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A CHEMICAL INVESTIGATION OF IMMOBILIZED BIOLOGICAL MOLECULES IN CLINICAL ANALYSIS

by

Gary Lee Smith

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF CHEMISTRY

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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THE UNIVERSITY OF ARIZONA

GRADUATE COLLEGE

I hereby recommend that this dissertation prepared under my direction by Gary Lee Smith

entitled A Chemical Investigation of Immobilized Biological Molecules in Clinical Analysis

be accepted as fulfilling the dissertation requirement for the degree of Doctor of Philosophy

George S. Wilson — 16 September 1977

Dissertation Director — Date

As members of the Final Examination Committee, we certify that we have read this dissertation and agree that it may be presented for final defense.

M F Burke	9-10-77
Quinton Fernando	Sept 10, 1977
R Glass	9/12/77

Final approval and acceptance of this dissertation is contingent on the candidate's adequate performance and defense thereof at the final oral examination.

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SIGNED: Gary L. Smith

To Katie and Gerrit

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ABSTRACT

The investigation of immobilization of antihuman gamma immunoglobulin to controlled pore glass (CPG) particles by covalent attachment was carried out. Optimum conditions for the silanizing, activating, and coupling reactions were determined. These conditions were utilized in the remainder of the investigation to yield an immobilized antibody exhibiting high amounts of biological activity. The stability of this immobilized antibody with respect to time and number of sample introductions was investigated.

Surface properties of the CPG particles were studied by acid base micro titrations using a modified Gran plot. A correlation between biological activity and number of reactive groups on the silica surface was established. Certain physical properties of CPG matrices were demonstrated to be beneficial in achieving maximum amount of biological activity/g of matrix.

Silanization efficiencies of between 6.5 and 19.5% with available silanol groups were observed. Calculations of a monolayer coverage of the surface of the CPG particles using X-ray crystallographic information together with data on amount of protein actually covalently linked to the particles as determined by amino acid analysis suggest formation of up to 20 monolayers of protein on the surface.

The immobilized antibody activity was investigated using direct and indirect spectrophotometric and fluorometric methods. Standard

reaction mixtures of human IgG were reacted with the immobilized antibodies to produce an antigen antibody complex. This complex was dissociated by a change in pH or introduction of a chaotropic agent and the soluble IgG assayed. A linear relationship between change in amount of IgG complexed and IgG concentration in standard reaction mixtures was observed. Chemical modifications of the antibody molecule on the surface of the CPG particles after covalent attachment were detected. The specificity and binding rate of the immobilized antibody were not significantly altered by this modification. Heterogeneity of the immobilized antibody binding site was determined by Scatchard and Sips plots to be 0.71, indicating a heterogeneous population.

A fluorescence quenching assay for human IgG was devised incorporating labeled antibody immobilized on CPG particles. Initial use of the particles in the assay gave detection limits of 0.25-0.50 μg/ml. A high pressure liquid chromatography (HPLC) column utilizing CPG particles containing immobilized antibodies as the packing material was implemented into an immunoassay method for human IgG. A linear relationship was observed between peak height of the free and bound fractions of IgG and initial concentration of IgG in the sample over the range of 0-10 μg/ml.

INTRODUCTION

The observation that individuals who recover from an infectious disease often acquire complete and long-lasting immunity to the disease has been documented many times during the early recorded history of man. In 1546, Fracastorius wrote a discourse entitled "de Contagiou." In this work, he suggested that "infectious diseases were the result of minute insensible particles and proceeds from them." It remained for Koch in 1876 to demonstrate scientifically that microbes cause infectious diseases. Around the late 1800's, scientists isolated a certain substance in the sera of experimental animals which had recovered from a particular infectious disease. This substance had the ability to neutralize a particular infectious disease *in vitro*. The name given this substance was antibody (Ab) because it was directed against the harmful "foreign body," i.e., the causative agent of the infectious disease. This harmful body was, in turn, called an antigen (Ag).

An antigen is defined as a substance which can stimulate the synthesis and release of proteins (antibodies) capable of reacting with it in a specific manner. The antibodies produced by a given antigen react only with that antigen or closely related antigens. This avidity of the antibody for a specific antigen is one of the special characteristics of this particular group of serum proteins previously identified by the general term immunoglobulins (Ig). Another trait which distinguishes antibodies from other biologically active proteins, for

example, enzymes, is their pronounced heterogeneity. The same antibody combining site, i.e., that part of the molecule which is responsible for specific conjugation with an antigen, can be found in molecules with distinctly different physical properties. This is primarily attributed to the fact that the same antibody to a given antigen can come from different immunoglobulin classes in different animal species. These classes were defined by Tiselius in 1936 who differentiated the various proteins in serum by electrophoresis. After placing a serum sample in an electric field, the protein component of the serum which moved the least in the anodic direction at alkaline pH was designated the gamma globulin fraction (current nomenclature designates this fraction as the immunoglobulin G (IgG) fraction). The other main classes are designated IgA, IgM, IgD, and IgE.

The presence of antigens in most animals usually causes the production of all five types of immunoglobulins (antibodies). The IgG class is the most prevalent, accounting for 80% of the total immunoglobulin population. Like any immunoglobulins, the IgG molecule is really a hybrid made according to the instructions of separate genes which code for the various heavy and light chains which make up the molecule (Figure 1). These chains, two heavy and two light, are held together by three inter-chain disulfide bridges. The overall shape of the IgG molecule is sometimes depicted in the "T" form as shown in Figure 1A; however, the molecule is flexible and other possible configurations seen in Figure 1B are possible. Electron microscopy indicates a more natural configuration is the "Y" form shown in Figure 1B. Also,

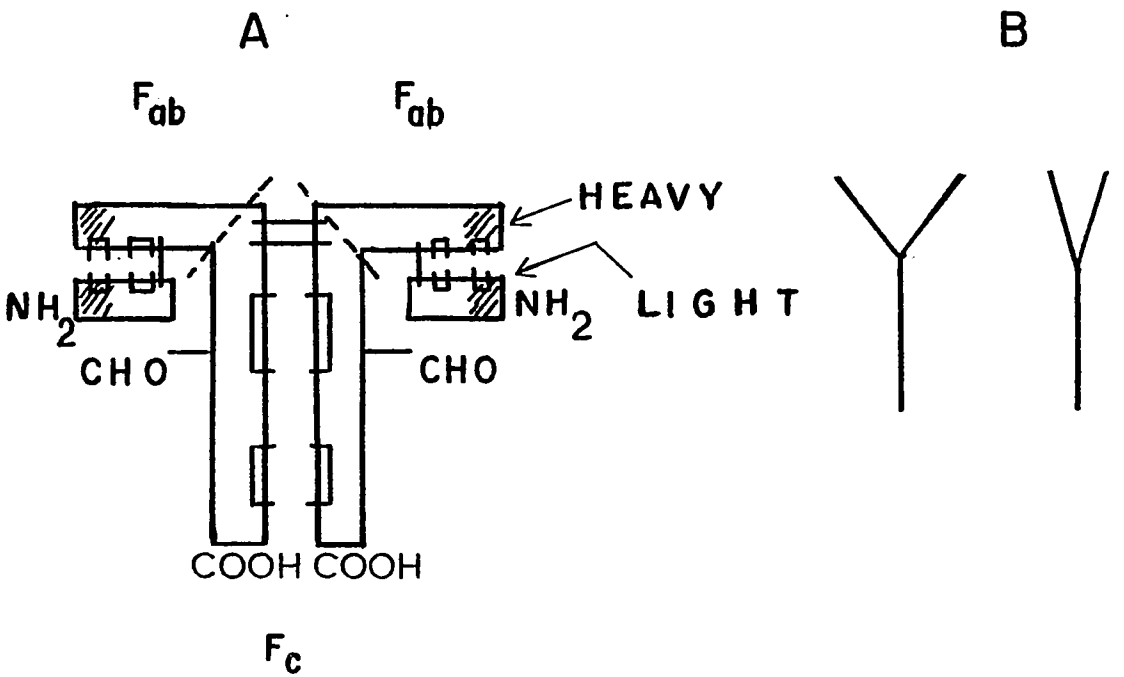


Figure 1. Structural arrangement of IgG molecule. -- (A) Overall arrangement of chains and disulfide bonds in IgG molecule. Shaded area represents variable region in heavy and light chains; dashed lines indicate position of proteolytic cleavage; CHO = carbohydrate region. (B) Possible overall conformations for IgG.

there are two important structural aspects of the immunoglobulin molecule not clearly reflected by Figure 1. First, there are a considerable number of intra-chain disulfide linkages which, in conjunction with hydrogen bonding, hold the chains together in a particular three-dimensional array. Secondly, these three-dimensional coilings of the individual chains and the juxtaposition of the amino acids on these chains result in the formation of the antigen combining site. This integral part of the immunoglobulin molecule is where the antigenic determinate on the antigen aligns itself with the antibody molecule. As the fit between the antigenic determinant and the combining site improves the strength of the non-covalent bond formed by hydrogen bonding increases. This leads to a higher union between the two molecules and more efficient antibody function in the body's immunological defense scheme.

There are two combining sites on the IgG molecule, both of which are exactly the same. It is possible to enzymatically cleave the IgG molecule into three parts. A portion of the heavy chain representing the downstroke of the "T" which has no combining site can be cleaved. This fragment is given the name F_c. The remainder of the IgG molecule can be split at the middle into two fragments, each containing a viable combining site which retains its antibody specificity. These two fragments are each labeled F_{ab} (Nezlin 1970). Only about 15 amino acids contribute directly to defining the combining site on each of the F_{ab} fragments. Yet this small area, less than 2% of the whole molecule, gives the molecule its specificity and variability. As seen in

Figure 1, both chains contribute to making the combining site, although the heavy chain plays a much more dominant role in determining the viability of the combining site. This is demonstrated by isolating the heavy chain from the remainder of the molecule and observing that it retains a reasonable proportion of the whole molecule's ability to recognize and react with the appropriate antigen. This is not, however, the situation with isolated light chains (Nisonoff, Hopper, and Spring 1975).

Only a few intact immunoglobulins have been subjected to extensive X-ray crystallographic analysis, and these studies have not yet obtained high resolution. Sarma et al. (1971) crystallized a human myeloma IgG(κ). At the highest resolution obtained, 6 Å, the overall shape of the molecule was estimated to be in the "T" shaped configuration with the two F_{ab} fragments comprising the arms of the "T." The horizontal length of the "T" is 142 Å and the vertical dimension is 85 Å. These values are larger than those obtained using electron microscopy (120 Å, 75 Å) (Labaw and Davies 1971). Studies at higher resolution have been precluded so far by the sensitivity of the crystals to X-radiation. The discrepancy in the results of the crystallographic and electron microscopy studies may reflect the hydration layer normally associated with proteins. The shape of the IgG may be an intermediate between the "Y" and "T."

Immunoassays

Much of the information concerning antibody specificity and the combining region has been acquired through experiments in which

different molecules of varying size, shape, and charge are allowed to react with the antibody. Various analytical procedures are then utilized in determining the extent of the interaction with these various molecules, i.e., goodness of fit. In order to gather useful information, the analytical method must possess the unique specificity and high sensitivity demanded by the very nature of the reactants being investigated. Because the antibodies and antigens are mixed in the serum matrix with other proteins, historically they were detected and differentiated from the other proteins by observing the secondary phase of their immunochemical reaction. The various observable physical properties marking the advent of this secondary stage of their reaction include: agglutination, lysis, or precipitation. Unfortunately, in using these techniques it is not possible to acquire information on the primary phase of the binding reaction, i.e., the initial rapid and normally spectrally invisible combination of antibody with antigen.

One of the first accurate precipitin methods used for following the course of an antibody-antigen reaction was developed by Heidelberger and Kendall (1935). This method was based on the determination of the amount of protein in a precipitate formed after the addition of a very slight excess of antigen to serum. As increasing amounts of antigen are added to serum containing antibody, the quantity of the precipitate increases to a maximum, and then decreases again as the result of the formation of soluble antigen/antibody complexes (Figure 2). All antibodies are precipitated by a small addition of excess antigen, and thus the exact quantity of antigen needed for this excess is determined. If

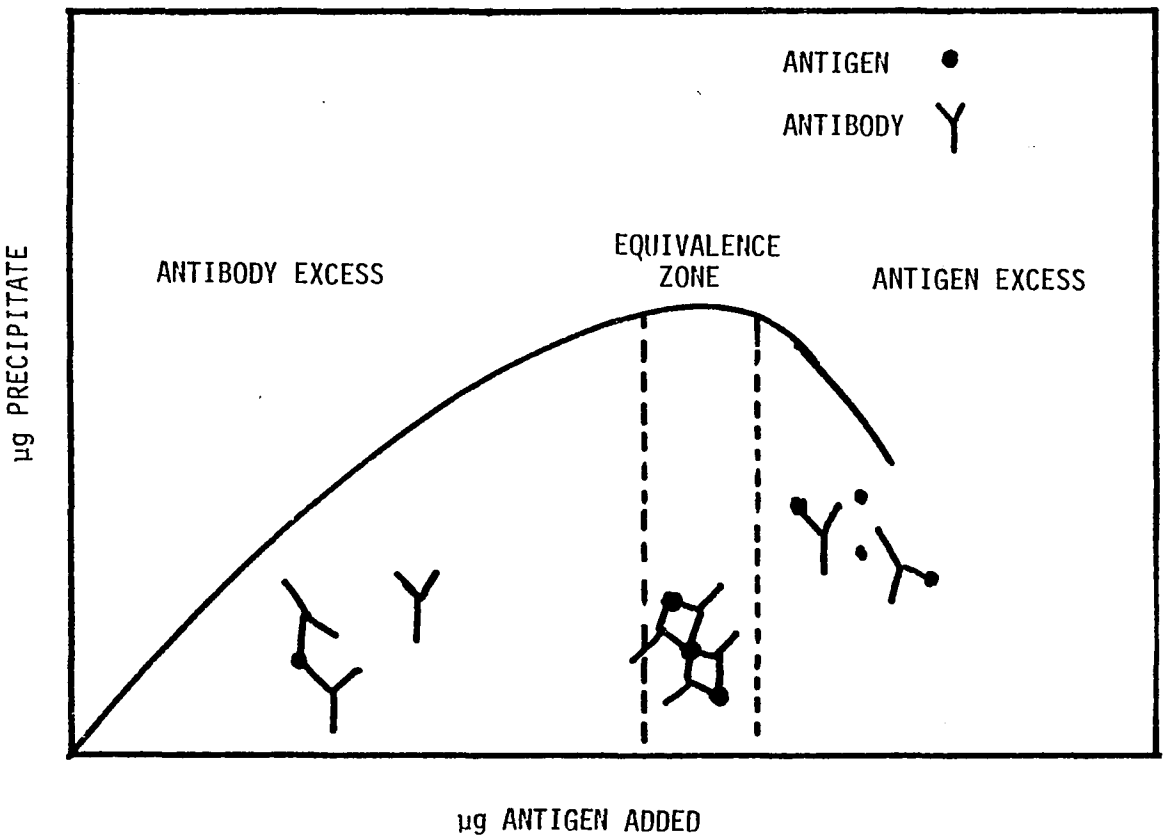


Figure 2. Reaction of antibody at varying concentrations of antigen. -- With excess Ab, lattice formation is inhibited, and thus no precipitate. Similarly, with excess Ag, the few Ab molecules find only one Ag. The other combining site is left free and no lattice network forms.

the antibody concentration is high, a known amount of antigen can be added in small portions to a definite serum volume, the precipitate being filtered off after each addition. The titration endpoint is reached when addition of antigen produces no further precipitate. A drawback of this method is the complicated preliminary estimation needed in order to determine the correct ratio of antigen to antibody in protein precipitate. This problem is avoided only by limiting this approach to previously well-studied antigen antibody systems in which the antigen-antibody ratio is known. Because of the necessity of washing the precipitate prior to the protein determination, a considerable amount of precipitate may be lost in the washing cycle, from 10-300 µg/ml (Nezlin 1970). Additional difficulties are encountered in attempts to determine the quantity of so-called non-precipitating antibodies, i.e., those which are not precipitated by addition of an antigen. In this instance, coprecipitation may be employed where an antigen and a precipitin, which yield a known quantity of precipitate, are added to a solution containing this type of antibody. Then, the quantity of nonprecipitating antibody is estimated from the increase in the amount of precipitate formed. This method, however, does not lend itself to quantitative determinations of these types of antibodies.

Solid Phase Immunoassays

In order to circumvent some of the previously mentioned problems associated with immunological assays based on the detection of secondary effects of the antigen-antibody reaction such as the requirement for large amounts of antibody and the solubility of the antigen-antibody

complex at elevated antigen concentrations, a more general approach was developed. This approach was concerned with following and quantitating the initial phase of the antigen-antibody binding reaction. Such assays used for qualitative and quantitative detection of antigens and antibodies employ immunoadsorbents. The term immunoadsorbent implies an antigen or antibody which has been rendered immobile by attachment to a water insoluble matrix. This method first allows the antibody from the serum sample to react with the matrix coupled antigen. Second, after washing the immunoadsorbent to remove non-specific proteins adsorbed on the support and to separate the antibody from other components of the assay, the bound antibodies are determined quantitatively either while bound to the support or after elution from the immunoadsorbent.

Some of the early work in immobilizing various antigens or antibodies covalently to water insoluble matrices was carried out by Campbell, Lerman, and Luescher (1951).

Diazotized p-amino benzyl cellulose was used as a matrix to which serum albumin was coupled. This immunoadsorbent was used to remove non-specific (non-antibody) serum proteins from various serum samples. Other such as Isliker (1957), Gurvich (1957), and Mondgal and Porter (1963) expanded these immobilization studies on cellulose by employing different reactions to derivatize the cellulose matrix. These newer methods were somewhat successful in reducing the non-specific adsorption of interfering substances (i.e., specificity for the substance which is to be complexed, under both adsorptive and desorptive conditions) (Weliky and Weetall 1965).

The initial pioneering work done on attaching immunoglobulins to solid phases (Campbell and Lerman 1949) was based on the physical adsorption of the protein to the matrix. This technique involved the use of physico-chemical forces such as Van der Waals forces, dipole-dipole interactions, and other similar properties which allowed formation of weak, non-covalent bonds between the protein and the matrix. An extension of this early work using cellulose particles resulted in the development of immuno-chemical molecules immobilized on other inert matrices such as agar, cellulose acetate, latex, and carboxymethyl cellulose using both non-covalent and covalent attachment procedures (Allen 1963, Paronetto 1963, Toussaint and Anderson 1965). At the time these different solid phases were investigated, no attempt was made to understand the basic chemistry involved. The emphasis was placed on the ultimate detection and quantitation of the particular immunological protein under investigation.

In the mid-1960's, the recognition of the contribution of the various complex chemical reactions to the whole immobilization process and to the retention of immunological activity was acknowledged. Toussaint and Anderson (1965) and others acknowledged that certain poorly understood chemical interactions in immobilization were important in producing a biologically active immobilized protein. A classic example is the incorporation of a 0.1% bovine serum albumin (BSA) solution in the coupling step. Without serum albumin it was demonstrated that a particular test incorporating immobilized material would not work. In order for the assay to produce results that were clinically

meaningful, the matrix containing the immobilized immunoglobulin had to be coated with a 0.1% bovine serum albumin (BSA) solution before exposure to the particular serum sample to be analyzed. This chemical involvement of the BSA was only vaguely alluded to in the report.

In the 1970's, more attention has been focused on the chemical reaction involved in preparing the matrix (Van Dalen 1965, Capel 1974, Siegel et al. 1973, Weetall 1975). These attempts to optimize coupling procedures were concerned with non-cellulose matrices. Using supports of varying chemical composition permitted investigators to employ a wide variety of chemistries in the coupling reaction. Essentially, immobilization of immunochemical species may be achieved by any of four methods: adsorption, entrapment, protein crosslinking, and covalent attachment. In Table 1 are listed some of the chemical methods used in immobilizing various immunologically important proteins on solid phases.

In general, the development of a new solid-phase immunoassay system involves two steps. The initial step involves selection of the matrix with which the immobilized substituent will be associated. Most of the typical materials can be classified into three main groups: carbohydrate polymers, other organic polymers, and inorganic materials. Each group has its own intrinsic characteristics, making it more suitable for certain problems than for others. Structural properties of the matrix, insolubility in the solvent system, and resistance to degradation and bacterial attack by enzymes and micro-organisms contained in the reaction mixture are important considerations. The mechanical stability of the matrix is also important. The chemical

Table 1. Some different chemical reactions involved in preparing immunoadsorbents.

Protein Covalently Linked	Matrix	Reactive Group on Matrix	Assay For	Detection Mode[a]
insulin	dextran	imidocarbonate	insulin antibody	RIA
Ab HCG[b]	CPG	isothiocyanate	HCG	EIA
Antiserum digoxin	CPG	diazonium salt	digoxin	RIA
Ab thyroglobulin	plastic tube	glutaraldehyde	thyroglobulin	RIA
Ab IgA			IgA	fluorescence
Ab IgG	dextran	imidocarbonate	IgG	
Ab IgM			IgM	

[a]RIA = radioimmunoassay; EIA = enzyme immunoassay.

[b]Human chorionic gonadotropin.

micro-environment at the combining site is significantly influenced by localized charge on the matrix. The hydrophobic or hydrophilic nature of the carrier, the surface area and degree of lattice porosity, and the charge characteristics of the solid phase in the macro- and micro-environment at the matrix surface must, therefore, be considered.

Coupling of Immunogens to Solid Phases

Physical Attachment

After selection of the matrix, the next step involves the selection of the attachment method. Adsorption was initially used because of its convenience and ease. The adsorbed substituent exhibited enough immunological specificity to allow a qualitative assay to be carried out (Campbell and Lerman 1949). This method requires the adsorbent matrix to have a high enough affinity for the protein to keep it bound, and yet not so high that its biological function is seriously impaired. In addition to cellulose and its derivatives, polystyrene and polypropylene discs and test tubes have been used as adsorbents for antibodies (Catt 1967, 1969). These surfaces are coated with antibody by allowing the antiserum to stand briefly in the tube or the discs to soak in 1 to 2 ml of serum. When the coating is carried out with diluted whole serum, the monolayer will be formed not only from the antibody immunoglobulin, but also with other serum proteins. This dilutes the immobilized antibody and leads to low activity. Also, cross reactivity and non-specific adsorption are enhanced in the presence of extraneous immobilized protein. To avoid these problems, Abraham and Odell (1970) treated the

antiserum with 2-ethoxy 6,9 diaminoacridine prior to coating to precipitate all serum proteins other than IgG. Using this procedure, they were able to coat 680 ng of IgG on the inner surface of the polypropylene tube. Another common adsorption enhancement procedure is to crosslink the previously purified protein prior to adsorption, or after adsorption of the protein onto the matrix. In both instances, this can result in some loss of immunological activity (Avrameas and Ternynck 1969). This step is often necessitated by the weak physico-chemical linkage between the protein and the matrix. Any change in ionic strength, pH, temperature, or immunogen concentration in the bulk solution will disrupt and dissociate this bond.

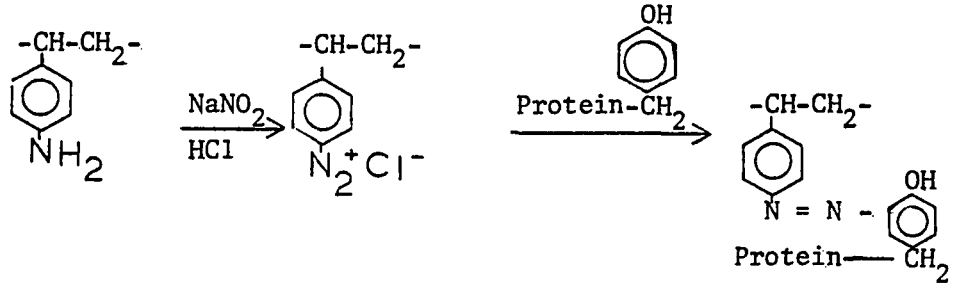
The immunoglobulins can also be immobilized by occlusion in crosslinked polymeric gels. The lattice network retains the much larger protein molecule, while allowing the smaller antigen to diffuse into the gel. Goodfriend, Ball, and Updike (1969) entrapped the antisera in polyacrylamide gels. The antigen is then incubated with the gel-entrapped antibody contained in small disposable columns. After incubation, the gel is washed and then the radioactively labeled antigen counted. Microencapsulation of antibodies has also been carried out using spherical ultrathin polymer membranes (Chang 1964). Each of these membranes envelops a microdroplet of antiserum, thus preventing the leakage of the antibody from the microdroplet. The thin membrane allows the antigen to diffuse rapidly (< 30 seconds) across the interface to react with the entrapped antibody. This causes the antigen-antibody

complex to become permanently entrapped in the microdroplet until the antigen-antibody bond is disrupted.

Covalent Attachment

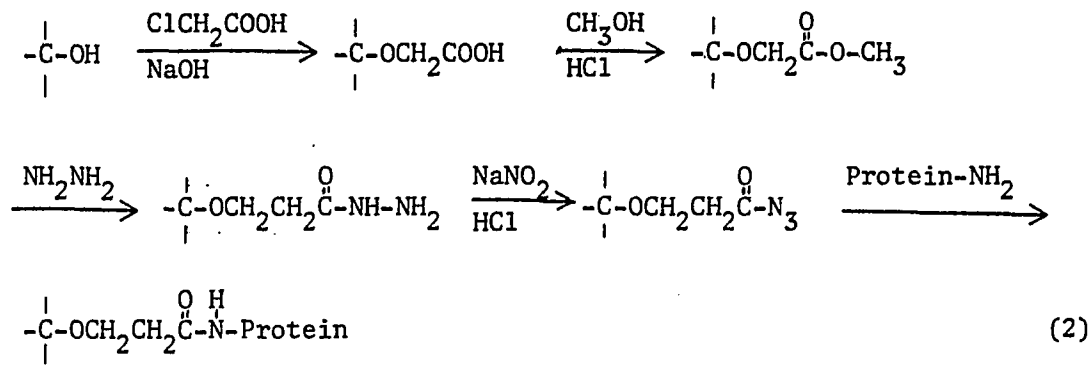
Covalent attachment often involves difficult chemistry but adsorption involves numerous variables, including those previously mentioned, that are difficult to define and control (Catt 1969). Conditions in the assay itself may be encountered that cause unexpected desorption (Ransom 1976). Different batches of material used may vary widely and unpredictably in their adsorbing activity.

There are several chemical methods of binding which can be characterized by the type of covalent bond formed. One of the first reported immunologically useful molecules chemically linked to a solid was Ovalbumin (Campbell et al. 1951). This protein antigen was immobilized on p-aminobenzyl ether cellulose matrix by a diazotization reaction. The formation of azo links via diazotization of aromatic amines usually occurs as shown in Reaction (1) with the amino acid tyrosine being the primary reactive amino acid. Other amino acids shown to be involved include lysine, arginine, tryptophan, and histidine (Cuatrecasas 1971).



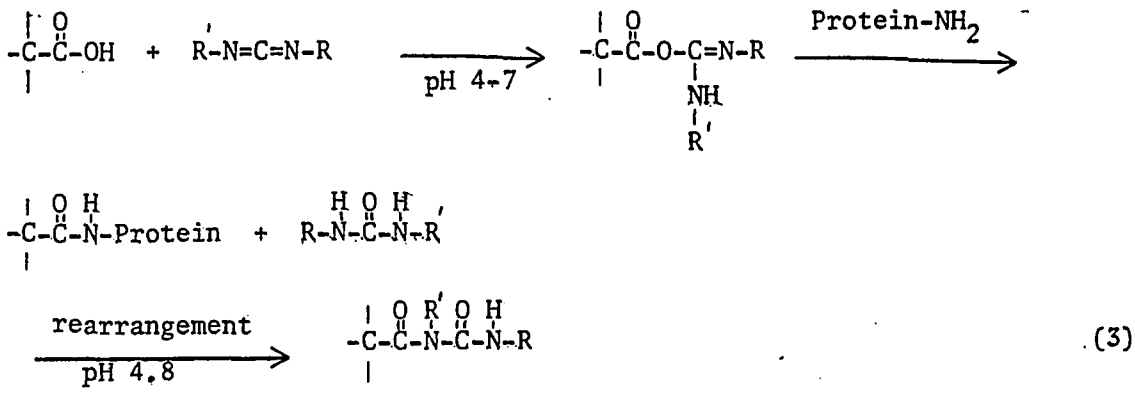
(1)

Another one of the older and more frequently employed methods uses the azide linkage. The natural support most often used is activated carboxymethyl cellulose (CMC), or a similar agarose or dextran matrix, activated and coupled to the protein as shown in Reaction (2).



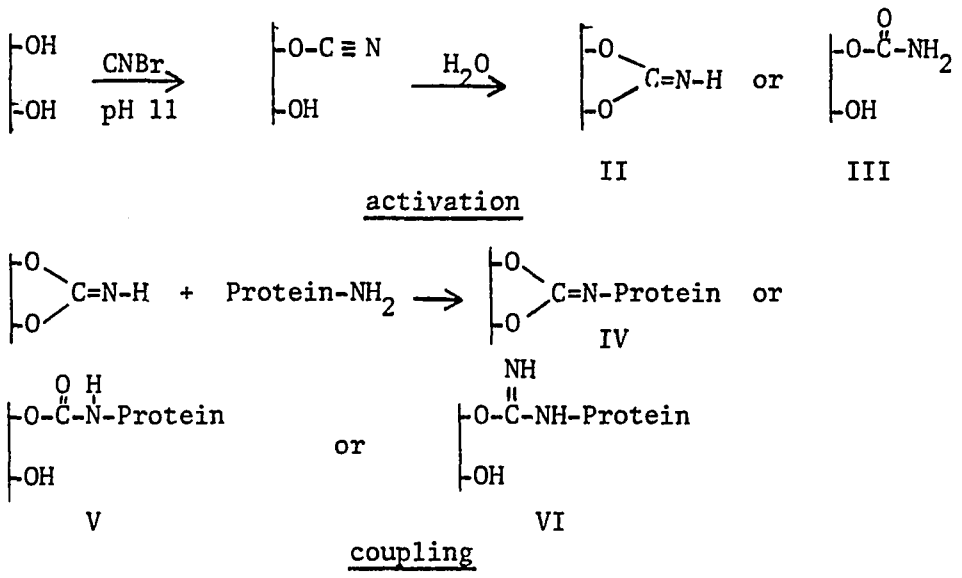
(2)

The formation of the amide bond using Reaction (2) is attractive because the link between the protein and the matrix is at least as strong as the amide bonds between the various amino acids in the protein. The carbodiimide method also yields an amide linkage between the immunogen and matrix as shown in Reaction (3) (Line, Kwong, and Weetall 1971). This well-characterized coupling reaction, adopted from solid phase peptide synthesis, can be carried out under extremely mild conditions (Marshall and Merrifield 1971). One possible problem is the rearrangement from the O-acylisourea to the N-acylurea. The extent to which this affects the coupling efficiency was studied by Swaisgood and Natake (1973). The results of this investigation indicated that at a pH greater than 4.8, this reaction is negligible.



(3)

Probably one of the most popular and well-publicized methods for covalently linking immunogens to crosslinked dextran (Sephadex) and agarose gels involves activation with cyanogen halides. These are capable of attaching the protein through its pendant primary amino groups (generally thought to be provided for the most part by the protein's side chain lysyl residues) to the hydroxyl groups on the matrix (Cuatrecasas 1971). Although the activation and coupling mechanism have not been firmly established, recent evidence (Axen and Vretblad 1971; Ahgren, Kagedal, and Akerstrom 1972; Weetall 1975) indicates that CNBr adds across vicinal OH groups (vicinal in three-dimensional space; i.e., they may be on the same or separate polymer chains) to form both the unreactive carbamate (Structure III) and the reactive imidocarbonate (Structure II) as shown in Reaction (4). Activation of agarose gels is generally carried out in aqueous media at pH 11 (Cuatrecasas 1970), while, with crossed linked dextrans, a lower pH of 9.5 to 10.0 is used to prevent large amounts of gel crosslinking at higher pH.

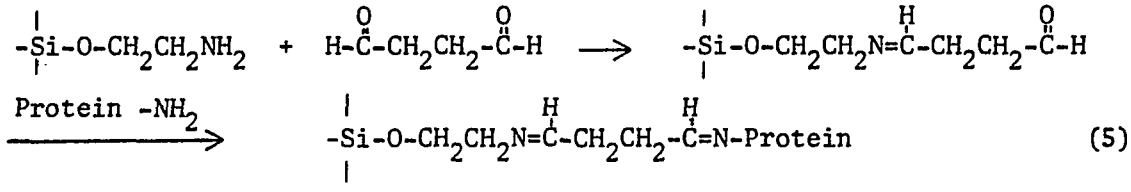


(4)

The activated gel (Structure II) then reacts with the protein to form either an N-substituted imidocarbonate (Structure IV), carbamate (Structure V), or most likely the isourea (Structure VI).

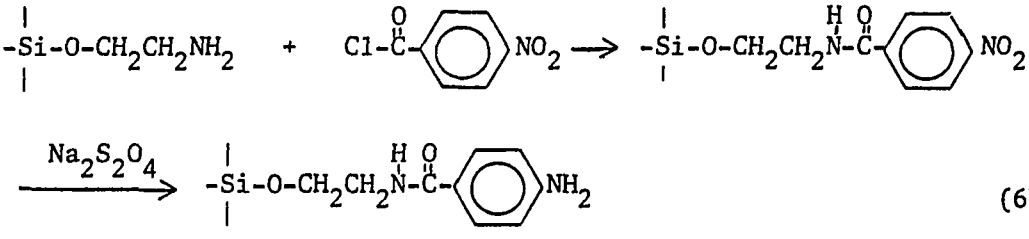
Like the aforementioned examples of Sephadex and agarose matrices, controlled pore glass (CPG) particles serve as supports for covalent attachment of proteins. The glass is first silanized to place an organic functional group on the surface. This is usually accomplished by reacting the CPG particles with a bifunctional silane. (This will be discussed in more detail in the Methods and Materials section.) Antibodies then may be directly coupled to the functional group on the CPG particles through the appropriate amino acid side chains. This can be accomplished by the reaction of glutaraldehyde with the derivatized silicon matrix as shown in Reaction (5). In this instance, glutaraldehyde is used only as a monofunctional reagent in its reaction with the protein. One part of the reagent is attached to the matrix and serves to place the reactive group, which later binds with the protein, away

from the matrix to prevent any spatial problems. Glutaraldehyde is also used as a bifunctional reagent where intermolecular covalent linkages between immunoglobulins are formed after the protein has been adsorbed or covalently linked on a suitable matrix (Avrameas and Ternynck 1969).



