO) prepared in the same buffer was then added such that the enzyme:IgG ratio was 1:33 (w/w). The mixture was incubated at 37°C, and the progress of the digestion was monitored over time by high performance size exclusion chromatography (HPSEC). When the digestion was completed as determined by HPSEC, the reaction was stopped by raising the pH to 8.0 with the addition of Tris-hydroxymethyl aminomethane solid. The resulting reaction mixture was then dialyzed against PBS pH 7.4 for 24 hrs with three changes of buffer.
4.2.3 Preparation of F(ab') Fragments

Preparation of F(ab') fragments by reduction of F(ab')2 was carried out according to the method of Nisonoff et al. (1961) with the following modification: First, the purified F(ab')2 solution was dialyzed against 0.1 M PB pH 6.0 containing 1 mM EDTA for 24 hrs. The concentration of the F(ab')2 was then adjusted to less than 10 mg/ml with the same buffer. The F(ab')2 solution (0.95 ml) was then incubated for 10 min. at 37°C and made 10 mM in MEA by the addition of 50 µl of 0.2 M MEA (prepared in the same buffer). The reaction mixture was incubated with stirring for 90 min. at 37°C after the addition of MEA. Resulting F(ab') fragments were then separated from the excess reducing agent by passing through a Sephadex G-15 column (30 x 1 cm), equilibrated with the PB at 1.0 ml/min. The number of sulfhydryl groups liberated in the F(ab') fragments was determined as described in section 4.2.6. In the preliminary experiments it was found that the deaeration of buffers with N2 did not have a significant effect on the reoxidation of resulting F(ab') in the presence of 1 mM EDTA. Therefore, the deaeration step was discontinued for all the other experiments.

4.2.4 Determination of the Progress of Digestion of IgG

The extent of digestion was monitored by high performance size exclusion chromatography (HPSEC). Samples were run on a Zorbax G-250 (DuPont, Wilmington, DE) column at a flow rate of 1.0 ml/min. with 0.2 M PB, pH 7.4 as the mobile phase. Digestion of IgG by pepsin was carried out as described in section 4.2.2. One hundred microliter aliquots were withdrawn from the reaction mixture at 0, 6, 12, 20 and 24 hrs after the addition of pepsin and mixed with 200 µl of 0.3 M Tris-buffer pH 8.5. The amount of IgG digested at time t is given by the difference in the peak areas (ΔA) of the
IgG peak at time $t = 0$ ($A_0$) and $t = t$ ($A_t$). Therefore, the percent IgG digested at time $t$ is given by the percentage of the ratio $\Delta A_t/ A_0$.

### 4.2.5 Determination of the Progress of Reduction of F(ab')$_2$

The progress of the reduction was monitored by HPSEC essentially as described in section 4.2.4. Reduction was carried out as described in section 4.2.3. Samples of 20 µl were withdrawn from the reaction mixture at specified times after the addition of MEA, and added to a solution containing 20 µl of 40 mM iodoacetamide and 160 µl of the PB. These samples were then subjected to HPSEC. The amount of F(ab')$_2$ reduced was determined by the decrease in the area of the F(ab')$_2$ peak.

### 4.2.6 Determination of the Number of Sulfhydryl Groups of F(ab')

Number of sulfhydryl (SH) groups in F(ab') was determined using 4-4' dithiodipyridine (4PDS) as described by Grassetti and Murray (1967) with the following modifications: A 5 mM solution of 4PDS (Aldrich, Milwaukee, WI) was prepared by dissolving 11.01 mg of 4PDS in 1.0 ml of absolute ethanol and making the volume up to 10.00 ml with PB, pH 6.0. A calibration curve was prepared by using standard solutions of MEA. One hundred microliter aliquots of increasing concentrations (0 - 1 mM) of standard MEA solutions were added to a solution of 900 µl of PB, pH 6.0 containing 50 µl of 5 mM 4PDS and incubated at 30°C for 10 min. A 4PDS blank was treated essentially the same as standards without the MEA. Experiments were carried out in triplicate for each standard. The absorbance of the reaction mixtures was then measured at 324 nm.

