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**Preparation and characterization of immunological reagents for
analytical applications**

Nielsen, Randall Gunnar, Ph.D.

The University of Arizona, 1988

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PREPARATION AND CHARACTERIZATION OF IMMUNOLOGICAL
REAGENTS FOR ANALYTICAL APPLICATIONS

by

Randall Gunnar Nielsen

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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SIGNED: Randall A. Nielsen

"And may you know WHO really gets the credit
deserves the credit given to HIM...God!"

Robert A. Kinsey, M.D.

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ABSTRACT

Immunochemical reagents were characterized under carefully controlled laboratory conditions using conventional high performance liquid chromatography instrumentation. The stationary phase was prepared by attaching antigen molecules to an insoluble support through a covalent linkage. Experiments were carried out by introducing antibody molecules into the mobile phase and monitoring their interaction with the stationary phase. Monoclonal antibodies were employed because of their more homogeneous properties compared to polyclonal antisera.

Radioisotopes were employed to study low level adsorption on the stationary phase. Recovery experiments were carried out in which it was possible to account for all of the material introduced into the mobile phase. Antibodies were purified over a preparative scale antigen affinity column following labeling to insure high immunoreactivity. Studied under normally dissociating conditions, irreversible adsorption of picomole amounts of protein on the antigen stationary phase was greater than on other ligand modified stationary phases. This accumulation decreased with repeated use of the affinity column. The present study provides a framework for evaluation of other immunoaffinity systems and demonstrates that reproducible

recovery of immunologically active material in high yield is possible.

Monoclonal antibodies labeled with fluorescein were different from unlabeled molecules in binding and physical characteristics. Computer simulations were used to describe binding behavior. Although fluorescein labels improve detection sensitivity over native protein absorbance, their use in this case decreased binding affinity significantly. Heterogeneity of affinity purified fluorescein labeled and unlabeled monoclonal antibodies was examined with two dimensional gel electrophoresis. In addition to increased charge heterogeneity in the labeled antibody fragments, both light and heavy chains possessed more negative character. These results agree with each other. Fluorescein contains a carboxylic acid group, and modification of antibody light chains may interfere with binding affinity. The number and location of labels covalently attached to antibodies must be carefully controlled to obtain maximum detection sensitivity and preserve immunoreactivity.

CHAPTER 1

INTRODUCTION

Various classes of proteins differ greatly from one another in size and charge characteristics. Traditionally size exclusion and ion exchange chromatographies have been successfully applied to separations of proteins from these different classes. The growing development and production of proteins for both medical and industrial applications presents new challenges for the purification and analysis of these macromolecules (Borman, 1987 and Warren, 1984). This chapter will focus on the general problem of protein separations citing numerous literature examples.

The ultimate chromatographic method would be one which could resolve native and denatured proteins. The term denatured refers to a broad range of altered protein forms (slight to extreme), some of which may reversibly return to the native form. The relationship between protein structure and function is often poorly understood at best. A protein may lose some of its tertiary or quaternary structure due to unfolding and this may affect the interaction of the protein with a chromatographic stationary phase, another biological molecule, or receptor. For example, the loss of a prosthetic group at the active site

of an enzyme may result in a biologically inactive apoenzyme which behaves identically to the native protein in chromatographic separations. The assessment of denaturation in a hormone represents a greater challenge. The evaluation of changes in protein structure and function as a result of a chromatographic separation is a complex task.

Affinity chromatography, one of many forms of chromatography applied to purify proteins, isolates molecules on the basis of some form of "biological recognition". This so-called "biological recognition" represents the optimum combination of weak chemical interactions(ionic and Van der Waals or hydrophobic forces). The use of immobilized antibodies to purify antigens(or vice versa) is a unique form of affinity chromatography known as immunoaffinity chromatography. The importance of this method has certainly progressed as antibodies have been developed which recognize and bind to specific portions of drug molecules, hormones, enzymes, and other proteins. Despite certain obvious advantages of affinity chromatography, there are also important disadvantages in comparison to other methods of purification. In order to provide a stronger and more objective foundation for the present work, current methods of protein purification will be discussed.

Changes in tertiary or quaternary structure which affect the size of the protein may be observed with size

exclusion chromatography. In the interest of separations with short analysis times, high performance size exclusion techniques have become popular. The development of rigid stationary phase supports with particle sizes and pore diameters of 10-40 microns and 100-500 angstroms, respectively, has made this possible. These supports are represented by two major groups, 1) hydrophilic polymers and 2) silica derivatized with glyceropropyl groups. Ideal size exclusion chromatography depends only on molecular protein size, and stationary phase particle and pore diameters. However, charges on the support surface will interact with mobile phase analytes by ionic attraction or repulsion. Protein surface domains lacking charged groups may interact by hydrophobic attraction. Due to these nonideal effects, detergents such as sodium dodecylsulfate, desoxycholate, or Triton X-100 are often included in the mobile phase(Henschen et al., 1985).

Denatured proteins often have new charged and nonpolar groups exposed on their exterior which makes ion exchange methods attractive. HPLC ion exchange has been very similar to its low pressure counterpart with the exception of stationary phase supports(Henschen et al., 1985). Silica- and methacrylate-based supports provide the necessary mechanical strength. Silica supports have been coated with polyethyleneimine(pellicular) or glyceropropyl groups. The polyethyleneimine derivatized silica behaves as

a weak anion-exchanger, similar to diethylaminoethyl(DEAE)-cellulose. The methacrylate- and glycochase-based supports have been modified with DEAE, methyldiethylaminoethyl-(QAE), carboxymethyl-(CM) or sulfonyl propyl-(SP) side chains.

The purification of apolipoproteins has usually been accomplished through some combination of size exclusion, ion exchange, chromatofocusing or isoelectric focusing chromatographies. Anion exchange fast protein liquid chromatography has recently been applied to the direct isolation of native apolipoproteins by Mezdour et al.(1987). The separated apolipoproteins compared favorably using sodium dodecylsulfate polyacrylamide gel electrophoresis(SDS PAGE) to apolipoproteins isolated by a combination of alternate methods. Immunodiffusion experiments showed that the apolipoproteins retained immunological reactivity.

The rapid isolation of monoclonal immunoglobulins G(IgG), IgG1, IgG2a, and IgG2b from ascites fluids in high purity and functional activity using an anion exchange column has been described by Clezardin et al.(1985). Antibody fragments from papain-treated monoclonal antibody preparations were also isolated using this method. Resolved protein fractions were identified using SDS PAGE and solid phase radioimmunoassay with ¹²⁵I-labeled sheep anti-mouse antibodies directed against total immunoglobulins. Rapid assessment of purity of the isolated monoclonal antibodies and their fragments was performed by size exclusion HPLC.

Rather than using sheep antiserum to total immunoglobulins, it would have been interesting to know if the isolated antibodies still reacted with their antigens. The solid phase radioimmunoassay was not applied to the isolated antibody fragments. Despite claims of high purity, the silver stained SDS PAGE results showed the IgG1 and IgG2b fractions to be significantly contaminated with transferrin and a 20,000 dalton impurity, respectively.

Electrophoresis has been applied to the separation of proteins on the basis of size and small differences in charge. Improvements in isoelectric focusing have made it possible to transfer focused protein zones out of the capillary bed without losses in resolution (Hjerten, Liao, and Yao, 1987). High resolution has been observed when electrophoresis combines separations by size and charge. O'Farrell (1975) resolved more than 1000 components from an Escherichia coli sample using two dimensional gel electrophoresis. Unfortunately, these separations are more suitable for qualitative than preparative or analytical work due to the difficulty of retrieving or quantitating the resolved samples.

Preparative chromatofocusing was recently applied to the separation of biologically active molecules from human and porcine follicular fluid (Campeau, Marrs, and Dizerega, 1983). Chromatofocusing has been described as carrying out isoelectric focusing on an ion exchange column. Biological

activity, measured by percent inhibition of ovarian weight, ranged from 0 to 90 percent in various fractions. The more laborious purification of apolipoproteins by chromatofocusing was carried out by McLeod et al.(1986). Reproducible elution profiles required the inclusion of dithiothreitol(sulfhydryl reducing agent) following delipidation. Polyvalent buffers were removed from apoproteins on short columns of hydroxyapatite. Biological activity was confirmed by the ability of apoproteins to activate lecithin:cholesterol acyltransferase, and functional activity was demonstrated by interaction with low density lipoprotein receptors. SDS PAGE showed these apoproteins were identical to apoproteins purified by more tedious techniques.

The power of chromatofocusing was demonstrated by Wingfield et al.(1987) in isolating and characterizing recombinant-derived proteins. Two forms of interleukin-1B differing only by the presence of initiating methionine(N-terminal alanine vs N-terminal methionine) were resolved by isoelectric focusing into peaks 1(75 percent) and 2(25 percent). These results were in good agreement with those obtained by gas-phase Edman degradation of the mixture with 70 percent polypeptides commencing in alanine and 30 percent in methionine. The difference in isoelectric points(pI) of peaks 1(6.7) and 2(6.4) allowed their separation by chromatofocusing but not by SDS PAGE.

The limit of chromatofocusing was approached by Wingfield in the next separation. Two major fractions of recombinant-derived bovine growth hormone (BGH) were determined to have pIs of 8.0 and 8.2, respectively, by isoelectric focusing. This difference was too small to be the result of deamidation, and it did not permit complete resolution by chromatofocusing under the conditions used. However, the difference in molecular size was sufficient to observe two bands on SDS PAGE. Fractions resolved by isoelectric focusing and chromatofocusing were shown by Edman degradation to have 3 different N-termini but the same C-terminal sequence. Based on these observations for recombinant derived proteins, Wingfield recommended chromatofocusing as a general separation technique for resolving biosynthetic polypeptides.

Reversed-phase (RP) chromatography has become an option for the separation of large molecules (molecular weight greater than 3000) with the use of larger pore size supports. Reversed-phase chromatography of proteins has been an extension of peptide separations. Unfortunately, the retention characteristics of large molecules are more difficult to predict due to errors in estimating their effective surface hydrophobicity and charge. Since short columns separate proteins as efficiently as larger columns, it has been reasoned that proteins interact with the support by multisite binding and are fractionated by

adsorption-desorption on the matrix surface rather than partitioning between the stationary and mobile phases(Henschen et al., 1985). This explains why gradient elution is superior to isocratic elution for protein HPLC. The most common organic modifiers for protein HPLC are acetonitrile, 1-propanol, 2-propanol, methanol, or various combinations of these. Volatile organic acids or volatile buffers are the most commonly used in reversed-phase HPLC of proteins. Dilute trifluoroacetic acid acts through ion pairing to change the hydrophobicity of the protein/mobile phase interaction thereby changing its retention behavior. Formic acid, pyridine-formic acid, and pyridine-acetic acid are examples of volatile mobile phase components. Nonvolatile salts such as phosphate, sometimes with perchlorate or salts such as tris acetate and sodium acetate are also employed.