(5)

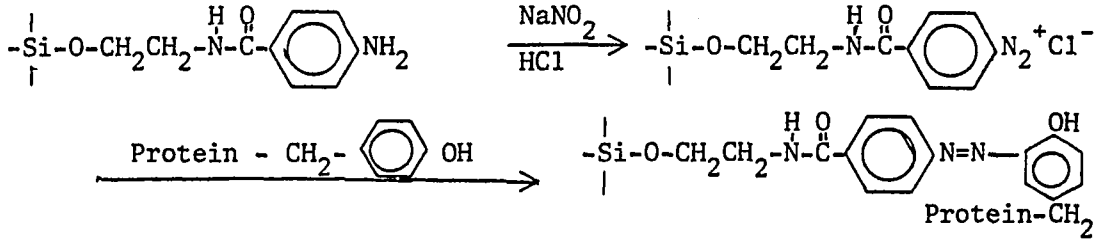
The aminopropyl CPG particles may be further derivatized to introduce other reactive groups. For example, reaction with p-nitrobenzoyl chloride to yield the p-nitrobenzamide (Reaction 6), from which the p-aminobenzamide is generated by reduction with $Na_2S_2O_4$. Diazotization of the aromatic amine and coupling of the protein through the tyrosine residue (Cuatrecases 1971) is shown in Reaction (7). Other amino acid residues thought to be involved in the azo linkage include tryptophan and histidine (Weetall 1975).



(6)

The reaction of succinic anhydride with the alkylamine derivative yields a covalently attached aliphatic ligand with an active carboxyl group removed from the silica matrix. This succinylated ligand can react with an immunogen (Reaction 8) or with N-hydroxy succinimide (Reaction 9) to yield an activated ester which permits

simple immobilization of proteins with free primary amine groups (Reaction 10).



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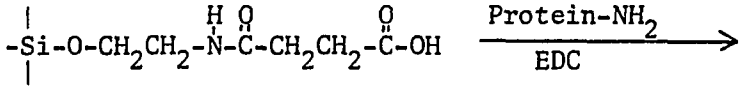
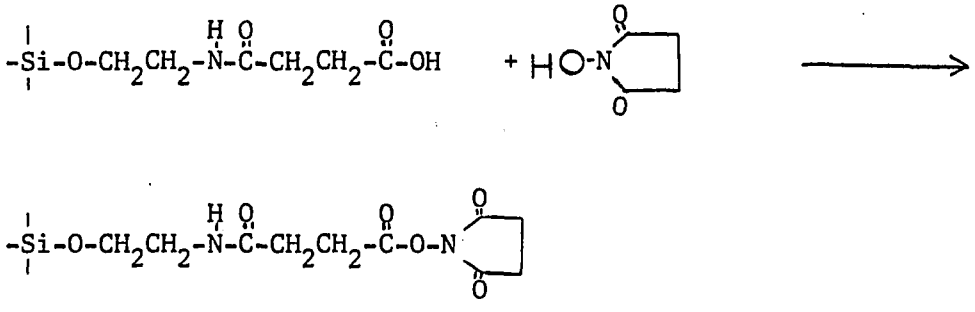
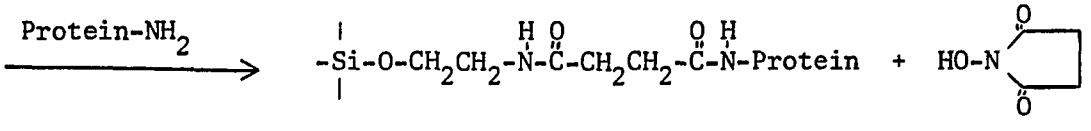


(8)



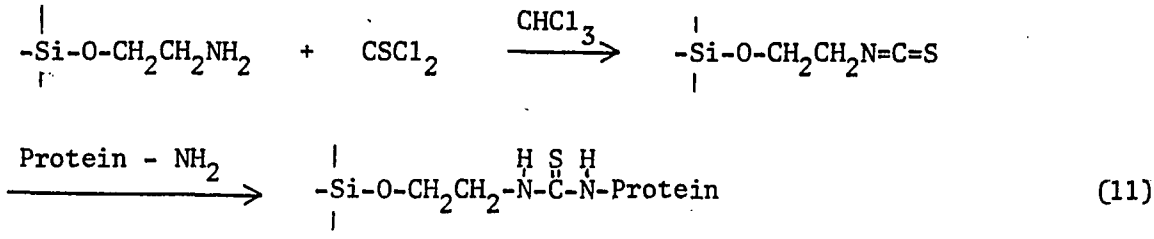
(9)



(10)

Antisera have been attached to isothiocyanate derivatives in high yield and with good retention of antibody activity (Line et al. 1973). The

isothiocyanate is generated from the alkylamine CPG particles by reaction with thiophosgene in $CHCl_3$ as shown in Reaction (11). The CPG isothiocyanate couples with proteins through their primary amino groups with the formation of the N,N' substituted thiourea adduct.



(11)

Implementation of Immobilized Immunogens into an Immunoassay System

Most types of immunological assays using solids phases except the homogeneous enzyme assay (EMIT) are competitive binding assays. In all of these assays, the labeled component of an antigen-antibody reaction binds to its complimentary binding site (Figure 3). The amount of bound material depends on the concentration of other components of the system and if one of these is varied a change in the distribution of the labeled component between the bound and unbound fractions results. The properties of the label are used to determine its distribution, and thus a calibration curve can be constructed relating concentration of the label to the concentration of the component varied. The assay requires that behavior between the standard and unknown antigen be identical in their ability to displace labeled antigen from a labeled antigen-antibody

Li = ligand capable of complexing to binder. In RIA, ligand is antibody.

Li^* = labeled ligand

Bi_s = binder molecule covalently linked to matrix with specific affinity for ligand. In RIA, binder is specific antibody for ligand.

$$Li + Bi_s \underset{k_1'}{\overset{k_1}{\rightleftharpoons}} LiBi_s \quad \text{and} \quad Li^* + Bi_s \underset{k_2'}{\overset{k_2}{\rightleftharpoons}} Li^*Bi_s$$

at equilibrium

$$K = \frac{[LiBi_s]}{[Li][Bi_s]} \quad \text{and} \quad K^* = \frac{[Li^*Bi_s]}{[Li^*][Bi_s]}$$

assuming Li and Li^* have identical physicochemical properties

$K = K^*$ and bound-to-free ratio (R) for Li^* is

$$R = \frac{[Li^*Bi_s]}{[Li^*]}$$

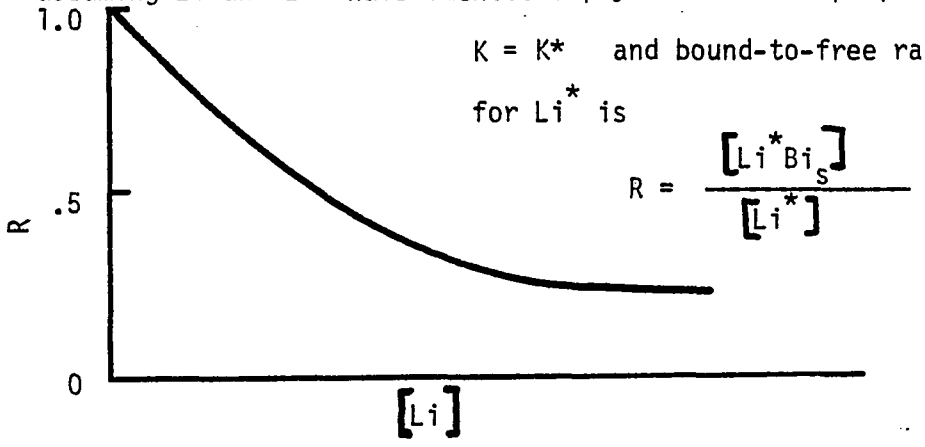


Figure 3. General mathematical expression for reaction stoichiometry involved in solid-phase competitive binding assay and result-calibration curve constructed from plotting (R) versus [Li].

complex. After incubation of the three essential components (Li, Li*, and Bi_s), the antigen-antibody complexes or bound antigens ($LiBi_s$, Li^*Bi_s) are separated from the free antigen (Li*, Li) and the label of either or both phases is measured. The ratio of bound to free (B/F) is usually plotted rather than just the bound or free fraction of the labeled ligand in order to improve upon precision of the assay (Besch 1976).

The development of various immunoadsorbents used in the solid phase competitive binding assays has provided for rapid and efficient separation (Daughaday and Jacobs 1971). In the solid-phase assay, the immunoadsorbent facilitates separation of bound and free fractions of the labeled immunogens. The advantage of solid-phase systems is that all three components of an assay (Ab, Ag*, and means of separating bound and free phases) are found within one unit. Binding of the antigen is rapid, virtually irreversible, and the two phases may be separated by a simple manipulation. The physical design of the system and the solid phase employed will determine the type of manipulation required to bring about complete physical separation of the bound and free phases (i.e., centrifugation, decanting, or filtering). The physical shape and composition of the matrix also dictate whether the system will function in a continuous or batch mode operation. The advantages and disadvantages of systems using such diversely shaped and sized solid phases as polystyrene test tubes, polypropylene discs, CPG particles, glass and plastic rods, latex spheres, and polysaccharide powders have been well-documented (Weetall 1975, Ransom 1976, Besch 1976).

After the integration of the solid phase containing the immobilized immunogen into an immunoassay system, the selection of a mode of detection is made. Because most solid-phase systems are used in clinical laboratories to detect low circulating levels (i.e., sub-microgram range) of such diverse biological materials as hormones, drugs, vitamins, and infectious agents, some type of natural or artificial label on the antigen or antibody is used. Thus, the mode of detection is governed by the particular label employed.

Currently, the most popular labeled immunoassay is the radio immunoassay (RIA) which employs an isotopically labeled immunogen. Radioimmunoassay is a form of competitive binding assay in which the ligand is an antigen and the binder is specific antibody to the ligand and is often attached to a solid phase. If the specific antibody is also a plasma protein, the assay is called a competitive protein binding assay (CPBA). Radioimmunoassay methods require only that the antigen combine with antibody; there need be no precipitate or visible evidence of this combination. (This is not the case when the RIA method does not employ the use of a solid phase. In that case, in order to separate the soluble binder (Ab) after reaction with Ag*I and AgII, the double antibody method of separation is commonly used. This involves the formation of a conventional antibody-antigen precipitate using a second Ab which then allows separation of the bound and free labeled fractions.) Various non-isotopic labels have also been investigated. These have included: an enzyme (EIA) (Engvall and Perlman 1972), a fluorescent group (fluoroimmunoassay) (Aalberse 1973), and a stable free

radical (spin immunoassay) (Wisdom 1976). The sensitivity of these labeled assays is primarily limited by instrumental considerations. However, an improvement in sensitivity can be achieved by concentrating the immunogen to be determined prior to the detection step by using an immunoadsorbent in the assay. When an assay involves a spectrophotometric protein determination, it is 400 times less sensitive than when it employs the use of a liquid scintillation counter (Table 2).

Table 2. The sensitivity of various methods of antibody determinations.

Detection	Method[a]	Sensitivity[b]	Reference
Spectrophotometric	Lowry	4 µg	Nezlin (1970)
	Lowry micro-method	0.2-0.02 µg	Nezlin (1970)
	Lowry (IM)	2 µg	Nezlin (1970)
	Lowry micro-method (IM)	0.4-0.04 µg	Nezlin (1970)
Scintillation counter	RIA	0.0001 µg	Skelley, Brown, and Besch (1973)
End-window counter	RIA	0.001 µg	Nezlin (1970)
Scintillation counter	EIA	0.003-0.0003 µg/ml	Wisdom (1976)

[a](IM), concentration of antibody by immunoadsorbent prior to detection step; RIA, radio immunoassay; EIA, enzyme immunoassay.

[b]Sensitivity expressed as µg of antibody nitrogen or µg/ml of antibody.

STATEMENT OF THE PROBLEM

The attachment of biological molecules to an inert matrix so that they exhibit optimal activity requires detailed understanding and control of the coupling conditions. Methods will be developed to assess the extent of attachment and to permit optimization of the reaction conditions. This will be accomplished for a number of different chemistries involved in the immobilization of biological molecules to an insoluble matrix. It is the intent of my research via this orderly investigation of the various chemistries used in the immobilization procedures to produce an immobilized material capable of functioning in a heterogeneous environment such as serum. Furthermore, the incorporation of the immobilized protein in an analytical detection system will facilitate measurement of a particular biological constituent. This system will allow for the detection of the substance in question in a complex medium and provide some indication of its concentration level relative to a "normal" concentration level.

The development of an optimum set of reaction conditions to yield such an immobilized material will be accomplished using classical analytical techniques. These techniques will allow the monitoring of important chemical parameters during the coupling of the protein to the matrix. The use of amino acid analysis, conventional electrophoresis, immunoelectrophoresis, and immunoprecipitin methods will help determine if the biological molecule is modified structurally or functionally

during the coupling process. Possible structural alterations of the biological molecule after exposure to chemical coupling reagents (such as the bifunctional reagents which promote both intra- and intermolecular crosslinking) may be determined by comparing certain of its physical properties to that of the native protein. Those properties which might be helpful in detecting structural changes include: electrophoretic mobility, gel filtration retention time, and molecular absorbance. An attempt to predict the influence of various parameters in the coupling reaction on the spatial orientation of the active site of the immobilized protein will be carried out. This will be accomplished by comparing biological activity of the immobilized immunoglobulin toward various immunologically active substrates to that exhibited by the native protein.

MATERIALS AND METHODS

Materials

The basic source of human IgG was Gamastan, Cutter Laboratories, Berkeley, California. The immune serum globulin contained 16.5±1.5% IgG in 0.3 M glycine with thimerosal added as preservative. This product was prepared from pooled units of human plasma and supplied in 10 ml vials. This IgG solution can be obtained locally from drugstores.

The other source of human IgG was purified from pooled whole blood donated by the experimenter. Reference human IgG serum was purchased from Behring Diagnostics, Somerville, New Jersey. This standard human serum has been stabilized by addition of albumin, 51 mg/ml. The serum contained 13.3 mg/ml IgG, 2.5 mg/ml IgA, and 1.13 mg/ml IgM. Standardized human reference serum was purchased from Kallestad Laboratories, Chaska, Minnesota, lot number R055J201-5 containing 24.5 mg/ml IgG, 4.20 mg/ml IgA, 3.5 mg/ml IgM, and 0.078 mg/ml IgD; lot R055J202-5 contained 9.6 mg/ml IgG, 1.5 mg/ml IgA, 1.4 mg/ml IgM, and 0.021 mg/ml IgD; lot number R055J203-5 contained 3.85 mg/ml IgG, 0.55 mg/ml IgA, 0.50 mg/ml IgM, and 0.0085 mg/ml IgD. All IgG solutions were stored at 4°C without any visual indications of product deterioration, even after storage up to 6 months. The precaution of centrifuging the IgG solutions prior to making dilutions was carried out once a month. Non-reference IgG was subjected to further purification as discussed in Procedure Section.

The antibody to human IgG was obtained from three different commercial sources. Antihuman rabbit IgG, gamma chain specific, lot number 2849A, in 5 ml vials was purchased from Behring Diagnostics, Somerville, New Jersey. This preparation contained additional excess antigen (human plasma proteins) and was removed according to methods described in Procedures Section. Antihuman goat IgG was a gift from Dr. W. Dito, Scripps Clinic, La Jolla, California. This antisera was prepared commercially by Technicon Instruments, Tarrytown, New York, lot number B4L401 and had passed the expiration data unopened and stored at 4°C by 3-6 months when opened and evaluated. Physical examination of the solution revealed that some denaturation had taken place, although assaying the antiserum on radial immunodiffusion (RID) plates indicated that sufficient antibody remained in a viable form to warrant use of the product. Antihuman goat IgG, gamma chain specific, log IGG-P004-S, grade S, in 0.05% NaN_3 was purchased from Atlantic Antibodies, Westbrook, Maine. This solution had undergone sterile filtration to remove particulate matter. This solution had antibody concentration of 1.01 mg/ml as determined by Protein Assay as outlined in Procedures Section. This was 13% of total protein content in the antihuman goat IgG solution.

Two different types of commercial RID plates were purchased. An LC Partigen RID plate for quantitating low levels of IgG in serum in the range 0.01-1 mg/ml was obtained from Behring Diagnostics. A RID plate for quantitating low levels of IgG in serum in the range 0.05-5 mg/ml was purchased from Kallestad Laboratories. All plates were stored upside-down to prevent condensation from collecting in the wells. RID

plates were also prepared in the laboratory as outlined in the Appendix. The materials used in their construction were: agarose, lot A786, Miles Laboratories, Elkhart, Indiana. This agarose had a sulfate content of < 0.5% and a clarity of 85% at 500 nm with a 1% solution which made it acceptable for immunodiffusion work. The electroendoosmosis purity value (M_r) was 0.20-0.23 for this lot. This value was slightly in the high range which could possibly result in increased non-specific protein binding in the gel. This appeared to be the case with protein solutions containing more than 15-20 mg/ml as seen after staining prior to washing the gel with high ionic strength buffer. Because most solutions assayed were in the range of 0.05-1 mg/ml, these gels using this agarose lot were satisfactory.

Polyacrylamide gels were constructed from acrylamide, 99%+ electrophoresis grade, gold label, Aldrich Chemical Co., Milwaukee, Wisconsin; ammonium peroxydisulfate, 98%+ purity, lot number E5K, Eastman Kodak, Rochester, New York; N,N,N',N' tetramethylethylenediamine (TMEDA), Aldrich Chemical Co., 99%+ purity. Coomassie Blue R (No. B-0630) was purchased from Sigma.

Column chromatography was carried out on 1.5 x 100 cm and 1.5 x 30 cm glass columns with adjustable plunger, plastic end plates and supports, Glennco Scientific, Houston, Texas. Packing materials employed were: DEAE cellulose powder, micro-granular form, 1.0 mEq/dry g, Whatman, Clifton, New Jersey; Sephadex G-200, 40-120 micron lot number 6175, and Sephadex G-75, 40-120 micron lot number 1866, Pharmacia Fine Chemicals, Piscataway, New Jersey; Bio Gel A-5m and

Affi-Gel Blue, 100-200 mesh, 11.5 mg/ml albumin capacity, Bio Rad Laboratories, Richmond, California. All packing except Affi-Gel Blue was in the dry, unswollen state prior to initial use. Affi-Gel Blue was supplied fully hydrated in deionized water, pH 7 with 0.02% NaN_3.

The CPG particles, 177-840 micron, lot numbers 0314 and 2G02, were purchased from Corning Biological Products Department, Medfield, Massachusetts, or from Pierce Chemical Co., Rockford, Illinois. The 177-840 micron particles were irregularly shaped and had a surface area of 70 m^2/g. Lot number 2G02 had organofunctional groups on the surface, 0.170 mEq of free amine/g of CPG (manufacturer's specifications). Micro-titration of these particles by method discussed in Procedures Section gave a value of 0.025 mEq free amine/g CPG. A possible explanation for these discrepancies is discussed in Results Section. The CPG particles, 200/400 mesh (37-74 microns) were obtained from Electro Nucleonics, Fairfield, New Jersey. These particles had a surface area of 50 m^2/g and had no organofunctional groups on the surface. Spherical, HPLC silica packing material SW5 and SW10, 5 and 10 microns, batch numbers 2.241 and 70/79, respectively, were gifts from Professor M. F. Burke. These are manufactured by A.E.R.E., Harwell, United Kingdom, and distributed in the United States by Spectra Physics. Silanization procedures were carried out on these silica and CPG particles using the following reagents: 3-aminopropyltriethoxysilane, purchased either from Aldrich Chemical Company, Milwaukee, Wisconsin, or from PCR, Gainsville, Florida, lot number 9007; trimethylchlorosilane, PCR, lot number 6593; and p-nitrobenzoyl chloride, Aldrich Chemical Co. Water soluble

carbodiimide 1-ethyl-3(3-dimethyl-amino-propyl)-carbodiimide (EDC) was obtained from Sigma Chemical Co., St. Louis, Missouri. It was stored in a dessicator at 0°C until used. Some deterioration in the EDC coupling efficiency was observed after prolonged storage, presumably due to its reaction with moisture. All EDC remaining after 6 months was discarded and fresh EDC was used in the coupling reactions. Chloroform was dried by distilling from P_2O_5 and stored in tightly stoppered container. Thionyl chloride was purified by distillation. Toluene was purified by distilling over sodium metal and stored over 4A molecular sieve. The buffers employed were: 0.01 M acetate, pH 5.6; 0.01 M phosphate, pH 6.9; 0.02 M phosphate in 0.15 M NaCl, 0.02 M phosphate, pH 7.6 in 0.15 M NaCl-phosphate buffered saline (PBS); PBS in 50% (v/v) glycerol; 0.02 M phosphate in 0.15 M NaCl, pH 8.0; 0.02 M phosphate in 0.15 M NaCl, pH 2.0; 0.02 M tri)hydroxymethyl) aminomethane (Tris) HCl, pH 8.0; 0.01 M citrate, pH 2.2 with thiodiglycol. All buffers were made from sodium salts except where noted, and were made with reagent grade chemicals and deionized water. For fluorescent and HPLC work, the water for the buffer solutions was doubly distilled, once from a glass-lined still and filtered through a 0.2 micron Millipore filter. The buffer solution was degassed under reduced pressure for 10 minutes. All buffers were stored at 4°C and were discarded upon visual indication of bacterial contamination (i.e., white, floculent material floating in solution). Functional group micro-titrations were carried out using 0.0154 M NaOH. This solution was prepared from 50% NaOH, Analytical Reagent grade, Mallinckrodt, St. Louis, Missouri, and standardized with potassium acid

phthalate, primary standard grade, G. Frederick Smith Chemical Co., Columbus, Ohio. Electrolyte solution for use in particle sizing studies was obtained from Coulter Electronics, Hialeah, Florida. Fluorescein isothiocyanate was obtained from ICN Pharmaceuticals, Inc., Cleveland, Ohio, and used without further purification. Immunobead RAH-3 containing 480 µg IgG/g bead, batch number 15752, was purchased from Bio Rad.

Apparatus

All spectrophotometric measurements were performed on a Cary 14R spectrophotometer equipped with dual range (0-0.1, 0.1-0.2 absorbance) or (0-1, 1-2 absorbance) slidewire. A Beckman Amino Acid Analyzer Model 43B provided amino acid analysis of immobilized protein hydrolysates. All fluorescence measurements were carried out on a Perkin Elmer Model 204A Spectrofluorometer equipped with a Linear Model 550 strip chart recorder. A special cell similar to that described by Gabel, Steinberg, and Katchalski (1971) (Figure 4) for measuring the fluorescence emitted from the surface of packed beds of immobilized antibodies was constructed. It consisted of a micro cell (quartz 5.0 mm O.D., 3.0 mm I.D.) obtained from Precision Cells, Hicksville, New York. The bottom was cut off and a top and bottom holder for the micro cell were constructed to fit into an ordinary 1.0 x 1.0 cm fluorescence cuvette. The cell holder was fabricated from nylon and two stainless steel 18 gauge syringe needles. The angle between the from face of the cell and the exciting beam was milled to 30±.5° on a round table mill. Because of excessive back pressure generated by the liquid passing through micro

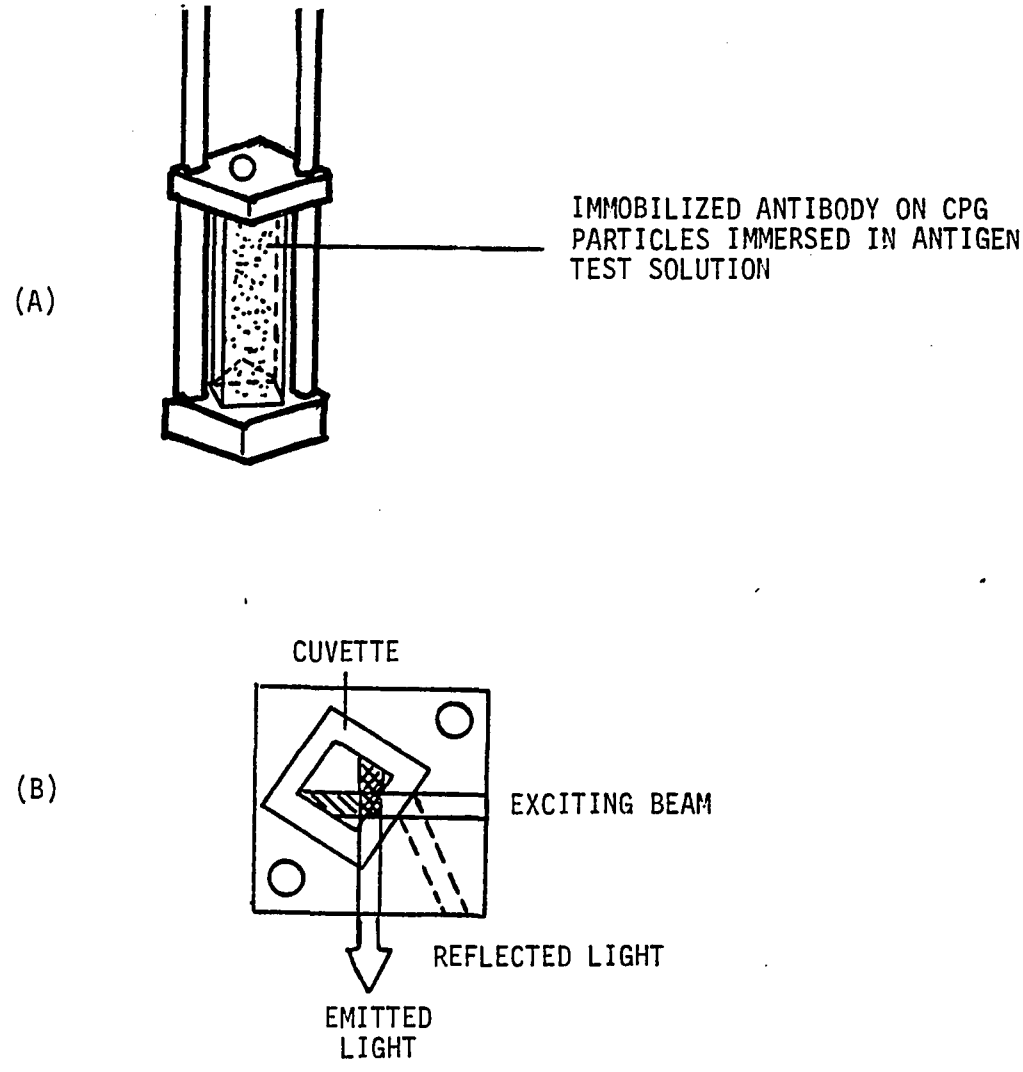


Figure 4. Cell for measuring fluorescence of immobilized antibody. -- (A) View of the cell. (B) Sketch of the optical pathway, viewed from above.

CPG particles, excess liquid was removed by aspiration using a Pasteur pipette with a porous glass frit epoxied over the opening to prevent loss of material during aspiration. The drain in the bottom of the cell as shown in the original design was not used. Silicone rubber sealer, Dow Corning, Midland, Michigan, was used to cement the bottom of the micro cell to the square nylon holder. This prevented leakage of the antigen test solution out of the micro cell into the cuvette filled with distilled water which acted as a constant temperature bath for the micro cell. Use of this cement did not appear to release fluorescent material into the aqueous medium.

Electrophoresis was performed on an Ortec Model 4200/4100 high resolution electrophoretic system with pulsed constant power supply. Electrophoresis of the proteins was carried out at 4°C in order to minimize denaturing of protein resulting from the heat generated during the separation. Buffers employed were 0.02 M, pH 8.0 PBS (lower reservoir); and 0.02 M, pH 7.6 PBS (upper buffer reservoir). The effluent from column chromatography was monitored by an ISCO UA-4 absorbance monitor connected to a dual beam Type 4 optical unit. Column fractions were collected in the time mode on a Gilson microfractionator using rimless 13 x 100 mm borosilicate culture tubes, Corning #99445 or Kimble #73000. High pressure liquid chromatography column work was carried out on a Spectra Physics Model 3520 gradient high pressure liquid chromatograph equipped with a Model 770 variable wavelength u.v. detector. Immobilized antibodies on SW5 and SW10 silica were packed in a 1/4 to 1/16 inch stainless steel reducing union fitting with 2 μ stainless steel frit and

connected to the Model 3520 using stainless steel Swagelok connections. The 2 micron stainless steel frit served as a bed support and prevented loss of the column packing material. Gravity flow liquid chromatography was performed on modified 1.0 x 20 cm glass tubing with fine porous glass frit as shown in Figure 5. The glass frit was pretreated with HNO_3, then soaked in a solution of albumin and washed extensively prior to use in immunoassays. A water aspirator was connected to the modified tube (Figure 5) to increase flow rate through the CPG particle column. Using this system, flow rates of 0.2-0.6 ml/min were obtained. All quantitative micro-titrations were carried out on a titration apparatus described elsewhere (Seymour, Clayton, and Fernando 1977). The qualitative micro-titrations were carried out in the same type of stainless steel column described above. The eluent was monitored by a Corning Model 110 digital pH meter interfaced to a data acquisition system (Ramaley and Wilson 1970) and a Hewlett-Packard 2100 computer. A gas-tight septum was fitted to the top of the column and a 5 ml constant delivery (volume) syringe inserted. In order to minimize fatigue and fracture of particles, the stirring bar in the titration cell was rotated at the slowest rate while N_2 purging pressure was increased to compensate for this decreased mixing efficiency of the stirring bar. Fluorescent microscopy was carried out on a Zeiss microscope equipped with a variable wavelength excitation source and monocular lens with adaptor for Nikon camera. High-speed Ektachrome, ASA 150, film was used. Development of the film was pushed to ASA 400. Particle sizing experiments were carried out on a Coulter Model T Particle Counter interfaced

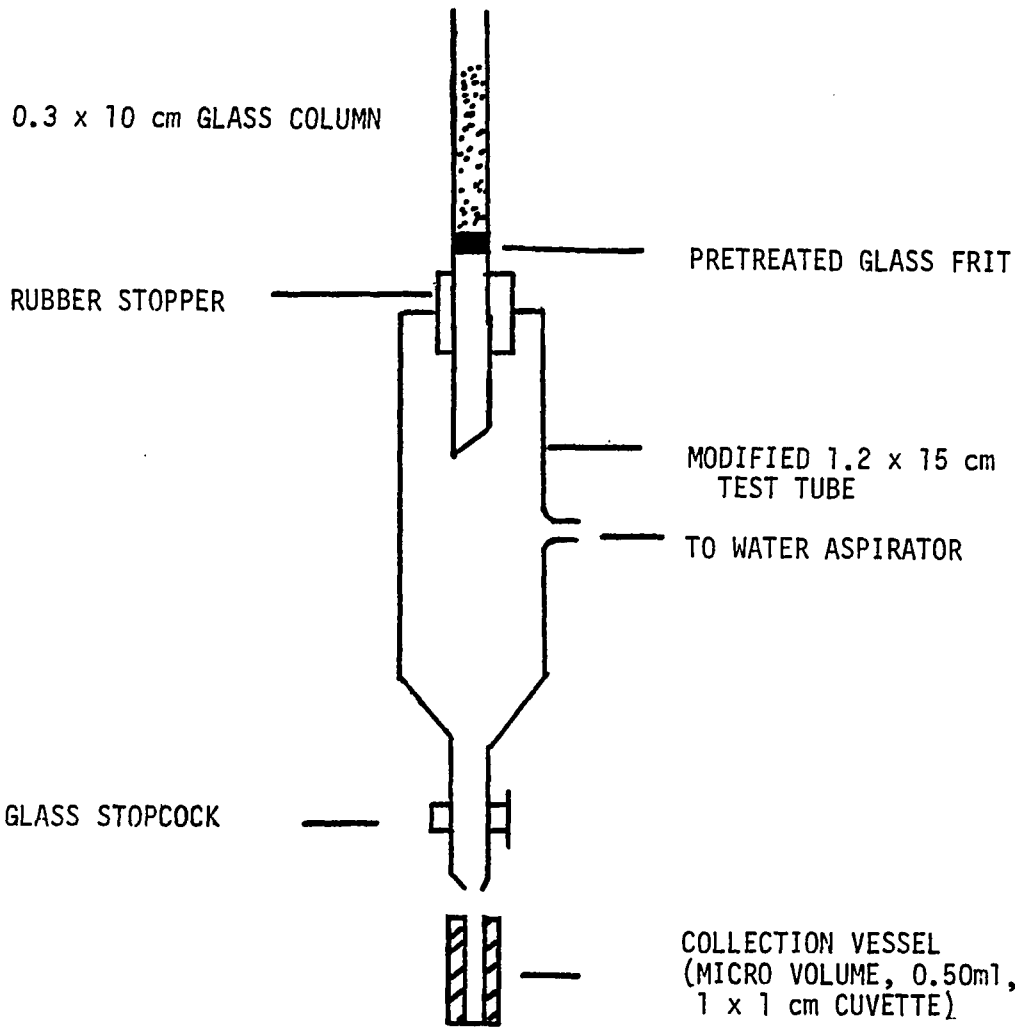


Figure 5. Atmospheric pressure liquid chromatography apparatus.

with a PDP/8 microprocessor and Houston high-speed XY recorder. Pre-sizing of particles before use in the Coulter Particle Counter was carried out on a Sonic Sifter, Bradley Company, Madison, Wisconsin. Pressure dialysis was carried out either on Amincon 50 ml Pressure Dialysis Cell with UM2 membrane, molecular weight exclusion limit 10,000; or on a Millipore 13 ml stirred cell dialysis unit with PTHK 02510 membrane, M.W. exclusion limit 20,000.