In the determination of SH groups in F(ab'), a 1.0 ml solution of F(ab') in PB, pH 6.0 (concentration less than 5 mg/ml) was incubated with 50 µl of 5 mM 4PDS for 10 min.
and the absorbance was measured at 324 nm. Experiments were carried out in duplicate for each sample.

Alternatively the number of SH groups was determined using 5,5' dithiobis (2-nitrobenzoic acid) (DTNB) (Sigma, St. Louis, MO) as the assay reagent according to the method of Ellman (1959) with the following modifications: A calibration curve was prepared as described above with MEA standard solution using DTNB. One hundred microliter aliquots of DTNB were added to each standard solution in place of 4PDS and the absorbance was measured at 412 nm. Samples were treated essentially the same as the standard solution. The DTNB solution was prepared by dissolving 39.6 mg of DTNB in 10 ml of PBS, pH 7.4. This was important because the solubility of DTNB in PB, pH 6.0 was poor.

4.3 Results and Discussion

The preparation of F(ab') from IgG involves the digestion of IgG with pepsin followed by the reduction of the resulting F(ab')2 with MEA. The first critical step in the procedure is the pepsin digestion of IgG. The reported procedures (Nisonoff et al., 1961; Paharm, 1983) for digestion of IgG did not always give optimum results. As depicted in Figure 4.1, anti-glucose oxidase and anti-peroxidase antibodies raised in goats had almost the same sensitivity to pepsin, having 92.2% and 92.6% of IgG digested respectively after 24 hrs of reaction. On the contrary only 50% of the goat anti-mouse IgG was digested under the same reaction conditions (pH 4.5, enzyme : IgG (w/w) = 1 : 33 ), showing high resistance to proteolytic cleavage. These results illustrate that even within the same species different antibodies could have different sensitivities to proteolytic cleavage by pepsin. Therefore, one must not use the reported reaction conditions to prepare F(ab')2 from IgG without optimization. The important parameters of the digestion are pH of the reaction,
Figure 4.1. Time Course of Digestion of IgG with Pepsin

- Goat anti-glucose oxidase
- Goat anti-peroxidase
- Goat anti-mouse
time of incubation, protein concentration and enzyme / IgG ratio. Therefore, to establish
the optimum conditions each of these parameters has to be optimized. In order to carry out
such a task it is very important to have a rapid, simple method to monitor the progress of
the reaction. The HPSEC is such a method and can be used for both quantitative and
qualitative monitoring. As shown in Figure 4.2, chromatography on Zorbax G-250
column has sufficient resolution to separate IgG and F(ab')_2 in a very short time (15 min.).
The area under each peak can be used as a measure of the relative amounts of IgG and
F(ab')_2, and consequently the extent of digestion can be quantitatively determined.

In the process of optimization, the pH of the reaction should be optimized first as it
is the most sensitive parameter in the whole process. The pH optimum for pepsin is 1.0
and therefore, as the pH decreases the rate of digestion increases. Since pH between 4.0
and 4.5 has been widely used for proteolytic digestion of many antibodies, it can be used
as a starting value. Similarly the enzyme : IgG (w/w) ratio of 1 : 50 can be used as a
starting value. Once the optimum pH is determined, the next step should be to optimize the
enzyme : IgG ratio. Subsequently the time of incubation and the concentration of IgG
should also be optimized. The concentration of IgG does not have a very significant effect
on the rate of digestion as long as it is less than 10 mg/ml and therefore, this parameter can
be considered last.