As a result of the popularity of RP HPLC methods for protein separations, much research has been directed towards understanding the interaction of proteins with a silica bonded hydrocarbon. Based on the kinetics of denaturation of papain, soybean trypsin inhibitor, and lysozyme on n-butyl-bonded silica gel surfaces, two steps were proposed for the unfolding process(Benedek, Dong, and Karger, 1984). The first step occurred rapidly upon contact of the protein with the stationary phase and the second step had a half-life of about 15 minutes. The first step was present for

incubation times following injection from 0 to 60 minutes before the start of the gradient elution and depended only on gradient conditions. The amount of denaturation in the slow unfolding step varied directly with the incubation time on the bonded phase surface. As long as the refolding kinetics were slow relative to the chromatographic elution, two peaks, native and denatured, would be observed. The type and concentration of organic modifier in the mobile phase can play a significant role in the extent of unfolding in the rapid denaturation step. 1-Propanol caused the bonded phase surface to behave less hydrophobically relative to dilute H_3PO_4 as the mobile phase. This was explained by the adsorption of the organic modifier on the stationary phase making it more polar. With respect to the slow unfolding process, results showed that shorter contact times between the protein and the bonded phase surface favored the elution of more protein in the native state.

Spectroscopic studies were performed on selected proteins containing alpha helical and beta structures under solution conditions used for RP HPLC to support structural studies of protein chromatographic behavior (Sadler et al., 1984). Protein denaturation as a result of exposure to various mobile phase compositions independent of the stationary phase was investigated. The selected proteins were studied under the following solution conditions 1-propanol (0-40 percent) in pH 4 and 7 mobile phases without

the stationary phase present. Circular dichroism(CD) spectral changes induced with propanol were more than 90 percent reversible at pH 4 and more than 80 percent reversible at pH 7. Fluorescence emission spectra of the proteins provided additional information about the tertiary structure in terms of changes in the environment of aromatic amino acid side chains. Removal of propanol produced at least a 90 percent return to the unperturbed spectra. The stability of proteins in the presence of propanol was also examined using the nonspectroscopic technique of differential scanning calorimetry. A linear decrease in the stability of the proteins with increasing percent propanol was observed in all cases. Assuming that these solvent conditions represented the environment inside the column, it was concluded that 1) RP HPLC did not bring about true denaturation but slight alteration to a different conformational state and 2) RP HPLC may be used to purify some biologically active proteins. Similar research(Lau, Taneja, and Hodges, 1984) using size exclusion and CD to examine 5 synthetic peptides(900-8000 molecular weight) before and after RP HPLC concluded that while tertiary and quaternary protein structure were disrupted, the alpha helix(supported by hydrogen bonding) was stabilized by increased nonpolarity of the mobile phase.

The extrapolation of the effects of the RP column environment on protein structure and their behavior in the

RP column environment was improved upon by probing directly the conformational changes of proteins bound to octyl silica in the presence of increasing concentrations of 1-propanol (Katzenstein et al., 1986). Photoacoustic(PAS), diffuse reflectance deconvolution Fourier transform infrared(FTIR), and front face fluorescence spectroscopic techniques were used to confirm the existence of surface-associated protein conformational changes induced by 1-propanol. Very good correlation was observed between the percent 1-propanol in the mobile phase required to elute various proteins and the percent 1-propanol at which transitions were observed in fluorescence or PAS experiments suggesting these transitions were related to surface adsorption. The surface and solution fluorescence data support partial alteration of protein conformation upon binding, probably due to hydrocarbon induced conformational change in the protein to permit significant apolar interaction. While CD results mentioned above reported altered solution conformations primarily of a helical nature, FTIR spectra of bound protein did not show any evidence of increased helical structure. These observations suggest at least a two step process for conformational alteration. Step 1 could be referred to as binding and step 2 could be desorption followed by coiling.

The effect of RP HPLC on protein structure and activity was evaluated using ion exchange HPLC and immunodiffusion, respectively, by Luiken, Van der Zee, and

Welling(1984). Bovine pancreatic ribonuclease(RNase), bovine serum albumin(BSA), horseradish peroxidase(HRP), and ovalbumin eluted over a wide range of organic solvent concentrations. Structural changes following RP HPLC affected significantly the elution of BSA and ovalbumin but only slightly that of HRP. Double immunodiffusion showed no changes in reactivity for RNase and BSA, while HRP reacted only weakly and ovalbumin not at all. The enzymatic activity of HRP was decreased 95-99 percent and RNase was not affected. In contrast to their conclusion, the data reported suggest that the separation of biologically active proteins by RP HPLC must be evaluated on a case by case basis.

Virus proteins isolated by size exclusion, ion exchange, and RP HPLC were identified using SDS PAGE and evaluated with a mouse antiserum based enzyme linked immunosorbent assay(ELISA)(Welling et al., 1984). Detergent extracted Sendai virus envelope proteins were chromatographed using different mobile phase modifiers for the various methods. Since the envelope protein was not tested with ELISA before extraction it is not clear that the antibody in the antiserum recognized the native protein. Size exclusion HPLC was performed on boiled samples and eluted with SDS in phosphate buffer. A gradient of sodium chloride in Tris-HCl buffer containing Triton X-100 was used to elute samples from an anion exchange HPLC column.

Before RP HPLC, detergent was removed with Amberlite XAD-2 and the proteins were reduced with dithiothreitol. The RP column was eluted with a gradient of acetonitrile(25-75 percent) in aqueous trifluoroacetic acid(0.05 percent). It may be a surprise that proteins isolated by any of these techniques showed any response in the ELISA. Exposure to detergents, organic solvents, or a temperature of 100°C would be expected to denature most proteins. Protein fractions isolated by size exclusion and ion exchange methods however, were immunologically active. The wide variety of mobile phase modifiers, not to mention pretreatment before RP HPLC likely support the lack of immunological reactivity.

The combination of ion exchange and reversed-phase based separation techniques has resulted in the development of hydrophobic interaction chromatography. In hydrophobic interaction chromatography the sample is introduced into a high ionic strength mobile phase with which an alkyl bonded stationary phase has been equilibrated. Elution is carried out by applying a decreasing concentration salt gradient. The denaturation of protein structure upon contacting a hydrophobic interaction column was studied with cytochrome c, myoglobin, lysozyme, and two synthetic analogues of tropomyosin(TM-22 and TM-36) by Ingraham et al., (1985). Operated in a hydrophobic interaction mode the column(Bio-Gel TSK-Phenyl-5PW) completely disrupted the quaternary

structure of TM-22 at all temperatures(0-50 °C) studied while TM-36, myoglobin and cytochrome c underwent various degrees of partial denaturation with increasing temperature. Operating the column in the RP mode cytochrome c, myoglobin, and TM-22 were eluted in denatured form throughout the temperature range studied(0-50 °C). In contrast lysozyme and TM-36 eluted primarily in native conformation at low temperatures, being partially or totally denatured at higher temperatures. The denaturation was attributed to the high phenyl ligand density of the column competing for hydrophobic groups buried within the protein or at subunit interfaces. Since the surfaces of most native proteins are covered with charged groups, interaction with a charged surface would be expected to minimize destabilization of the molecule. It was concluded that hydrophobic interaction chromatography will succeed when the protein has accessible hydrophobic residues on its surface.

Few references report on both the recovery of protein mass and native conformation or enzyme activity. Kato, Kitamura, and Hashimoto(1983) prepared rigid supports for hydrophobic interaction chromatography by coupling butyl or phenyl groups to TSK-Gel G 3000SW, a silica based column material normally used for size exclusion. Mass recoveries of 90 to 100 percent were obtained on eight proteins evaluated. Of three enzymes included in the eight proteins the enzyme activity recovered was only reported for

chymotrypsin which retained 95-100 percent activity. The proteins were applied to columns equilibrated with 0.1 M phosphate buffer (pH 6.0) containing 2 M ammonium sulfate. Unfortunately, tests evaluating protein conformation or function were not applied to the other proteins.

BSA adsorbed to gels having hydroxyl, butyl and phenyl groups on the surface in highly concentrated solutions of ammonium sulfate and in the presence of dilute solutions of perchloric acid, trichloroacetic acid and trifluoroacetic acid (Imai, Tamai, and Sakura, 1986). The mechanism of adsorption was attributed to hydrogen bonding, made possible by slight denaturation of proteins in solutions of high ionic strength. Elution was carried out with mobile phases containing organic solvents (30 percent methanol or acetonitrile), salt (0.18-0.2 M phosphate), urea (5-7 M), ethylene glycol (10 M), and triethylamine (0.1 percent). Organic solvents or salting in brought recoveries of 85-95 percent. Concentrated urea was necessary to disrupt hydrogen bonding and to attain quantitative recovery. Conformational changes in the alpha helical structure of BSA detected by CD were slight (except for urea). The conformation was reversibly recovered after removing ammonium sulfate and perchloric acid but not urea.

Generally affinity chromatography refers to the weak interactions of mobile phase molecules with immobilized ligands such as concanavalin A (Con A), enzyme inhibitors,

adenosine triphosphate(ATP), or protein A. Often these columns may be used for the purification of a particular group of molecules.

The binding of specific enzymes to Con A-Sepharose was studied as a function of cell growth by Crean and Rossomando(1977). Activities of plasma membrane-associated enzymes, alkaline phosphatase, 5'-nucleotidase, and cAMP phosphodiesterase remained constant during the period(16 hours) of cell growth under study, but the amount of activity residing in the affinity bound fraction decreased 50 percent. In each experiment there was at least a 15-fold excess of Con A present in the column in comparison to the amount of protein applied to the column. PAGE of bound Con A fractions were relatively constant in composition throughout the experiment. Unbound fractions showed no binding on fresh Con A columns. The effects of periodate oxidation on the ability of the enzymes to bind to the affinity column were examined. Con A bound fractions were treated with periodate and reapplied to a second Con A column. The recoveries varied from 40 to 95 percent activity in the second bound fraction while the controls all recovered more than 90 percent activity.

The known secretion of glycosidases by cells when true exponential growth ceases was suggested as one mechanism for explaining the observed changes in binding to the Con A column. Incubation of the enzymes in media from

starved cells would have been expected to quickly reduce the enzyme activity in bound Con A fractions in a short time(5 hours) but this was not observed. The decrease in glycosylation of various enzymes with growth in the cell population was concluded to be associated with two separate mechanisms. Both enzymatic and nonenzymatic mechanisms were involved in the 50 percent decrease in activity of the bound fraction following Con A affinity chromatography.

Another application of Con A affinity chromatography was the purification of acceptor glycoproteins from human T cell leukemia and endocervical adenocarcinoma cell lines(Koch and Smith, 1986). Rat antisera were prepared using the purified glycoproteins. The resulting antisera were used to classify tumor and normal cell differentiation. Immunofluorescence studies showed that the antigens were preferentially expressed on a sub-population of immature thymocytes. T cell leukemia antigens were fully expressed(antisera recognized) on T cell tumor lines and normal thymocytes, but not on non-T cell tumor cell lines, peripheral blood lymphocytes or other blood cells. Endocervical adenoepithelium associated antigens were expressed on normal endocervical adenoepithelium but not on ectocervical, endometrial or intestinal epithelia.