Procedures

Silanization

Silanization of the CPG particles was carried out by two methods, organic and aqueous, both of which used gamma aminopropyltriethoxysilane as the silanating reagent. In the organic procedure, the silanization reaction was carried out by equilibrating the CPG particles in 5-10% HNO_3 solution at room temperature for 4 hours or under reduced pressure for 20 minutes. In the silanization and activation procedures, the CPG particles are initially placed under reduced pressure to ensure complete coverage of the particle, including inside the pores, with the silanating or activating reagent. Previous experiments with CPG (Smith 1975) have shown that complete equilibration with these reagents at atmospheric pressure requires up to 20 hours. The glass particles are then boiled in the HNO_3 solution for 1 hour on a hot plate with overhead stirring of the HNO_3 solution. Then they are dried in a vacuum oven at 110°C for two hours in a steep-walled container with cover to prevent loss of particles if bumping occurs during the drying process. After drying,

they are rinsed with 200 ml of dry toluene per gram of particles, air dried, placed in a vacuum oven at 110°C overnight. The glass is then refluxed for 6 hours in a 10% solution of gamma aminopropyltriethoxysilane in dry toluene, 100 ml solution volume/g CPG particles. After refluxing, particles are washed with 200 ml dry toluene and dried in vacuum oven at 110°C for 4 hours. The glass is then refluxed again in a 10% solution of gamma aminopropyltriethoxysilane for 6 hours, washed, and dried. The final silanization step is optional depending on the desired final concentration of free amine groups on the surface of the CPG particles. This step can be carried out in two different ways. Either treat the glass with 5% solution of triethoxyethyl silane under reflux conditions for 6 hours in dry toluene, wash, and dry overnight; or use a procedure similar to Halász and Sebastian (1969) where the glass is placed in a 9×10^{-3} M solution of trimethylchlorosilane (TMCS), purged with N_2, refluxed for 6 hours, washed, and dried overnight. All glassware used in the silanization procedure was rinsed in $CHCl_3/CH_3OH$ before and after use and stored in oven when not in use. Glassware used in this procedure was saved just for that purpose when possible. The procedure for preparing water silanized CPG particles consists of boiling glass in 5% HNO_3 solution for 1 hour, dried in oven at 110°C for 4 hours, and then refluxed in 20% solution of gamma aminopropyltriethoxysilane. This solution is adjusted to pH 3.5 with HCl until the pH stabilizes. The glass is then washed with copious quantities of water and allowed to dry overnight in the oven.

To produce the arylamine derivative, the alkylamine glass is refluxed gently overnight in dry chloroform containing 10% triethylamine and p-nitrobenzoyl chloride (PNBC), 0.30 g PNBC/g CPG particles. The glass is then filtered, washed with 200 ml dry chloroform, and dried in an oven at 110°C for 4 hours. The nitro group is then reduced by boiling the glass in a 10% solution of $Na_2S_2O_4$ for two hours. After filtering and washing with distilled water, the glass is placed in distilled water under reduced pressure for thirty minutes and then allowed to air dry.

Activation Reactions

Additional activation procedures were carried out to produce particles with either longer spacer arms or reactive groups other than $-NH_2$ at the ends of these spacer arms, or both. Initially, the CPG particles were thoroughly washed to remove any residual organic compounds remaining from the silanization procedure. The CPG particles were then equilibrated with coupling buffer, first under reduced pressure for 30 minutes, and then at atmospheric pressure at 4°C for 4-6 hours. During the equilibration at 4°C, the beads were gently agitated by end-over-end mixing to ensure thorough mixing with the minimum of fatigue to the glass matrix. A magnetic stirring bar is not suitable for this purpose as it increases fragmentation of the CPG matrix. The buffering solution was filtered, and the moist CPG particles were then activated by addition of the various reagents according to the methods listed below.

Glutaraldehyde. To 0.25 g of the moist aryl or alkyl amine CPG particles, 5 ml of 25% aqueous glutaraldehyde (Aldrich) in 25 ml of water was added. The 25% glutaraldehyde solution was stored frozen at 0°C in the dark to minimize polymerization. Recent evidence (Kamin 1977) indicates that between 6-12% (w/v) of this solution has polymerized even after being stored under these conditions for 6 months. The mixture was allowed to react under reduced pressure and temperature for 20 minutes. The glutaraldehyde solution was decanted and the CPG particles washed three times, twice with water and once with 0.02 M phosphate buffer used in the coupling reaction. Other buffers were used in coupling reaction where indicated but did not contain any primary or secondary amines or other strongly nucleophilic groups. The protein solution of optimum concentration was mixed with enough buffer to cover the activated CPG particles (usually 1.0 ml) and added to screw cap vials, 2 dram, followed by 15 minutes of degassing. The vial was then placed in the end-over-end mixer and gently mixed for the specific time.

The uptake of glutaraldehyde was followed according to the procedure of Hajdu and Friedrich (1975). An aliquot of the activation mixture is reacted with an excess of hydroxylamine. The glutaraldehyde remaining in the activation mixture forms an intermediate with the hydroxylamine, ε_{240} 9.3 $(M)^{-1}(cm)^{-1}$. This change in absorbance during the activation process is related to the uptake of glutaraldehyde over the course of the reaction. No interferences from the protein, protein complex, buffer, or polymerized glutaraldehyde were noted using this method.

Diazotization. To 0.5 g of moist arylamine CPG particles, 8 ml of cold 2 N HCl is added to the glass and reacted under reduced pressure for 15 minutes. Then 150 mg $NaNO_2$ is added to the mixture immediately after the vacuum is released. The mixture is then placed under reduced pressure again and allowed to react for 30 minutes. The reaction vessel is brought to atmospheric pressure, another 0.150 mg $NaNO_2$ added, and the solution allowed to react another 2 hours. The particles are then filtered and washed with cold 5×10^{-3} M sulfamic acid, 200 ml, and cold water, 800 ml. The efficiency of the diazotization procedure is followed qualitatively by visually observing color of the CPG particles as they change from light yellow to dark yellow/brown indicative of the diazotized particles. This is more clearly seen under the fluorescent microscope where the non-diazotized particles appear blue in contrast to diazotized particles which appear dark brown.

Carboxyl Intermediate. This coupling was carried out using CPG alkyl or aryl amine particles in two different protocols. The first involved activating the glass with succinic anhydride; the second involved adipic acid activation. In the first protocol, 8 ml of 5% aqueous succinic anhydride solution was added to 0.5 g of the amine CPG particles with concomitant monitoring of the pH of the reaction with a Fisher microelectrode. The pH was maintained at 6.5 by manual addition of concentrated NaOH. This usually required approximately 5 minutes before the pH of the solution stabilized, after which the reaction mixture was allowed to react under reduced pressure for 15 minutes, followed by return to atmospheric pressure. After checking the pH, the

reaction was allowed to continue for 2 hours at room temperature with gentle mixing. The glass particles were washed in the usual manner using 200 ml of water and air dried. An 8 ml portion of 15% $SOCl_2$ in dry $CHCl_3$ was added and refluxed gently for 4 hours. The product was washed in the usual way using 300 ml of dry $CHCl_3$ and then immediately dried in the vacuum oven at 90°C for 30 minutes followed by immediate coupling of the protein. The second procedure involves substitution of 5×10^{-3} M solution of adipic acid for the 5% solution of succinic anhydride; otherwise the procedure is identical.

Coupling Procedures

Glutaraldehyde or 1-ethyl-3(3 dimethylaminopropyl) carbodiimide (EDC) were employed as coupling reagents to bring about covalent linkage of the protein to amine groups on amine groups on the CPG matrix. When glutaraldehyde was used as coupling reagent, the moist CPG particles, 0.5 g, were suspended in enough PBS, pH 6.5, to cover the glass particles. Then 10% glutaraldehyde solution, 1 ml, was added and allowed to react under reduced pressure for 15 minutes. The solution of optimum concentration of protein was added, allowed to react at room temperature for 30 minutes. Then the reaction was placed in the mixer and rotated gently at 4°C for the specified time. When EDC was used as the coupling agent, the moist CPG particles were suspended in enough PBS, pH 8.0, to cover the glass particles. In an additional 1 ml of buffer, 2 mgEDC/mg protein in coupling solution was dissolved, added to the glass particles and allowed to react at reduced pressure for 15 minutes. After return to atmospheric pressure and room temperature, the particles were allowed

to react for an additional 30 minutes. The protein solution then was added and allowed to react 30 minutes at room temperature before being placed in the mixer and rotated gently at 4°C for the specified time. The reaction of the aryl or alkylamine CPG particles was carried out with protein in the absence of EDC or glutaraldehyde to determine the extent of coupling occurring without the use of the bifunctional coupling reagents. This was carried out by placing the CPG particles in 2.0 ml of PBS, pH 8.0, and equilibrating under reduced pressure for 15 minutes. After returning to atmospheric pressure, the protein solution was added and the coupling carried out as described previously.

The same coupling procedures were used with CPG particles having reactive carboxyl groups on the surface. The direct coupling of protein to these particles without the use of the coupling reagents EDC or glutaraldehyde was attempted.

Termination of all coupling reactions were carried out in the same manner. First, the coupling solution was filtered off through a 200 mesh nylon screen placed on top of a coarse sintered glass frit. Then 2.0 ml of 1 M glycine was added to the reaction vessel and allowed to react for 30 minutes at room temperature with any remaining free amine groups on the matrix. This presumably prevented multiple attachment of the protein to the matrix after removing the coupling solution.

Titration of Reactive Groups on CPG Particles

Titrations of acidic organic groups on the CPG particles were carried out using standardized NaOH. The glass particles were initially

dried in the oven overnight at 110°C. After cooling, between 0.100 and 0.200 g of the matrix were weighed out and placed in the washing apparatus. This consisted of a 30 ml filter funnel with a fine sintered glass frit. Across the glass frit was placed a 72 micron nylon screen, Spectrum Medical Industries, Los Angeles, California, which prevents clogging of the frit and enhances filtration rates. To further increase the rate of filtering, the filter funnel stem is placed in a one-holed #4 rubber stopper and then placed in the modified tube (Figure 5). The glass particles were pretreated with a 100 ml wash of 1×10^{-3} M HCl solution in order to assure all reactive carboxyl groups were in the protonated form. In order to assure thorough mixing, approximately 30 ml aliquots of the HCl were added to the sintered glass funnel containing the CPG particles and a piece of parafilm stretched over the top. The apparatus with the vaccum line disconnected was then inverted two or three times until the particles were all suspended in solution. After 5 minutes, the solution was drawn off and the process repeated twice more. Then the CPG particles were washed with 1000 ml of 0.15 M NaCl, quantitatively transferred to the titration vessel with minimum of 0.15 M NaCl. After the particles had settled, excess 0.15 M NaCl was removed and the remainder of the titration carried out according to the procedure developed by Seymour et al. (1977). For titration of basic organofunctional groups, the procedure is exactly the same except for the substitution of 1×10^{-3} M NH_4OH for 1×10^{-3} M HCl in the pretreatment step. A blank titration consisting of underivatized CPG particles in 0.15 M NaCl was performed with each batch of titrations for acidic and

basic groups. A control titration of just the 0.15 M NaCl solution was also carried out to determine contribution of background electrolyte.

Protein Purification

Purification of human IgG was carried out on a 1.5 x 100 cm DEAE cellulose column. The precipitation of the IgG by $(NH_4)_2SO_4$ was not carried out for two reasons. Corrosion of the centrifuge caused by $(NH_4)_2SO_4$ is not desirable, and secondly, if overloading of the DEAE column is avoided, the separation and purification of serum IgG is improved over the precipitation method (Deutsch 1967). Approximately 250 ml of 0.01 M phosphate buffer, pH 8.0, was allowed to equilibrate with the packing material overnight. The column was poured and rinsed with two column volumes of pH 8.0 buffer at 4°C. The flow rate was adjusted to 0.6 ml/min before addition of a sample of human serum. The sample had been previously dialyzed overnight against three changes of 500 ml of 0.01 M phosphate buffer and clarified by centrifugation at 5,000 rpm for 20 minutes. After placing the sample on top of the column, approximately 5 ml of the 0.01 M, pH 8.0 buffer was accomplished by a continuous buffer gradient with a change in phosphate molarity from 0.01 to 0.30 M and a concomitant fall in pH from 8.0 to 4.5 (Figure 6A). This was carried out with the system depicted in Figure 7B consisting of a mixing chamber and a buffer reservoir connected by siphon to the DEAE cellulose column. Effluent fractions of 4-6 ml were collected in the Gilson microfractionator. All IgG fractions were pooled, concentrated to approximately 10 mg/ml by ultrafiltration, and assayed according to the protocol listed in "protein assays" in this chapter. The myeloma

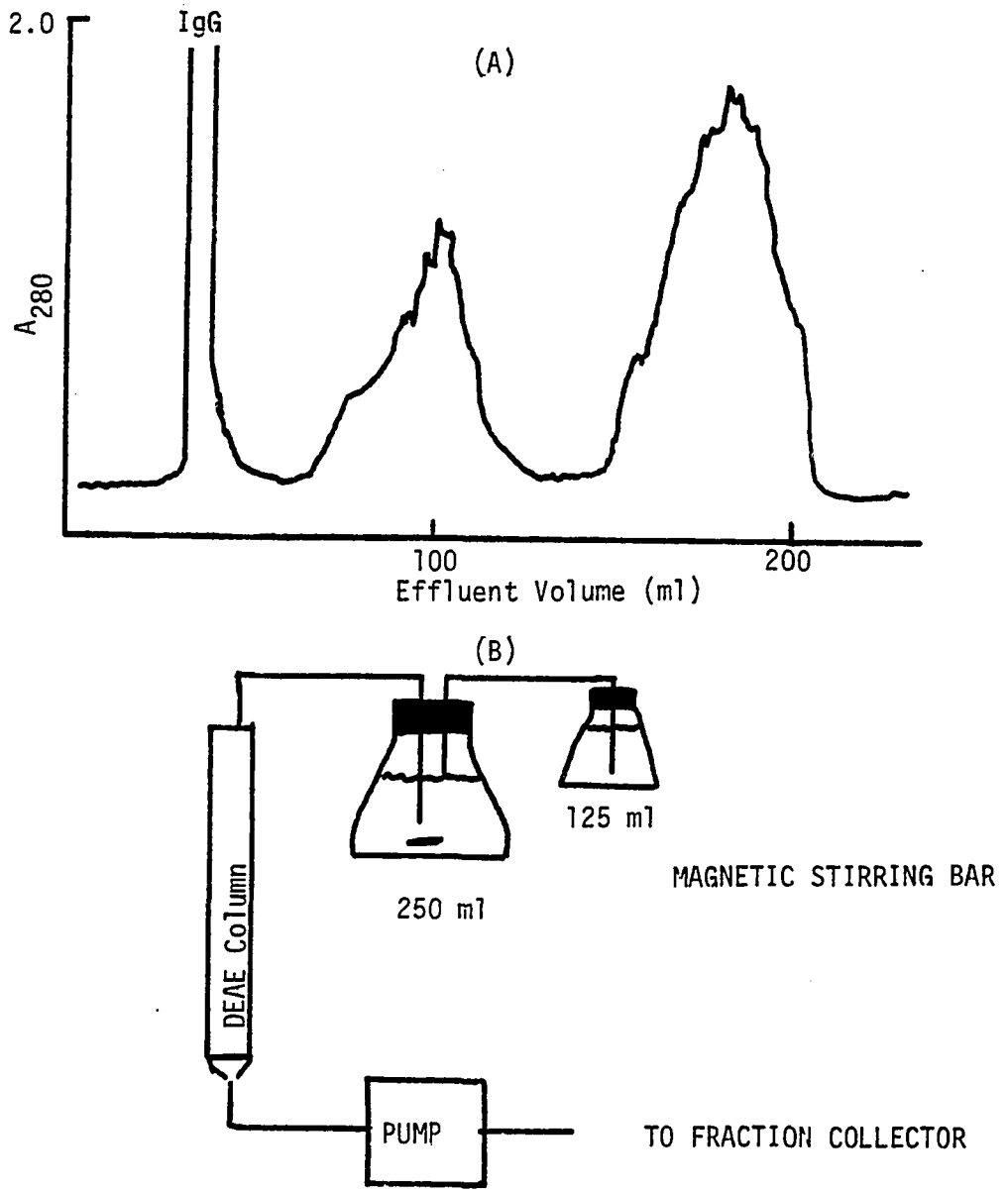


Figure 6. Chromatographic purification of IgG. -- (A) Elution profile of human serum using DEAE cellulose column 1.0 x 30 cm, pH 8.0-3.5 phosphate buffer gradient, and 0.20 ml/min flow rate. (B) Continuous buffer gradient system to separate various immunoglobulins from human serum. At beginning, the larger flask contains 0.02 M pH 8.0 phosphate and smaller one 0.30 M phosphate, pH 4.5.

serum protein IgG sample, kappa chain, a gift of Dr. Byers, Arizona Medical Center, was purified on a 1.5 x 30 cm column packed with Affi-Gel Blue, 50-100 mesh. The packing material was presoaked in 0.02 M phosphate buffer, pH 7.1, overnight at 4°C. The column was poured and washed with two column volumes of pH 7.1 buffer before addition of the serum sample (0.2 ml serum sample/ml Affi-Gel Blue). The serum sample had been previously dialyzed overnight against 500 ml of pH 7.1 buffer with a dialysate change every 8 hours. The sample was washed into the column with 5 ml of pH 7.1 buffer. The column was washed with two bed volumes of pH 7.1 buffer and the effluent from this step collected as it contained the monoclonal IgG. The effluent was concentrated by ultrafiltration, and assayed according to the protocol listed in "protein assays" in this chapter. The serum albumin was eluted with 0.02 M phosphate buffer in 0.15 M NaCl, pH 7.1, collected, concentrated, and measured at 280 nm. This column was then regenerated by first adding two column volumes of 8 M urea in pH 7.1 buffer, followed by a column rinse with 250 ml of pH 7.1 buffer.

Fluorescent Labeling of Proteins

The method of labeling with fluorescein isothiocyanate (FITC) was adopted from Clark and Shepard (1963) and tends to minimize non-specific staining. Concentration of the protein solution was adjusted to 20 mg/ml with 0.15 M NaCl. To 1 ml volume of protein solution in dialysis tubing (M.W. cut off 20,000) was added 0.50 ml of 0.5 M carbonate buffer in 0.15 M NaCl, pH 9.5. The dialysis tubing was then placed in an ice bath for 10-15 minutes. During this time, 20 ml of 0.5 M

carbonate buffer was placed in a 50 ml graduated cylinder and 2 mg of FITC powder added and mixed thoroughly with magnetic stirrer. Then the dialysis tubing was removed from the ice bath and placed in the graduated cylinder and allowed to react at room temperature for 15 minutes. The reaction was then allowed to proceed overnight at 4°C. The unreacted FITC and small molecule derivatives were removed at the conclusion of the labeling process by passing the reaction mixture through a G-50 gel filtration column. The conjugated protein was quickly eluted using PBS, pH 7.5 buffer. The remaining components of the mixture were washed from the column with some difficulty. The column was washed with 2000 ml of PBS, pH 7.5, over a two-day period in order to completely remove any traces of fluorescence in the effluent. After concentrating the conjugated sample to its original volume of 1 ml, it is placed in dialysis tubing and allowed to dialyze against PBS, pH 7.5 buffer until the dialyzate appears free of fluorescence. It normally takes about 48 hours to reach zero fluorescence level. All fluorescence measurements were carried out at 490 nm excitation, 510 nm emission for FITC labeled proteins. To determine the dye content of conjugated protein, it is necessary to take into consideration the different absorption maxima of free and bound dyes, and the change in the molar absorbtivity. In the case of FITC maximum absorbance is 490 nm for unbound dye and 496 nm for dye bound to protein. The molar absorbtivity of the bound dye is approximately 75% of the free dye (Jobbagy and Kiraly 1966; Wells, Miller, and Nadel 1966). Thus, FITC content can be solved for by equation (1) (Goldman 1968):

$$\text{Concentration of FITC bound to protein } (\mu g/ml) = \frac{\text{Concentration FITC standard}}{A_{490}\ \text{FITC standard}} \times \frac{A_{496}\ \text{conjugate}}{0.75} \quad (1)$$

Protein Assays

Analysis of soluble immunoglobulins was accomplished by two techniques. The concentration of an IgG solution was determined spectrophotometrically at 280 nm using an absorbtivity coefficient of 1.39 cm^{-1} $(mg/ml)^{-1}$ (Crumpton and Wilkinson 1963). Antihuman IgG concentrations were determined spectrophotometrically in an analogous manner using 1.42 cm^{-1} $(mg/ml)^{-1}$ as the absorbtivity coefficient (Kabat 1976). Measurements were made at 320 nm on these solutions to determine if light scattering from particulate matter in the solution was falsely elevating the absorbance values. If A_{340} was greater than 0.5% of A_{280}, the solution was clarified by centrifugation. A second method used for determining immunoglobulin concentrations was radial immunodiffusion (RID). Commercial RID plates were used in calibrating RID plates produced in the laboratory. Standard IgG reference solutions were diluted to form the appropriate calibration curves. For routine work, four dilutions of the reference IgG solution were made to fall in the range 0.005-1 mg/ml IgG. Five µl of these dilutions were applied to the sample wells in the RID plates and allowed to develop at 4°C for 48 hours. The diameter of the precipitin ring was measured (mm) from outer edge to outer edge and a plot of diameter^2 versus IgG concentration was constructed. The y intercept of the line will be equal to the

square of the diameter of the sample well (Crowle 1973). Because of low antigen concentration, the diameter of the disc will be essentially that of the well. If the intercept value is off by more than 10% of this value, or the calibration line was not linear over the normal range, the plate was recalibrated using different IgG reference solutions. Samples of unknown IgG concentration were first clarified by centrifugation if necessary, and then duplicate 5 µl aliquots were applied and allowed to develop at 4°C for 48 hours. After 48 hours, the plates were soaked in 0.15 M NaCl solution for 8 hours with two changes of solution and then in PBS, pH 8.0, overnight. This removed any non-specific protein that may have been adsorbed on the gel or the precipitate itself. A 10% solution of Coomassie Blue R in 10% acetic acid was then used to stain the plates. This was carried out until the gel adopted the bluish color of the stain. The RID plates were then destained in three changes of 10% acetic acid with stirring until the background color of the gel was clear. The diameter of the precipitin ring was measured using a monocular eye piece with 0.1 mm grid scale superimposed on the lens. Construction of the gel containing the antibody for the RID assay was carried out according to the method described in the Appendix.

Analysis of immobilized immunoglobulins was carried out by two techniques. The total protein bound to the CPG particles was determined by amino acid analysis on the Beckman Amino Acid Analyzer Model 43B. The glass particles were first thoroughly washed with 5 liters of PBS, pH 8.0, and 1 liter of 0.02 M phosphate buffer, pH 2.0. After washing, the particles were dried overnight in the oven, weighed, and placed in

small ampoules. To these 0.50 ml of constant boiling HCl was added along with enough water to cover the glass particles (approximately 0.25-0.50 ml). The ampoule was sealed, placed in a 110°C oven and hydrolysis carried out for 48 hours. The seal was broken, and the sample lyophilized to dryness. The glass particles and walls of the ampoule were washed free of the hydrolyzed protein with 3.0 ml pH 2.2 citrate buffer. This was filtered through a 0.2 micron Millipore filter into a test tube. The amount of vacuum applied to carry out this step was closely monitored to avoid foaming of the hydrolysate. This was lyophilized to dryness and resuspended in exactly 1.00 ml of .02 M citrate buffer, pH 2.2. It was then loaded onto the amino acid analyzer column using a sample loading loop, 300 µl. Standard samples for IgG and antihuman IgG were run in the absence of CPG particles and the peak areas for lysine and arginine for these known concentrations of protein were determined. This resulted in a linear relationship which could be used in determining the amount of covalently attached protein. The second method for assaying immobilized immunogens employed the Folin-Lowry method. The glass particles were hydrolyzed as discussed above, and after the sample was lyophilized to dryness, the particles and the walls of the ampoule were washed with 3 ml of PBS, pH 7.5. This solution was lyophilized to dryness and then resuspended in 2 ml of PBS, pH 7.5. From this solution a 100 µl sample was mixed with 3 ml of Lowry reagent and allowed to stand 10 minutes. This consisted of mixing one part 2% NaK Tartrate$\cdot 4H_2O$ and one part 1% $CuSO_4 \cdot 5H_2O$ and 98 parts 2% Na_2CO_3 in 0.1 M NaOH. Then 0.10 ml Folin-Ciocalteu reagent (Sigma) was

added. After 40 minutes, the solution was read at 625 nm. Standard IgG and antihuman IgG solutions were analyzed and the absorbance versus concentration graphed to yield a calibration curve.

Electrophoresis of protein solutions was carried out on polyacrylamide/agarose gels prepared in the laboratory. Preparation of these gels was carried out according to the method described in the Appendix. Samples were dialyzed overnight against PBS, pH 8.0. Then 100 µl of the sample was diluted with an equal volume of 40% (w/v) sucrose and placed in sample well. After the capping gel has solidified, the entire gel is placed in the cooling buffer and electrophoresis carried out. For a qualitative separation, the following schedule was used:

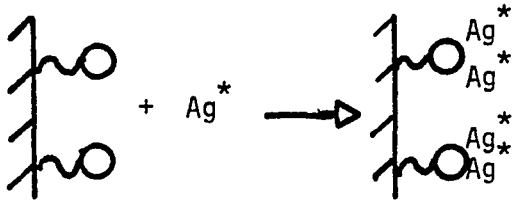
275 V	75 pulses/sec	15 min
300	125	1 hour
300	225	1 hour
325	300	15 min

If more resolution is needed, longer exposure to the electric field is required. For serum samples containing around 5 mg/ml protein, the following schedule was used:

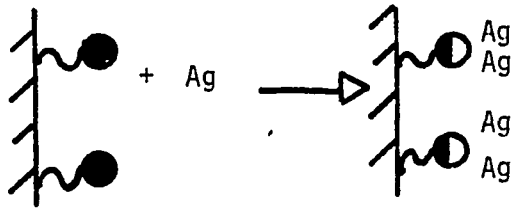
225 V	75 pulses/sec	20 min
275	125	2 hours
300	175	4 hours
325	225	2 hours
350	300	30 min

Determination of Biological Complexing
Ability of Immobilized Immunogens

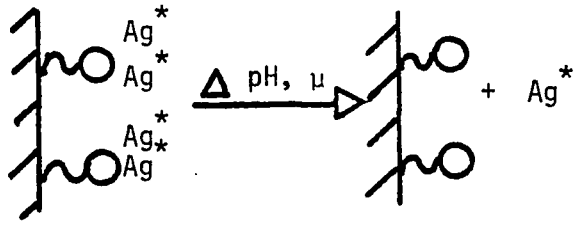
Fluorescent determinations were carried out on labeled proteins using four different techniques (Figure 7). These can be generally divided into the direct and indirect methods. In the single-layer direct method, the unlabeled immobilized antibody is exposed to a solution of labeled specific antigen. The excess labeled antigen is washed from the column and the fluorescence of the CPG matrix quantitated in the specially designed cell (Figure 4). The single-layer direct quenching method in which labeled specific antibody is covalently linked to the CPG matrix, and its initial fluorescence measured. Then a solution of unlabeled antigen is circulated over the CPG matrix, the matrix washed, and the fluorescence measured. The decrease in fluorescence is then related to the amount of antigen binding. In the indirect single-layer method, the unlabeled immobilized antigen is exposed to the labeled specific antibody. After excess labeled antibody is washed from the column, the bound labeled specific antibody is eluted from the matrix by changing pH, ionic strength, or introduction of a chaotropic agent. The fluorescence of the eluent is measured and related to the amount of binding. With the indirect sandwich technique, an unlabeled antibody is immobilized on the CPG particles, exposed to unlabeled antigen, and thoroughly rinsed to remove excess antigen. Then labeled specific antibody is passed through the column, and binds with any antigenic determinates not occupied on the complexed antigen molecule. After washing excess labeled antibody from the column, both the bound antigen and labeled antibody are eluted from the column by the same methods as



(A) SINGLE-LAYER DIRECT - measure bound labeled antigen



(B) SINGLE-LAYER DIRECT QUENCHING - measure quenching of complexed immobilized labeled antibody



(C) SINGLE-LAYER INDIRECT - measure soluble labeled antigen

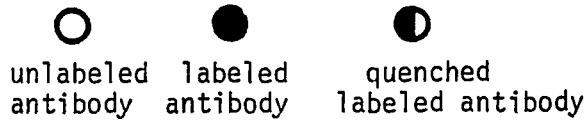


Figure 7. Direct and indirect techniques for fluorescent determination of biological assay of immobilized immunogen complexing ability.

previously mentioned. The fluorescence of this solution is measured and related to antibody binding which is proportional to antigen binding to unlabeled immobilized antibody. The competitive binding assays on the fluorescein labeled CPG particles and Immuno Fluor particles were carried out according to Immuno Fluor IgG Protocol (Bio Rad 1975).

The spectrophotometric determination of biological activity of the immobilized immunogens was carried out by the indirect single-layer technique. The eluent was measured at 280 nm and, using the appropriate molar absorbtivitties, the concentration of ummunogen determined.

A difference method based on the decrease in absorbance of a solution of known concentration of IgG after passing it through a column of immobilized antibody was also used. In this instance, a known rinse volume was applied to the column, collected, and measured at 280 nm. This value represented the amount of unbound protein and was subtracted from the initial solution absorbance to give a value which represented amount of IgG bound to the column. A background correction for non-specific protein adsorption was carried out by passing a 20 mg/ml solution of BSA through the column and measuring the difference in initial and final solution absorbances.

RESULTS AND DISCUSSION

Optimization of Covalent Attachment of Antihuman IgG to CPG Particles

The optimization of the chemistry involved in the immobilization of antihuman IgG on the CPG matrix was carried out in a systematic manner. The first variables to be investigated were those relating to the CPG matrix itself: surface area, pore volume, and particle size. The number of reactive groups on the surface and the efficiency of the silanization procedure will be discussed later in this section. A standard coupling procedure involved silanization of 0.5 g of CPG by the organic protocol, followed by coupling of the protein to the matrix using EDC as the coupling and activating agent. The resulting biological complexing ability via the indirect single-layer U.V. method was determined for four different CPG matrices. The relationship of surface area, pore volume, and mean particle size to the biological activity exhibited by the immunoadsorbent is shown in Figure 8. The CPG particles investigated were:

Source	IgG Complexed (μg/g)	Designation	Size Range (μ)	Surface Area (m^2/g)
Spectra Physics	63	SW5, SW10	5	200
Electro Nucleonics	49	CPG-HS1400	30-37	18
Corning	7	GAO-1350	133-840	34
Corning	4.5	MAO-550	133-840	70

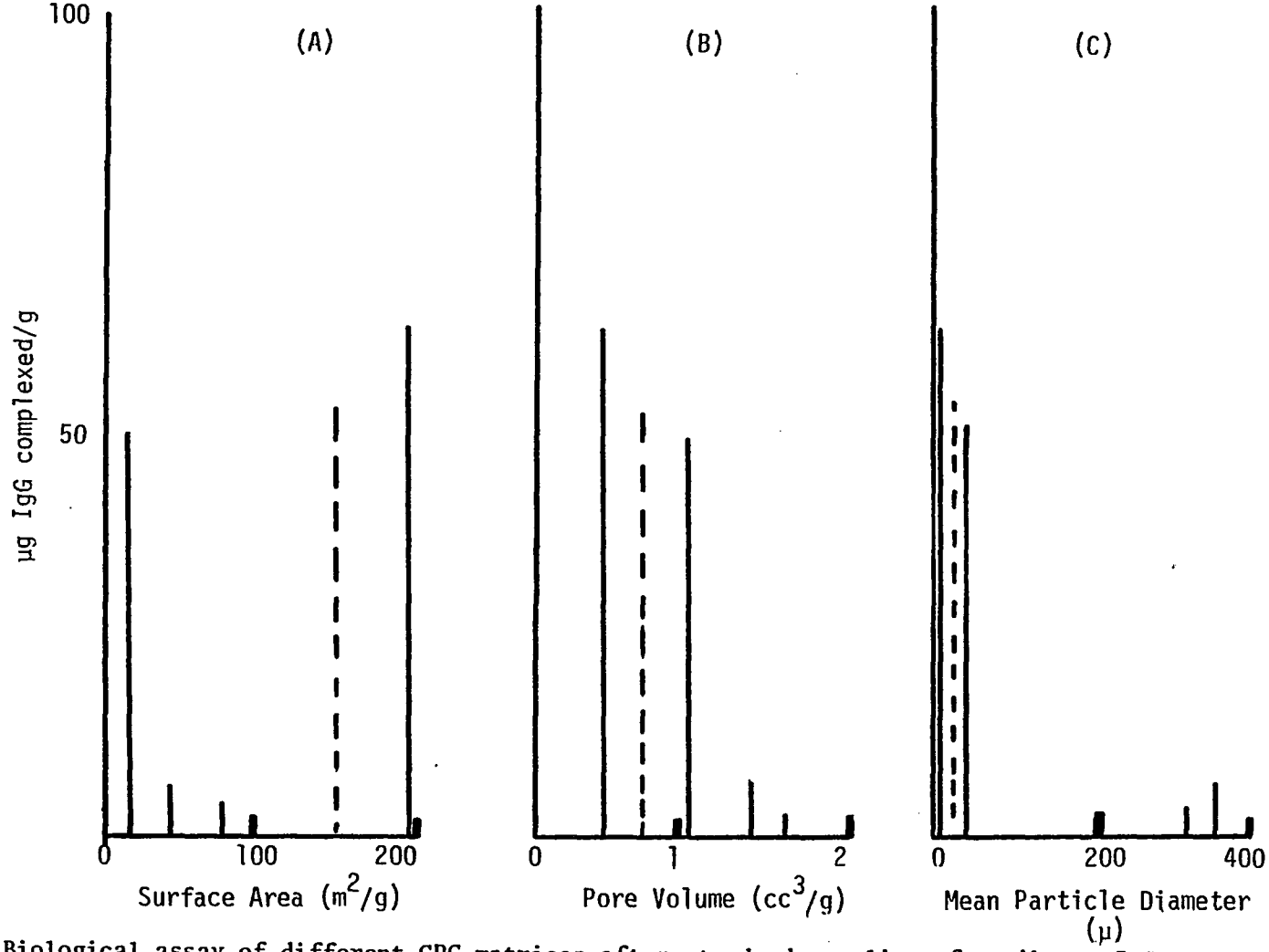


Figure 8. Biological assay of different CPG matrices after standard coupling of antihuman IgG to the matrix. -- Antihuman IgG immobilized on Bio Gel P-4, >400 mesh.