The next critical step in the preparation of F(ab') is the reduction of F(ab')_2
fragments. Even though several disulfide reducing agents have been used for the
reduction, MEA has been the most widely used reagent. A concentration of 10 mM MEA
has been reported to be the most appropriate concentration (Mandy and Nisonoff, 1963).
As depicted in Figure 4.3, 10 mM MEA is not able to completely reduce goat anti-mouse
F(ab')_2 even after 180 min. of reduction. Usually the reduction of F(ab')_2 is carried out
for 90 min. at 37°C. The amounts of F(ab')_2 reduced after 90 min. and 180 min. were
Figure 4.2 HPSEC of Digestion of IgG
Figure 4.3. Time Course of Reduction of Goat Anti-Mouse F(ab')$_2$
85.6% and 89.5% respectively (Figure 4.3). Increase in the time of reduction from 90 min. to 180 min. had very little effect on the amount of $F(\text{ab}')_2$ reduced. This suggests that about 10 - 15% of goat anti-mouse $F(\text{ab}')_2$ is resistant to the reductive split. It has been reported that the amount of $F(\text{ab}')_2$ resistant to the reductive split varies depending on the host species (Clausen, 1988). In rabbit this amount is very small but in goat and guinea pig it is usually 5 - 10%. The amount could be even larger in $F(\text{ab}')_2$ of rat and mouse because in the latter case there is more than one inter heavy chain disulfide bond. Sometimes optimization of the reducing conditions may help to decrease the amount of $F(\text{ab}')_2$ resistant to reductive split. However, if the amount of unreduced $F(\text{ab}')_2$ is significant, then the resulting reaction mixtures have to be subjected to gel filtration chromatography in a column that provides adequate resolution (i.e. Sephacryl HR 200, HR 300, Sephacryl S 300 or Ultragel AcA 44).

The reduction of $F(\text{ab}')_2$ can also be monitored by HPSEC on a Zorbax G 250 column. As shown in Figure 4.4, the resolution and sensitivity is quite adequate. The area under the peaks corresponding to $F(\text{ab}')_2$ and $F(\text{ab}')$ can be used to determine the relative amounts of these fragments present during the course of the reaction. It should be noted that the reduction reaction has been stopped and the SH groups formed have been protected by the addition of iodoacetamide immediately after the withdrawal of samples. Therefore, the relative amounts of these fragments as determined by chromatography represent the correct amounts present at the specified time. It is also important to note the difference between the extent of reduction and the amount of $F(\text{ab}')_2$ reduced. The amount of $F(\text{ab}')_2$ reduced is determined by the decrease in area under the peak (HPSEC) corresponding to $F(\text{ab}')_2$. On the other hand, the extent of reduction is determined by the number of moles of SH groups liberated per mole of $F(\text{ab}')$ formed. The total protein concentration of a mixture of $F(\text{ab}')_2$ and $F(\text{ab}')$ can be determined by the absorbance at 280 nm. The
Figure 4.4 HPSEC of Reduction of F(ab')$_2$
chromatographic data would allow determination of the ratio of F(ab')$_2$ : F(ab') in the mixture and consequently the amount of F(ab') in the mixture can be determined. According to the definition, to determine the extent of reduction it is necessary to know the number of SH groups liberated in the F(ab') formed.