Human pancreatic function is critical to the digestion of carbohydrates, lipids, and protein. The purification of human proteolytic enzymes and protein

inhibitors from human pancreas was carried out by a combination of chromatographic techniques(Feinstein et al., 1973). Two trypsins, two chymotrypsins and one elastase were isolated in the following procedure. Human pancreas extract was passed over a lima bean-trypsin inhibitor Sepharose affinity column. The column was washed of contaminants, and the enzymes were eluted with dilute acetic acid. These proteins were resolved on a cation exchange column using a salt gradient. Purified enzymes produced one major band on disc gel electrophoresis and were further characterized on the basis of amino acid composition and molecular weight. Inhibitors were extracted from pancreatic tissue and separated from the larger molecular weight enzymes by size exclusion chromatography. The inhibitor fraction was identified by the capacity to inhibit trypsin activity. The inhibitor fraction was purified on a trypsin-Sepharose column. The washed column was eluted with dilute hydrochloric acid. Isoelectric focusing was applied to separate 4 inhibitor components. Ampholytes and sucrose required for the isoelectric focusing were removed from the inhibitors by affinity chromatography on trypsin-Sepharose.

Human carboxypeptidase A was isolated in a pure form and in reasonable quantities for detailed examination through the work of Peterson, Sokolovsky, and Vallee(1976) using a competitive inhibitor, benzylsuccinic acid as an affinity ligand. The enzyme had been isolated from several

other species, bovine pancreases being particularly available. Human pancreatic juice, clarified by centrifugation was applied directly to an aminobenzylsuccinic acid-Sepharose column. Washing with 0.5 M sodium chloride removed chymotrypsin, and l-arginine eluted carboxypeptidase B. Carboxypeptidase A was eluted with 2.0 M sodium chloride with an overall purification and yield of 70-fold and 105 percent, respectively. Producing a single band on disc gel electrophoresis, the purified protein was further characterized with respect to molecular weight, amino acid composition, zinc content, and peptidase activity.

Another application of affinity chromatography has been the purification of ATP-modified hemoglobin. ATP-modified hemoglobin is important as a blood substitute due to the following properties 1) extremely low oxygen affinity and 2) ability to deliver oxygen at low temperature. Not only was separation of ATP-modified and unmodified hemoglobin difficult by cation exchange chromatography, extensive oxidation(30 percent) of the modified hemoglobin to methemoglobin took place. Passing the reaction mixture of periodate-oxidized ATP-modified hemoglobin over an ATP-agarose affinity column permitted isolation of ATP-modified hemoglobin contaminated with less than 2 percent unmodified hemoglobin(Hsia et al., 1986). The oxygen binding

affinities of the separated hemoglobins were distinct from one another as well as that of a mixture of the two.

Immunoglobulins from many species have been prepared through isolation on protein A (a coat protein from Staphylococcus aureus) modified stationary phases. Forsgren and Sjoquist (1966) investigated the interaction between protein A and various fragmented and whole IgGs from myeloma and normal human sera. On average, it was shown that protein A could precipitate 45 percent of a pooled normal gamma globulin preparation. Myeloma antibodies not specific to protein A as an antigen inhibited the binding of normal antibodies. The reaction of antibodies with protein A was determined to be nonspecific and dependent upon some structural feature not present on the Fab fragment where the antibody binding sites are located. It is known today that carbohydrate moieties on the Fc fragment of immunoglobulin molecules are bound by protein A.

Hjelm, Hjelm, and Sjoquist (1972) reported on the affinity chromatographic purification of protein A, and its use as an immunosorbent for isolation of immunoglobulins. Immobilized nonimmune IgG was used to purify protein A from a bacteria digest rather than conventional purification involving acid and salt precipitation, ion exchange chromatography, and gel filtration. The isolated protein A was covalently attached to Sepharose and evaluated for purification of human IgG. The IgG fraction was eluted with

glycine-HCl buffer(pH 3). The recovery of IgG was estimated to be 95 percent by immunoelectrophoresis.

Although not a general affinity technique, the next example represents a group of naturally occurring binding proteins which some researchers(Bachas, Tsalta, and Meyerhoff, 1986) believe to be superior to immunochemicals for developing diagnostic tests. Careful experimental design by Santa-Coloma et al.(1987) allowed the purification of toad sex steroid binding protein(SBP) using affinity chromatography. SBP yields of less than one percent were observed by conventional biospecific elution chromatography using a steroid as an affinity ligand. This was due to the presence of the difficult to remove free steroid(dihydroxytestosterone) in the samples competing with the immobilized steroid for SBP. A dextran treated charcoal Sepharose column was connected in series with the steroid affinity column to pick up the free steroid. Monitoring the mobile phase tritium activity as a sample containing tritium labeled steroid and SBP recycled through the two columns, a steady state was reached after 9 hours. The charcoal column was then removed and free steroid introduced to elute SBP from the affinity column in 55-60 percent yield. The SBP purified in this manner was homogeneous by PAGE under nondenaturing conditions as well as SDS PAGE.

The formation of reversible covalent bonds between specific functional groups serves as the basis for covalent

chromatography. Conventional chromatographic and electrophoretic methods have found little success in isolating glycosylated hemoglobins present in small amounts(5-10 percent) in hemolysates without contamination from nonglycosylated proteins. Nonenzymatic glycosylation of proteins is widespread in mammalian tissues and may be responsible for certain long term complications of diabetes mellitus. Boronate affinity chromatography was used to separate glycosylated and nonglycosylated hemoglobins by Garlick et al.(1983). Glycosylated hemoglobin was eluted from the column with sorbitol in dilute ammonium sulfate solution. Enzymatic and nonenzymatic attachment of sugars was distinguished by treatment with glycosidic enzymes, and the distribution of glucose adducts at various positions was evaluated with ion exchange chromatography.

Covalent chromatography was successfully applied to the separation of DNA(deoxyribose nucleic acid) from RNA(ribose nucleic acid) by Ackerman, Cool, and Furth(1979) with a boronate cellulose column. The difficulty of this separation becomes more apparent when one considers the close contact between these molecules while RNA is being transcribed from DNA. The column specifically bound ribose sugars with cis-diol groups. Both tritium and carbon-14 labels were incorporated in experiments which showed than DNA and RNA could be quantitatively separated by this technique.

An organomercurial-agarose column was used to successfully purify two synthetic peptides (14 and 37 residues in length) by Kreiger, Krickson, and Merrifield (1976). Solid-phase peptide synthesis often results in the desired peptide plus various defect byproducts. Terminated peptides are defects which occur when growth during synthesis stops permanently. While still attached to the solid-phase, the desired synthesis products were covalently tagged with a dipeptide affinity reagent, cysteinyl-methionine (Cys-Met), through the methionine carbonyl group. Attachment to the synthetic support was cleaved and the synthesis products were passed over an organo-mercurial agarose column which bound the sulfhydryl group of cysteine. Terminated peptides washed through the column, and the remaining bound product eluted with an organic thiol. The desired peptide was obtained upon cleavage of the Cys-Met affinity reagent by cyanogen bromide followed by gel filtration. The presence of Cys and Met amino acids within polypeptide chains has complicated this method, requiring sophisticated group blocking strategies. Amino acid analysis confirmed the structure of the two peptides. The 14 unit synthesis peptide was combined with the complement structure of ribonuclease and recovered 85 percent ribonuclease activity compared to highly purified control by ion-exchange chromatography.

Developments in the area of covalent chromatography have been concerned with the optimization of reversible covalent bond formation for selective isolation. Hannestad, Lundqvist, and Sorbo(1982) evaluated an arsenical modified agarose for the purification of mono- and dithiols as an alternative to organo-mercurial columns. Thiols adsorbed to the arsenical agarose could not be eluted with neutral or acidic pH buffers even at high ionic strength. However, monothiols could be eluted more easily than dithiols with dilute sodium hydroxide solutions. Mono- and dithiols could be eluted under acidic conditions by other thiol compounds. Among the dithiols it was particularly interesting that those which formed five- or six-membered rings with the arsenical agarose were held much more strongly than those which formed seven-membered rings.

Immunoaffinity chromatography has made possible the isolation of molecules otherwise often considered inaccessible by conventional purification means. Immunoaffinity chromatography involves antibody-antigen binding constants ranging from 10^6 to 10^{10} L/mole. Binding constants less than 10^6 L/mole may be easily confused with nonspecific protein-protein interactions. Dissociation of antibody-antigen complexes with binding constants greater than 10^{10} L/mole may not be possible or at least not without using protein denaturing conditions. Examples are the saliva-interacting cell wall protein from bacteria,

Streptococcus mutans(Ackermans et al., 1985), human myelin basic protein in highly purified form(Tigyí et al., 1984) and immunoglobulin E(IgE). The bacterial protein was isolated by immobilizing a rat monoclonal IgM on cyanogen modified agarose. The human myelin basic protein retained functional activity(ability to elicit allergic encephalomyelitis) and was purer than conventional multistep preparations as evidenced by a single band on SDS PAGE with silver stain detection. While IgE is found in blood it is often present in concentrations ten-fold less than that of IgG. IgE was quickly isolated using a monoclonal antibody immobilized on protein A coated glass beads(Phillips et al., 1985a). The isolated IgE was evaluated using a nonisotopic version of radioimmunoassay and radial immunodiffusion. Protein A coated glass beads were suggested as a universal support for high performance immunoaffinity chromatography using monoclonal antibodies(Phillips et al., 1985b). This was explained on the basis that protein A binds to the carbohydrate moiety on the tail or Fc portion of the IgG molecule. The protein A was coupled to carbonyl diimidazole activated glycoPhase controlled pore glass. After mixing the protein A coated glass beads with the desired immunoglobulin, followed by dialysis, the immunoglobulin was crosslinked to protein A with a carbodiimide. Unfortunately the IgG protein A coated glass bead columns decreased in capacity with use. The presence of protein A probably

caused many undesired proteins to adsorb to these immunosorbents eventually interfering with the specific antibody-antigen interaction. Chaotropic elution (sodium thiocyanate, polyvinylpyrrolidone-iodine, or sodium chloride) yielded 40-50 purifications before the column failed but with acid elution (citrate, glycine, tris-glycine, or acetate buffer) the columns survived only 20-25 purifications. Rather than using protein A to orient the antibody molecule it would have been better to attach the antibody to the support through a covalent linkage away from the binding sites. In addition, most monoclonal antibodies produced are of the IgG1 class which exhibit a low affinity for protein A.

Often immunoaffinity chromatography has been combined with another separation technique. The rapid detection (2.5 hours) of subnanogram amounts of a Sendai virus protein has been reported (Van der Zee and Welling, 1985). The virus protein was isolated using a polyclonal antibody immobilized (attached through carbohydrate moiety on Fc fragment) on protein A-Sepharose affinity column. The identity of the protein was confirmed with RP HPLC.

Warden and Giese (1984) reported the use of soluble affinity chromatography in preference to solid phase affinity chromatography (immobilized ligands) in the isolation of ^{125}I -thyroxin. Their reasoning was that the immunosorbent in solid phase affinity chromatography suffers

from problems associated with capacity, diffusion, leakage, and elution. Conventional chromatographic techniques such as ion exchange, hydrophobic interaction, and size exclusion can be used to isolate antigens which behave much differently when complexed with a soluble antibody. The complexities of the immunosorbent support are eliminated, and the antigen will be recovered with a soluble antibody under constant elution conditions. The major drawback of this technique would be the difficulty of controlling the rapid dissociation of antigen antibody complexes in solution.