The factor of 15 difference in surface areas between SW5 and CPG-HS1400 matrices makes it difficult to assume any linear relationship between available surface area and biological activity. The pore diameter of SW5 is 2-3 times too small for an IgG molecule to penetrate (Haller, Tympner, and Hannig 1970), and thus any critical relationship between activity and pore size and volume would seem to be different. The data in Figure 8b and 8c suggest there is an inverse relationship between mean particle size and pore volume and the resulting biological activity exhibited by the immobilized antibody. Thus, a small particle with shallow pores would be the optimum matrix. As will be discussed later, this idea is further corroborated by kinetic studies using CPG particles of varying size. While the physical properties and resulting activity of immobilized proteins on non-silica versus silica matrices are somewhat different, it is worth noting that antihuman IgG immobilized on Bio Gel P4 tends to follow the pattern suggested above.

After characterization of the matrix was carried out, the optimization of the different coupling chemistries was investigated. This required optimization of the individual reaction conditions including pH, ionic strength, concentration of coupling and activating reagent, reaction time, and amount of protein offered to the matrix in the coupling reaction. The results of previous optimization studies were employed for most of these same reactions (Smith 1975). The coupling of antihuman IgG to the CPG matrix using the EDC method was initially studied because of earlier success with this procedure in immobilizing enzymes to CPG particles. In Figure 9, the effect of changing the

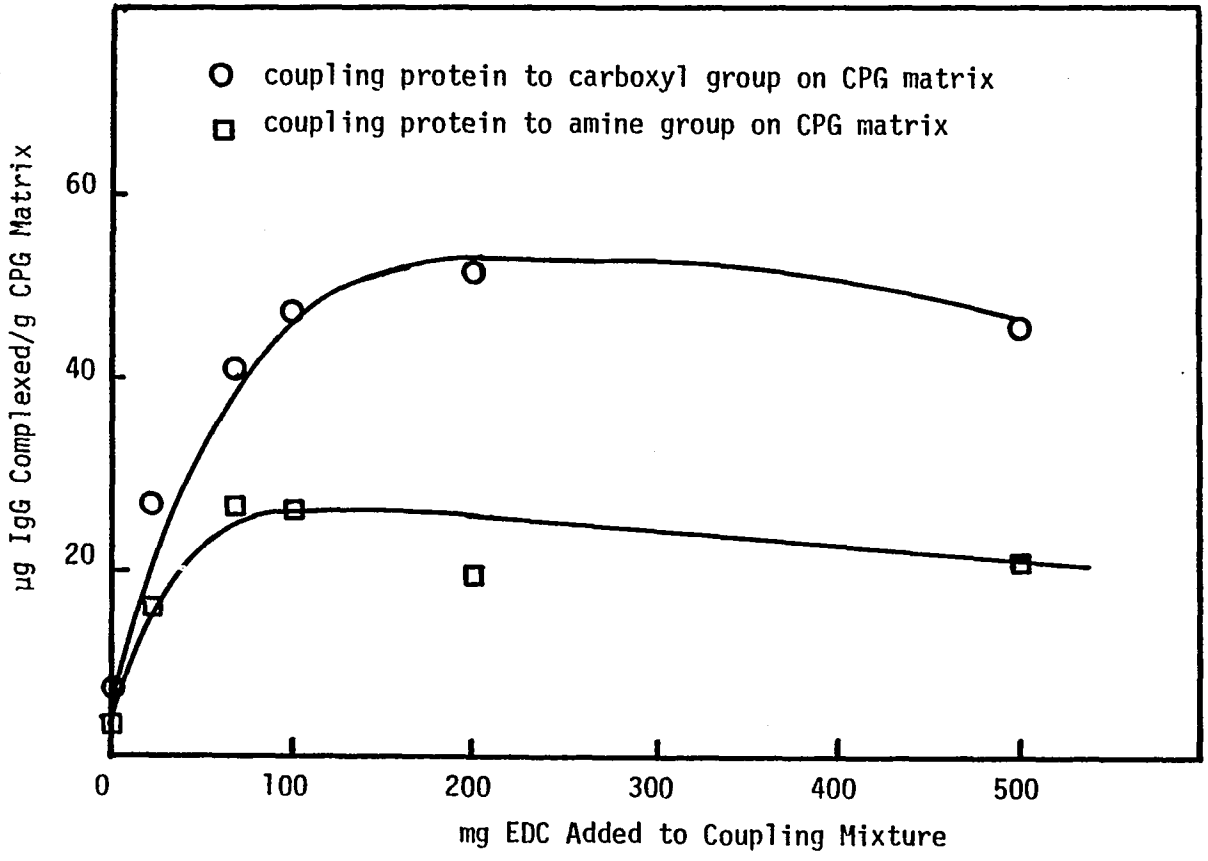


Figure 9. Optimization of EDC concentration in coupling IgG to CPG matrix.

concentration of EDC in a standard coupling reaction mixture is shown. These curves do not pass through the origin as there is some coupling of the protein in the absence of EDC. At high concentrations of coupling reagent, the biological activity decreases by 25% for the coupling of protein through its free amine groups and 15% for the coupling through its carboxyl groups to the matrices. Samples of the coupling supernatant were taken at different EDC concentrations. They were measured spectrophotometrically at 280 nm and failed to show more than a 5-8% difference between the absorption values at EDC levels of 100 mg and 500 mg, and 70 mg and 500 mg for coupling to CPG-NH_2 and CPG-COOH matrices, respectively. Samples of the coupling solution supernatant at high EDC concentrations were analyzed by electrophoresis. Because of the innate heterogeneity of the antibody, it is difficult to discern if the protein moiety staining faintly at the very top of the gel was polymerized antihuman IgG or residual IgM from the antiserum preparation. Gel filtration of the supernatant on G-200 Sephadex produced inconclusive results. The chromatogram showed one large peak. That this peak may be the summation of two smaller, poorly resolved peaks containing antihuman IgG and partially polymerized antihuman IgG is suggested by other experiments.

The EDC coupling method resulted in considerably larger quantities of antibodies remaining after 24 hours in the coupling solution (Wash 0, Figure 10) as detected by spectrophotometric analysis compared to that found when glutaraldehyde is used as the coupling agent under comparable conditions. When 96 mg/ml glutaraldehyde is used in

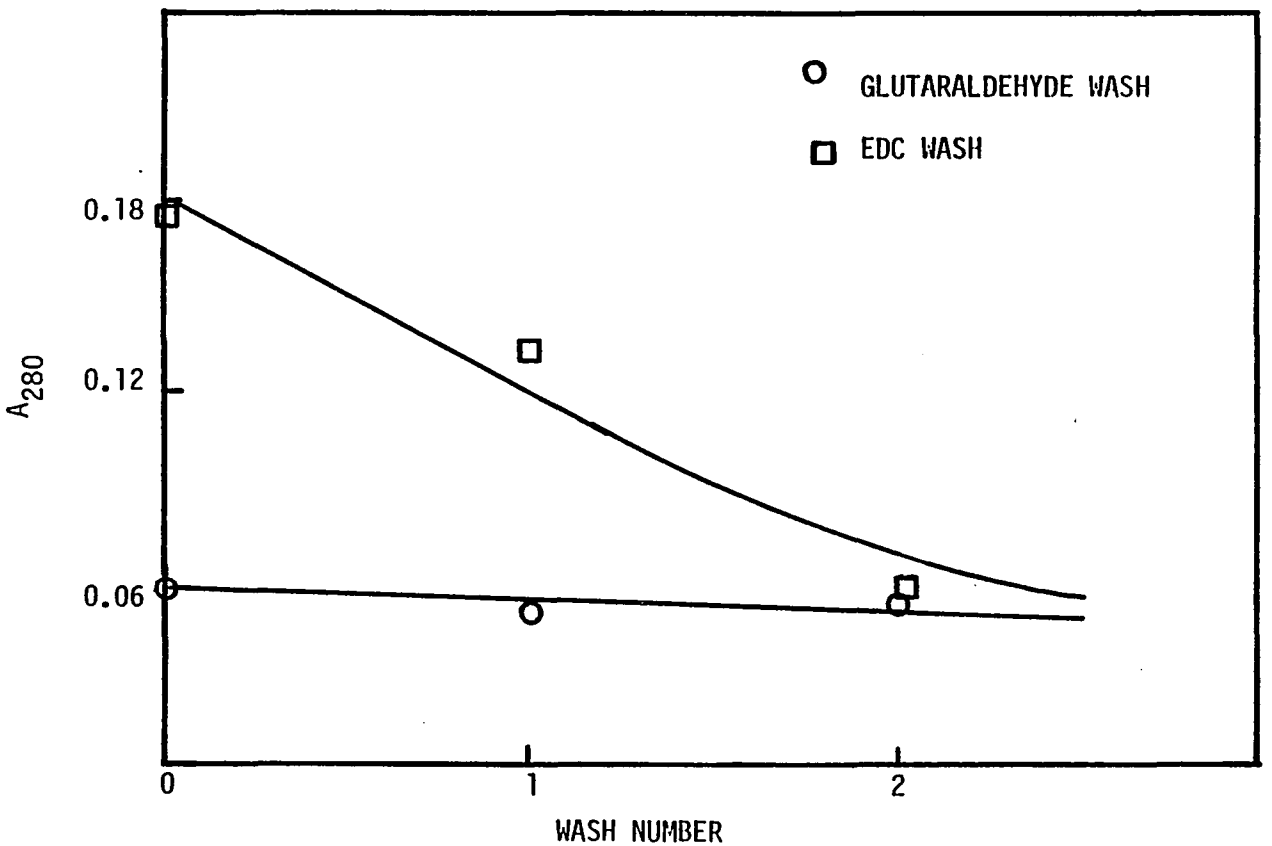


Figure 10. Monitoring of Ab remaining in coupling solution after 24 hours (- wash 0), and the desorption of Ab from the inorganic supports after removal from the coupling solution.

the standard coupling reaction mixture, there is 79% decrease in the initial absorbance; while with 200 mg/ml EDC there is 42% decrease (Figure 10). The antihuman IgG remaining uncoupled is determined by taking 10 µl sample and diluting to 1 ml, measuring absorbance at 280 nm (corrected for Rayleigh light scattering at 320 nm) (Shapiro and Waugh 1966) and comparing to a calibration curve for antihuman IgG (Figure 11). The slope of the line yields an absorptivity value of 1.39 $(mg/ml)^{-1}$ $(cm)^{-1}$ which is comparable to the value determined by Kabat (1976). This procedure gives the amount of soluble antihuman IgG remaining in the native and/or denatured state, but does not differentiate between them. This procedure does not appear to be influenced by crosslinked antihuman IgG polymers. As mentioned, all solutions were checked for particulate scattering and centrifuged at 10,000 rpm for 20 minutes to remove the very large polymeric species. The centrifugation step was not necessary when the coupling solution supernatant was analyzed immediately after sampling. However, this step was used periodically when assaying the supernatants which had been stored over a period of time. This spectrophotometric assay as carried out on freshly extracted aliquots from the coupling solution is sensitive to both the active and inactive forms of antihuman IgG.

A sensitive assay for distinguishing between the active and inactive forms is radial immunodiffusion (RID). A 10 µl sample containing antigen is placed in a well cut in an agarose gel containing antibody uniformly dispersed. The antigen diffuses radially into the gel and combines with the internal reactant or antibody to form a disc

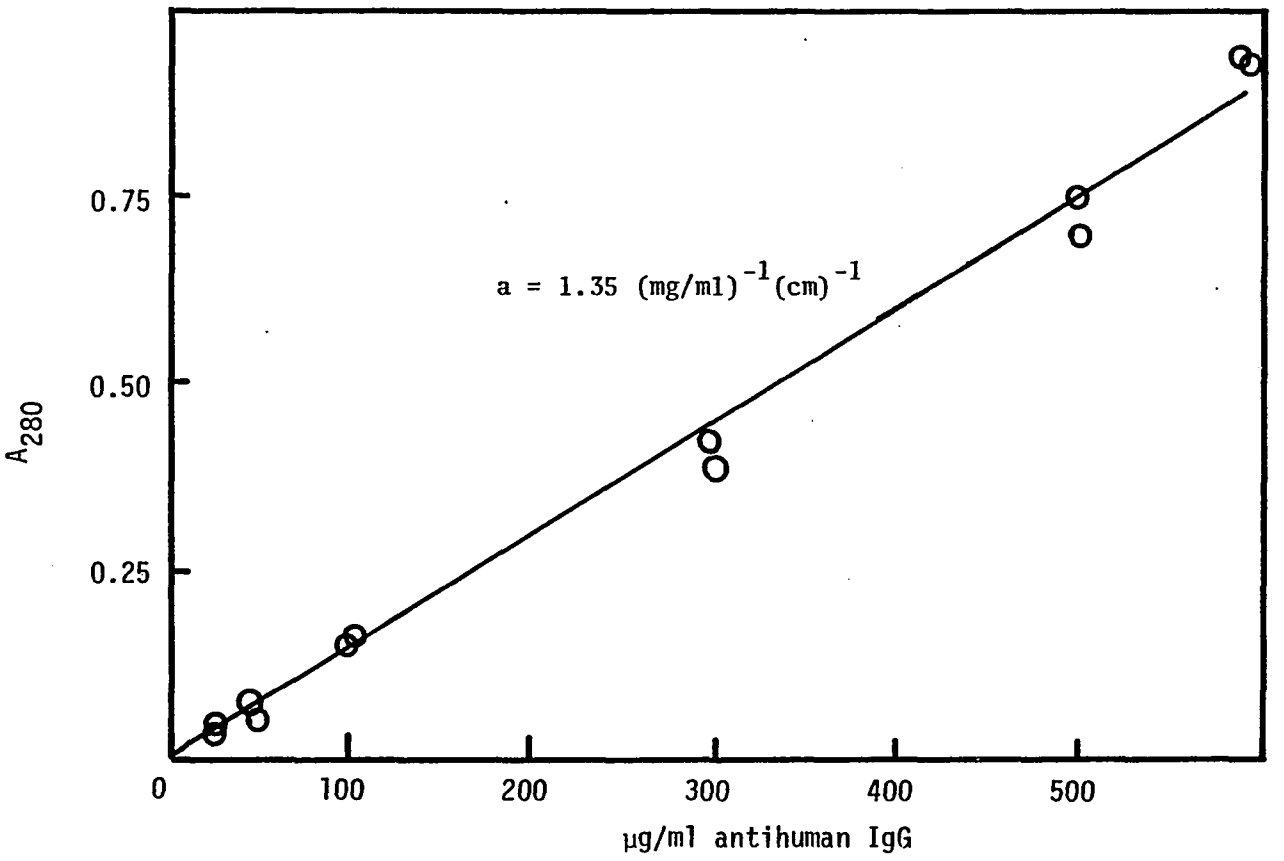


Figure 11. Calibration curve from spectrophotometric determination of soluble antihuman IgG at 280 nm.

of precipitate that expands out into the surrounding medium until at maximum size the front of the diffusing external reactant has reached equilibrium with the surrounding antigen and the disc stops growing. The radius of the disc precipitate varies linearly with the initial concentration of the antigen and is inversely related to the concentration of the antibody dispersed in the agarose gel. The linear relationship between the concentration of antigen or any external reactant and the radius of the precipitate that it forms will fail if the external reactant is composed of physically heterogeneous subpopulations. Other interferences include concentration-dependent interactions between external reactant molecules, external molecules, and accompanying solutes; and antibodies which are not monospecific for the external reactant (Crowle 1973). For example, antibody preparations specific for IgG, kappa type, will produce a range of IgG values due to the different proportions in antisera (containing antibody) of precipitins with gamma specificity (Fahey and McKelvey 1965).

The RID technique will differentiate biologically active antihuman IgG from inactive antihuman IgG, since the precipitin disc is a function solely of the concentration of protein which is able to complex with agarose entrapped antibody (i.e., biologically and immunologically active molecule). From the spectrophotometric analysis, total protein content of the active and inactive antihuman IgG can be determined while RID analysis will give total active, non-polymerized antihuman IgG concentration. As seen in Figure 12, there is substantial inactivation (i.e., disappearance of the biologically active form) even though there

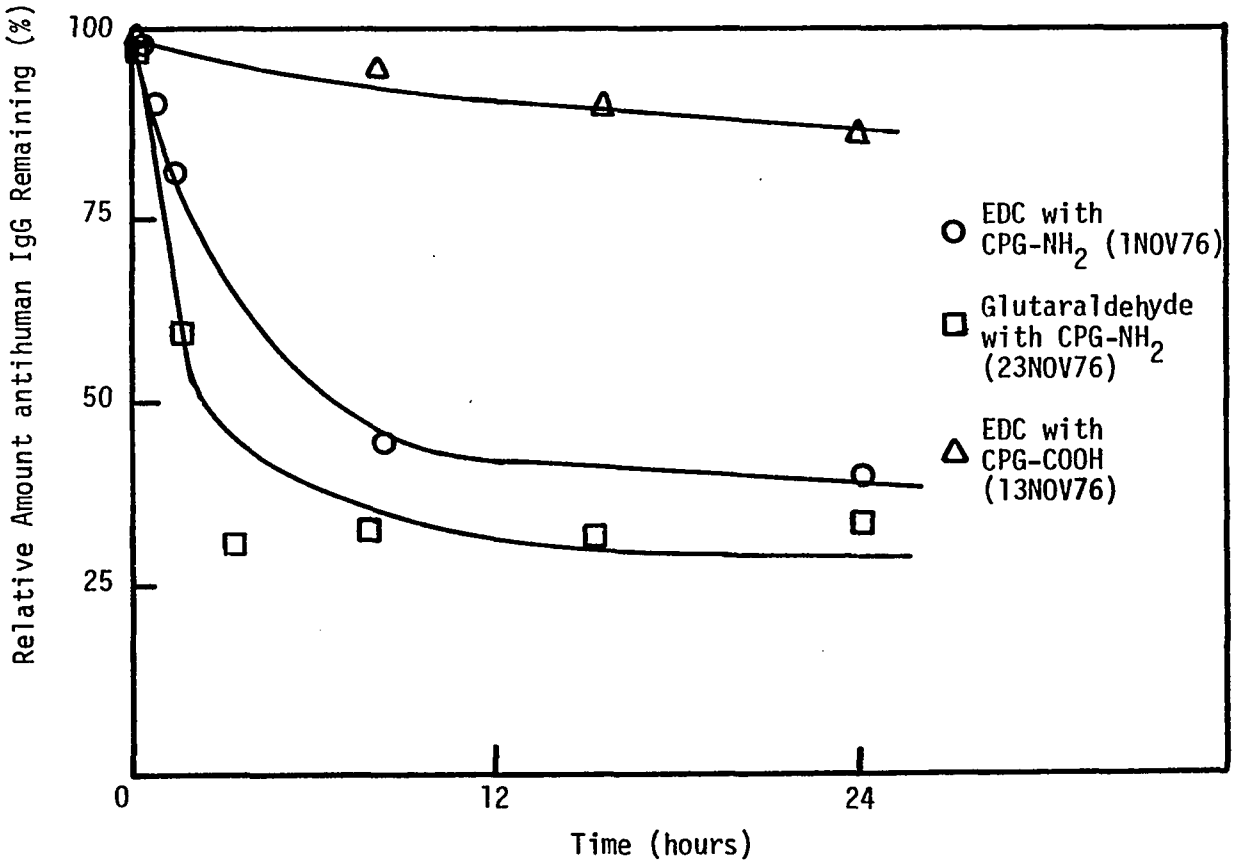


Figure 12. Decrease in antihuman IgG concentration in coupling solution as function of time with different coupling reagents.

is little net change in total absorbance at 280 nm beyond approximately 5 and 10 hours for the glutaraldehyde and EDC coupling reaction supernatants, respectively. This disappearance of the active form may be due to coupling of the antibody to the matrix or inactivation of the soluble form by some polymerization mechanism (Line et al. 1973). The observation of no net change in total absorbance at 280 nm coupled with decreasing biological activity suggest some irreversible conversion of active IgG into a biologically inactive form. From Figure 10, it is apparent that there is considerable non-covalently linked antihuman IgG associated with the CPG matrix. An adsorption equilibrium between available sites on the CPG matrix and the protein may exist. Later experiments show that increasing the antihuman IgG concentration in solution beyond a certain concentration does not increase the amount of biological activity of the covalently bound material. Also, there is very little extraneously adsorbed protein on the CPG matrix when glutaraldehyde was used as the coupling reagent (Figure 10). From Table 3, it appears that a large part of the protein covalently linked to the CPG matrix by the glutaraldehyde coupling method is inactive. This suggests that the protein adsorbed on the surface is eventually crosslinked to covalently attached protein. In both coupling reactions, the spectrophotometric monitoring suggests that progress in the coupling of protein to CPG matrix has ceased, while monitoring of both the biological activity and coupling reagent indicates that this may not be the actual situation.

Table 3. Amount of antihuman IgG covalently linked to CPG matrix and its biological activity (µg IgG complexed/g matrix).

Matrix	Coupling Reagent	Length of Coupling (hours)	Protein Covalently Attached (µg/g)[c]	Complexing Ability of Immobilized Binding Site (µg IgG/g)
7NOV76 CPG-COOH	EDC[a]	4	5 (0.03)[d]	3.5 (0.02)[e]
10NOV76 CPG-COOH	EDC[a]	12	54 (0.34)	8.0 (0.05)
13NOV76 CPG-COOH	EDC[b]	24	268 (1.68)	12.0 (0.08)
23APR77 CPG-COOH	EDC[b]	36	281 (1.75)	13.0 (0.08)
23NOV76 CPG-NH_2	glutaraldehyde[f]	24	437 (2.73)	9.0 (0.06)
3APR77 CPG-NH_2	glutaraldehyde	36	441 (2.76)	7.5 (0.05)
12APR77 CPG-NH_2	none (control)	24	4 (0.03)	3.0 (0.02)

[a]Coupling reagent concentration 4.0 mg EDC/mg protein.

[b]Coupling reagent concentration 2.0 mg EDC/mg protein.

[c]Determined by Folin-Lowry method after repeated washing.

[d]Nanomoles of antihuman IgG in parentheses.

[e]Nanomoles of IgG in parentheses.

[f]Procedure as given in Methods section, p. 41.

Because the absolute decrease in absorbance does not correspond directly to the amount of protein covalently immobilized on the inorganic carrier (Smith 1975, Weetall 1975), additional analysis of the particles after thoroughly washing overnight with high ionic strength solutions to remove adsorbed proteins permits determination of the amount of protein covalently linked to the carrier by the Folin-Lowry method of amino acid analysis. The data in Table 3 show the results of these studies on five different CPG matrices to which protein had been attached covalently using the glutaraldehyde or EDC coupling methods. The amount of protein covalently coupled to the 23NOV76 CPG-NH_2 particles using glutaraldehyde as the coupling reagent represents 52.9% of the total amount of antihuman IgG in the standard coupling solution (Table 3). From Figure 12, the amount of antihuman IgG remaining in the coupling solution after 24 hours suggests that 70% of the initial concentration has been removed leaving 17.1% unaccounted for. In the case of the 13NOV76 CPG-COOH particles linked to antihuman IgG by EDC, 13.9% of the initial amount of protein is found to be covalently attached, while spectrophotometric analysis shows 62% of the initial concentration has been removed. This leaves 48.1% of the protein unaccounted for in the EDC coupling reaction. Some of this protein is adsorbed on the CPG matrix, but as Figure 10 indicates for the glutaraldehyde coupled CPG matrix, it is unlikely that all 17.1% of the unaccounted for protein can be attributed to adsorption on the matrix.

As discussed later in this section, the amount of protein on the CPG matrices reflects optimization of many other aspects of the coupling

reaction besides the physical properties of the matrix. Since the amount of protein used in these coupling procedures in the initial coupling solution was identical, the data from Table 3 and Figure 10 would seem to indicate that increasing the reaction time or coupling reagent concentration beyond a limiting value would not increase net amount of covalently attached material. The optimized coupling reaction conditions for the EDC and glutaraldehyde reactions extracted from these studies were: 150 mg EDC with 33 mg protein (4.5 mg EDC/mg protein) when linking antibody carboxylate groups to the CPG matrix; and 70 mg EDC with 33 mg protein (2.1 mg EDC/mg protein) when coupling protein amino groups to the CPG matrix. These conditions were employed in the remaining experiments except where noted.

The effect of changing the pH of the coupling mixture is shown in Figure 13. There appears to be a relatively large range of pH values over which the immobilized immunogen can be attached to the matrix without losing a significant amount of its original activity. In the acidic range, there is a decrease in the amount of biological activity remaining as coupling pH drops below pH 5. This has been observed previously when attaching antibodies to CPG particles (Line et al. 1973) and was speculated to be due to the extensive crosslinking of the antibody by the coupling agent. In the alkaline region, past pH 8.0 the loss in activity drops drastically with increasing pH. In this region, the EDC is probably not coupling any protein to the matrix, and any residual biological activity is the result of direct coupling of antibody to the matrix (Swaisgood and Natake 1973). The reaction of the

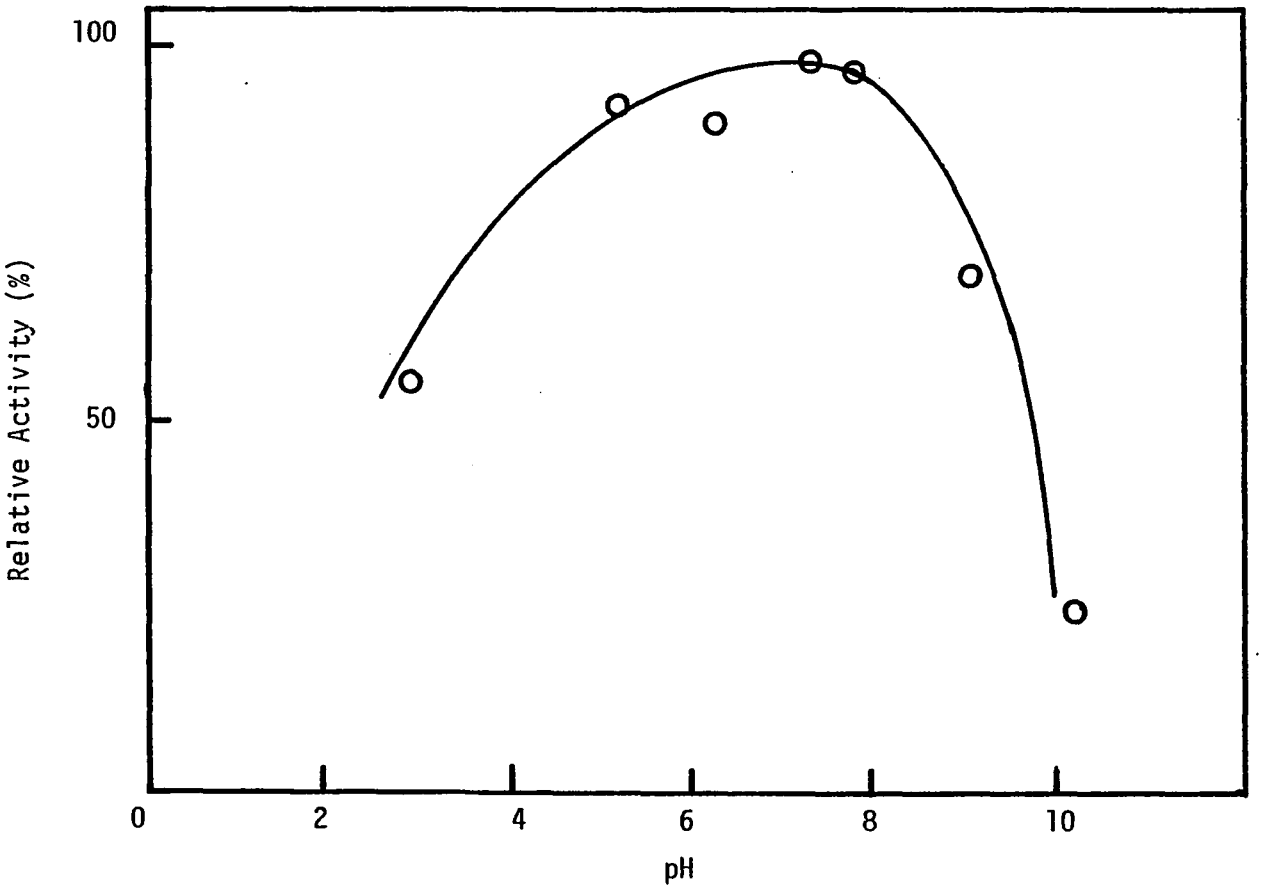


Figure 13. Effect of changing pH in EDC standard coupling reaction of antihuman IgG to CPG-COOH matrix on activity.

protein with the carboxyl groups on the CPG matrix in the absence of EDC yields activity on the order of 2-4 µg IgG complexed/g matrix. A similar wide range of pH optimum values is obtained when using EDC to couple protein to amine functions on the CPG matrix. The resulting biological activity is less than that obtained from carboxylate coupling to the matrix. Direct coupling of the protein to the matrix with free amine groups yields activity in the range of 0.3-0.5 µg IgG complexed/g matrix.

The time of coupling reactions for EDC has usually been given as 18-24 hours or shorter (Cho and Swaisgood 1972, Line et al. 1973, Smith 1975). The extension of reaction time does not result in any initial increase in biological activity (Table 3). However, over an extended period of time, biological activity of the immobilized antihuman IgG matrices exposed to the coupling reagent for less than 24 hours steadily decreased. It was not unusual for immobilized proteins to lose some activity as a result of initial exposure to test solution containing antigen (Weetall 1970). This non-linear decrease in activity after the initial coupling is usually attributed to the loss of adsorbed protein on or in the matrix which is not removed by the washing procedure. Once the adsorbed protein has been removed, the activity tends to stabilize. If activity decreases over a period of time with use, this decrease usually occurs at a linear rate considerably smaller than the initial rate of decrease in activity associated with loss of adsorbed material. This was not the situation with the EDC coupled antihuman IgG (13NOV76) linked to CPG particles as shown in Figure 14. A steady decrease in

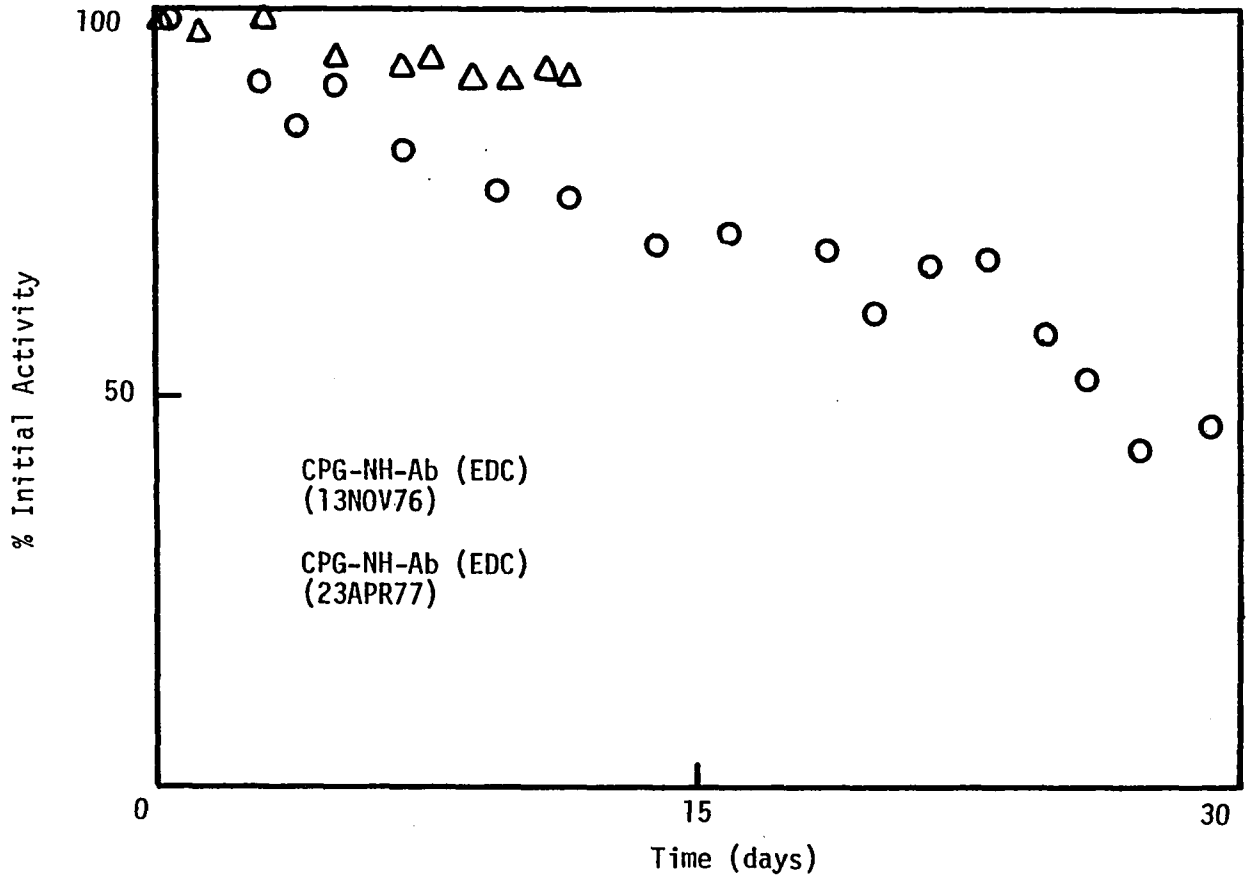


Figure 14. Comparison of IgG complexing ability of two different CPG-NH-Ab matrices over period of time.

activity was observed after 20 determinations over a thirty-day period. The eluent from overnight washing of the CPG (13NOV76) particles was concentrated and checked for adsorbed protein. No soluble protein within the concentration limits of the detection method was found. Using the same coupling procedure with extended coupling time gave CPG (23APR76) particles which displayed a more typical biological activity versus time relationship (Figure 14). Approximately 89% of the initial activity of the immobilized protein that was coupled for 36 hours remained after 12 days while only 52% of the initial activity remained for the CPG (13NOV76) particles. This further substantiates the previous findings that the coupling reaction should be allowed to continue past the point at which the spectrophotometric analysis indicates the uptake of protein for covalent attachment to the matrix has ceased.