There are several reagents to assay the SH groups liberated. Spectrophotometric assays of thiols using either 4PDS or DTNB (aromatic disulfides) have been widely used for this determination (Clausen, 1988). The DTNB and the 4PDS react with accessible SH groups in proteins to form a mixed disulfide (protein-S-S-Ar) and an aromatic thiol or thione. Therefore, the aromatic thiol or thione formed is stoichiometric with the SH content. Use of the 4PDS has certain advantages over the DTNB. When the DTNB is used as the assay reagent, the absorbance depends on the concentration of 5-mercapto-2-nitrobenzoate (MNB) anion. The pK$_a$ of the SH group of MNB is 4.4 (Brocklehurst and Little, 1973). Therefore, the value of $\varepsilon_m$ declines with decreasing pH below 6.0. The thiol to be assayed itself reacts as RS$^-$ and thus the rate also declines with decreasing pH (the pK$_a$s of most biological thiols are found to be between pH 8.0 - 9.5). However, as the DTNB hydrolyzes spontaneously above pH 9.0 (Hiramatsu, 1977), the permissible pH range of the assay medium is limited to 6.0 - 8.5. On the contrary, the pK$_a$ value of the corresponding thiol of 4PDS is 8.7 (Brocklehurst and Little, 1973), but the absorbing species is a thione rather than a thiol anion. Therefore, the $\varepsilon_m$ does not depend on the pH of the assay medium. The choice of pH depends only on the rate of the reaction over the range 2.0 - 8.5. Furthermore the $\varepsilon_m$ value of the thione from the 4PDS is nearly 1.5 times that of the MNB anion from the DTNB, and thus 4PDS is more sensitive. The calibration curves for thiols using the 4PDS (Figure 4.5) and the DTNB (Figure 4.6) as the assay reagents are very linear (correlation coefficient 1.00) over the concentration range 0 - 100 $\mu$M. The MEA standard solutions are very stable over a period of 96 hours in PB, pH 6.0,
Figure 4.5. Calibration Curve for Thiols using 4PDS
Figure 4.6. Calibration Curve for Thiols using DTNB
1 mM EDTA. Since oxidation of sulphhydryl compounds are frequently catalyzed by traces of metals, the addition of 1 mM EDTA to the assay medium is very important. It has been reported that the addition of 10 mM EDTA has markedly decreased (only 15% reoxidized over 72 hours) the reoxidation of F(ab') (Mandy and Nisonoff, 1963). The 4PDS assay reagent was very stable as evidenced by the increase in the absorbance of the reagent blank by a small margin, from 0.07 AU to 0.10 AU, over a period of 4 days. The reproducibility of the SH determinations was excellent (CV < 2% for three determinations).

As shown in Table 4.1 the moles of SH groups liberated per mole of F(ab') after the reduction of F(ab')2 for 90 min. and 180 min. were 1.25 and 1.17 respectively. The results suggest that the reduction of goat anti-mouse F(ab')2 has resulted in the reduction of disulfide bonds other than the most labile inter heavy chain disulfide bond (goat IgG has only one inter heavy chain disulfide bond). The liberation of more SH groups could be a problem for the reactivity of these F(ab') fragments with cross linking reagents such as bismaleimides. In the chemical preparation of bispecific antibodies homobifunctional bismaleimides are used to cross link F(ab') from two different antibodies. The liberation of more SH groups could be a reason for the observed low yield (< 25%) of chemical preparation of bispecific antibodies. Therefore, it is necessary to reinvestigate reduction conditions of F(ab')2 of individual antibodies to prepare well characterized F(ab') using the techniques developed in the present study.

In order to establish the optimum conditions for the reduction, several parameters have to be investigated. The pH of the reduction is an important parameter because the structure of the F(ab')2 is a function of the pH. At lower pH values it is believed that the protein is unfolded and thus the exposure of the disulfide bonds is greater. But the reactivity of the reducing agent also depends on the pH, because the thiol-disulfide exchange reaction requires that the thiol to be in the RS- form. Therefore, the pKₐ of thiol
Table 4.1
Amount of Thiol Groups Liberated per mole of F(ab') Formed

<table>
<thead>
<tr>
<th>Time of Reduction^a</th>
<th>% of F(ab')\textsubscript{2}\textsuperscript{b}</th>
<th>Amount of F(ab')\textsuperscript{c} nmoles Liberated per mole of F(ab')</th>
<th>Moles of SH\textsuperscript{d}</th>
</tr>
</thead>
<tbody>
<tr>
<td>90 min</td>
<td>85.6</td>
<td>25.7 (30.0)</td>
<td>1.25 (32.2)</td>
</tr>
<tr>
<td>180 min</td>
<td>89.5</td>
<td>25.3 (28.3)</td>
<td>1.17 (29.5)</td>
</tr>
</tbody>
</table>

^a goat anti-mouse F(ab')\textsubscript{2} reduced with 10 mM MEA at pH 6.0

^b determined as defined in the text

^c values in parenthesis are total amounts of F(ab') and F(ab')\textsubscript{2} in the reaction mixture determined by the absorbance at 280 nm.