It was observed by Bachas et al.(1986) that many antibodies produced to hapten-conjugate immunogens often bound more strongly to the hapten-enzyme conjugates in enzyme immunoassays than to the haptens alone. This was attributed to bridging group recognition on the modified haptens. For this reason naturally occurring binding proteins other than immunoglobulins were suggested for use in enzyme linked competitive binding assays of biological molecules.

The various methods of protein purification described in this introduction lead to some concluding thoughts 1) the terms functionally active and nonfunctionally active protein are more appropriate than native and denatured, respectively, 2) the efficient protocol for separating functionally active and nonactive

proteins will include a minimum of one conventional chromatographic method and one affinity chromatographic method, and 3) one separation protocol will not be generally applicable to all proteins. The major difference between affinity chromatography and nonaffinity chromatography techniques lies in the use of a biologically tailored combination of weak chemical forces versus trying to mimic this interaction chromatographically. Conventional and affinity chromatographic techniques each have their place. The logical process for isolating a functionally active protein could take place in 2 steps. One step should discriminate between proteins on the basis of general physical and chemical properties (size, pI, and hydrophobicity) using conventional chromatography. The other step to be applied would separate molecules on the basis of functional activity using some form of affinity chromatography.

Examples of separations of biological molecules presented in this introduction illustrate the many challenges involved. Careful and systematic evaluation of the chemical processes underlying these separations will lead to improvements in their methodology. Affinity chromatography employing immunochemical reagents has often been associated with several common problems. These include: poor geometry of immobilized ligand (attachment prevents antibody-antigen complex formation); irreversibly

blocked sites; leakage of immobilized ligand; and low biological activity of purified material. While these problems affect preparative affinity chromatography, their presence can be disastrous for analytical measurements where analyte concentrations are often at the nanomolar level. This dissertation will address these problems in order to facilitate the optimization and implementation of affinity chromatography.

CHAPTER 2

EXPERIMENTAL SECTION

Materials

Reagents

Mouse monoclonal anti-bovine immunoglobulin G1(IgG1, kappa type), rabbit anti-mouse IgG(heavy and light chain specific), horseradish peroxidase(RZ approximately 3.0, 180 units per mg protein, substrate pyrogallol), and beta-galactosidase(E. coli, 600 units per mg protein, substrate o-nitrophenyl-beta-D-galactoside) were obtained from American Qualex International, Inc., La Mirada, CA. Goat anti-human IgG(gamma chain specific) was purchased from Cooper Biomedical, Inc., Malvern, PA. Bovine IgG, Cohn Fraction V, was purchased from Fluka Chemical Corp., Hauppauge, NY. Bovine and porcine insulin were donated by Lilly Research Laboratories. Anti-bovine insulin IgG1 monoclonal antibodies(VB9 and IVH) were prepared by Tammy Terhune of this laboratory using standard hybridoma technology(Kohler, G. and Milstein, C., 1975). Bovine serum albumin(BSA), phosphoglycerate kinase(PGK, bakers yeast, 2000 units per mg protein, substrate 1,3-diphosphoglycerate), actin(rabbit), fluorescein isothiocyanate(FITC), 4,4'-dithiodipyridine, 4-methylumbelliferone,

4-methyl-umbelliferyl-beta-D-galactoside, 4-hydroxy-3-methoxyphenylacetic acid(HVA), and 4-hydroxyphenylacetic acid(HPA) were purchased from Sigma Chemical Co., St. Louis, MO. Servalyt polyampholines(pH 2-11) were purchased from Serva Fine Biochemicals, Inc., Garden City Park, NY. Reactigel 6X(a 6 percent cross-linked agarose bead derivatized with 1,1'-carbonyldiimidazole) and m-maleimidobenzoyl N-hydroxysuccinimide ester(MBS) were purchased from Pierce Chemical Co., Rockford, IL. MBS used was also prepared by de Alwis(1984) in this laboratory according to the procedures of Parola(1934), Searle(1948), and Kitigawa and Aikawa(1976). Sephadex G-25(coarse), G-75, Sepharose 4B, and Sephacryl S-300 were purchased from Pharmacia, Inc., Piscataway, NJ. Immunolon Removawells were purchased from Dynatech Laboratories, Inc., Chantilly, VA. Sodium iodide, [¹²⁵I](25 mCi/ml), was purchased from ICN Radiochemicals, Irvine, CA. Bolton and Hunter Reagent for protein iodination(N-succinimidyl, 3-(4-hydroxy, 5-[¹²⁵I]iodophenyl, 5 mCi/ml) propionate was purchased from Amersham Corp., Arlington Heights, IL. All other reagents were ACS grade.

Equipment

Protein solutions were concentrated with a Biomolecular Dynamics Model MPDC-115(Beaverton, OR) negative pressure concentrator using a 15,000 molecular weight cutoff

membrane. Equilibrium dialysis was carried out using 12,000 - 14,000 molecular weight standard cellulose dialysis tubing, purchased from VWR Scientific, Los Angeles, CA.

Preparative affinity columns and size exclusion columns were monitored with a Dupont Model 835 photometer, modified with a larger volume flow cell(100 ul) and a 280 nm interference filter(10 nm FWHM). Fractions eluting from chromatographic columns were collected with a Gilson Model FC-80K fraction collector. The concentration of protein solutions was determined by measuring their absorbance using a Varian Model 219 spectrophotometer. The absorption of IgG($a = 1.4 \text{ ml mg}^{-1} \text{ cm}^{-1}$) and insulin($a = 1.06 \text{ ml mg}^{-1} \text{ cm}^{-1}$) were measured at 276-280 nm. The absorption of 4-thiopyridone was measured at 324 nm($e = 1.98 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$). The absorption of horseradish peroxidase($a = 2.5 \text{ ml mg}^{-1} \text{ cm}^{-1}$) was measured at 403 nm. Fluorescent labels, fluorescent products of enzyme catalysis, and enzyme labels were characterized using a Perkin Elmer Model 204-A double monochromator fluorometer.

HPLC experiments were carried out with the following apparatus(see Appendix A for operation/maintenance notes). Mobile phases were delivered at 0.5 ml/min(unless otherwise noted) by a Spectra Physics Model 8700 pump with a low pressure ternary solvent mixer. Introduction of samples was made by a Valco Instruments Model C6PX manual injector(20 ul loop) which was later replaced with a Pharmacia Model ACT-

100 autosampler(25 ul loop). The fluorescence detector was custom built by Sportsman(1982). Only the major components are described. The excitation energy for the fluorescence detector was produced by a Varian Eimac 150 watt xenon arc lamp focused onto an Instruments SA Model H-20 monochromator. The ultramicro fluorescence flowcell(Perkin Elmer) had a volume of 20 ul. The fluorescence emission passed through a narrow band interference filter[filter wavelengths - 390 nm high pass(HVA and HPA), 450 nm(5.2 nm FWHM, Ealing Corp., So. Natick, MA, 4-methylumbelliferone), and 520 nm(10 nm FWHM, Corion Corp., Holliston, MA, FITC)] and was focused onto a RCA Model IP-28 photomultiplier tube. The raw data were plotted on a Hewlett Packard Model 3390A recording integrator. The autosampler functioned as the master(Appendix A - Automation) to control the pump, integrator and fraction collector unattended for periods limited only by the number of samples or amount of mobile phase.

High performance immunoaffinity chromatography experiments were conducted using a stainless steel(4 cm X 0.2 cm id) or glass column(described below). The temperature of the stainless steel column was maintained at 25.4 °C with a Forma Scientific Model 2095 refrigerating and heating circulating water bath. In order to measure the amount of radioisotope labeled protein residing in the column directly, a glass column transparent to the 35 KeV

gamma emission from ^{125}I was used. Porous polyethylene plastic discs (Isolab Inc., Akron, OH) were pushed into the ends of a piece of glass tubing to make a column (4.5 cm X 0.25 cm). The glass column was connected to stainless steel HPLC tubing using polyvinyl chloride tubing.

Radiometric determinations of ^{125}I labeled proteins were carried out on an LKB Model 80000 automatic gamma counter and a Nuclear Chicago Model 4454 manual gamma counter. Often it was necessary to make correlations between activity measurements made on the two different counters. Counter geometry was different and calibration standards of similar activity and geometry were placed in the counting wells of both detectors and calibration factors were determined. The glass column described above was placed in the well of the manual gamma counter.

Electrophoresis was performed using a Bio-Rad Model Protean II electrophoresis cell along with a LKB Model 2197 power supply.

Methods

Buffer Solutions

Non-HPLC solutions were made using distilled deionized water. HPLC buffer solutions were prepared with doubly distilled water and passed through a 0.45 micron nylon filter before use. All buffer solutions contained 0.01 percent sodium azide as a preservative.

Preparative Affinity Immunosorbent

Approximately 80 mg of bovine IgG was covalently attached(Kummel, Daus and Mauch, 1979 and Colowick and Kaplan, 1976) to 20 g of activated Sepharose 4B using the following procedure.

1. Prepare 1 ml of cyanogen bromide(CNBr) solution(Perform this step in a good fume hood.). Add 0.75 ml of acetonitrile to 1 g CNBr weighed out using a glass stoppered container.
2. Wash 20 g of agarose with 100 ml of distilled water three times using a sintered glass funnel(coarse) and remove the excess water.
3. Suspend the gel in 30 ml of 0.33 M potassium phosphate buffer solution(pH 11.9) in a stoppered erlenmeyer flask. Add the BrCN solution to the gel suspension and agitate vigorously for 2 min on a vortex mixer.
4. Transfer the solution to a sintered glass funnel and wash three times with 100 ml of each of the following: a) ice cold 0.25 M potassium phosphate buffer(pH 6), b) distilled water, and c) 0.1 M NaHCO_3 , 0.5 M NaCl(pH 8.5) coupling buffer.
5. Remove the coupling buffer(Do not suck the activated gel dry.) and suspend the gel in the protein solution(approximately 5 mg/ml protein dialyzed against the coupling buffer). Rotate end-over-end for 46 hr at 4 °C.
6. Add 1.5 g of glycine to protein gel suspension to

deactivate the remaining imido carbonate and continue gently mixing for another 24 hr.

7. The coupled deactivated gel is washed twice with 200 ml of each of the following: a) water, b) 0.1 M NaHCO_3 , c) 0.001 M HCl, d) 0.5 M NaCl, and e) distilled water.

8. The gel was next washed alternately with sample buffer, pH 7.4 and elution buffer, pH 2.2 phosphate(0.05 M) buffered saline(0.145 M). The washings were collected for the determination of uncoupled protein by absorbance. Coupling efficiency was routinely observed to be greater than 90 percent.

The immunosorbent was packed into a 25.0 ml buret and operated at a flow rate of 0.3 ml/min at ambient temperature. The preparative column was stored at 4 °C in pH 7.4 PBS.

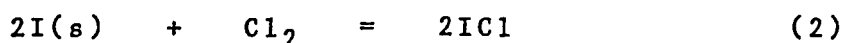
HPLC Immunosorbents

Protein dialysed against 0.1 M BBS, pH 9.5 was coupled to Reactigel 6X according to Sportsman(1982). Studies of nonspecific adsorption were carried out with gels made according to the following directions. Following the washing with BBS coupling buffer and decanting the excess buffer, the wet gel(weighing 0.75 g) was suspended in 2.0 ml of BBS coupling buffer, pH 9.5 containing 100 nanomoles of ligand. The solution was mixed by end-over-end rotation for 48 hr at 4 °C. The coupled gel was washed with 50 ml of

distilled deionized water, followed by several alternate washes of pH 7.4 and 2.2, PBS. The collected washings were saved for the determination of uncoupled protein. HPLC immunosorbents were packed into columns and used at 0.5 ml/min. HPLC columns were stored the same way as preparative columns when not in use.