Surface Functional Group Analysis of CPG Matrices by Acid-Base Micro-Titrations

Acid-base titrations of functional groups attached to CPG matrices provide valuable information concerning its efficiency of various silanization and other surface activation procedures. The main technique used to investigate CPG surface properties was the micro-titration method of Seymour et al. (1977). This involves a potentiometric titration of the CPG matrix in which a modified Gran plot is used to locate the equivalence point. The slopes and x intercepts of the linear plot give the concentration of the titrant and the equivalence point. With this technique, it is possible to assess whether a particular matrix will have sufficient accessible carboxylic and/or

basic organofunctional groups to react with a particular immunogen in the coupling reaction.

The two silanization reactions investigated were carried out either using the organic (Weibel and Bright 1971, Weetall 1972) or the aqueous protocol (Weetall 1972). Initially, the importance of anhydrous conditions in the organic silanization reaction was underestimated. The reaction was carried out in glassware that had not been properly pretreated or dried. The CPG matrix was not preconditioned with the HNO_3 wash which serves to maximize the silanol groups on the glass particles prior to silanization. As a result of this inattention to reaction conditions, the organic method initially yielded only a small excess (approximately four times) of free amine equivalents/g matrix as compared with the aqueous method. This is reflected in Table 4 which also shows that the titrable basic groups on the CPG matrix before activation with either the glutaraldehyde or p-aminobenzoyl chloride is significantly smaller in each instance when the CPG particles have been silanized using the aqueous protocol. The influence of this lack of reactive free amine groups on the surface of the aqueous silanized particles is reflected in the biological activity exhibited by both the diazotized and glutaraldehyde coupled antihuman IgG. Regardless of the coupling procedure utilized, if there are not enough reactive organic groups initially, then little protein is coupled to the matrix. The extent to which these silanization reactions occur with a given CPG matrix is controlled by the reactivity of the groups present and their

Table 4. Comparison of the number of reactive groups and biological activity of two batches of CPG particles[a] silanized by different methods.

Method	Number of Reactive Groups (μEq/g)	Coupling Method Used	IgG Complexed after Coupling Antibody to Matrix (μg/b)[b]
Aqueous silanization at 80°C for 6 hours	0.76	glutaraldehyde	0.05 (0.03)
	2.71	diazotization	0.30 (0.18)
Organic silanization in dry toluene	28.7	glutaraldehyde	7.76 (4.85)[c]
	193.0	diazotization	93.0 (58.1)[c]

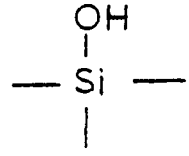
[a] CPG-HS1400.

[b] x 10^{-11} moles IgG/g CPG in parentheses.

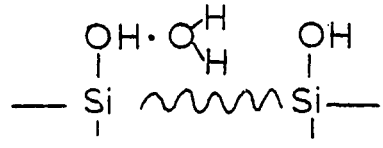
[c] Assuming a molecular area for IgG of 50 x 85 Å, monolayer surface coverage of IgG of these particles should yield 11.1 n moles of IgG of matrix. These values larger than 0.13 μmoles indicate substantial intermolecular crosslinking of the antibody has occurred.

steric availability. It is postulated that three types of groups are present on the surface (Iler 1955):

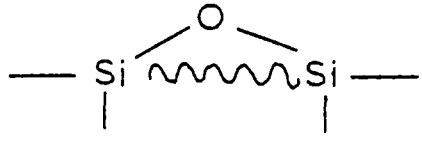
1. Silanol or "bound water",



2. Silanol with physically adsorbed water,



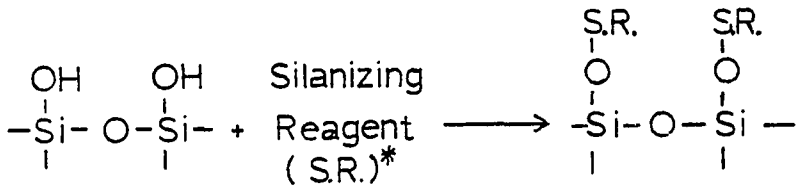
3. Dehydrated oxides,



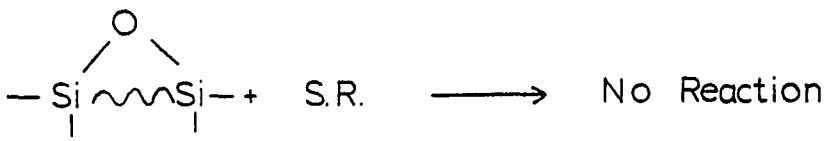
The silica surface is ordinarily considered to be covered with a monolayer of silanol groups (Carman 1940). Values which have been estimated for the number of silanol groups/nm^2 range from 5-8 (Iler 1955, Kiselev and Yashin 1969). The number of these silanol groups which can react with the silanizing reagent also depends on dehydration state of the silica. As described in the Methods section, the CPG particles are pretreated with HNO_3 wash to maximize to number of silanol groups and then heated at 110°C in a vacuum oven. This directly influences the dehydration state of the silica surface. Heating the particles below 150°C results in loss of physically adsorbed water (Lowen and Broge 1961), while heating them above 150°C may result in the partial removal of the

"bound water." This leaves a dehydrated oxide condition which is not chemically reactive with the silanizing reagents used in the investigation (Gilpin 1973):

1. Hydrated surface,



2. Dehydrated surface,



Thus, by controlling the pre-reaction dehydration, varying numbers of organic groups may be attached to the surface.

However, the number of activated sites produced as a result of the particular activation procedures used in this study can not be greater than the initial number of reaction sites produced by the silanization procedure. Often the inefficiency of the activation procedure will result in only a fraction of the number of initial reactive groups being activated. In Figure 15, a modified Gran plot of the potentiometric titration of CPG-NH_2 particles (AB) is compared with that of CPG-CHO particles (CD). THE CPG-NH_2 particles were initially silanized using the aqueous method and then activated using the glutaraldehyde protocol. The difference in the number of μEq/g free amine slope (AB)-Slope (CD) represents the number of aldehyde groups which have replaced the free amine groups. These groups do not react

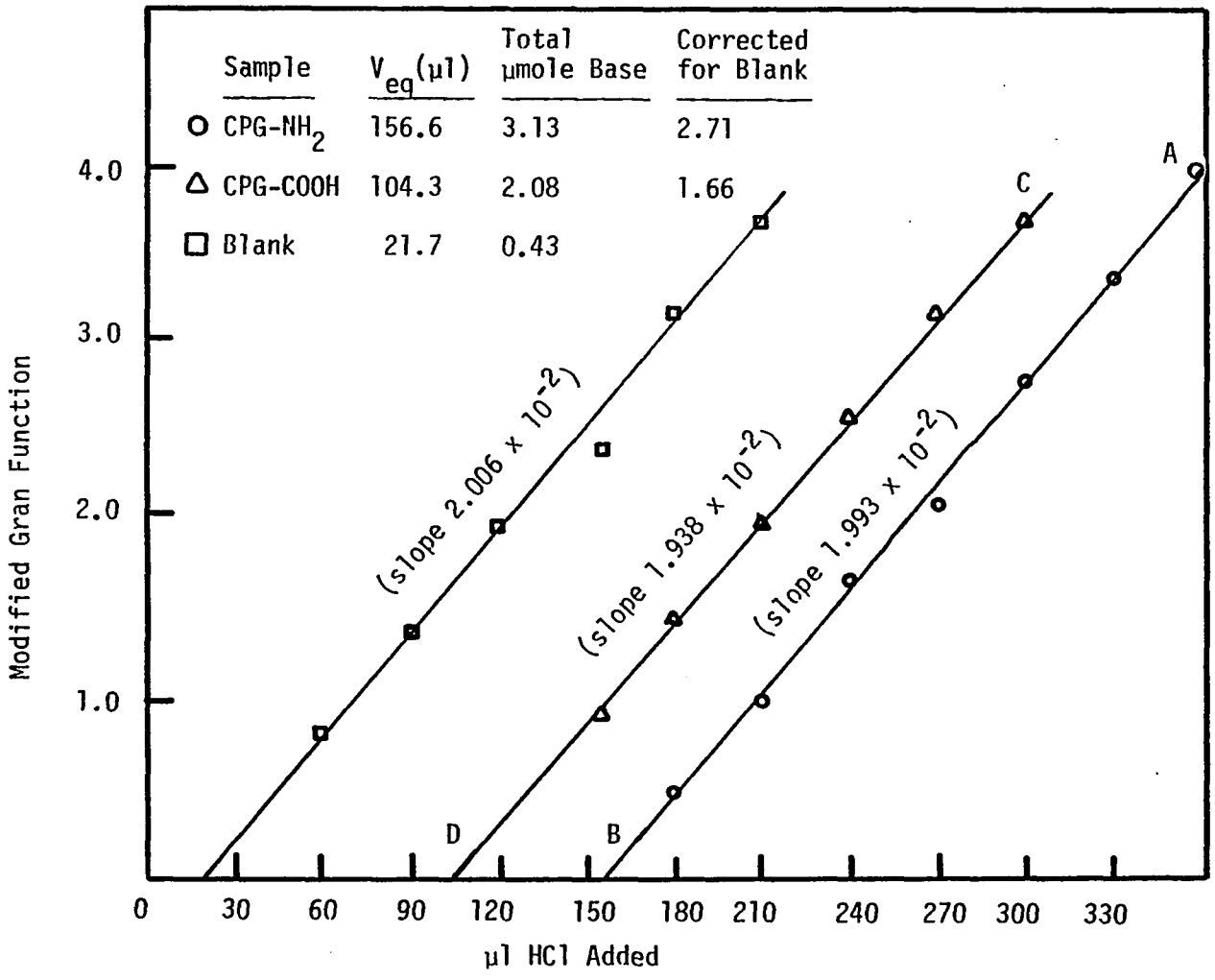


Figure 15. A modified Gran plot of potentiometric titration of 0.30 g of CPG matrix with 2.002×10^{-2} M HCl in 8.0 ml of 0.10 M NaCl.

with the titrant in the titration and thus do not contribute to the total μEq of free amines found. The number of activated aldehyde groups is about 61% of the initial number of free amines (Figure 15). The biological activity of the glutaraldehyde activated, aqueous particles is somewhat lower than the activity resulting from activation and coupling using the diazotization method with the same particles. Neither of these activities compares to those obtained using the CPG matrix prepared by organic silanization method with activation and coupling of the protein carried out using the diazotization protocol. The small biological activity exhibited by the glutaraldehyde coupled antihuman IgG on the same matrix is a reflection of the chemistry rather than the silanization process. This is more clearly seen in Figure 16 which depicts the biological activity of immobilized antihuman IgG coupled through different chemistries to the same CPG matrix which had been silanized by the organic method. All matrices exhibit a slight propensity toward losing activity during the first assays, but further losses reflect an inactivation of the immobilized protein.

As previously mentioned, small particle size seems to be conducive to obtaining high biological activity. As seen in Table 5, the area-to-volume (A/V) ratio for these smaller particles is almost two orders of magnitude greater than for the larger particles. Thus, approximately 34 times more functional groups would be expected to be on the 5 μ particles than on the larger 170 μ particles. As Table 5 indicates, this theoretical increase is observed experimentally. Assuming the following distribution of silanol groups on the CPG particles

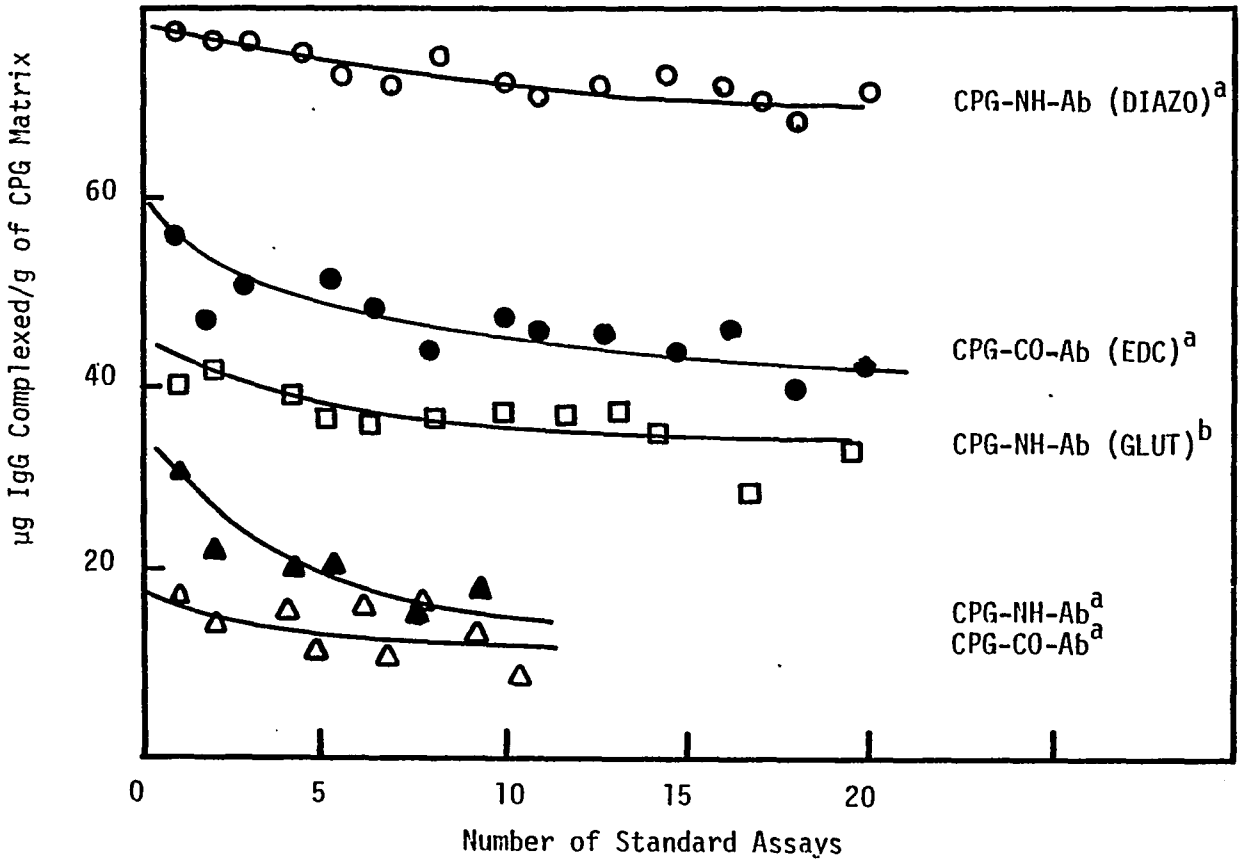


Figure 16. Complexing ability of immobilized antihuman IgG after exposure to standard solution of IgG. -- Test solution: 4 mg IgG/ml, 0.20 g of CPG matrix. (a) CPG matrix, 30-37 micron, organic silanization. (b) CPG matrix, 30-37 micron, aqueous silanization.

Table 5. Area to volume (A/V) ratio for various sized silica matrices.

Assuming Spherical Shape	Sphere Area (cm^2)	Volume (cm^3)	A/V $(cm)^{-1}$
5 μ	7.86×10^{-7}	6.54×10^{-11}	1.2×10^{4}
30 μ	2.82×10^{-5}	1.41×10^{-8}	2.0×10^{3}
37 μ	4.30×10^{-5}	2.65×10^{-8}	1.6×10^{3}
170 μ	9.08×10^{-4}	2.57×10^{-6}	3.5×10^{2}
840 μ	2.22×10^{-2}	3.10×10^{-4}	7.2×10^{1}

(Kiselev and Yashin 1969, Iler 1955), the maximum theoretical number of free amines can be calculated:

$$\frac{5 \text{ silanol groups}}{nm^2} \times \frac{10\ m^2 \text{ surface area for } 5\mu \text{ particles}}{g}$$

$$\times \frac{10^{18}\ nm^2}{m^2} = \frac{5 \times 10^{20} \text{ silanol groups}}{g}$$

$$\times \frac{1 \text{ mole silanol}}{6.0 \times 10^{23} \text{ molecules}}$$

$$\frac{8.3 \times 10^{-4} \text{ moles silanol}}{g} = \frac{830\ \mu\text{moles silanol}}{g \text{ of } 5\ \mu \text{ matrix}}$$

Comparison of this 830 μEq/g to the actual value determined in Table 6 indicates approximately 6.5% of the theoretically available sites on the 5 μ CPG particles are silanized (this assumes a 1:1 stoichiometry between silanizing reagent and available silanol groups; however, this could be a 3:1 ratio (Weetall 1972) giving a value of 19.5% for the number of theoretical sites silanized). This is in contrast to the 170-840 particles which have used approximately 1.8% (5.4%) of the theoretically available sites.

This increase in number of reactive sites on the smaller particles may also reflect an inefficiency in the silanization process when larger sized CPG particles are involved. In order to prevent bumping during the silanization process, the particles were gently stirred during refluxing of the silanizing reagent. This provided for maximum exposure of the CPG particle's surface to the silanizing reagent.

Table 6. Number of basic functional groups on different sized CPG particles silanized by the organic procedure.

Particle Size (μ)	Number of Titratable Basic Groups			
	Total (μEq/g)	Blank (μEq/g)	Corrected (μEq/g)	Ratio[c]
5	56.3	1.61	54.7	1.0
30-37	31.3	1.61	28.7	1.9
170-840[a]	3.1	0.42	2.7	20.3
170-840[b]	3.3	0.42	2.9	18.8

[a]Pore size 1350 Å.

[b]Pore size 550 Å.

[c]Number of corrected μEq/g into 54.7 μEq/g.

Even with such mixing, the larger and heavier CPG particles tended to settle to the bottom of the reflux flask and may not have experienced optimal contact with the silanizing agent.

The number of titrable free NH_2 groups on the CPG matrix was also determined using an automated titrating and data collecting system. A constant delivery syringe (50 µl) was attached to a stainless steel liquid chromatographic tubing connector fitted with a 2 micron stainless steel frit as the bed support (Figure 17). The connector held approximately 50-100 mg of derivatized glass particles with a void volume of 210 µl. Prior to the titration, the particles were treated as described in the Methods section. A Corning Model pH meter with Fisher microelectrode was used to obtain potentiometric data during the titration. The data were collected on a computer interfaced A/D converter system (Ramaley and Wilson 1970) and stored on paper tape using software written by Langhus (1975). At the conclusion of the titrations, a plotting routine produced a hard copy of the data stored in two one-dimensional arrays on paper tape. This system was used to gather qualitative information concerning the number of reactive sites and relied on visual estimation of the equivalence point using the titration curve plotted on the HP X-Y recorder.

The importance of carrying out even qualitative titrations in an inert atmosphere is shown in Figure 18. The titrations conducted without purging the titration vessel or titrant yields a curve without any pronounced inflection to signify the equivalence point. However, purging the titration system and titrant of CO_2 for 10-20 minutes prior to the

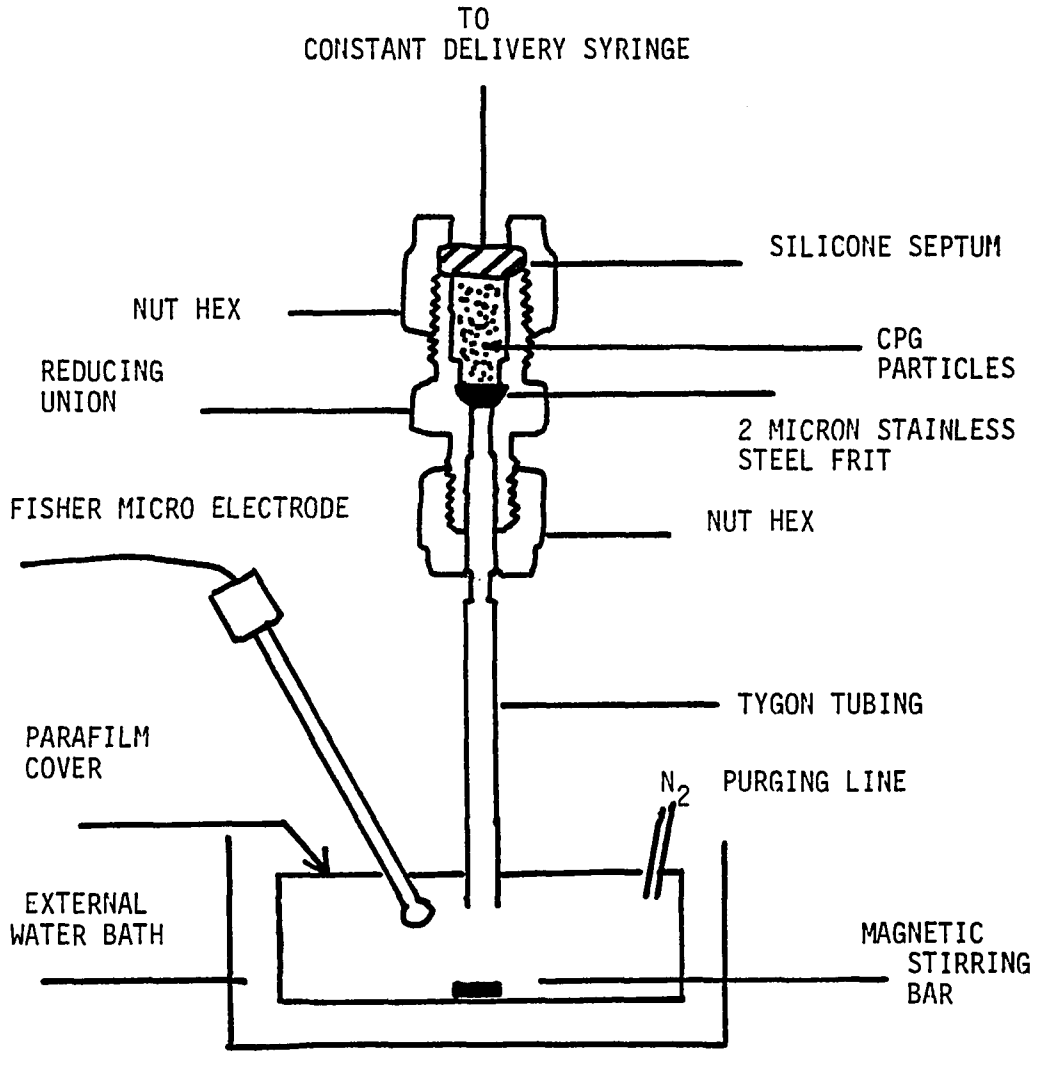


Figure 17. Qualitative micro-titration apparatus.

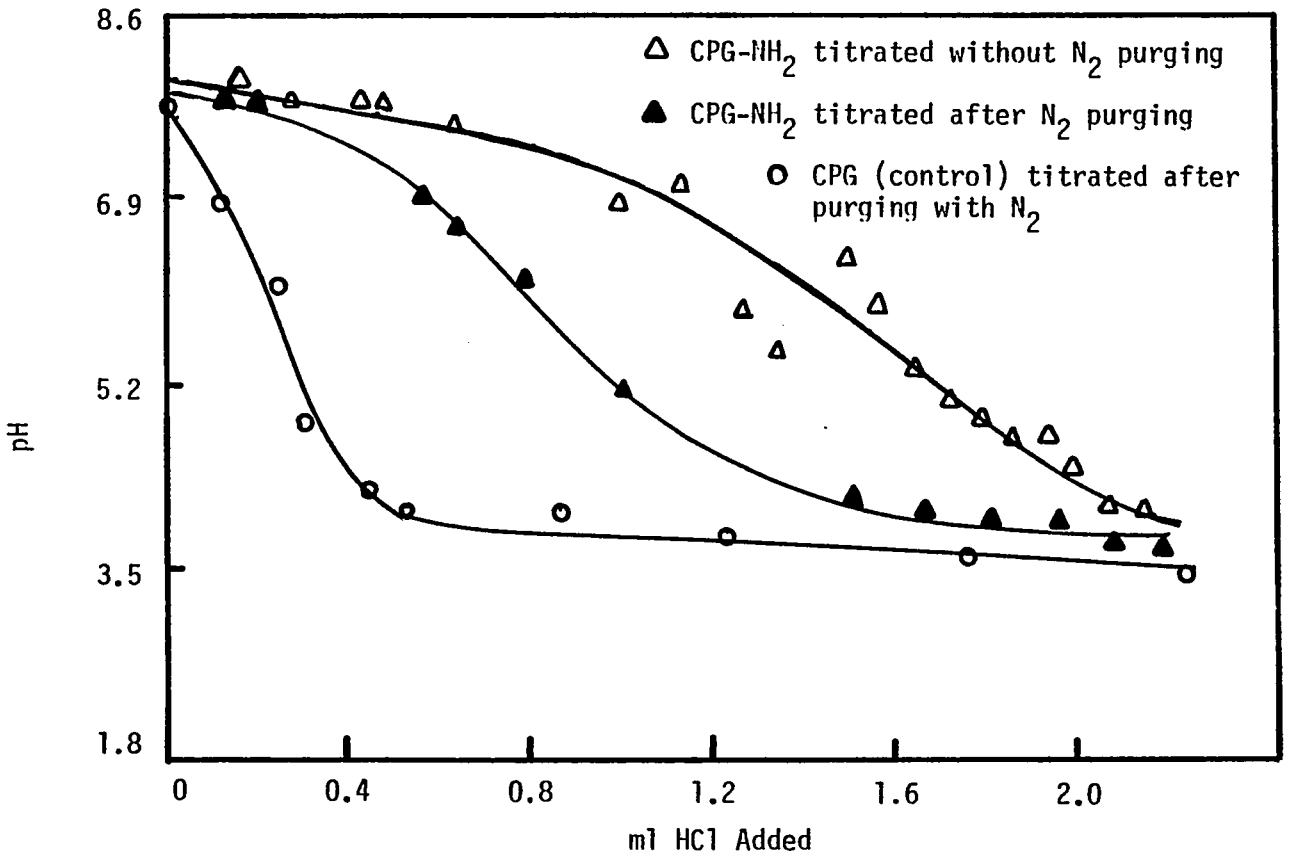


Figure 18. Effect of CO_2 on qualitative titration of CPG-S10 particles silanized by aqueous method.

start and during the course of the titration yields a curve in which a qualitative estimate of the equivalence point can be made. For more precise information, the micro-titration method employing the modified Gran plot was used. In comparing Figures 18 and 19, the factor of three increase in the number of reactive amine groups placed on the CPG matrix by the organic silanization procedure is qualitatively seen. The apparent pKa of the amine on the CPG matrices (5FEB76 and 11JAN76) as determined from the qualitative titrations is approximately 7.0. Using a modified Gran plot for the titration of CPG particles (10MAR76) with HCl (Figure 20) gave a value of 156 µl for the equivalence point. Using this value and Figure 20, an apparent pKa of 7.4 for the titrable amine groups was estimated.

A titration of the silanized CPG particles prepared by Corning Glass was carried out. The manufacturer reported 0.09 mEq/g free amine groups. This was in contrast to the values found by our qualitative and quantitative micro-titrations. The qualitative determination gave 0.05 mEq/g estimation of the number of reactive free amine groups on the surface, while the quantitative method using the modified Gran plot gave a value of 0.02 mEq/g. Possible deterioration of the CPG particles may have occurred since they were stored for 18 months prior to use.

Detection of Secondary Phase of Antigen-Antibody Reaction Using Immobilized Antibody on CPG

Two criteria must be established prior to soluble antigen testing of the antibody coupled to CPG particles. First, qualitative and quantitative potentiometric titration methods are used to verify the

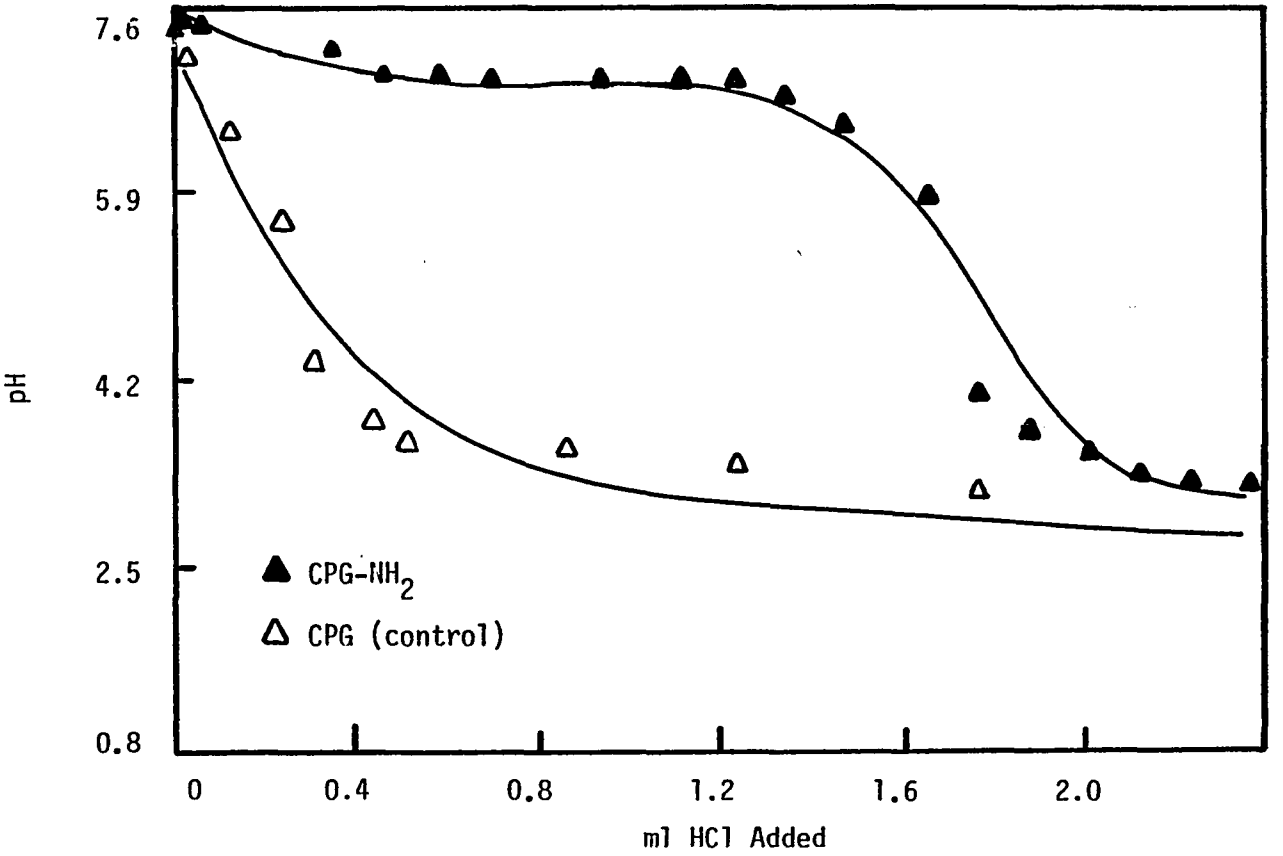


Figure 19. Determination of free amine groups on silanized (organic) CPG-S10 particles using qualitative acid base titration method.

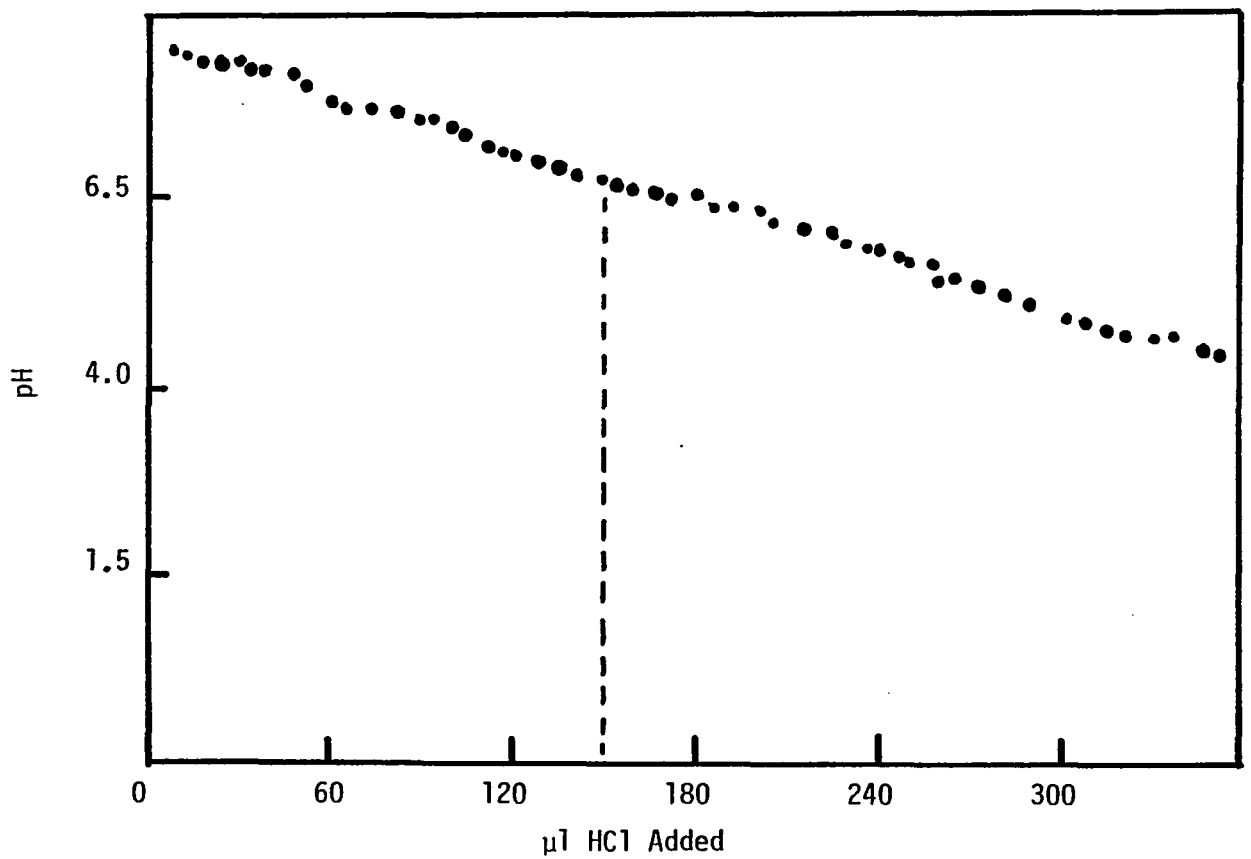


Figure 20. Quantitative titration of CPG-HS-1400 particles after aqueous silanization treatment.

presence of sufficient numbers of reactive coupling sites on the surface of the particles. After coupling a determination of whether sufficient antibody had been covalently attached. This was made by either amino acid analysis or spectrophotometric analysis using the Folin-Lowry determination of the acid hydrolysates of the CPG matrix in question. Once these criteria were met, testing of the particles in the presence of antigen could be carried out with assurance of adequate biological activity from the immobilized antihuman IgG. The reaction of the immobilized antibody was monitored in both the initial and secondary stages. The secondary phase of the antibody-antigen reaction involves the formation of a larger, more easily perceived physico-chemical complex. Because this occurs at higher concentrations than the initial reaction phase, detection of the secondary phase requires less sensitive and sophisticated equipment in general (i.e., visual determination).