^d values in parenthesis are total amounts of SH groups liberated in nmoles
group of the reducing agent is also an important factor to be considered. The pK$_a$ values of the thiol groups of the DTT are in the range of 8.0 - 8.5 and thus the maximum reducing power of this reagent can be achieved only if the reduction is carried out at or around pH 8 to 9. Since the pK$_a$ of the thiol group of MEA is 10.5, it is also very effective at high pH. However, the reduction with MEA is usually carried out around pH 5 - 6. This could be to avoid the reduction of disulfide bonds other than the most labile inter heavy chain disulfide bond in the molecule. These factors have to be considered when conditions are optimized for the reduction of F(ab')$_2$. The other parameters that may effect the reduction of F(ab')$_2$ are the temperature, the time of incubation and the concentration of both the reducing agent and the protein. All these parameters can be systematically optimized using the HPSEC to monitor the progress of the reduction and the spectrophotometric assay to determine the extent of reduction.
Chapter 5

Future Directions

The conclusions of the preceding chapters show several possible directions that future work might take. These include fundamental studies as well as development of existing analytical techniques.

In the preparation and characterization of monoclonal antibodies to enzymes it has been shown that the screening of hybridomas has an influence on the properties of the antibodies selected. At present, screening of hybridomas is mainly carried out by coating the antigen on the surface of an ELISA plate. The monoclonal antibodies selected by this method may preferentially recognize the antigen adsorbed on the ELISA plates over the antigen in solution. Coating of an antigen on a solid surface could cause conformational changes not only to fragile molecules such as peptides, but also to very robust molecules as GOx. Therefore, it is important to develop solution phase screening procedures to obtain antibodies with desired properties for solution phase applications.

If the antigen is an enzyme which would give a colored product with its substrate then it is much easier to develop a solution phase screening procedure (Alkaline phosphatase and horseradish peroxidase). A screening method can be developed by coating the ELISA plates with polyclonal goat anti-mouse Fc specific antibody to capture the antibodies of interest. The captured antibody could then be allowed to react with their antigens in solution. Subsequently, the substrates can be added to identify the positive wells, producing hybridomas of interest. One of the fundamental limitations of this method would be the antigen capture capacity (AgCC) of the coated antibody (CAb). The CAb should be able to bind all classes of mouse immunoglobulins and should have high enough capacities to capture all the immunoglobulins in the hybridoma supernatant. Usually when the plates are coated with 10 μg/ml of CAb the amount of antibody coated on the plate would be 2 pmol/cm² or 320 ng/cm² (Cantraero et al., 1980). When 100 μl aliquots of
solutions are used the available surface area per well would be 0.94 cm$^2$. Therefore, the amount of antibody coated per well would be 300 ng. It has been reported that the AgCC of approximately 100 ng of CAb is 10 - 20 ng (Butler, 1988). The concentration of immunoglobulins in matured hybridoma supernatants would be approximately 10 - 50 μg/ml (Tijssen, 1985). If we assume, that at the beginning of the cultures the concentration of immunoglobulins is 2 μg/ml, then the amount of antibody in 100 μl aliquots would be 200 ng. Since the capacity of the CAb is about 30 - 60 ng it would not be able to extract all the antibodies in the supernatant. The efficiency of capturing would be even lower from the supernatants of matured hybridomas. Therefore, if the abundance of the antibody of interest is low this method would give false negative results. However, if only one type of antibody is produced (monoclonal) then this method can be used with a detection system that has a very low detection limit. Thus, one of the future directions should be to improve the AgCC of the screening method. One alternative would be to immobilize a biotinylated protein on the surface and then use biotinylated CAb with avidin or streptavidin as the bridging agent. This method has shown significant improvement in the percentage AgCC (when the CAb is a monoclonal antibody), but the amount of CAb that can be immobilized is significantly small (Suter and Butler, 1986). The other alternative is to use solid phase surfaces with active groups for covalent attachment of CAb. This method would greatly increase the CAb concentration, but may not show a proportional improvement in AgCC. Therefore, one must optimize the concentration of CAb immobilized on the surface to obtain maximum AgCC. In doing this it would be necessary to consider the chemistry of immobilization and the type of solid support used for immobilization. The site directed immobilization of antibodies may be a better alternative to improve the AgCC, which should be considered in the future.
X-ray analysis indicated that the IgG molecule is a cylinder 240 x 57 x 19 Å (Edelman and Gally, 1964; Kratzky et al., 1955). If we assume that the axis of the cylinder is perpendicular to the surface when it is immobilized, then the surface area occupied by one molecule of IgG could be $2.55 \times 10^{-13}$ cm$^2$. Since the available surface area for 100 µl aliquots of solution per well, is 0.94 cm$^2$, the maximum amount of IgG that can be immobilized, for monolayer coverage would be $3.68 \times 10^{12}$ molecules or 0.98 µg. If only 20% of the immobilized IgG is immunologically active, then the AgCC of the CAb would be 196 ng. This is barely sufficient to capturing the antibodies in immature hybridoma supernatants. However, if the screening method is intended to screen the hybridoma supernatants containing more than 2 µg/ml of IgG, then it could be necessary to develop solid surfaces which can accommodate higher amounts of CAb. One could consider the development of solid spheres to achieve this goal in the future.