Iodine Monochloride Synthesis

ICl was synthesized by generating chlorine gas according to reaction 1 (Gmelins, 1927) and passing the gas through a bed of finely ground iodine crystals as in reaction 2 (Buckles and Bader, 1967). The HCl was added



dropwise from a separatory funnel into an ice-cooled side arm flask containing the KMnO_4 as drawn in Figure 1. The generated chlorine gas passed out of the flask side arm and bubbled through a H_2SO_4 trap (constructed from a T-tube) into a bed of finely ground (use mortar and pestle) iodine (located in a second T-tube). The most important part of the synthesis centered around the use of a fluted delivery tube into the iodine crystals (Flare the end of a piece of soft glass tubing until it will barely fit into the T-tube. Once the fluted glass tubing has been placed into the T-tube, add iodine crystals above the fluted portion of the tubing.). A

Figure 1. Iodine Monochloride Synthesis Apparatus.

- A. HCl reservoir-separatory funnel
- B. KMnO_4 reaction flask
- C. Ice water bath
- D. H_2SO_4 trap
- E. Solid Iodine above fluted delivery tube
- F. Exit to fume hood

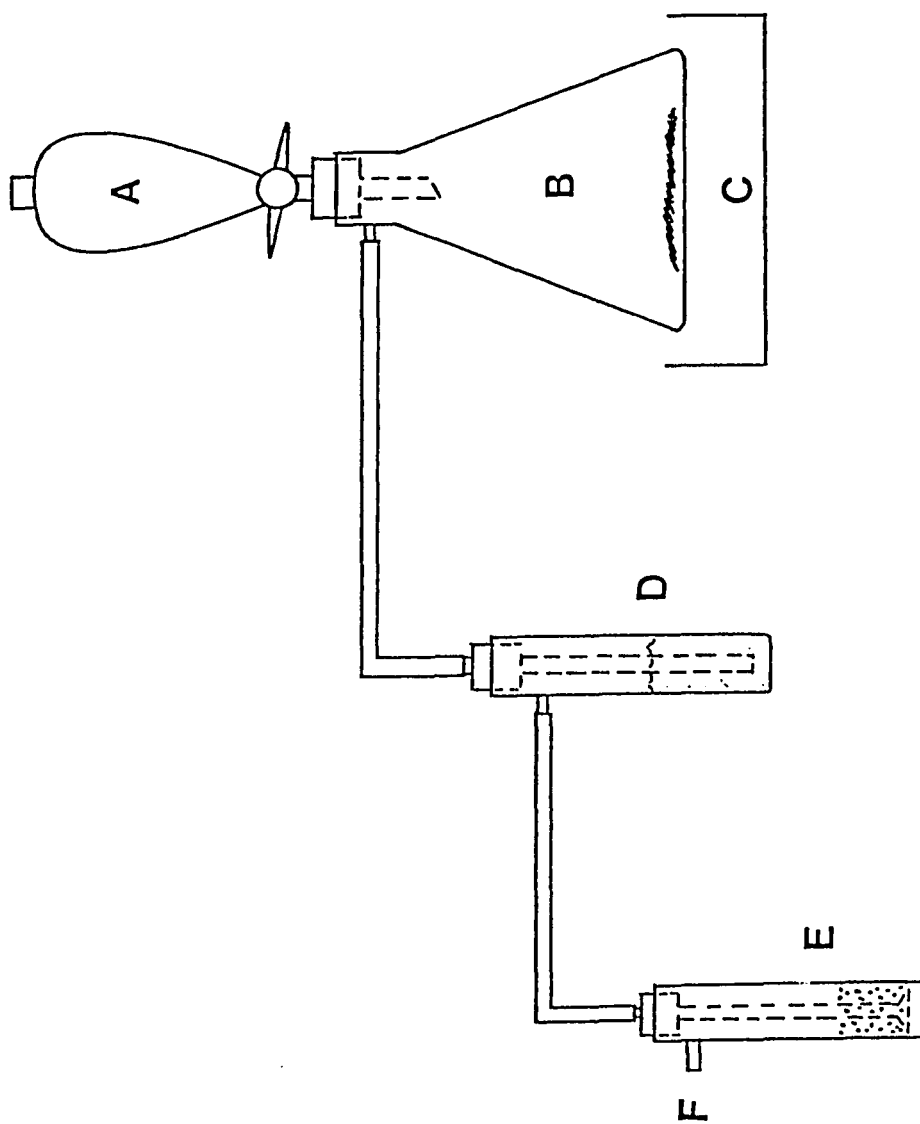


Figure 1. Iodine Monochloride Synthesis Apparatus.

straight delivery tube clogged quickly sealing the tube off. Liquid ICl was transferred into 1 ml glass ampoules, sealed, and stored in the dark at room temperature. When the synthesis was carried out with 60 ml 12 M HCl, 10 g KMnO_4 , and 20 g I(s) the yield was approximately 8 ml of ICl.

Radioisotope Labeling

The labeling of proteins with ^{125}I using the ICl method was described by Sittampalam(1982). Protein solution(100 ul) previously dialyzed against BBS, pH 9.0 was reacted with the desired amount of Na^{125}I (not diluted with cold NaI) in the presence of 80 ul of 0.5 mM ICl in 2 M NaCl with the room lights off for 2 minutes (with agitation). The amount of ^{125}I reacted with 1 mg protein varied from 0.1 to 1 mCi. Current developments in the use of this method have been described by Contreras, Bale, and Spar(1983).

The method of Bolton and Hunter(1973) was also used to label protein with ^{125}I . The Bolton and Hunter reagent was supplied in dry benzene. Divide the reagent up into several aliquots suitable for later iodinations before proceeding.

1. Prepare protein solution(0.5 mg/ml) previously dialyzed against 0.1 M BBS, pH 8.5 and a Sephadex G-25 size exclusion column, previously equilibrated with the desired working buffer. The closer the volume of the protein solution to 100 ul the more efficient the labeling.

2. Pass a gentle stream of nitrogen gas delivered from a syringe needle over the Bolton and Hunter reagent(Perform only in a fume hood.). Residue scattered upon the container walls may be washed to the vial bottom with a small amount of dry benzene and evaporated.

3. Add the protein solution(chilled on ice) to the dried iodinated ester and agitate the mixture intermittently for 30 min at 0 °C(ice water bath).

The purification was carried out by passing the label-protein mixture over a Sephadex G-25 column(1 cm X 21 cm, flowrate 0.3 ml/min), previously equilibrated with pH 7.4 PBS. Every 1 ml fraction eluting the column was collected and counted for radioactivity. The protein peak eluted first followed by a large excess of free radioisotope. The protein fraction was collected and concentrated. If the protein was to be affinity purified, then the protein fraction(without concentrating) was applied to a large scale affinity column(1 cm X 21 cm, preparative affinity immunosorbent described above) and washed with PBS continuously(0.3 ml/min) for about two days until the background decreased to a steady level. The bound protein was eluted with desorption buffer, PBS pH 2.2, and neutralized with an equal volume of 0.3 M PBS pH 7.4. The labeled protein was then concentrated and stored at 4 °C. The determination of the percentage of unbound radioisotope in the labeled protein solution was described by Sittampalam

(1982). A small sample (20 ul) of the concentrated radioisotope labeled protein solution was diluted to 0.5 ml with PBS. Two microcentrifuge tubes, labeled A and B received 200 ul each of this diluted solution. BSA solution(100 ul of 3 percent solution) was added to each tube. Trichloroacetic acid(TCA, 150 ul of 10 percent solution) was added to tube B, and 150 ul of 0.15 M NaCl was added to tube A. The tubes were centrifuged for 5 minutes at 13,000 X gravity(Dummy microcentrifuge tubes from each lot used should be filled with water and tested to assure one that they will not rupture under this treatment.). To check for complete precipitation, an additional 100 ul of TCA solution is added to tube B, and 100 ul of NaCl solution is added to tube A. Two aliquots (100 ul each) were removed from the supernatants of the clarified solutions(A,B) for counting. The ratio of activity in supernatant B to the activity in supernatant A represents the fraction of free radioisotope present in the labeled protein solution. The percentage of free ^{125}I in the protein fractions was routinely observed to be less than 5 percent.

Fluorescein Labeled Protein

FITC stored at room temperature for several years retains its chemical structure and reactivity. This was established after examining the NMR(Sadler Index, 1977) and IR spectra of old(5 years), purified(Kawamura, 1977), and

recently purchased FITC with Sadler Index(1977) spectra. Ascites fluid(2 ml) containing approximately 4 mg of specific antibody, was dialyzed against three changes of 1.5 L of BBS pH 9.5, over three days at 4 °C. FITC(6 mg solid) was added to the dialyzed ascites fluid(initial sample volume was 2 ml) in an ice-water bath over 20 min with stirring(Nairn, 1976). The mixture was allowed to react at 4 °C for 48 hr on an insulated stir plate. The protein solution was applied to a G-25 Sephadex column(1 cm X 21 cm, flowrate 0.3 ml/min) previously equilibrated with PBS, pH 7.4. Monitoring the absorbance at 280 nm, the protein fraction eluted first, and it was applied directly to a preparative affinity column. The bound fraction was eluted with desorption buffer, PBS pH 2.2, neutralized with an equal volume of 0.3 M PBS pH 7.4, and concentrated. The ratio of the number of moles of label attached per mole of protein(F/P) was determined according to Nairn(1976) using a corrected absorbance for protein-bound FITC. The absorbance, A of FITC was measured at 495 nm, and Cp

$$\frac{\text{moles FITC}}{\text{moles IgG}} = \frac{2.8(A)}{C_p} \quad (3)$$

equals the concentration(mg/ml) of the labeled protein. Molar ratios of FITC to IgG obtained by this method were supported by results obtained by Brighton and Johnson(1971) using ¹⁴C-labeled FITC. Since the absorbance of FITC

interferes with the absorbance of protein(280 nm), the concentration of labeled protein solutions was determined by a modified Folin Lowry Assay(Peterson, 1977). The molar ratios of FITC to IgG typically observed varied between 4 and 6.

The relative fluorescence of FITC in pH 7.4, PBS was more than 95 percent quenched in pH 2.2 PBS. The bound fraction in FITC-labeled protein experiments was eluted with 25 percent acetonitrile in 0.05 M phosphate buffer, pH 7.4 and 0.5 M sodium chloride. The fluorescence emission of FITC was enhanced in 25 percent acetonitrile PBS solution compared to PBS without acetonitrile. The peak areas of chromatographic experiments were multiplied by appropriate response factors for the mobile phases used.

Insulin - Horseradish Peroxidase Conjugate

The procedure for covalent coupling of horseradish peroxidase to antibodies(Wilson and Nakane, 1978) was adapted for coupling with insulin.