In monitoring the secondary stage, the use of a particle sizing instrument, a Coulter Particle Counter Model Z_f, was employed.[1] The diameter of the CPG particles with immobilized antibody was measured before and after exposure to the appropriate antigen in the test solution. The change in the distribution of the mean particle diameter was compared to changes in the mean particle diameter exhibited by similar CPG particles exposed to a control solution (negative) void of the particular antigen in question. The difference in the mean particle

---

1. In particle sizing counters, materials in a fluid suspension flow through a small orifice or aperture, causing a change in the electrical resistance of the aperture. This change is sensed by an external circuit and translated into a voltage pulse. This pulse is proportional to the volume of the particle in the aperture.

size was used in qualitatively or quantitatively determining the presence of the antigen in the test solution.

The first area of investigation was the determination of the overall size distribution of the inorganic supports. A difference between the particle diameter or mean cell volume (MCV) of a particle coupled with an antibody and a particle coupled with an antibody complexed with an antigen will be observed only if the initial distribution of particle size is narrow. The magnitude of the change anticipated in the particle diameter is shown by the following example: antibody is covalently linked to the particle in the optimal orientation as shown in Figure 21, yielding a net change in particle diameter of approximately 0.04 microns. The particle is then reacted in the presence of antigen in the test solution and the antigen-antibody complex is formed. The net result is an increase in particle diameter of approximately 0.08 microns as compared to a control particle with no antibody attached. This net change may be large if the antigen binding to the antibody is multivalent. This allows formation of an antigen-antibody lattice which would be considerably easier to detect using the Coulter Particle Counter. Normally, if the antibody involved in the lattice were soluble, and in excess, the antigen-antibody lattice would dissolve in the test solution. Because the antibody is covalently linked to the CPG matrix, the dissolution of this complex does not take place. Thus, with multivalent antigens such as IgG, size changes greater than 0.08 microns would not be unusual.

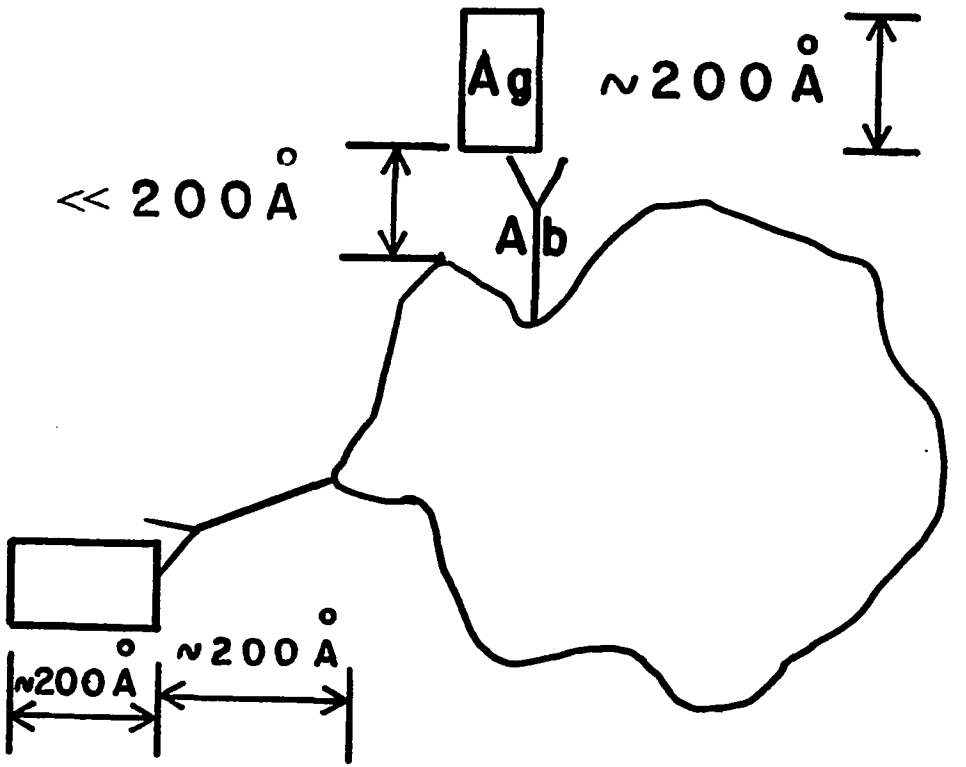


Figure 21. Expected change in particle diameter after Ab-Ag complex formation in optimal situation (left) of .08 microns/monolayer of complex formed, and in less than optimal situation (upper) of much less than .08 microns/monolayer of complex formed. -- Note that this is for a situation in which only one monolayer of complex is formed. Because of the polymerization aspects of the coupling reaction discussed later, it is anticipated that the increase in mean particle diameter will be much greater than 0.08 microns.

Analysis of the commercially available Electro Nucleonics glass particles by the Coulter Particle Counter gave a size distribution similar to that indicated by the manufacturer's specification (33-74 microns). As shown by Figure 22, the majority of particles is spread over 36-42 micron range. Pretreatment of these particles by the Sonic Sifter, Bradley Company, decreased the size distribution somewhat compared to the unsized particles. However, as can be seen in Figure 23, the distribution of presized particles is rather broad with significant tailing. Adjustment of various instrumental parameters on the Coulter Particle Counter was made to obtain a more discrete separation of the modes for each sized fraction. However, because of the background noise, there was no improvement in the separation of these curves even at a more sensitive detection setting.

The failure of the Sonic Sifter to produce a significantly narrower distribution such as given by the internal reference in Figure 23 may be attributed to several problems. First, the weight of the particles acquired for each of the various size ranges was dependent not only on the physical transfer of particles to and from the Sonic Sifter, but also on the time of sifting. As the sifting time increased, the amount of smaller particles (less than 30 microns) decreased while concomitantly the number of particles in the 37-44 micron range increased as determined by weight. Additional investigations using the Coulter Particle Counter showed that once the particles from this range were placed in solution, a much wider distribution of particles was obtained than was anticipated based on the sizing criteria of the Sonic Sifter.

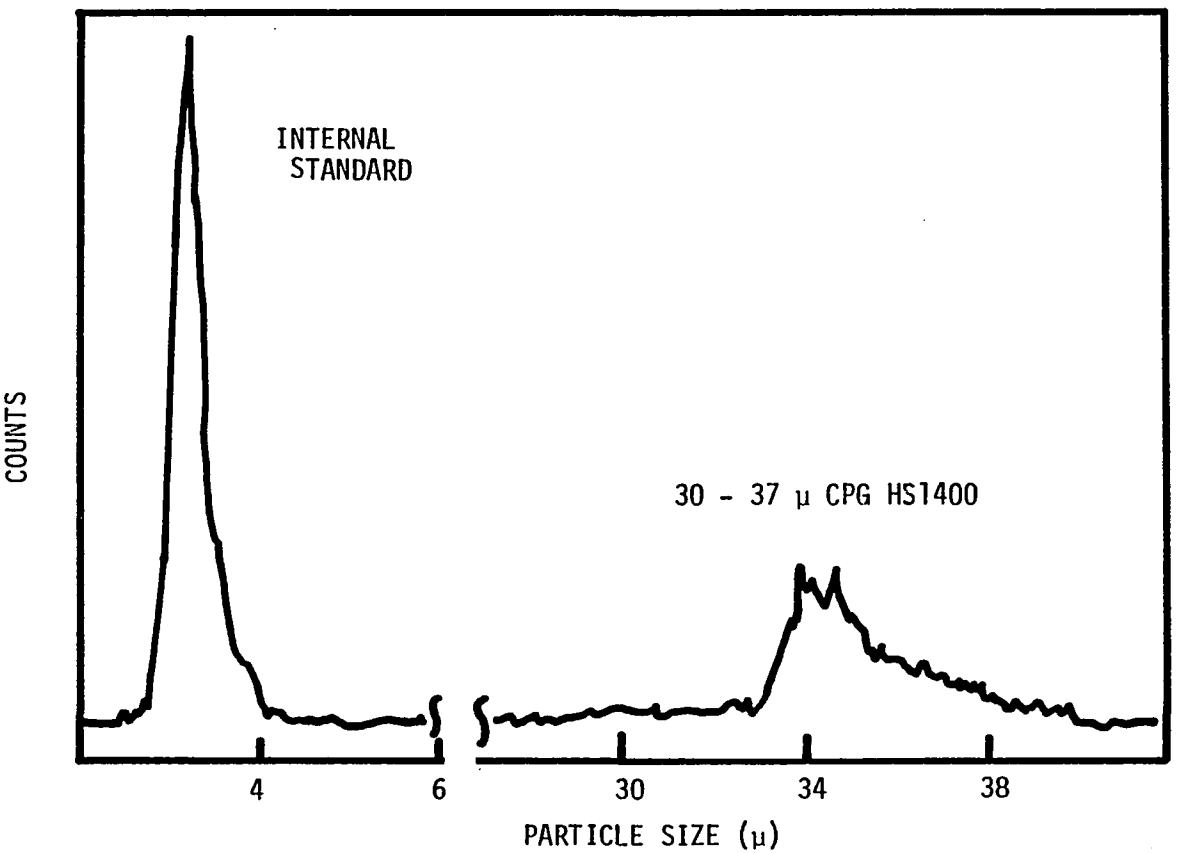


Figure 22. Narrowness of size distribution range of internal standard compared to CPG-HS1400 particles presized by Sonic Sifter.

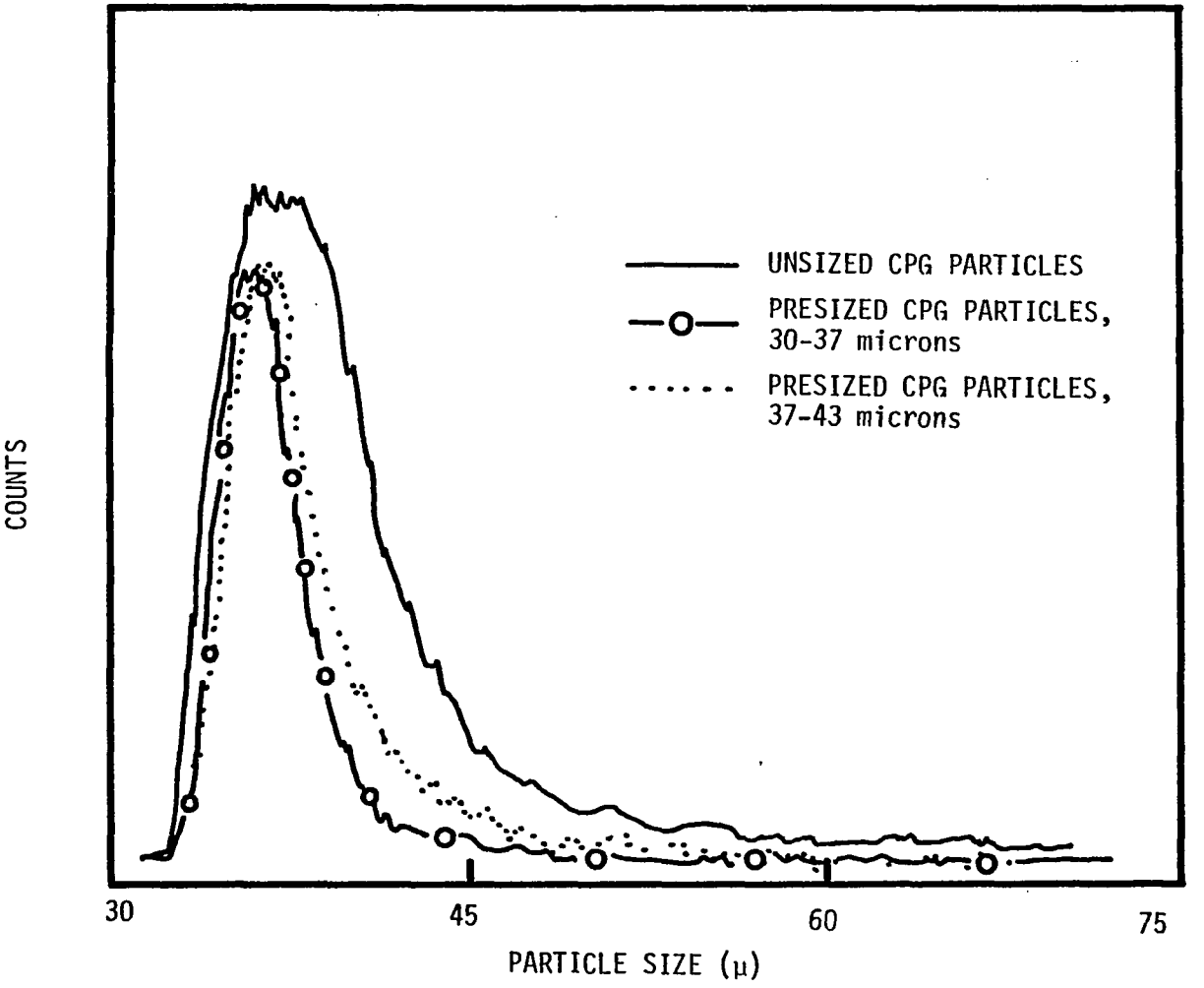


Figure 23. Size distribution of CPG-HS1400 particles before and after presizing treatment.

The increase in number of particles collected in the 37-44 micron range is puzzling in view of Ford's (1973) report which indicated that these particles have a high rate of attrition when placed in a rigorous environment such as the Sonic Sifter. An accumulation of particle "clumps" adsorbed underneath the sieving scrrens was observed, suggesting particle attrition of the larger particles that had been sized already. These "clumps" of particles, when placed in a polar medium such as water, readily dispersed, indicating they were held together by some type of surface charge. Derivatization of the CPG particles to reduce surface charge prior to the sizing process resulted in some improvement. However, as discussed later, this introduced the problem of not having enough reactive groups on the surface of the CPG particles.

Because of the difficulties encountered in sizing the irregularly shaped inorganic supports, attempts were made to acquire other types of supports which would have an innately narrow size distribution. Samples of non-porous glass spheres in the 10-30 micron range were investigated. Earlier work had shown that the porosity of the carrier is not an essential requirement for obtaining a matrix which exhibits high biological activity. There was a significant difference in precision when using the non-porous particles in the Coulter Particle Counter as compared to using the porous particles. Most notable was the development of a streaming potential by the non-porous particle. This caused the discriminator in the Coulter Particle Counter to overestimate the size of the non-porous particle (Waterman et al. 1975). The highly charged surface of these non-porous particles again resulted in their "clumping"

together, even after attachment of antibody. These particles tended to clog the orifice of the counting electrode of the particle counter.

A third type of support investigated was the Spherisorb particles, A.E.R.E., Harwell, United Kingdom. They are a microporous, totally spherical silica particle. According to manufacturer's literature, these particles have an extremely narrow size range, 90% are within ± 20% of the mean particle diameter. Experiments performed on the Spherisorb 5 micron (SW5) and the Spherisorb 20 micron (SW20) particles using the Coulter Particle Counter showed 84.7% of the particles actually sized by the Coulter Particle Counter (approximately $10^{4.5}$ particles) were within ± 15% of the mean diameter of 5 microns as shown in Figure 24. Also shown in Figure 24 is the distribution for the SW20 particles (as labeled by the manufacturer). This distribution is significantly better, 98.3% of the particles were within ± 5% of the mean particle size -- which, according to the Coulter Particle Counter, was 8.46 microns, with a mean cell volume (MCV) of 341.8 cubic microns. The apparent discrepancy between the manufacturer's value and the one determined with the Coulter Particle Counter is difficult to explain. Further analysis on a different batch of particles supplied by the manufacturer indicated the SW20 particles were mislabeled and, in fact, were SW10 particles. Because of the narrow size distribution, the SW10 particles were used in the remaining experiments involving the Coulter Particle Counter.

After determining the initial makeup of the glass particles, various activating and coupling steps were carried out using

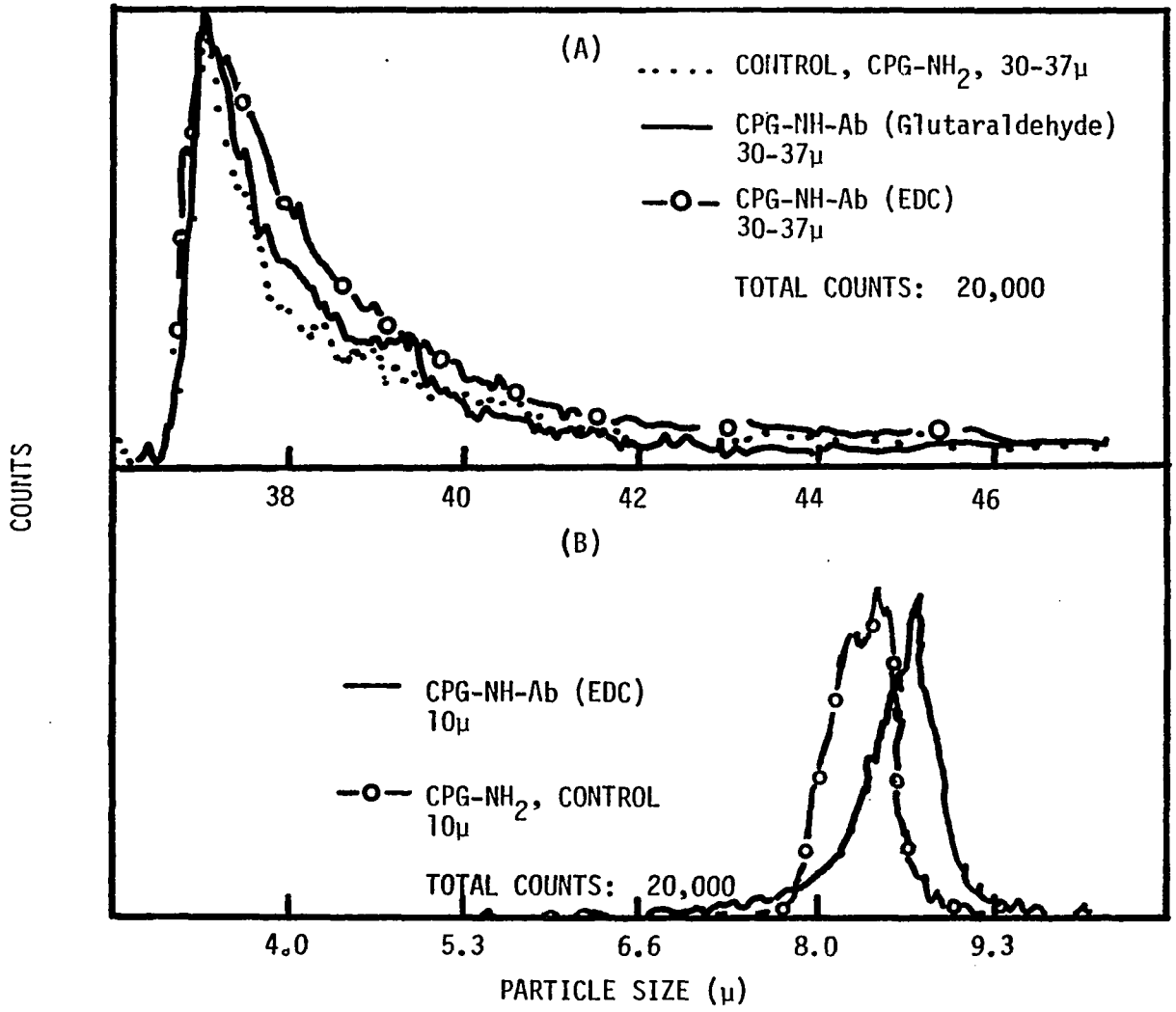


Figure 24. Change in mean particle size distribution after coupling antibody to CPG particles. -- (A) 30-37 μ, CPG-HS1400. (B) 10 μ, CPG-SW10.

glutaraldehyde or EDC on the particles. These particles were then analyzed by the Coulter Particle Counter. After coupling of antibody to the carrier, only a small increase in particle diameter is expected (approximately 0.04 microns per monolayer of antibody covalently attached). Because of the presence of the bifunctional coupling reagent, crosslinking between molecules is a possibility. The amount of coupling reagent was increased five-fold to see if this would result in the formation of a multi-layered, crosslinked antibody network covalently attached to the CPG matrix. As seen in Figure 24A, both glutaraldehyde and EDC, in excess in the coupling reaction, produced an immobilized antibody layer much greater than 0.04 microns, indicating that polymerization of the antibody is occurring in the coupling reaction. The shortcoming of the 37-44 micron particles is also displayed in Figure 24A. The severe tailing and very minimal shift between modes of the uncoupled and antibody coupled CPG particles are undesirable. The possibility of visually confirming a shift in mean particle size to larger values after reaction of the immobilized antibody with antigen is eliminated if the 37-44 micron CPG particles are used as the support. However, with the SW20 (really SW10) particles substituted in place of the 37-44 micron CPG particles and reacted with the antibody in exactly the same EDC procedure, a significant, visually detectable difference in the mean particle size of the uncoupled and antibody coupled particles is seen (Figure 24B). Essentially the same narrow distribution is retained after coupling the particles with the antibody as was obtained with the control particles which were activated and allowed to react

with the coupling reagent in the absence of any antibody. There was some "tailing" in the antibody linked particles at diameters smaller than the mean particle diameter. This may indicate that the cross-linking of the antibody and attachment of this lattice to the particles occurs to varying degrees depending on the various physical parameters of the matrix, i.e., charge, number of reactive sites.

Serum containing human IgG was prepared in various dilutions and then reacted for a specific period of time with the CPG particles containing immobilized antibody. Subsequent tests with the Coulter Particle Counter indicated a slight increase in mean diameter. This increase, however, was used only in a qualitative fashion. It was not possible, for example, to differentiate between a solution containing 300 µg/ml of IgG and one containing no IgG using the change in mean particle diameter as the sole criterion. It was possible to differentiate between a solution containing 300 µg/ml and one containing 600 µg/ml. These and other tests using this system indicated an increase in mean particle diameter of 0.4 microns as compared to the control group (Table 7). There was no significant improvement in mean partizle size over the control group when the immobilized antibody particles were allowed to react for longer periods of time (up to an hour) at room temperature.

This inability to detect larger changes in particle diameter after increased exposure time may be a limitation imposed by monitoring the secondary phase of the antigen-antibody reaction (i.e., waiting for formation of a large antigen-antibody complex). After formation of the

Table 7. Monitoring of the reaction of CPG particles[a] with varying concentrations of IgG by Coulter Particle Counter. -- Those samples in group A were allowed to react for one hour at room temperature before undergoing analysis. Those in group B were allowed to react for 10 minutes at room temperature.

Group	Sample #	Dilution	µg/ml	Mean Particle Size (µ)[c,d]
A	1	1:20	600	6.399
	2	1:40	300	6.041
	3	1:80	150	5.976
	4	1:160	75	5.983
	control[b]	-	0	5.991
B	1	1:20	600	6.546
	control	-	0	6.156

[a] 5 µ, CPG-SW5.

[b] CPG, 5 µ, after silanization.

[c] CPG, 5 µ, before silanization, MPS 6.004.

[d] CPG, 5 µ, after covalent attachment antibody, MPS 6.156.

initial complex on the surface of the CPG matrix, further cross-polymerization through adsorption and formation of hydrogen bonds between IgG molecules is possible. This network is held together by rather tenuous non-covalent bonds and may be partially destroyed by the shearing force exerted by the 70 micron aperture of the Coulter Particle Counter as it passes through. The radial shear at the extreme edge of the aperture ranges from 3×10^3 dyne/cm^2 to 1×10^4 dyne/cm^2. (These forces are sufficient to bring about hemolysis of red blood cells.) (Nevaril et al. 1968).

Also, any change in the surface properties of the CPG matrix will influence the sizing determination. Because the output signal is governed in part by electric field effects, the surface charge on the CPG particle will directly influence the size distribution of the particles as determined by the Coulter Particle Counter. Modification of the charge characteristics of the silica surface by silanizing reagents is well-documented (Gilpin 1973, and references therein). The extent to which these surface hydroxyl groups and their localized positive charge are modified, however, depends on the type of silanizing reagent employed and the steric availability of these groups.

Monitoring of the Primary Phase of the Reaction of Antigen with Immobilized Antibody on CPG

The primary reaction between antigen and antibody can be followed by physical techniques such as light scattering, fluorescence polarization, or fluorescence quenching; or by chemical techniques such as equilibrium dialysis. The technique of fluorescence quenching has

been used extensively with certain immunological systems for studying the binding of purified antibody to haptens (Eisen and Siskind 1963; Harisdangkul and Kabat 1972; Velick, Parker, and Eisen 1960). Unlike equilibrium dialysis, it is essentially an empirical chemical method and usually has to be standardized by carrying out an additional chemical measurement on the same solution. Proteins irradiated with U.V. light at a wavelength corresponding to their absorption maxima, 280 nm, will fluoresce and emit radiation in the 330-350 nm region. The fluorescence spectrum for various antibodies has been shown to correspond to that of tryptophan although tyrosine also fluoresces in this region (Kabat 1976, Velick et al. 1960). In the interaction of a hapten with an antibody combining site, some of the energy that would ordinarily be emitted as fluorescence from tryptophan is transferred to the bound hapten. The quenching of the tryptophan fluorescence of antibody hapten or antibody-antigen complexes is more likely to occur when the molecule or a part of the molecule involved in the complex has an absorption band in the 300-400 region. An example of this is the prosthetic group of hemoglobulin. This heme group quenches nearly all of the fluorescence of the globulin and would be expected to quench the fluorescence of an antibody in a hemoglobulin antibody complex (Velick et al. 1960).

The usefulness of the quenching of a soluble antibody fluorescence is severely limited in crude systems such as serum where the antibody contributes only a small fraction of the total protein emission. The measurement of fluorescent quenching of an antibody which is covalently bound to a matrix is not limited by this problem as it may

readily be separated from other fluorescing components of the sampling mixture. The measurement of fluorescence quenching of immobilized anti-human IgG after exposure to human IgG was carried out in the specially constructed cell discussed in the Methods section. One of the problems with this type of assay is that the small geometric area of the CPG matrix which is monitored for fluorescence contains only a limited number of antihuman IgG molecules. A 1 mm^2 viewing window will have 9×10^4 30 micron particles in it. Assuming an optimum loading of 1 mg protein immobilized/g matrix:

$$\frac{1 \times 10^{-3}\ g}{g\ matrix} \times \frac{1\ mol}{1.6 \times 10^5\ g} = \frac{1 \times 10^{-8}\ moles\ protein}{g\ matrix}$$

$$\frac{1 \times 10^{-8}\ moles}{g\ matrix} \times \frac{1\ g\ matrix}{10^8\ particles} = \frac{1 \times 10^{-16}\ moles}{particle}$$

then

$$\frac{1 \times 10^{-16}\ moles}{particle} \times \frac{9 \times 10^4\ particles}{viewing\ window\ (1\ mm^2)} = \frac{9 \times 10^{-12}\ moles}{viewing\ window}$$

Assuming these particles spherical and in a normal packing density, approximately 25% of the surface area will be irradiated by excitation beam:

$$\frac{9 \times 10^{-12}\ moles}{in\ viewing\ window} \times .25 = 2.25 \times 10^{-12}$$ moles immobilized antibody in viewing window in fluorescence micro cell

This assumes excitation light does not penetrate the packed micro cell beyond the first layer, although Gabel et al. (1971) assume a penetration of 0.5 mm in their experiment with Sephadex on which antibodies were covalently bound. This would correspond to approximately 5 layers of CPG particles, and would increase the number of molecules available for monitoring of their quenched native fluorescence by a factor of five.

The quenching sensitivity limit is also dependent upon the efficiency of excitation energy transfer from antibody to antigen. Immunochemical fluorescent quenching experiments have been previously carried out with predominantly hapten-sized molecules (Eisen and Siskind 1963, Velick et al. 1960). In this case, the fit of a hapten to an antibody binding site generally is much tighter, allowing the part of the hapten molecule which absorbs in the 300-400 nm region to be within 50 Å of the tryptophan residues on the antibody. The quenching efficiencies for these hapten systems range upwards from 65%. The heterogeneity of the antibody binding site along with the physically much larger antigen molecule does not usually provide for these types of optimum conditions for efficient quenching. This results in low quenching efficiency which is defined as:

$$\%Q = \frac{f_f - f_b}{f_f} \times 100$$

where f_f is the fluorescence of the unbound, free antibody, and f_b is the fluorescence of the antibody when bound by hapten or antigen. A %Q

reported for a soluble antibody-antigen system that did exhibit some quenching was between 5-10% (Tengerdy and Small 1966).

To compensate for low quenching efficiency and the relatively small number of molecules in the fluorescent viewing window, the antibody was labeled with fluorescein isothiocyanate (FITC). This labeling process increases the relative difference in the fluorescence between the non-quenched and quenched antihuman IgG covalently linked to the CPG matrix. This is accomplished by increasing the number of fluorescing groups in the viewing window and the fact that these groups have much higher quantum efficiencies than the native fluorescing amino acids.

The labeling of the protein was carried out according to the protocol listed in the Methods section with FITC. Fluorescein-labeled antihuman IgG had a molar conjugation ratio of 14.1 moles FITC/mole antihuman IgG. A series of sequential additions of 5 µl of antigen was made to 30 µg of antihuman IgG immobilized on CPG matrix inside the micro cuvette where the temperature was maintained at 22°±.5°C. After each addition, the solution was allowed to react for 5 minutes and then the fluorescence read. As discussed in the next section, binding of the soluble antigen to immobilized antibody is complete within 1-2 minutes. Then the solution and CPG particles were removed from the micro cell, the cell rinsed, and new aliquot of particles, 0.2 ml of 50 mg/ml CPG particles in pH 8.0 PBS was added. The initial fluorescence was measured, the antigen solution added, and the process repeated. The fluorescence of a blank, 10 mg of underivatized CPG particles without protein in pH 8.0 PBS is subtracted from all values. As the

concentration of antigen solution increased, the fluorescence decreased (Figure 25). The non-linear decrease at the higher concentrations of IgG reflects the saturation of the binding sites on the CPG particles. The linear portion of this assay lies in the 0-5 µg/ml IgG concentration range. This is substantiated by the HPLC column results discussed in the following section using the same CPG particles. The concentration of IgG at which one-half of the initial IgG is bound to the immobilized antibody and the remaining portion unbound was determined to be 1.53 µg/ml. This midpoint value suggested a potentially useful range of 0.25-7.15 µg/ml (Besch 1976). The sensitivity limits of this assay with initial use of the CPG particles containing immobilized labeled antibody are 0.25-0.50 µg IgG/ml. As the concentration of the antigen solution increased, the fluorescence decreased (Figure 25). The sensitivity limits with the initial use of the CPG particles containing immobilized labeled antibody are in the range of 0.5-1.0 µg/ml IgG. The fluorescence of a blank, 10 mg of underivatized CPG particles without protein in pH 8.0 PBS is subtracted from all values.

The use of the same CPG particles for a second determination resulted in a considerable decrease in the %Q. This large change in %Q after one use of the labeled antibody linked CPG particles may be attributed to the large number of non-covalently linked, labeled antibodies on the surface of the CPG matrix. Fluorescent microscopy of these particles has shown that extensive washing in high ionic strength buffer in absence of soluble antigen does not remove all adsorbed antibody. Even after exposure to antigen there still remains a very small

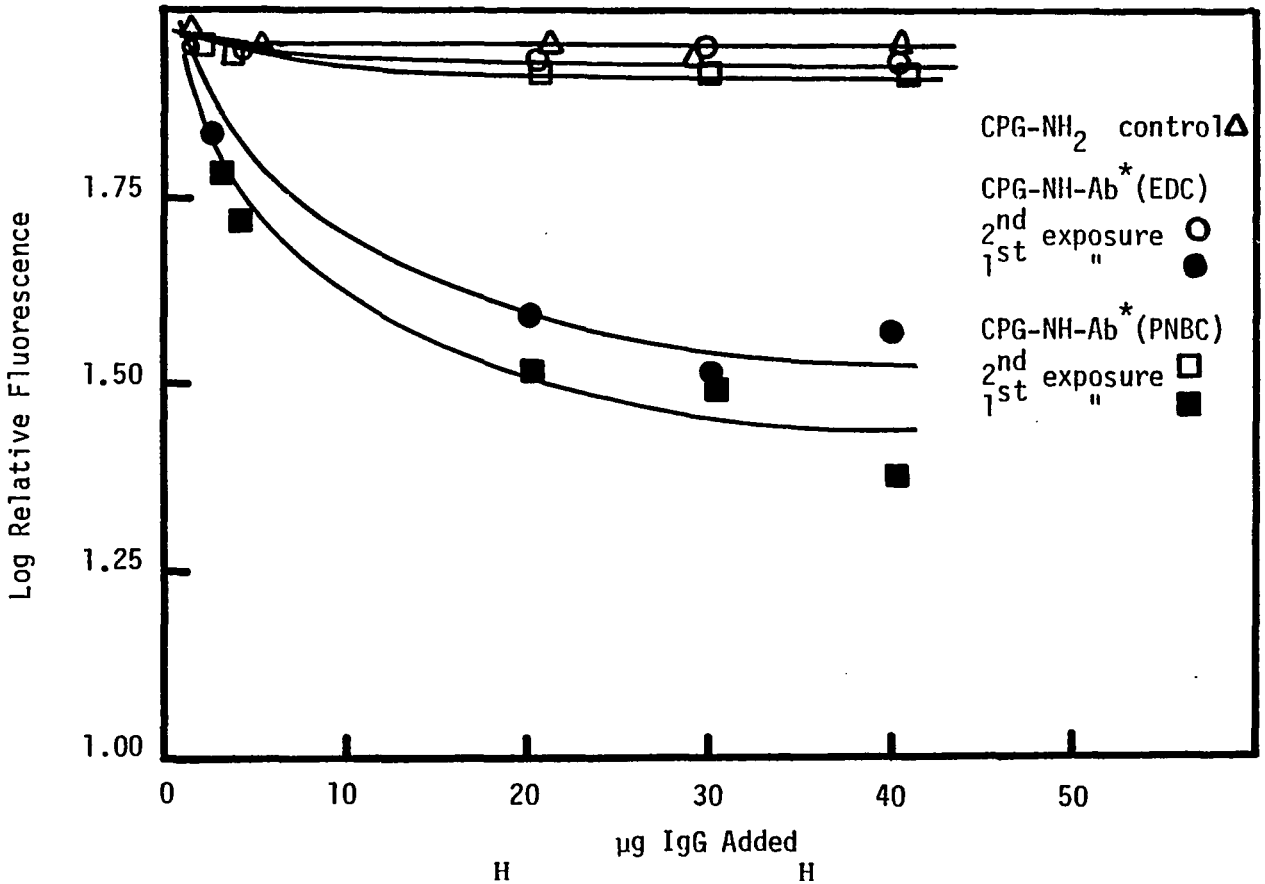


Figure 25. Fluorescent quenching of CPG-N-Ab* (EDC) and CPG-N-Ab* (PNBC) with IgG.

amount of adsorbed antibodies on the matrix. This is readily observed under the fluorescent microscope. In the initial use of these labeled antibody CPG particles, a considerable number of non-covalently attached antibodies on the surface react with antigen from the test solution. The antigen-antibody complex desorbs from the matrix either in the pH 8.0 rinse or the pH 2.0 wash, at the same time the antibody's fluorescence is being quenched efficiently by the bound antigen. In the subsequent use of the matrix there are not that many labeled adsorbed proteins to complex with antigen and have their fluorescence quenched, thus the %Q is small to zero.