In the present study of the preparation of immunosorbents it has been shown that the specific activity can be improved by site directed immobilization if the density of the antibody on the support matrix is low. The support matrix used for the study is a porous matrix with 1000 Å pores (HW 65). Therefore, it is not possible to unequivocally conclude that the loss of immunological activity at high antibody densities is due to steric hindrance caused by blocking of pores or due to the crowding of the antibody. In order to understand the phenomenon of crowding of the antibody it is necessary to carry out the immobilization on a solid surface. Therefore, in the future, site directed immobilization should be carried out on solid spheres. In order to avoid the effect of bivalency, the immobilization of F(ab') would be preferred to immobilization of oxidized IgG. Thus the solid sphere should be activated with iodoacetyl groups. This could be carried out with tosyl chloride (Kirkguard and Perry, Bethesda, MD) according to the procedure described in chapter 3. Using the dimensions (from X-ray analysis) of the IgG molecule it would be
possible to make an assumption for the surface area occupied by a F(ab') fragment on a planer surface. If the F(ab') would immobilize on the surface such that the imaginary axis of symmetry of the IgG molecule is perpendicular to the surface then it would occupy a surface area of $1.27 \times 10^{-13}$ cm$^2$ (equal to the area of one half circle with a diameter of 57 Å). Therefore, the maximum amount of F(ab') that can be immobilized for monolayer coverage on a 3.2 µm solid sphere would be 4.2 attomoles or 0.19 pg. Therefore, the maximum amount of F(ab') that can be immobilized per ml of these solid spheres would be 11.0 mg. In practice, if at least 10% of the maximum can be obtained, it would be sufficient for most analytical and semi preparative scale preparation of immunosorbents.

In the preparation of F(ab') fragments from IgG it has been shown that the described procedures are suboptimal. Therefore, in the future it is necessary to optimize these methods as suggested in Chapter 4 to achieve high quality F(ab') fragments with high yields. The analytical methods developed in Chapter 4 would be very useful for these optimizations. Once the preparation of f(ab') fragments is optimized then it would be much easier to optimize the conditions for preparation of bispecific antibodies.
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