1. React horseradish peroxidase(8 mg in 1 ml deionized water) with sodium periodate(200 ul of 0.2 M) for 20 min at room temperature with stirring.
2. Dialyze the oxidized protein solution against 1 mM acetate buffer, pH 4.4, overnight at 4 °C.
3. Bovine insulin(5 mg) was dissolved in 0.01 M HCl(400 ul). The protein concentration was verified by absorption

spectroscopy. The insulin solution(300 ul) was diluted with 100 ul of 0.1 M bicarbonate buffer, pH 9.5. With pH paper confirm that the pH is above 9.

4. Add the basic insulin solution to the horseradish peroxidase solution. Immediately adjust the pH of the resulting mixture above 9 by addition of 240 ul of 0.1 M bicarbonate buffer, pH 9.5. React for 2 hr with stirring at room temperature. This step has been reversed from the literature procedure(Wilson and Nakane, 1978) to greatly reduce the amount of homodimer enzyme formed.

5. Freshly prepared sodium cyanoborohydride solution(200 ul of 7 mg/ml) was added to the insulin horseradish peroxidase solution. React for 2 hours at 4 °C with stirring.

6. The reaction mixture was separated on a Sephadex G-75 size exclusion column(2 cm X 64 cm, flow rate 0.25 ml/min). The protein peak which corresponded to unconjugated horseradish peroxidase in elution volume possessed both enzymatic and immunological activity. Although the conjugate bound reversibly to an antibody affinity column, the bound fraction showed no absorbance at 403 nm and no enzymatic activity due to the loss of ferriprotoporphyrin III(protohemin). According to Whitaker(1972) the enzymatic activity can be recovered by replacement of the heme group.

Insulin - Galactosidase Conjugate

Insulin was coupled to beta-galactosidase with m-maleimidobenzoyl N-hydroxysuccinimide ester(MBS) following the method of Kitagawa and Aikawa(1976).

1. Dissolve insulin(3 mg) in 200 ul of 10 mM HCl followed by dilution with 200 ul of 0.1 M phosphate buffer, pH 8.0.
2. Add 50 ul of MBS solution(2 mg MBS per 0.5 ml tetrahydrofuran) and stir for 30 minutes at room temperature.
3. The MBS acylated insulin was precipitated by addition of 1 ml of 1 M citrate phosphate buffer, pH 5.0. Centrifuge for 10 minutes at 1000 X gravity and remove the supernatant.
4. The protein was resuspended with 1 ml 0.01 M citrate phosphate buffer, pH 5.0. The protein was collected by centrifugation, and the supernatant discarded. Repeat this step 3 times.
5. Remove the remaining moisture by lyophilization. Store the dry product at 4 °C. The amount of MBS coupled to insulin was determined according to the procedure below.
6. One ml galactosidase solution(1 mg dissolved in 0.01 M phosphate, 0.01 M MgCl₂, pH 7.0) was reacted with 0.6 ml insulin MBS solution(0.5 mg/ml) for 45 min at room temperature with stirring.
7. The mixture was applied to a Sephacryl S-300 size exclusion column(1.5 cm X 60 cm, flowrate 0.25 ml/min),

previously equilibrated with 0.01 M phosphate(pH 7.0) containing 0.01 M $MgCl_2$ and 0.05 M NaCl.

The number of MBS molecules attached per molecule of insulin was determined by measuring the uptake of a thiol compound, cysteine, by the MBS coupling agent. A known excess of cysteine was reacted with insulin-MBS. The remaining thiol was determined by reaction with a known excess of 4,4'-dithiodipyridine(4DPS)(Grasseti and Murray, 1967) which undergoes cleavage to form 4-thiopyridone(4TP). The absorption maxima for 4DPS(247 nm) and 4TP(230,324 nm) were quite distinct. The increase in absorbance at 324 nm was monitored to measure the amount of 4TP formed. Due to the strong UV absorbance of MBS it was important to make up a blank consisting of insulin-MBS and 4DPS but no cysteine. The reagent quality as well as the weighing and dilution of cysteine were very good as demonstrated by the construction of a linear calibration curve with dilute cysteine solutions, their concentrations determined by measuring the absorbance of the cleavage product of 4DPS.

Electrophoresis

Two dimensional gel electrophoresis was performed on ascites fluid, and affinity purified antibodies(FITC labeled and unlabeled). Non equilibrium pH gradient electrophoresis(NEPHGE) was performed according to O'Farrell, Goodman, and O'Farrell(1977) in the first dimension using pH

2-11 ampholines. The rod gels(2.5 mm X 100 mm) were electrophoresed without a prerun at the following limiting voltages: 200 volts(10 min), 300 volts(10 min), and 400 volts(5 hours). Sodium dodecyl sulfate(SDS) gel electrophoresis was run in the second dimension(1.5 mm X 120 mm) with a 5 percent crosslinked polyacrylamide stacking gel and 12 percent crosslinked polyacrylamide separating gel. The slab gels were electrophoresed at a limiting current of 20 mA/gel for 4 hr followed by staining with coomassie blue.

Batch(equilibrium) Protein Binding Experiments

Experiments were performed using an immunosorbent as described by Sportsman(1982) to determine the titer relationship(between immunosorbent and antigen) and binding parameters(equilibrium constant and number of binding sites).

Without an immunosorbent, immunochemical reactions were performed on polystyrene Removawells(Individual wells have geometry similar to those on an ELISA plate.). Due to the variability in protein adsorption to commercial polystyrene all experiments were performed in 5 fold or greater duplication.

The following procedure determines the maximum amount of labeled analyte which can bind to the adsorbed polystyrene.

1. Apply 100 ul of affinity purified antibody(10 ug/ml) in coating buffer(0.1 M carbonate buffer, pH 9.35) to each well. Allow protein to adsorb to the wells for 1 hr at 37 °C or overnight at 4 °C.
2. Wash the wells with a steady stream of wash buffer(0.01 M phosphate buffer, pH 7.4 containing 0.15 M NaCl and 0.5 percent Tween 20) being careful not to contaminate adjoining wells(wells come in strips of 12.). Repeat this step twice.
3. Incubate the antibody adsorbed wells with radioisotope labeled antigen(dilution series) in assay buffer(0.05 M phosphate buffer, pH 7.4 containing 0.15 M NaCl and 0.1 percent BSA) for 1 hr at 37 °C. The dilution series should be made up assuming approximately 1 picomole of antibody has been adsorbed to each well(Chapter 4).
4. Remove free radioisotope labeled antigen by suction with a disposable pipet connected to vacuum(water aspirator) using an appropriate trap. Apply 150 ul wash buffer to each well and remove by suction. Repeat this step twice more. This procedure avoids splashing of radioactive material.
5. Place each Removawell into a glass disposable test tube(13 mm X 100 mm) and determine the amount of radioisotope labeled antigen. Plotting bound radioactivity versus total labeled moles reacted should determine the concentration of antigen required to saturate the available binding sites.

The competitive binding experiment may be performed once the saturation experiment has been conducted. The number of moles of labeled and unlabeled antigen must always be equal to or greater than that required to saturate the available binding sites.

1. Apply 100 μ l of affinity purified antibody(10 μ g/ml) in coating buffer to each well. Incubate for 1 hr at 37 $^{\circ}$ C or overnight at 4 $^{\circ}$ C.
2. Wash the Removawells with a constant stream of wash buffer being careful not to contaminate adjoining wells. Repeat this step twice.
3. Prepare a dilution series of unlabeled antigen in assay buffer and several blanks. Add to each dilution and blank an appropriate amount of labeled antigen also in assay buffer. Apply 100 μ l of sample(labeled and unlabeled antigen) or blank(labeled antigen only) to each well. Incubate for 1 hr at 37 $^{\circ}$ C.
4. Transfer the solution(free analyte) from each well to a separate labeled test tube with an adjustable pipet set to 150 μ l.
5. Replace the pipet tip, adjust to 175 μ l and apply wash buffer to each empty well. Transfer the wash buffer from each well to the test tube which received the free analyte in step 4. Repeat this step twice.
6. Place each Removawell into a test tube and measure the radioactivity(bound). Count the test tubes containing free

analyte and washings(free). Plots of bound versus total(dose) and free versus total should be made to aid in the interpretation of an unusual plot of bound/free versus total.

Column Protein Binding Experiments

Reactigel immunosorbents were diluted on the basis of the amount of analyte required to saturate the number of specific sites available. A dilution of immunosorbent(6 mg IgG protein/ml gel) 1/10 to 1/50 may be an appropriate starting place(The following assumptions were made. 1. A column of approximately 200 ul volume. 2. About 10 percent of the immobilized protein will be immunologically active.). A series of injections of labeled antigen(dilute in mobile phase containing 0.1 percent BSA) should be introduced into the column. If the signal due to many repeated injections of analyte remains constant(equal to that on the first or second injection), then one of two possible conditions exist. The column has a large capacity for analyte allowing it to bind the same amount of analyte following each injection or the column may be so dilute that it was saturated upon the first injection of analyte. Injecting the same concentration of labeled analyte into the mobile phase without a column present(or a column containing unmodified stationary phase) will allow one to decide

whether the previous column was saturated or not. This last suggestion will not apply to the use of enzyme labels.

Introducing the label alone, not the labeled analyte, into the column should be performed at concentrations similar to those for the amount introduced when performing the saturation experiment above. This will be especially important for enzyme labels.

Once an appropriate column dilution has been determined, competitive experiments are possible. The moles of analyte (labeled and unlabeled) must always equal or exceed the amount required to saturate the column. A dilution series of unlabeled analyte should be prepared in the appropriate mobile phase containing 0.1 percent BSA. To each dilution and blank an equal amount of labeled analyte will be added.

CHAPTER 3

CHARACTERIZATION OF STATIONARY PHASE ADSORPTION

Research on the adsorption of proteins to commercial reversed-phase stationary phases (Sadek et al., 1986) represents an important contribution to the improvement of their separation efficiency. When the stationary phase consists of an immobilized protein with which the mobile phase analyte undergoes an immuno- or biochemical reaction, the study of stationary phase adsorption becomes more complex (Kennedy and Barnes, 1983). Unfortunately, protein modified stationary phases have often been associated with poor recoveries and the isolation of material with low biological activity. This may be due in part to adsorption effects related to unmodified or improperly blocked immunosorbent sites. An immunoaffinity column has been evaluated using affinity purified radioisotope labeled monoclonal antibodies. Monoclonal antibodies have been employed in the present study because of their more homogeneous properties compared to polyclonal antisera.

A model for antibody-antigen reactions taking place in affinity chromatography was developed by Sportsman and Wilson (1980) and Sportsman, Liddil, and Wilson (1983). Binding constants determined under flow conditions were

extrapolated to zero flow rate yielding comparable constants to those determined in the batch mode (equilibrium). This model has been tested using radioisotope labeled protein to study low level adsorption effects. Results from recovery experiments including continuous immunosorbent monitoring will be presented.

Affinity chromatography has been used here to prepare immunologically active antibodies in high purity. Figure 2 shows the affinity chromatogram of previously affinity purified monoclonal antibody. The free or inactive fraction represents less than one half of one percent of the total protein eluted, suggesting that this approach gives high yields of biologically active antibody. This preparative column contains several thousand fold excess of binding sites compared to the amount of protein applied. The affinity purified, radioisotope labeled reagent was used to perform the experiments described.