The possibility of a higher %Q for the few immobilized antibodies labeled with FITC may be realized if fewer FITC molecules are linked/antibody molecule. With 14.1 molecules of FITC/molecule of antibody, it is doubtful the antigen can fit into the binding site such that it is within 50 Å of all 14 molecules of FITC. Thus, if fewer molecules of FITC are covalently linked to the antibody, the spatial demands on the antigen when it complexes with the antibody would not be as great. This would result in having to quench fewer FITC molecule, which in turn would result in a higher %Q.

The proportion of sites bound is taken as the degree of quenching observed (Q_i) relative to maximum quenching (Q_m) obtained for the antihuman IgG-CPG matrix system when all antigenic determinates on the immobilized antibody are occupied. If Q_i/Q_m is multiplied by number of moles titrated, multiplied by valence, which assumed to be 2 with the

antihuman IgG, the result is the number of moles of bound antigen. This is subtracted from total antigen concentration to give free antigen.

A similar titration of immobilized antibody with labeled IgG conjugated with FITC was carried out using the single-layer indirect fluorometric technique for determining the amount of bound protein. The sequential addition of 5 µl aliquots was made to 200 mg of CPG matrix containing immobilized antihuman IgG inside a 0.9 x 10 cm glass column (Figure 5, Methods section). After each addition the solution was allowed to react for 5 minutes, the column rinsed with 30 ml of pH 8.0 PBS, and then the labeled antigen eluted with pH 2.0 PBS. In this series of titrations, a single batch of CPG particles was used for determining bound antigen at each of the different antigen concentrations. As in the quenching experiment, the number of moles of free antigen were determined by subtracting number of moles of bound from number of moles initially added. The difference in this number and that arrived at by directly measuring the rinse solution containing unbound antigen represents the amount of specifically adsorbed protein. The average value for adsorbed protein in these titrations remained around 4-6% total antigen added.

The equilibrium constant for the antigen-antibody reaction expressed as the association constant is:

$$\frac{[Ag\ B]}{[Ag][B]} = K \qquad (2)$$

where [Ag] represents the molar concentration of free antigen and [B] represents the molar concentration of antibody combining site remaining

unoccupied at equilibrium. If the reaction of antigen at one site does not influence the reaction at another site, and if all sites are assumed to have the same K, an equation derived by Klotz (1953) may be used to plot the data from the titration of antigen with antibody obtained either by the fluorescent quenching or indirect single-layer fluorometric technique. In the Klotz equation:

$$\frac{r}{c} = nK - rK \tag{3}$$

K is the association constant, n the valence of the antibody, r the ratio of moles of antigen bound/mole antibody, and c the free antigen concentration. An alternate form of the equation is:

$$\frac{1}{r} = \frac{1}{nK}\frac{1}{c} + \frac{1}{n} \tag{4}$$

in which a plot of 1/r against 1/c would give a straight line for a homogeneous antibody. However, a straight line is generally not obtained with an antibody. This is due to the fact that the antibody molecules do not have the same K, either because of their heterogeneity or because the binding of an antigen at one antibody binding site induces some type of conformational change at the other binding site. In Figure 26, different concentrations of labeled IgG were exposed to immobilized antihuman IgG on CPG matrix at two different temperatures. The amount of bound labeled antigen determined by the indirect single layer fluorometric technique. This information was then used in equation (3), and the information obtained listed in Tables 8 and 9. The

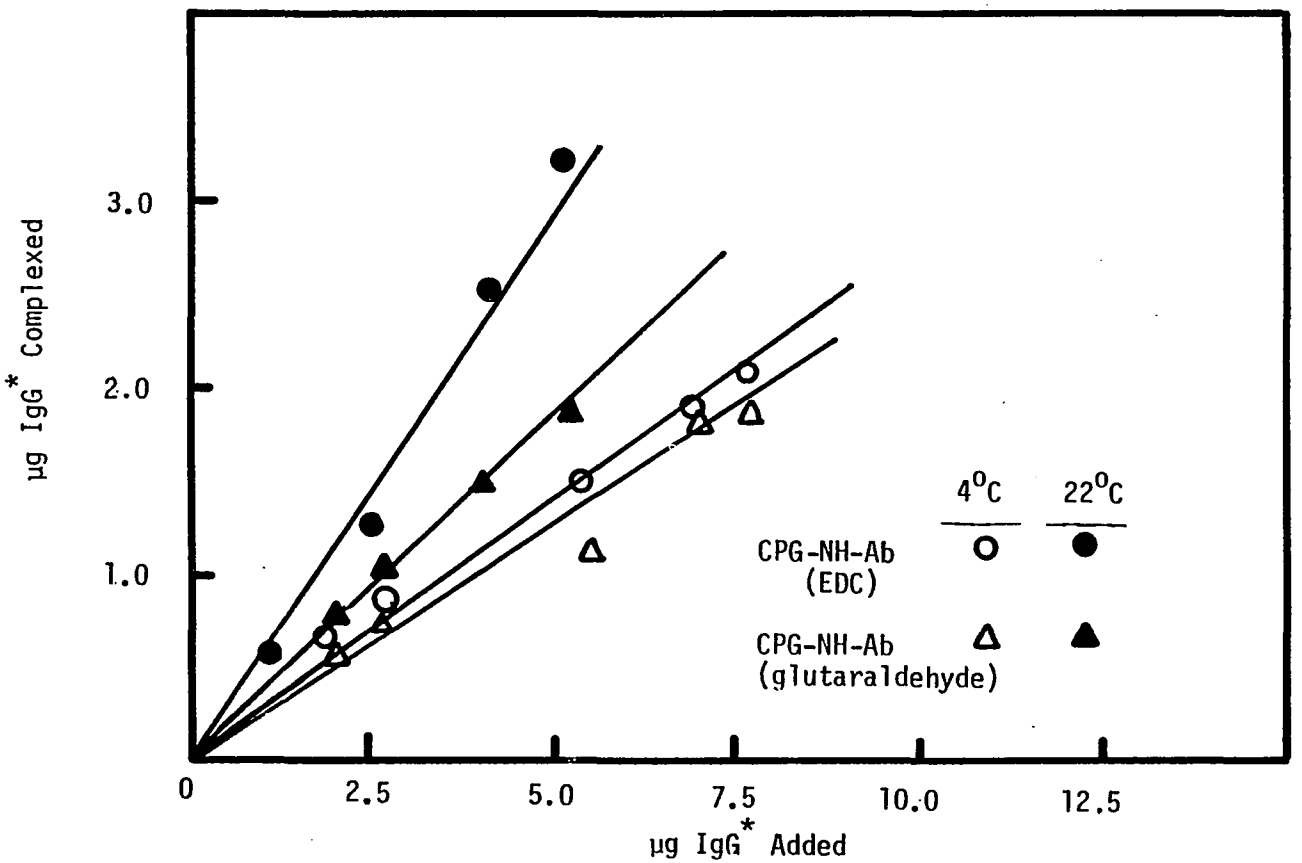


Figure 26. Comparison of binding properties of two different matrices with immobilized antihuman IgG at 22° and 4°C using indirect single-layer fluorometric assay.

Table 8. Determination of free and bound IgG fractions during titration of antihuman IgG immobilized to CPG at 4° and 22°C.

µg IgG (moles)	µg Antibody on CPG Matrix (moles)	r (µg bound)	r/c
	22°C		
5 (3.3×10^{-11})	30 (2.0×10^{-10})	1.63×10^{-1} (4.9)	3.26×10^{10}
10 (6.6×10^{-11})	30 (2.0×10^{-10})	3.2×10^{-1} (9.6)	1.6×10^{11}
15 (9.9×10^{-11})	30 (2.0×10^{-10})	3.0×10^{-1} (12)	2.1×10^{10}
25 (16.5×10^{-11})	30 (2.0×10^{-10})	5×10^{-1} (15)	7.69×10^{9}
100 (6.6×10^{-10})	30 (2.0×10^{-10})	7.6×10^{-1} (23)	6.55×10^{8}
400 (26.4×10^{-10})	30 (2.0×10^{-10})	8.33×10^{-1} (25)	3.36×10^{8}
600 (39.6×10^{-10})	30 (2.0×10^{-10})	1.06 (32)	2.83×10^{8}
1000 (6.6×10^{-9})	30 (2.0×10^{-10})	1.43 (43)	2.23×10^{8}
	4°C		
100 (6.6×10^{-10})	30 (2.0×10^{-10})	3.33×10^{-1} (10)	5.55×10^{8}
200 (13.2×10^{-10})	30 (2.0×10^{-10})	4.0×10^{-1} (12.5)	3.22×10^{8}
400 (26.4×10^{-10})	30 (2.0×10^{-10})	5.5×10^{-1} (16.5)	2.17×10^{8}
600 (39.6×10^{-10})	30 (2.0×10^{-10})	6.6×10^{-1} (20)	1.74×10^{8}
1000 (6.6×10^{-9})	30 (2.0×10^{-10})	7.16×10^{-1} (21.5)	1.11×10^{8}

Table 9. Determination of heterogeneity of antibody binding site using Sips equation. -- Log (r/n-r) = a log c + a log K_o.

(r/n-r)	log (r/n-r)	log c (μm)
	22°C	
3.33×10^{-1}	-.477	4.1
3.88×10^{-1}	-.411	3.3
6.12×10^{-1}	-.213	2.9
7.09×10^{-1}	-.149	2.6
1.12	.049	2.4
2.50	.397	2.2

typical Scatchard plot of r/c versus r was plotted in Figure 27, from the data in Table 8. This relationship seen in Figure 27 shows an "n" value in the range of 1.7 and 1.4 for the binding of labeled IgG to immobilized antihuman IgG at 22° and 4°C, respectively. The heterogeneity of the immobilized antibody is suggested by these "n" values of less than 2. The heterogeneity of the immobilized antibody was also shown by a Sips plot (Figure 28). The slope of the line in a Sips plot is an index of the heterogeneity and ranges from 0-1. The deviation from linearity at the lower concentrations in Figure 28 is due in part to the difficulty of measuring the fluorescence of very low concentrations of antigen by the indirect single-layer technique. If only the $\log \frac{r}{n-r}$ values for the higher concentrations of antigen are used in determining the slope, a value of 0.71 is obtained for the heterogeneity index. This indicates that 71% of the antibody sites have a K value between 1/16 and 16 times that of K_o where K_o is the average of all K values. From Figure 27, a K_o value of 7.0×10^8 1/mole at 22°C and 4.3×10^8 1/mole at 4°C is obtained. Using these values, the enthalpy, $\Delta H°$, and entropy, $\Delta S°$, for the reaction of antigen with immobilized antibody are calculated to be -17.3 Kcal/mole and -17.7 entropy units/mole, respectively. Because these values ($\Delta H°$ and $\Delta S°$) were calculated from K_o values, they have an indeterminate error associated with them. This is the result of the heterogeneity of the antibody. Since K values for the antibody binding site are necessarily an average value, it is not possible to predict how temperature will affect the average. Secondly, calculation of this average K value requires an a priori

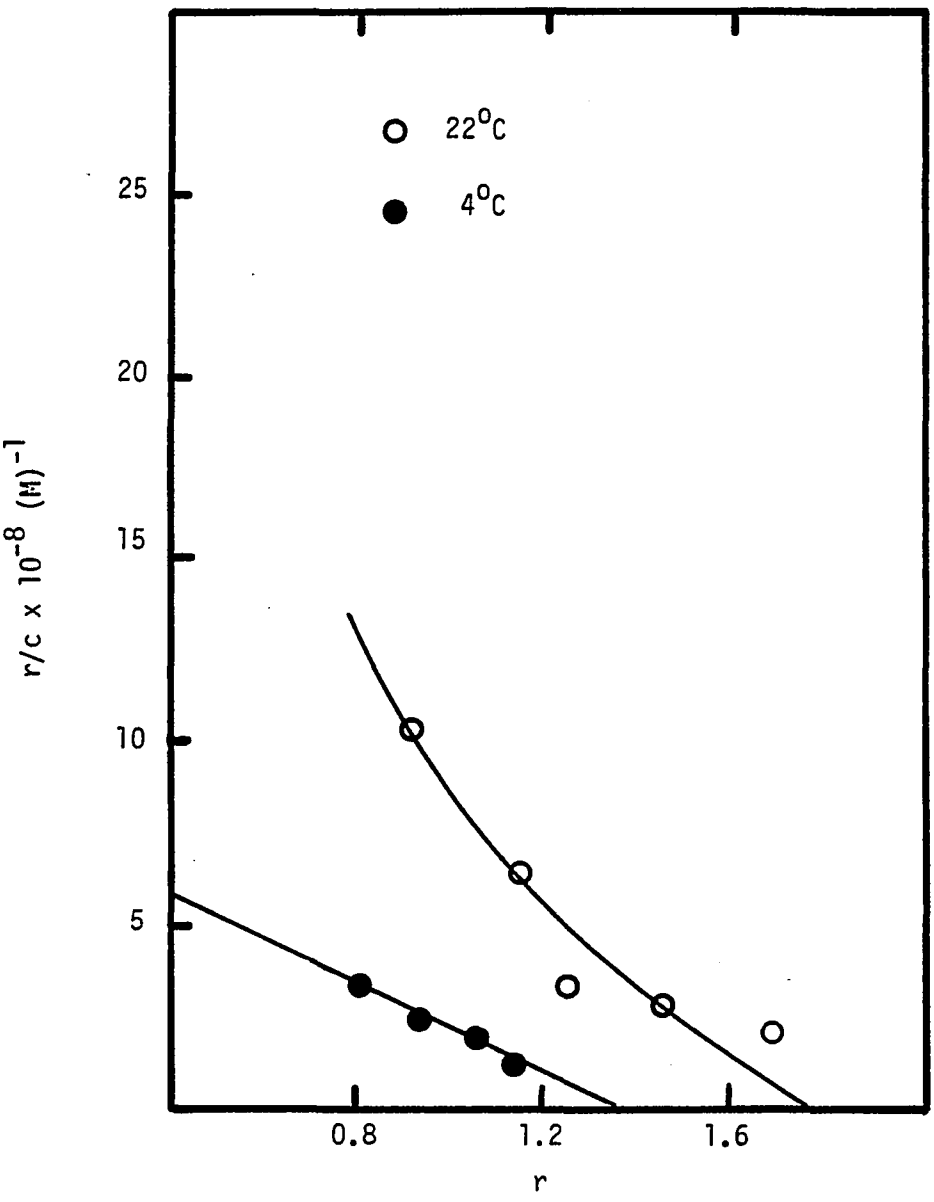


Figure 27. Scatchard plot of IgG binding to immobilized antibody on CPG matrix at 4° and 22°C.

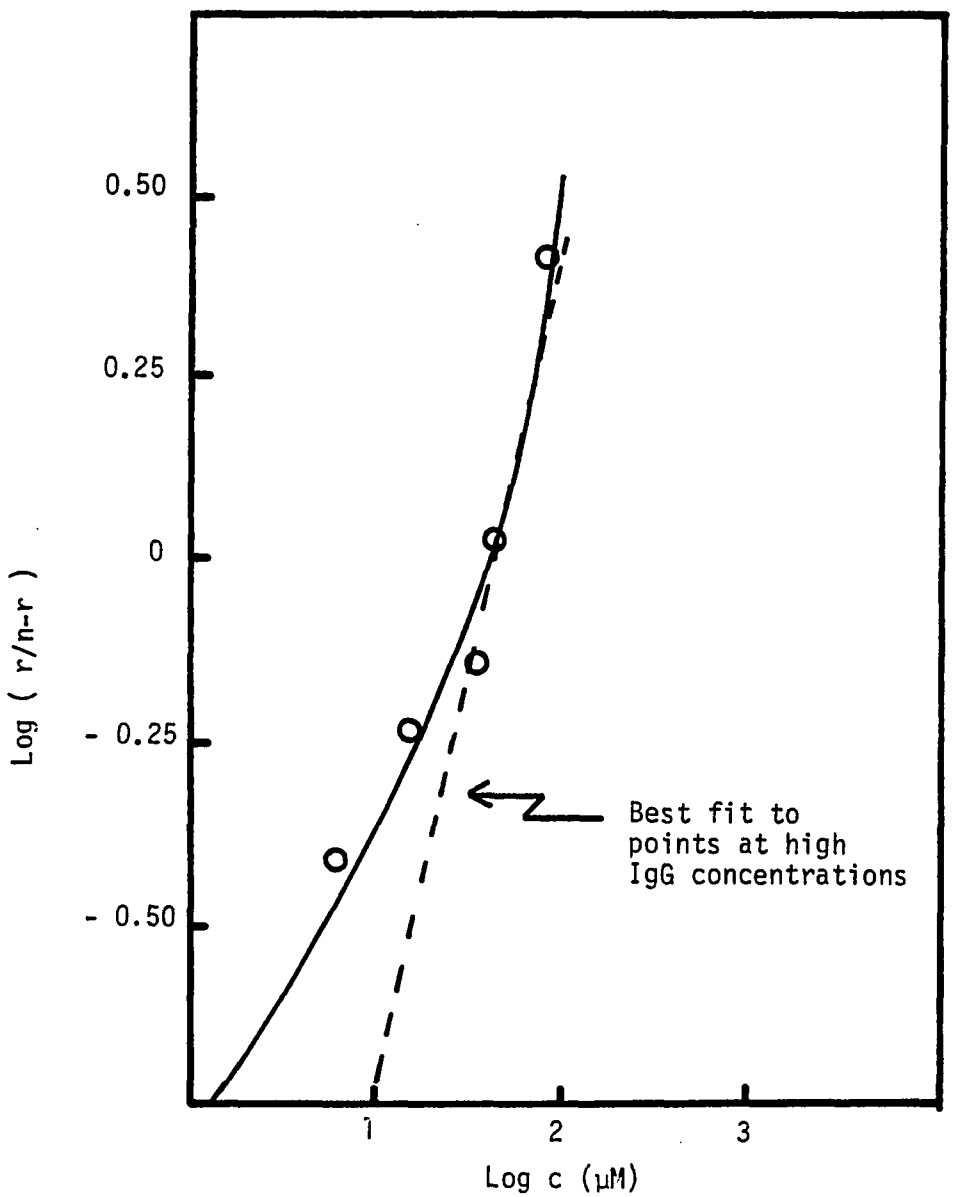


Figure 28. Sips plot of IgG binding to immobilized antihuman IgG at 22°C.

assumption as to the distribution of the heterogeneous antibody molecules with respect to their binding affinities. A commonly used approximation is that the free energy or ln K follows a Gauss error function. In the Gaussian distribution, all values of K are represented, and the "average K," K_o, is that corresponding to the peak of the distribution curve. However, recent experimental work on the distribution of antibody molecules has demonstrated that a few homogeneous subpopulations account for the majority of the antibody binding activity of a particular antiserum (Johnston, Barisas, and Sturtevant 1974; Barisas, Sturtevant, and Singer 1971). A qualitative assessment of this enthalpy/entropy information would be that the large entropy change would strongly oppose the binding of the antigen to the binding site; however, it is more than offset by the large decrease in enthalpy. The value of -17.5 Kcal/mole is a very large value relative to other values for soluble antigen-antibody or hapten-antibody systems (Nisonoff et al. 1975) -- most values for hapten-antibody systems are in the -5 to -12 Kcal/mole range (Kabat 1976).

Studies on the soluble antibody molecule have confirmed that the non-linearity of both the 4° and 22°C curves in Figure 27 are the result of the different amino acid sequences in the variable region on the heavy chain of the antibody (Kabat 1976). The values obtained for the number of binding sites/molecule are anticipated to be less than those obtained for the soluble molecule in equilibrium dialysis studies. This is because during the immobilization of the antibody, the random distribution and orientation of the molecules on the matrix is such that

part of the time the binding site is either involved in the covalent linkage to the matrix or in a sterically hindered region.

Clinical Importance of Immobilized Antibodies

Serum immunoglobulins are measured in a variety of ways in the clinical laboratory. One of the more common techniques involves the use of gel diffusion (RID) based on the procedure of Mancini, Carbonara, and Heremans (1965). The use of rocket immunoelectrophoresis (Axelson 1973) which is essentially a one-dimensional diffusion process utilizing an electric field as the driving force behind the rapid migration of antigen through the gel, has reduced the excessive time requirements of RID from 18 hours (non-equilibrium) or 48 hours (equilibrium) waiting periods to 2-3 hours before measurement of the "rocket" shaped precipitin line can be made. Unfortunately, automation of this technique is difficult and impractical. Radio immunoassay (RIA) employing competitive binding systems with soluble reagents is very sensitive, but requires considerable skill and is generally too slow for analysis of serum immunoglobulins in a service-oriented laboratory (Cawley et al. 1974, Fahey and Lawrence 1963). The improvement brought about by chemically immobilizing the appropriate antibody on a solid phase has overcome some of the innate limitations of RIA. The expense in instrumentation and extensive safety precautions required when dealing with radio labeling techniques are still present with the solid phase RIA. The use of fluorescence labeling eliminates these additional problems.

The CPG particles with immobilized antibody were used in a competitive binding assay with fluorescently labeled antigen.

Commercially available antibody bound to Bio Gel P4 particles, Immuno-Fluor, were evaluated in the same series of experiments as the CPG particles. The protocol followed for these tests was based on the directions supplied by the manufacturer of the Immuno-Fluor particles with a recommended total assay time of 2.5 hours. The results of this comparison are shown in Figure 29, for the assay of different concentrations of IgG, and indicate the CPG particles bind approximately 2.5 times more protein per gram of matrix than the commercial particles. There is some non-linearity of the CPG particles at concentrations of IgG greater than 30 µg/ml, indicating saturation of the binding sites of the immobilized antibody. To assay these concentrations either more CPG particles need be added, or the sample solution diluted so that the antigen concentration falls in the linear portion of the calibration curve. Some of the difference in binding capacity of the particles is related to the specificity and homogeneity of the particular antibody immobilized (Weetall 1975). Analysis by RID and gel electrophoresis of the antigen complexed by these two different types of particles indicated that both were complexing and releasing the same part of the antigen population.

There is essentially no improvement in the overall time required to carry out an immunoglobulin assay using the solid-phase technique with a fluorescent labeled antigen as compared to doing the same analysis using rocket immunoelectrophoresis. This is disconcerting since the actual time required for the binding of IgG to antihuman IgG immobilized on a solid phase is on the order of 30-60 seconds (Figure 30) (Weetall 1972). This is not surprising as the half-life for binding of antigen

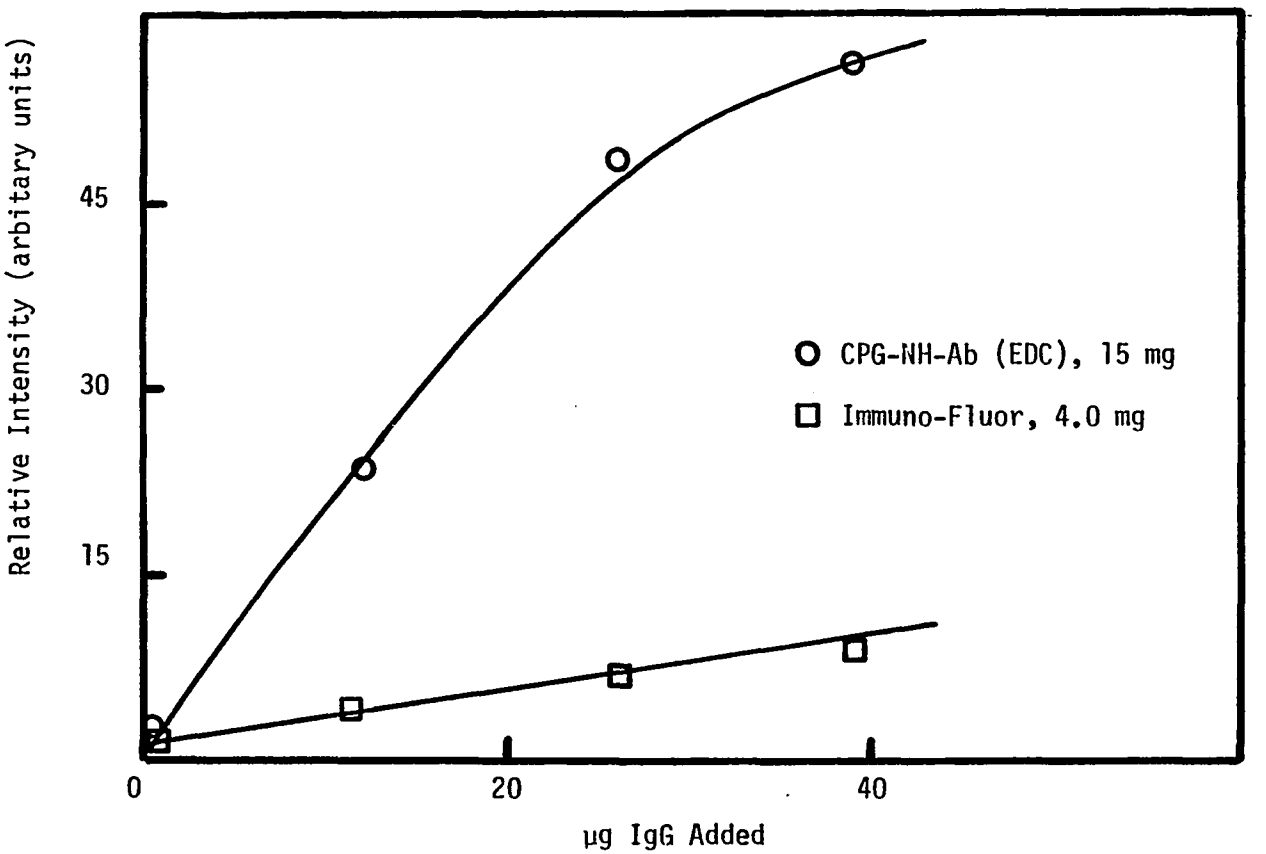


Figure 29. Comparison of Immuno Fluor with CPG antibody particles in determining different concentrations of IgG in a competitive binding assay.

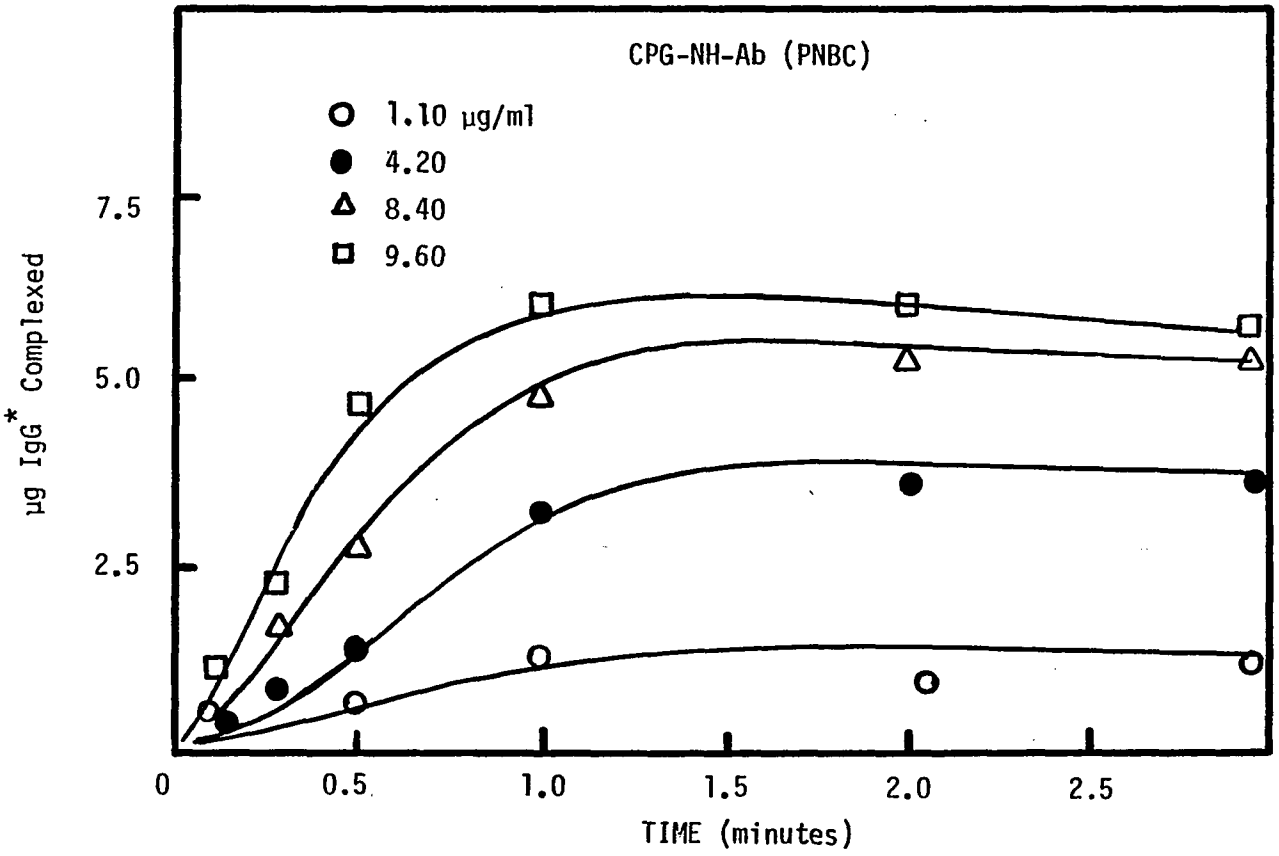


Figure 30. Binding of different concentrations of labeled IgG to immobilized antibody on CPG-NH-Ab (PNBS) matrix as a function of time.

to soluble antihuman IgG is in the millisecond range (Savory, Buffone, and Reich 1974). The time dependence of this binding of antigen to immobilized antibody exhibits an apparent initial lag phase of approximately 5-10 seconds. This may be an artifact of the monitoring or mixing technique. After approximately 60 seconds there is no additional change in the amount of bound antigen, and the curve reaches a plateau. In between these extremes there is an apparent linear region where amount of antigen complexed is directly proportional to the amount of time it is exposed to the immobilized antibody. In general, these curves are not of much value in gaining any fundamental information on the reaction between antigen and immobilized antibody because of the heterogeneity in the immobilized protein composition, binding site, and association constant. It can be concluded that the binding of antigen to immobilized antibody is a fairly rapid process and that leaving the antigen exposed to the immobilized antibody for any extended period of time does not lead to any increase in the amount of antigen complexed.

In an attempt to utilize this rapid binding of soluble antigen to the CPG matrix containing immobilized antibody, a high pressure liquid chromatographic column was packed with antibody CPG particles. Samples of varying concentration of antigen were injected onto the column from a 10 µl sample loop. The unbound antigen was washed from the column using pH 8.0 PBS and detected as the first peak by a variable wavelength spectrophotometer set at 280 nm. Using the buffer gradient capabilities of the instrument, a plug of pH 2.0 PBS was injected onto the column and caused the bound antigen to be released and detected as

the second peak of the chromatogram (Figure 31). The total time of the assay varies depending on the flow rate of the column, but can be carried out in five minutes as shown in Figure 31. Of these five minutes, 70% of the total time is used to establish the baseline for the pH 2.0 buffer after the peak from the eluted antigen has past, and to re-establish the pH 8.0 baseline before the next injection. In an attempt to overcome these problems associated with refractive index changes, which are very significant at the sensitivity setting employed (0.08 absorbance units full scale), a chaotropic agent, KCN, was used in place of the pH 2.0 PBS. This reagent has been shown to be as effective in dissociating bound antigen from immobilized antibody at concentrations lower than the 0.02 M pH 2.0 PBS (Figure 32). The use of KCN resulted in just as large a refractive index difference, even at concentrations lower than 0.005 M, without any improvement in reducing total assay time.