The data in Figure 3 show the importance of matching the sample size and the column. When three picomoles of purified antibody (above) were applied to an analytical column (Figure 3), the observed elution behavior was more complex. The difference in flow rates (0.2 ml/min) alone was one factor which contributed to the difference in elution behavior (Figures 2 and 3). The most important factors, however, were the differences in the size of the columns and the concentrations of binding sites present. The

Figure 2. Evaluation of Reagent.

Approximately 3 picomoles of affinity purified ^{125}I labeled anti-bovine IgG(0.2 uCi/mg) was applied to the preparative affinity column(1 cm X 21 cm, bovine IgG-Sepharose 4B described in Chapter 2, flow rate 0.3 ml/min).

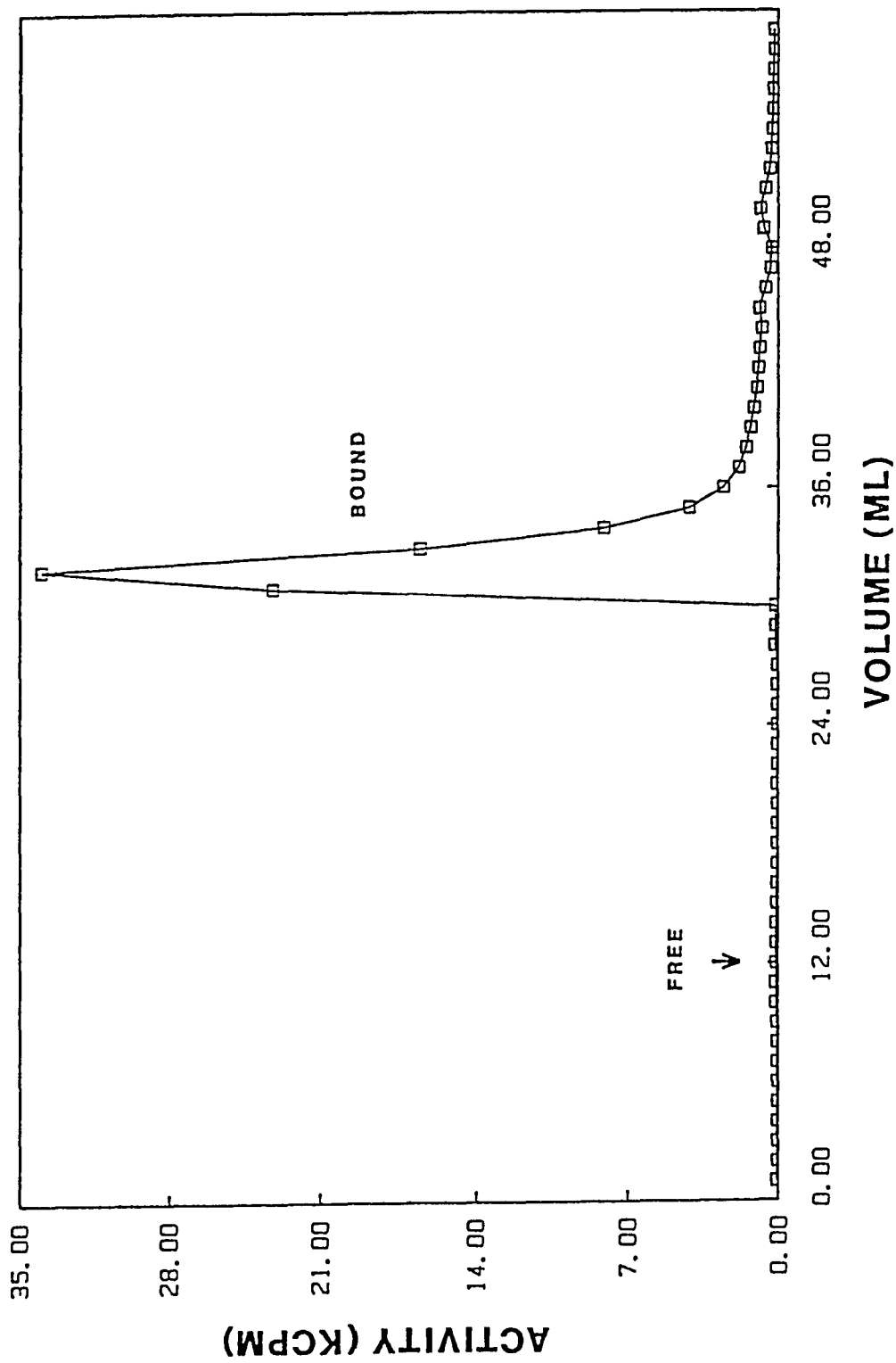


Figure 2. Evaluation of Reagent.

Figure 3. Analytical Scale Chromatography.

Approximately 3 picomoles of affinity purified ^{125}I labeled anti-bovine IgG (0.1 uCi/mg) was applied to an affinity column (0.2 cm X 4 cm, undiluted bovine IgG Reactigel described in Chapter 2, flow rate 0.5 ml/min). KCPM = 1000 X CPM.

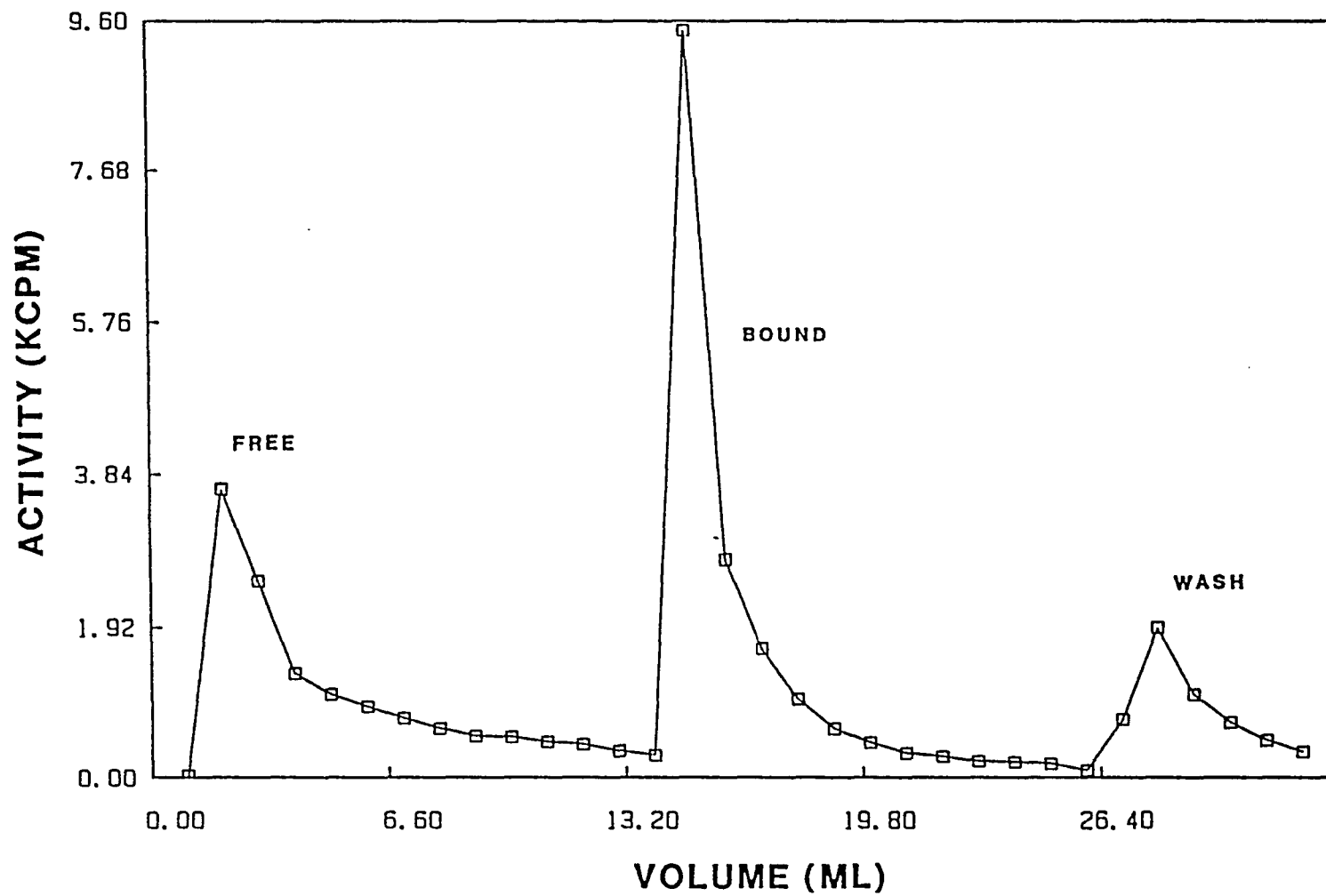


Figure 3. Analytical Scale Chromatography.

preparative column had a bed volume of 20 ml to which 80 mg of bovine IgG was covalently attached (4 mg/ml). The analytical column had a bed volume of 250 μ l with 2 mg of immobilized bovine IgG (8 mg/ml). Assuming approximately 10 percent of the immobilized material was active, the densities of protein in the immunosorbent were 0.4 and 0.8 mg/ml for the preparative and analytical columns, respectively. The ratio of binding sites to analyte molecules is much smaller in the analytical column than in the preparative column leading to a large "free fraction". The analytical column of Figure 3 also contains a "wash peak" which appears as the column is being re-equilibrated with reaction buffer, pH 7.4 PBS.

Figure 4 explains the occurrence of the "wash peak". Upon introduction of antibody molecules into the mobile phase, some form complexes with stationary phase antigens. Starting with the first line a step change in the mobile phase to pH 2.2 PBS has begun to dissociate these complexes, producing free mobile phase antibodies. The concentration of antibody molecules increases in a very narrow band at the solvent front of the low pH buffer as it moves through the column. At some point (line 3) the solubility of the antibody is exceeded and some molecules precipitate on the stationary phase. Only after the mobile phase has returned to pH 7.4 PBS does the adsorbed material dissolve and elute. Even though this material is solubilized on raising the pH,

Figure 4. Representative Elution Profile.

The following are represented:
column(rectangle); progress of desorption
buffer(arrow); presence of mobile phase
antibody dissociated from
immunosorbent(peak); and solubility
limit(S).

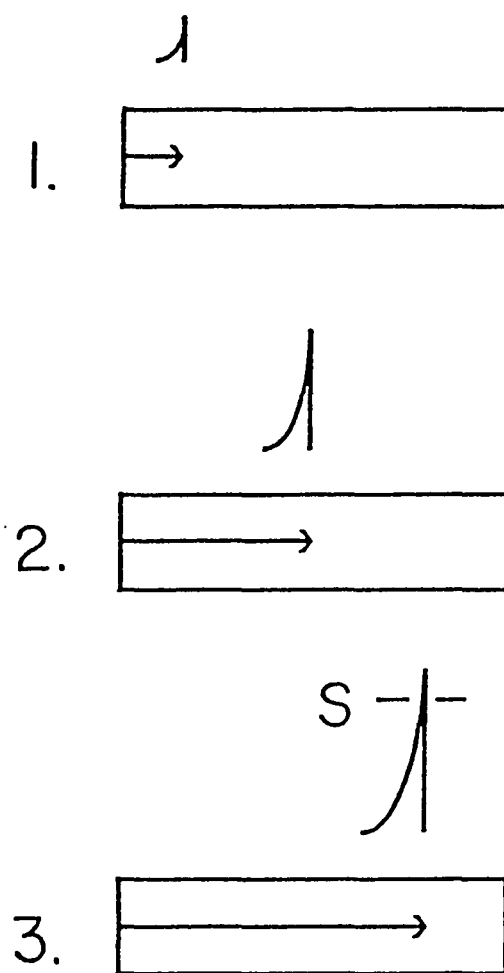


Figure 4. Representative Elution Profile.

it does not interact with the support because insufficient time has elapsed for the protein to renature. Looking back now at the baseline in Figure 2 following the elution of the "bound peak" there appears to be a small "wash peak". The position of this peak corresponds to the approximate mobile phase composition as that for elution of the "wash peak" in Figure 3.