The sample was placed on the column and allowed to react in the column with the immobilized antibody for 10-20 seconds. The pH gradient was started one minute after the injection. After bound antigen had been eluted and the baseline for pH 2.0 buffer had been established, the gradient mixer was programmed to return to the initial buffer and another sample injection was carried out one minute later. The relationship between peak height of the free fraction of antigen and concentration of antigen injected onto column; and peak height of the bound fraction of antigen and the concentration of antigen initially injected onto column are shown in Table 10 and plotted in Figure 33. The error bars in

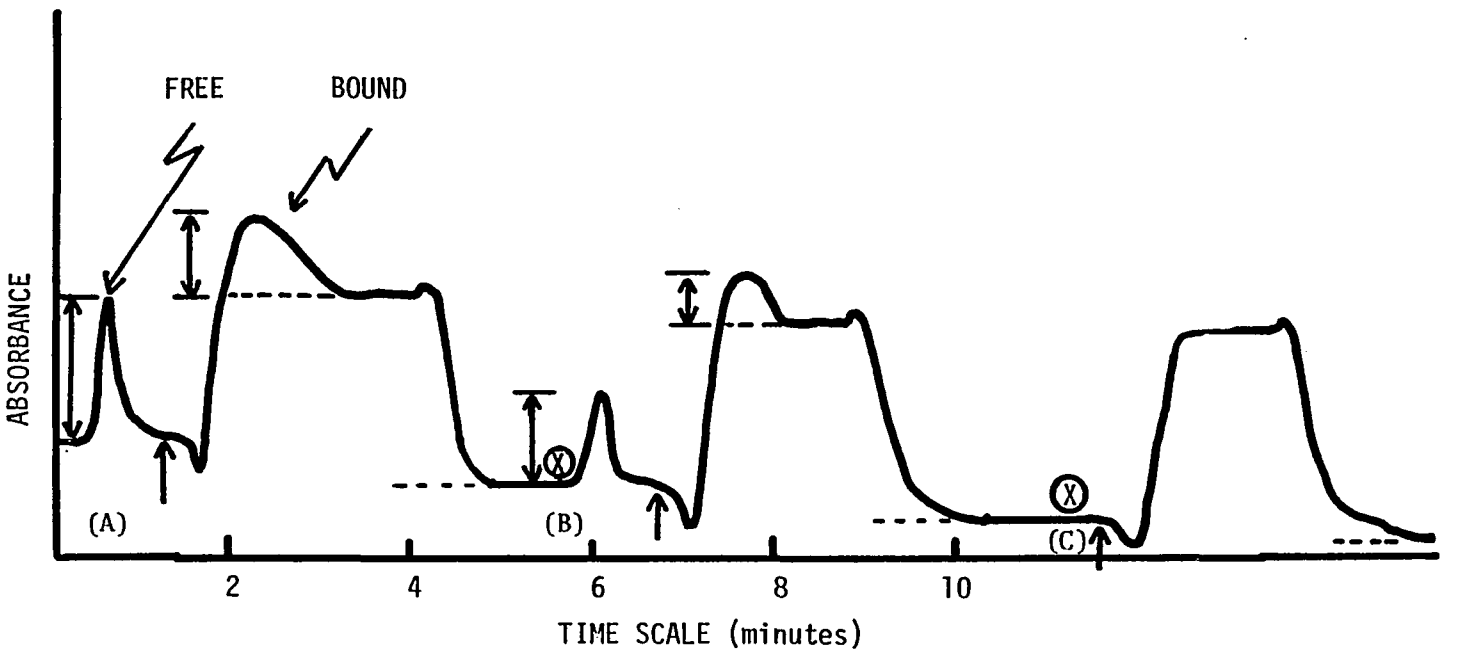


Figure 31. Chromatogram of different concentrations of IgG injected into HPLC column packed with CPG-NH-Ab (PNBC). -- Samples injected X , (A) 4.08 µg/ml, (B) 2.04 µg/ml, (C) blank; buffer changed †. Time between sample injections is five minutes. Multiwavelength detector on 0.08 abs units full scale. Flow rate 0.80 ml/min.

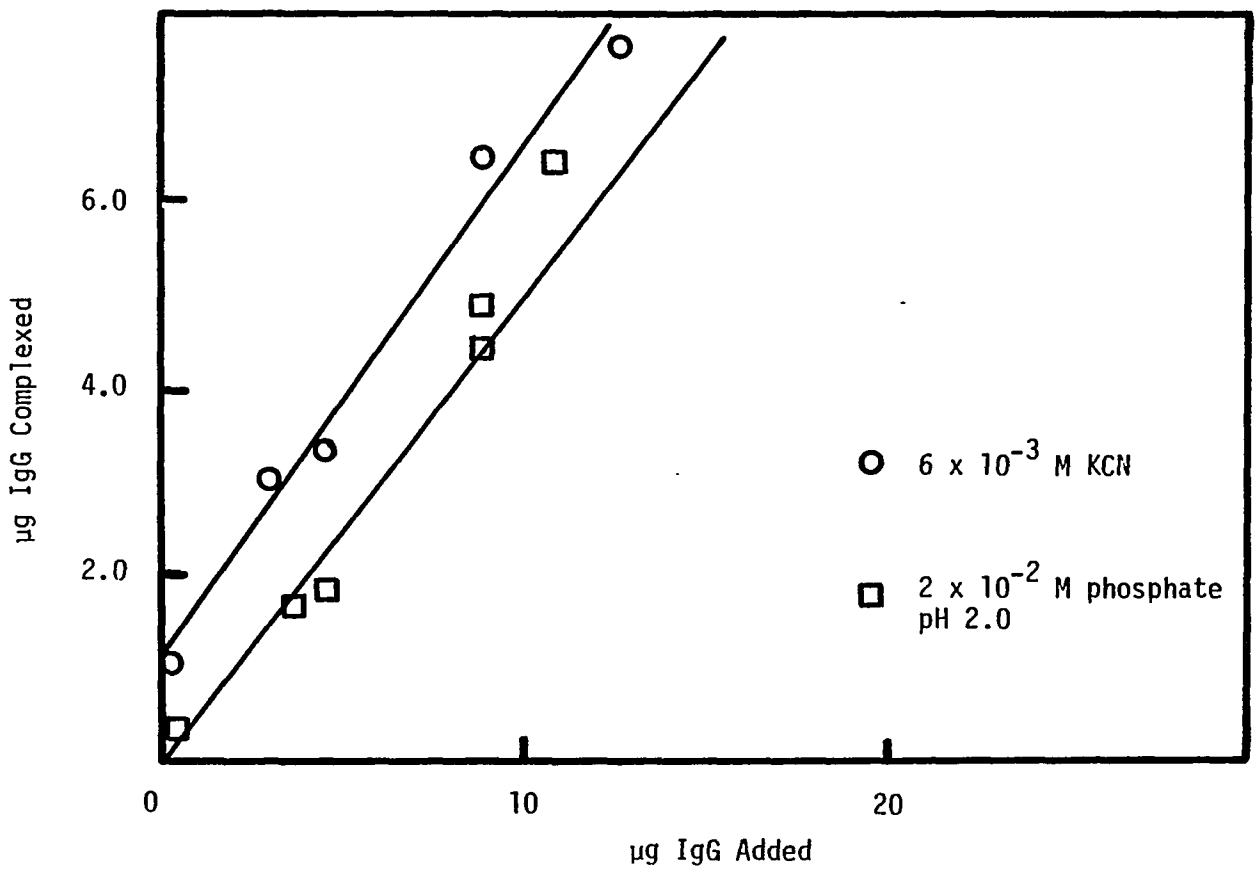


Figure 32. Comparison of elution properties of 2×10^{-2} M pH 2 PBS and 6×10^{-3} M KCN in dissociating antigen-antibody complex in HPLC column method.

Table 10. Concentration of IgG as measured by HPLC with CPG column containing immobilized antihuman IgG on CPG-COOH. -- All determinations carried out at room temperature. Column pressure was 816 psi and flow rate for pH 8.0 and 2.0 buffers was 0.80 ml/min.

Sample	Concentration IgG (μg/ml)		Peak Height (free)	Peak Height (bound)	Ratio B/F
1	8.3				
2			19.5	6	0.31
3			17	6	0.31
4			19	7	0.36
		av	18.5	6.3	0.28
5	4.15		8.5	4	0.50
6			9.5	3.5	0.37
		av	9.0	3.75	0.43
7	2.08		4.2	2.5	0.595
8	1.08		2.75	2.0	0.727

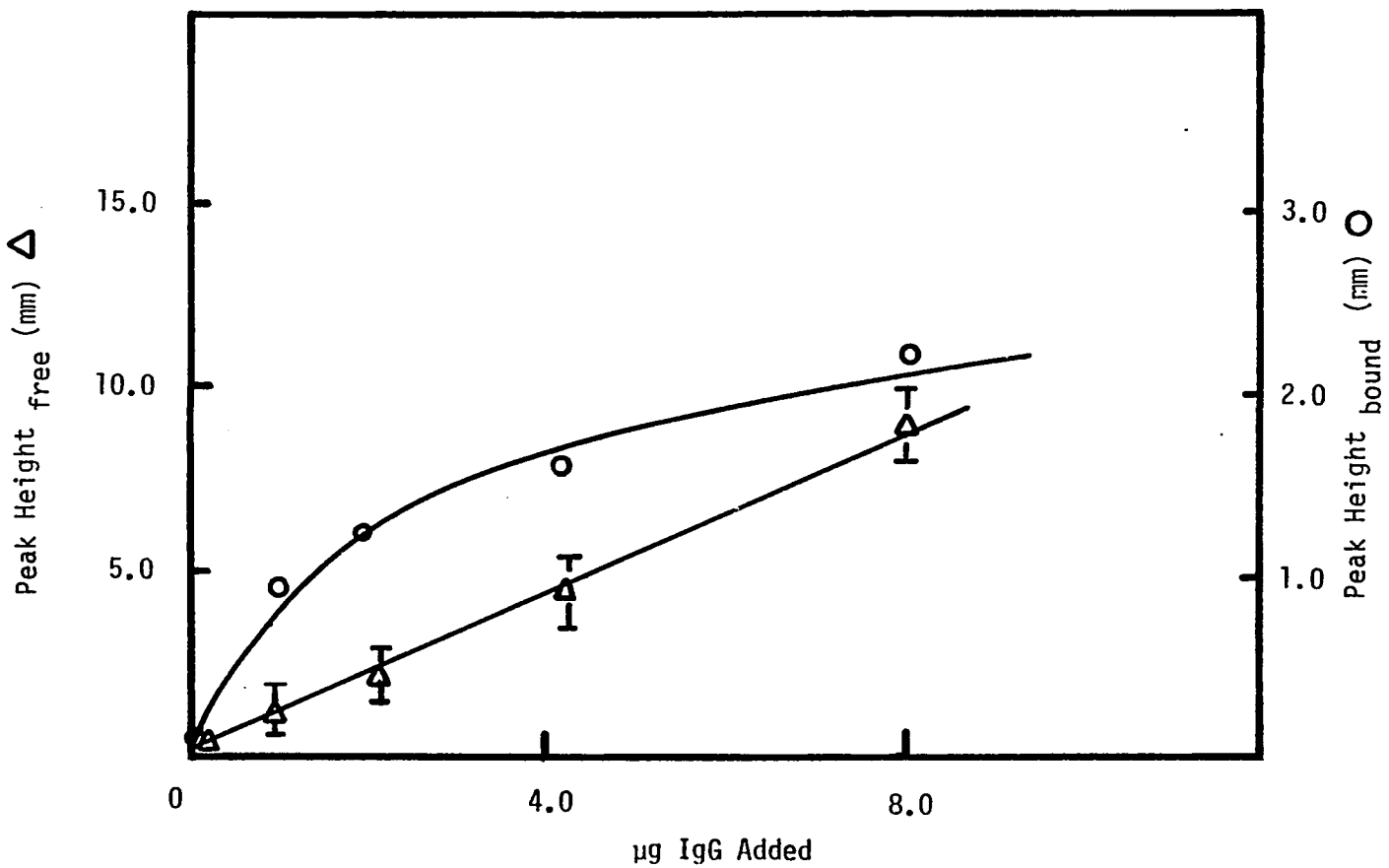


Figure 33. Relationship between peak heights of the bound and free fractions of IgG and the initial IgG concentration.

Figure 33 indicate a deviation from the mean value of the initial antigen concentration occurs at the higher concentration levels. This is probably due to the fact that a large sample concentration is passing by the immobilized antibody binding sites rapidly. Because this binding of antigen to antibody in the column is not in true equilibrium, binding of high concentrations of antigen is not complete at these short reaction times as seen in Figure 30. The linear relationship between amount of antigen complexed and time exposed to immobilized antibody is abbreviated at elevated concentrations of antigen.

The immobilized antihuman IgG was shown by micro Ouchterlony gel diffusion not to react with IgM, IgA, or BSA to a detectable extent, suggesting good specificity. These proteins were added to the sample in place of IgG and passed through the column to check for cross-reactivity and non-specific binding. The IgM and IgA did not bind to any binding sites in the column and thus appear not to compete in the reaction of IgG with antihuman IgG immobilized on the CPG matrix. Trace amounts of BSA combined with immobilized antibody or the CPG matrix in the column. The ratio of bound BSA to unbound BSA was 0.005 as determined spectrophotometrically. This indicated that non-specific binding was present but not to the degree observed in the indirect single-layer fluorometric assay. A possible explanation for this difference in non-specific adsorption is due to the types of bed supports employed. In the indirect single-layer fluorometric assay, a thicker silica (fine) sintered glass frit is used, while in the HPLC column technique a thin stainless steel 2 micron frit is used. When the CPG matrix was silanized and then

inactivated by reaction with trimethylchlorosilane (TMCS), the activity of the CPG particles dropped significantly. This is reflected in Figure 34, where only the peak height of the free unbound antigen is plotted because with the TMCS treated particles the peak height of the bound antigen was less than 1% of full scale and difficult to distinguish between noise and drift of the baseline. This treatment of the CPG particles with TMCS did reduce the B/F (non-specific adsorption ratio) for BSA added to the column to less than 0.001.

Comparison of HPLC Column Technique with Other Methods for Determination of IgG

Comparison of the RID and the HPLC method using immobilized antibody for IgG determinations gave good agreement for IgG reference serum (Table 11). The HPLC column method gave lower values than the RID assay which is consistent with results from other solid phase/RID comparison studies for determining IgG in serum (Cawley et al. 1974). The explanation offered for this discrepancy was that the IgG in the serum was being catabolized into F_{ab} and F_c fragments. This catabolism is concentration-dependent so that at higher concentrations of IgG its turnover is more rapid.

Gel diffusion (RID) is concentration-dependent and assumes all molecules are the same size and have the same diffusion coefficient. Fast diffusing biologically active fragments such as F_{ab} could cause an expanded zone of precipitation. This would cause overestimation of disc size, leading to erroneously high IgG results (Cawley et al. 1974). Breakdown of IgG in pathologic serum is a more common occurrence than

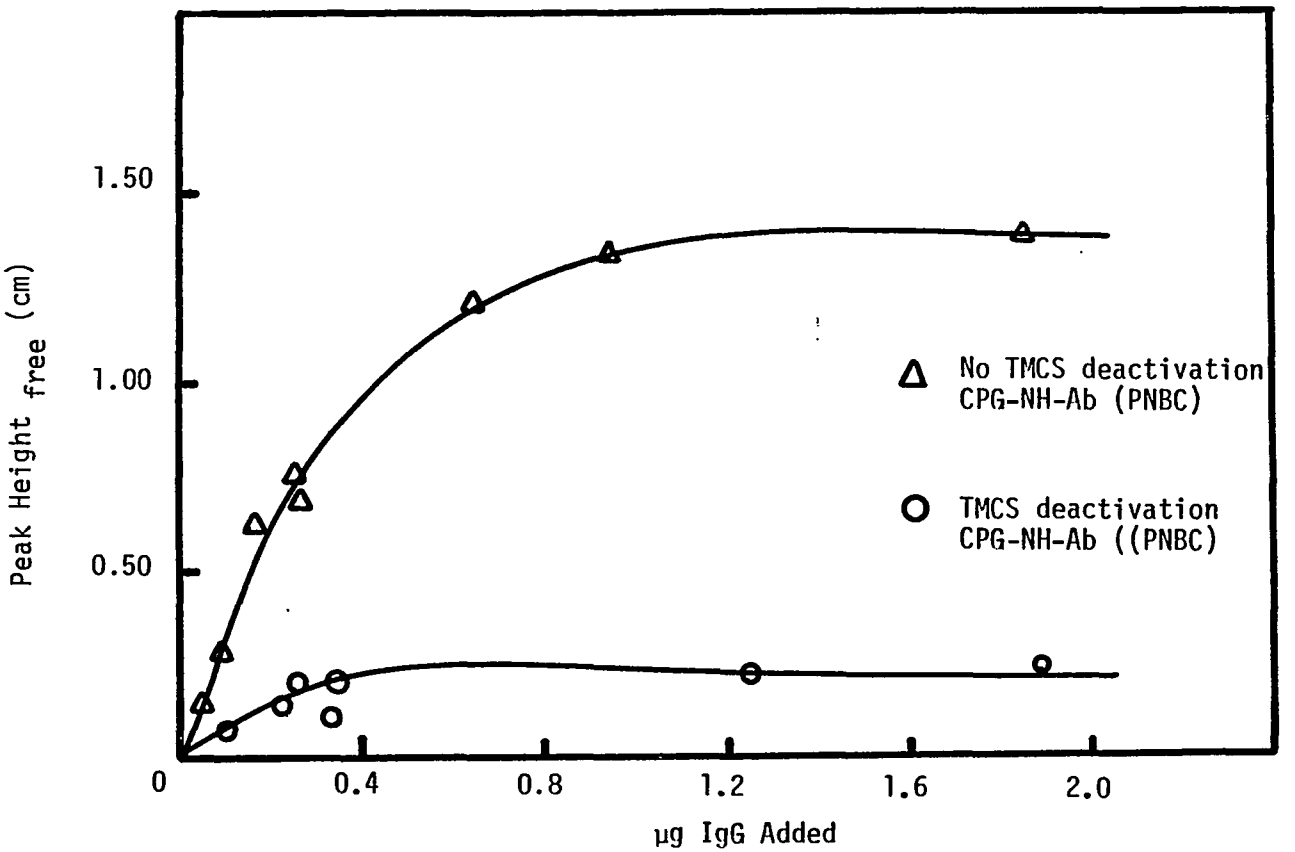


Figure 34. Comparison of biological activity of CPG-SW5 particles deactivated with those that did not undergo deactivation with TMCS in HPLC column assay of IgG.

Table 11. Comparison of concentration of IgG by HPLC with immobilized antibody packing and gel diffusion (RID). -- All determinations were carried out in duplicate and the average reported in mg/ml. RID measurements were made 48 hours after introduction of sample.

Gel Diffusion (RID)	HPLC with Immobilized Antibody
0.014	0.011
3.880	3.710
14.80	13.50

normally thought (Waller and Duma 1972). The production of $(F_{ab})_2$ fragments of severely burned patients is elevated (Goldberg and Whitehouse 1970), indicating perhaps that fragmentation of IgG is not only a natural function, but that it is accelerated under certain conditions.

The sensitivity of the HPLC method is compared to other methodologies in Table 12. Its advantages are speed and ease of the whole analysis procedure; no sample preparation, ease of automation, and re-useability of the matrix. A similar version of the HPLC method has been marketed by Becton Dickinson. The immobilized antibodies are covalently attached to micro-particles and packed in a chamber in which the sample is injected. The commercial HPLC method uses a competitive binding approach with a radio-labeled antigen. The manufacturer claims to have made over 3,000 assays on the same column packing material without any significant loss in biological activity of the immobilized antibody (Auto-Assay 1977). Total time for an assay in this system is increased by a factor of 3 or 4 over the HPLC method which uses elevated pressures to drive the test solution (the commercial antibody chamber develops pressures on the order of 70-100 pis). It also requires a labeled antigen with similar binding properties as the test antigen.

Further improvements in the HPLC method's sensitivity can be accomplished by using a fluorescent dye. This dye would react with the eluted antigen either free or bound after its exit from the column. In a flow-through system with this type of assay, sensitivities in the range of 1-10 ng IgG/ml should be possible. This would also decrease

Table 12. Comparison of methods for measuring serum immunoglobulin. -- Procedures are all manual, except for Sturgeon, Hill, and Kwak (1969), which is automated. RT = room temperature.

Reference	IgG Sensitivity (µg/ml)	Incubation Time	Type of Procedure
Ritchie (1967)	0.5	1 h RT	Turbidimetric
Sturgeon et al. (1969)	0.15	0.5 h 37°C	Hemolysis inhibition
Mann, Granger, and Fahey (1969)	0.02	3 h RT	Competitive binding, solid phase (bromacetyl cellulose-bound Ab)
Bombardiere and Christian (1969)	0.1	1 h 37°C	Competitive binding, solid phase (polyamino polystyrene bound Ab)
Smith (1977)	0.5	0.1 h RT	Indirect U.V. monitoring eluent from HPLC column packed with CPG containing immobilized Ab

the total assay time since it would no longer be necessary to wait for establishment of the pH 2 or pH 8 baselines. As seen in the re-traced chromatogram, there is a need for improvement in the shape of the bound antigen peak. Presently, it is difficult to quantitate the amount of IgG in the assay solely from the peak height of the bound fraction. Attempts to improve the peak shape of the bound fraction were carried out by altering the flow rates. As the flow rate increased from 0.20 to 0.80 ml/min, the peak broadening was reduced by a limited amount. This peak shape problem may be improved if the column capacity is increased, giving more binding sites/column. As suggested by the indirect single-layer fluorometric monitoring of the binding of labeled IgG to these same CPG particles used in the HPLC column method (Figure 30), the limited number of binding sites on the particles were saturated. Increasing the number of binding sites in the column should also improve the dynamic range of the HPLC column method. Prior to implementing these changes, the column optimization ratio (column diameter/particle diameter) will be improved. The present ratio of 1.05×10^2 is below the ratio of HPLC columns with similar sized packing material. Changing to 1/16 inch diameter column will improve this ratio by a factor of 5.

CONCLUSIONS

The many experiments concerning the attachment of different proteins such as enzymes and immunogens to silica surfaces has revealed a wealth of information on surface characteristics. These proteins via their biological activity have acted as sounding boards in relaying information on alterations of the CPG surface. When the surface is too hydrophobic, void of reactive groups for covalent attachement of the protein, or too small in area, the lack of biological activity reflects this situation. Similarly, when there is overloading of the surface with covalently attached or extensively crosslinked protein, the diminished specific biological activity of the immobilized protein suggests steric problems (Figure 35). The silanization efficiency of between 10 and 30% with available silanol groups yields between 0.8 and 2.4 free amine groups/nm^2 on CPG particles. Thus, during the protein coupling reaction 200 free amine sites are sterically blocked for every covalently coupled protein molecule. The assumptions for maximum monolayer coverage given in Table 4 and Figure 35, coupled with the data from Table 3, indicates only 25% of available surface area associated with the CPG particles is covered with immobilized antibody. This would represent an increase of only 0.08 microns in particle diameter which is considerably different from the value observed in the particle sizing experiment (Table 7). This difference may be attributed to formation of crosslinked antibody "chains" which become covalently attached to CPG particles. The

Figure 35. Qualitative estimate of the surface coverage on CPG particle by antibody.

By knowing size, shape, and area of antibody molecule after attachment to the CPG particles, it is possible to calculate the theoretical maximum number of antibody molecules per CPG particle.

Antihuman IgG dimensions (Nisonoff et al. 1975) = $140 \times 85 \times 50$ Å

Diameter of the CPG particle = 30 μ = 3.0×10^5 Å

CPG surface area = $(4)(3.14)(1.5 \times 10^5 \text{ Å})^2 = 2.8 \times 10^{11}$ Å^2

The area of space that an antibody molecule would occupy in the vertical position would be:

Area = $(8.5 \times 10^1 \text{ Å})(5.0 \times 10^1 \text{ Å})$

$= 4.2 \times 10^3$ Å^2

The maximum number of antibody molecules/CPG particle

$$= \frac{2.8 \times 10^{11} \text{ Å}^2}{4.2 \times 10^3 \text{ Å}^2} = 6.7 \times 10^7 \text{ molecules/CPG particle}$$

Assuming 10^8 particles/g

$= 6.7 \times 10^{15}$ molecules/g of CPG, or

= 11.1 n moles/g of CPG, or

= 3.91 n moles/g CPG

in the horizontal position, the number of μg antibody/g of CPG

$= (1.6 \times 10^5 \text{ g/mole})(1.1 \times 10^{-8} \text{ moles})$

= 1.76 mg antibody/g CPG, or

$= 4 \times 10^{-12}$ moles/cm^2

The above value of 11.1 n moles antibody/g CPG for a monolayer coverage of the surface of the CPG particles is 4 times larger than the maximum number of moles covalently linked (Table 3). However, the sizing experiments indicate the formation of 20 monolayers of antibody after covalent attachment. This difference may be explained by the fact that the antibody does not distribute itself evenly over the CPG surface, but instead forms crosslinked "chains" of antibody molecules which eventually become covalently attached to the CPG particles.

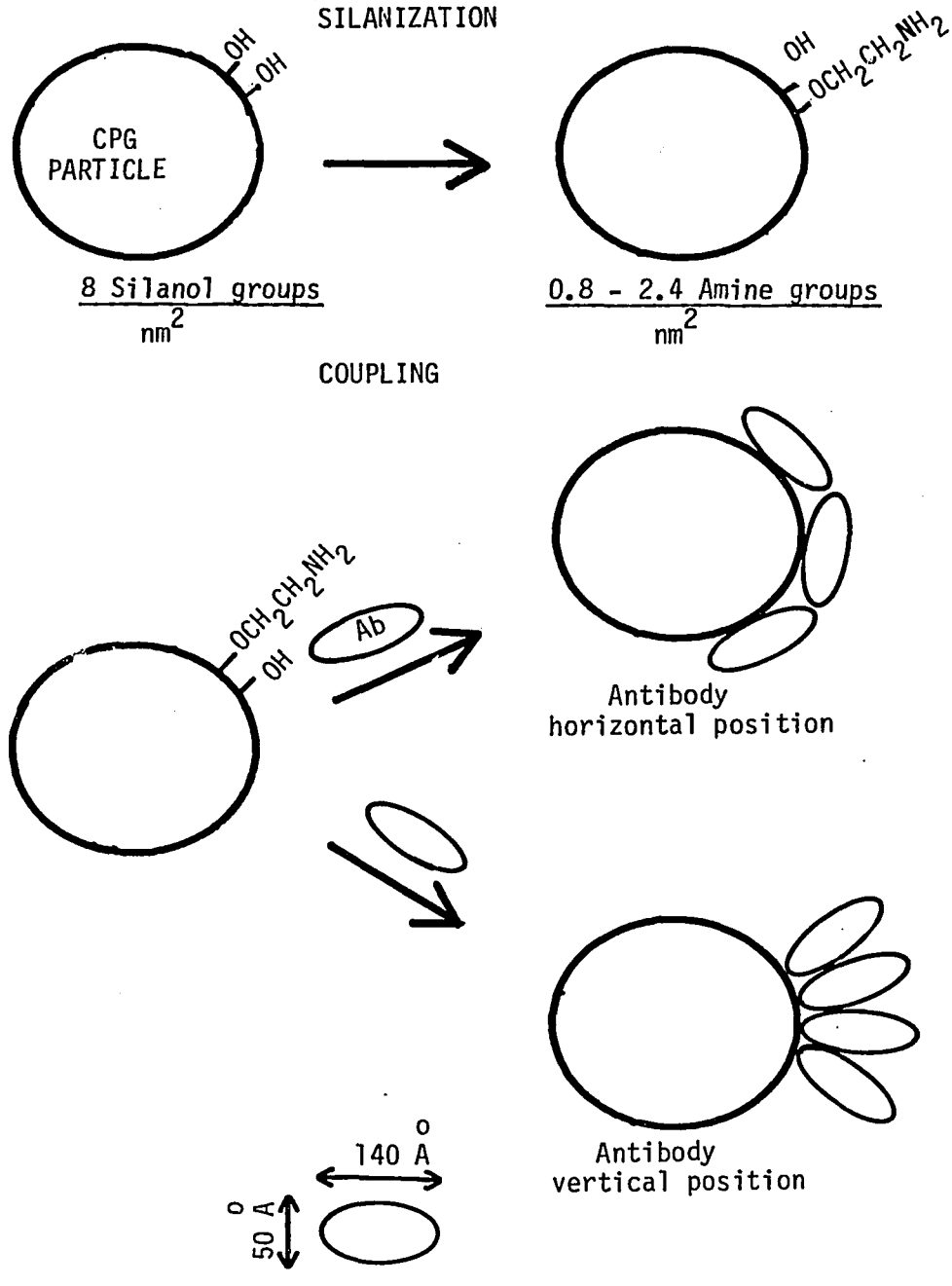


Figure 35. Qualitative estimate of the surface coverage on CPG particle by antibody.

more subtle changes in selectivity or binding rates are indicative of the small structural perturbations of the surface which occur as a result of the various chemical manipulations in the immobilization process, i.e., changes in the electrostatic properties of the surface after silanization. Acid base micro-titrations have shown the physicochemical alterations of the silica surface to follow a similar pattern suggested by experiments in other areas of chemistry such as chromatography.

The results gathered indicate that the improvement in specific biological activity of immobilized immunogens lies not in attaching more and larger amounts of material to the surface, but rather in devising a selective mechanism for orienting and tethering the molecules to these surfaces. The ultimate result of accomplishing this task will be an immobilized protein which is truly exhibiting maximum sensitivity and selectivity.

Once these unique properties of sensitivity and selectivity of the native protein have been captured by the process of immobilization, implementation of the re-usable CPG particles into an automated analytical detection scheme can be carried out. Previous work has demonstrated the sensitivity of the immobilized antibodies in detecting 10^{-11}-10^{-12} moles of antigen in a highly complex matrix. Further improvements in sensitivity can be made. Binding the antigen to the antibody prior to the coupling step will protect the binding site and improve biological activity. The complex is dissociated after coupling and the amount of antigen and antibody released is determined. This gives information on the amount of antibody attached covalently in active form. The

antigen-antibody ratio of released protein will be indicative of the orientation process occurring during coupling. This ratio will indicate if one protein is favored over the other in the coupling process. The ratio in this case would be significantly different from the ratio of one obtained for the random process. Covalent attachment of a homogeneous preparation of F_{ab} fragments could conceivably result in more biological activity. Although there remain many improvements to be made in the chemical aspects of the coupling process (less cross-polymerization) which will increase the sensitivity of a particular assay using immobilized proteins, presently it is the instrumental considerations that are the limiting factor. The HPLC column technique can detect 0.25 µg/ml, while solid phase RIA using the same particles are able to detect 0.001 µg/ml, indicating the biological sensitivity of the immobilized protein exceeds the state-of-the-art in most detection systems by two orders of magnitude.

APPENDIX

PREPARATION OF RID AND ELECTROPHORETIC GEL

RID plates

0.20 g agarose

10 ml 0.02 M phosphate buffer, pH 8.0

12-96 µl of antibody, 0.125-0.00125 mg/ml

1. Weigh out agarose and add to 10 ml of buffer that is boiling in 500 ml beaker with magnetic stirrer. Do not mix at a fast speed as this tends to introduce air bubbles.
2. Monitor temperature of agarose solution until it is around 45°C, add antibody and mix well. The concentration of antibody in gel depends upon concentration range of antigen to be assayed. At high antiserum concentrations, all the ring precipitates are relatively small and intense. With lower antiserum concentrations, the precipitates become less distinct and their proportions (1:5, 1:10, 1:20, 1:40, 1:80) and these dilutions mixed in 1:1 or 1:2 proportion with agarose solution. A representative test sample was serially siluted and applied to the agarose-antiserum mixture poured on a microscope slide and the precipitate characteristics evaluated at 48 hours. A desirable antiserum concentration was one which used the least amount of specific antiserum and still gave ring precipitates which were

easily readable in the range of protein concentration to be tested.

3. Pour this mixture immediately as it tends to solidify quickly at this temperature. Make sure the mold that the agarose is poured into is dry and clean. At this point the RID plate may be stored by wrapping in Saran wrap tightly to keep the plate from drying out.
4. Before using the RID plate, allow it to warm to room temperature and punch a well approximately 2-2.5 mm in diameter into it for sample solution. This can be done by placing a plastic template across the top of the agarose gell and through guide holes in the template excise agarose plugs 2-2.5 mm by connecting cutter to vacuum line. A convenient cutter can be made from a disposable pipette tip with the lip edge sharpened by trimming with a razor blade. Other similar mechanisms have been described in the literature (Fahey and McKelvey 1965). These wells should be large enough to hold between 5-15 microliters of sample.

Electrophoresis Gels

Acrylamide, 48% solution

Tris HCl, pH 8.4, 1.5 M in 0.48% tetramethylethylenediamine

Ammonium persulfate, 2%

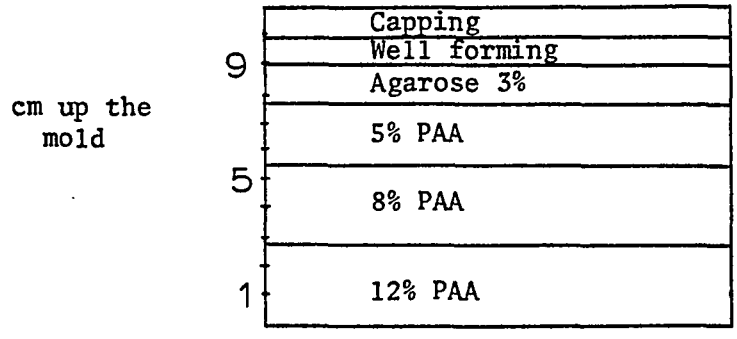
Gel Layer	12%	8%	5%	4%	Well Forming and Capping
Acrylamide, 48%	4 ml	2.7 ml	1.5 ml	0.4 ml	0.66 ml
Water	-	6.7	1.4	0.8	-
Buffer	4	4	2.4	1.2	2
Persulfate	8	2.6	2.4	2.4	5

Agarose

0.3 g agarose in 10 ml pH 8.4 Tris buffer

Mix together the acrylamide 48%, water, and buffer (mix well). Then add the persulfate to the solution, and mix for 30 seconds, pour or pipette into the mold the polyacrylamide (PAA) solution.

Gel format



This procedure was adapted from Ortec (1973). The agarose layer was added to allow initial migration of large molecular weight species into separating gel layer (5%). At the same time, the agarose lent stability to the gel so that after completion of the experiment, the gel could be removed from the gel mold without physically being destroyed. The

inclusion of an 8% separating gel layer allowed better resolution between proteins of 50,000 and 100,000 M.W., i.e., F_c fragment and intact IgG molecule.

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