The free, bound, and wash fractions eluting from the analytical column were collected separately and concentrated. Their reactivity upon reinjection into the mobile phase of the analytical column is displayed in Figure 5. It is interesting that each collected sample produces a major bound and wash peak rather than only one of either a free, bound, or wash peaks. Although the amount of material injected in each case was not the same, it was concluded that all material recovered from the column including that from the "wash peak" retains essentially full biological activity. The "wash peak" phenomenon can be eliminated by lowering the concentration of antibody in the mobile phase. Since the "wash peak" results from biologically active material which was bound to the column, the wash peak has been combined with the bound fraction for the remaining experiments.

The interaction of a range of antibody concentrations with the analytical column is shown in Figure 6. The increase in both the free and bound fractions as the

Figure 5. Evaluation of Fractions.

Free, bound, and wash fractions were collected from an analytical scale affinity column(Figure 3), concentrated, and reintroduced into the same column.

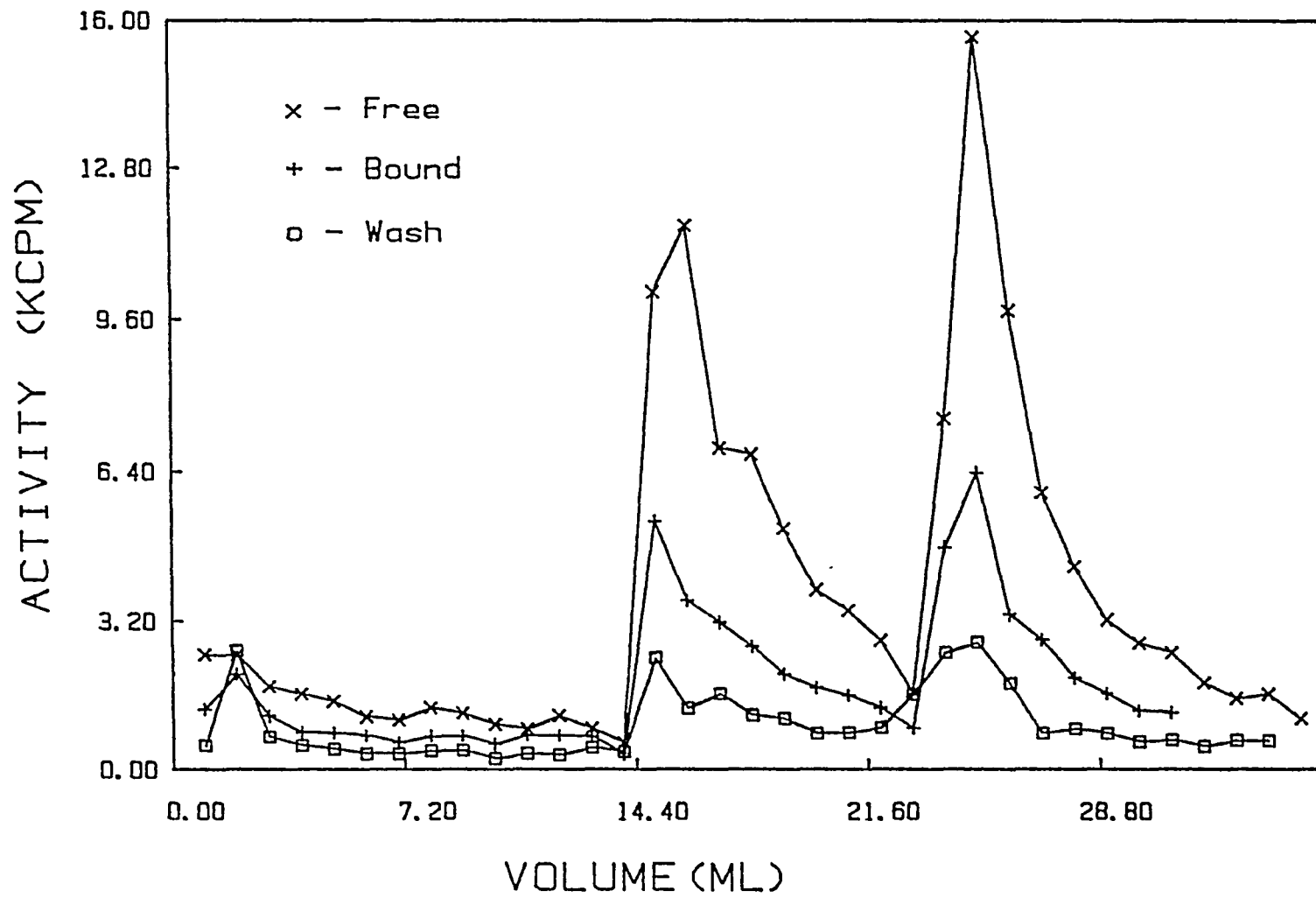


Figure 5. Evaluation of Fractions.

Figure 6. Antibody Partition into Various Phases.

The amount of radioisotope labeled antibody eluting the column(0.2 cm X 4 cm, undiluted bovine IgG Reactigel, flow rate 0.5 ml/min) in various fractions(free, bound, and wash) versus the amount introduced into the mobile phase.

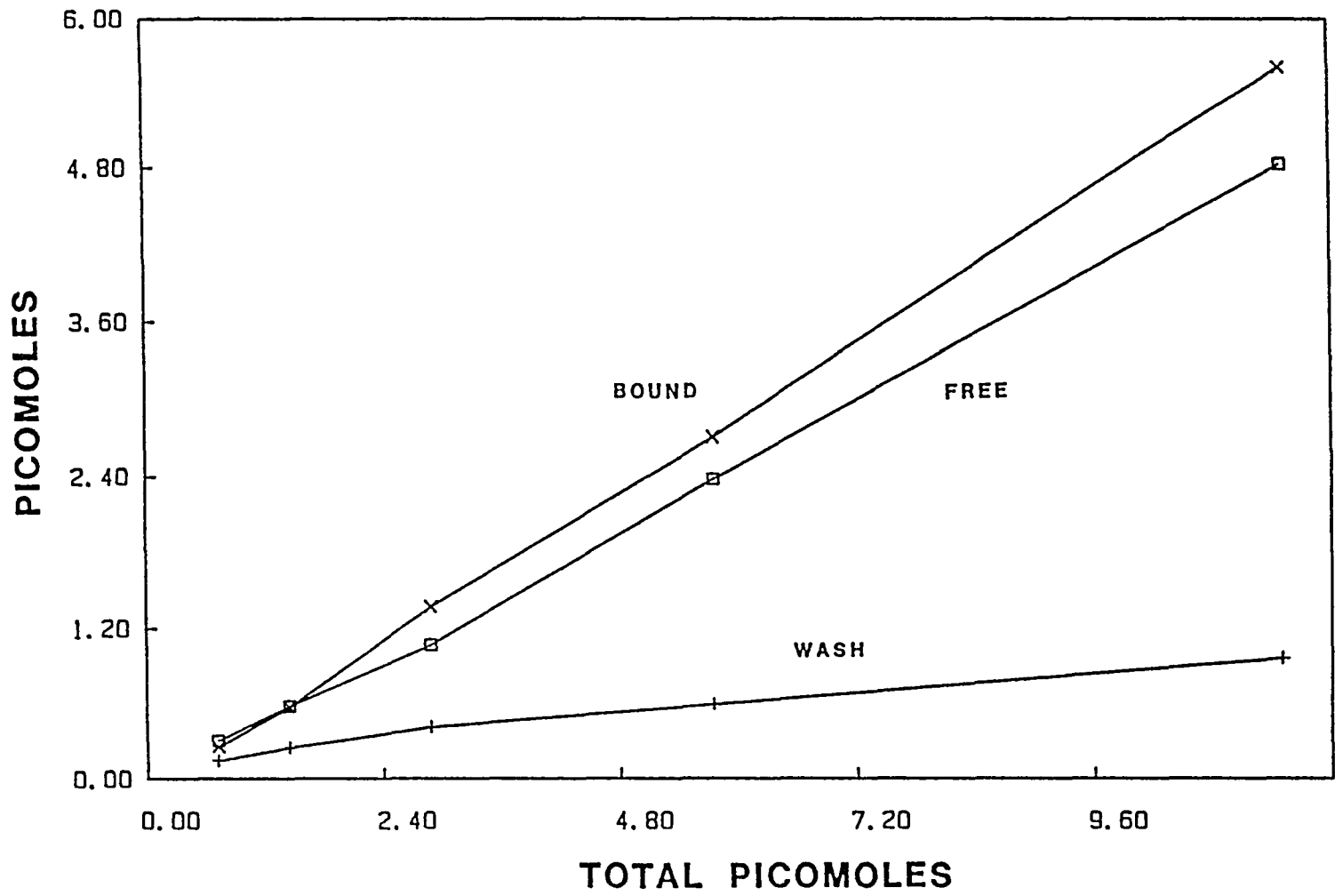


Figure 6. Antibody Partition into Various Phases.

amount of antibody introduced into the mobile phase increases agrees with the apparent equilibrium established under the conditions of this experiment. The steady increase in the "wash peak" corresponds to the elution of greater amounts of antibody from the analytical column where antibody solubility has apparently been exceeded.

Trying to further probe the interaction between the mobile phase antibody and the immunosorbent, a pH gradient was applied to elute the bound antibody from the column(Figure 7). The multiple peaks are certainly the result of differing rates of dissociation of the immobilized antibody antigen complexes. It is not possible at this point to decide whether this heterogeneous behavior is due to heterogeneity of stationary phase binding sites(antigenic) or to a heterogeneous population of antibody molecules. A heterogeneous population of monoclonal antibodies may result from the radiolabeling process or from heterogeneity introduced in the antibody biosynthesis. Within a monoclonal antibody population, heterogeneity may result from differences in phosphorylation, methylation, deamidation, or glycosylation(Pearson and Anderson, 1983). Evidence for charge heterogeneity is presented in Chapter 4 accompanied by a more thorough discussion.

Optimum performance of immunochemical measurements, especially competitive binding assays(CBA), often

Figure 7. Effect of pH Gradient upon Antibody Elution.

Mobile phase pH was changed in a gradient manner to evaluate elution behavior. Approximately 10 picomoles of radioisotope labeled antibody(0.1 uCi/mg) was introduced into the mobile phase. Column(0.2 cm 4 cm, 0.5 ml/min) consisted of undiluted bovine IgG Reactigel. KCPM = 1000 X CPM.

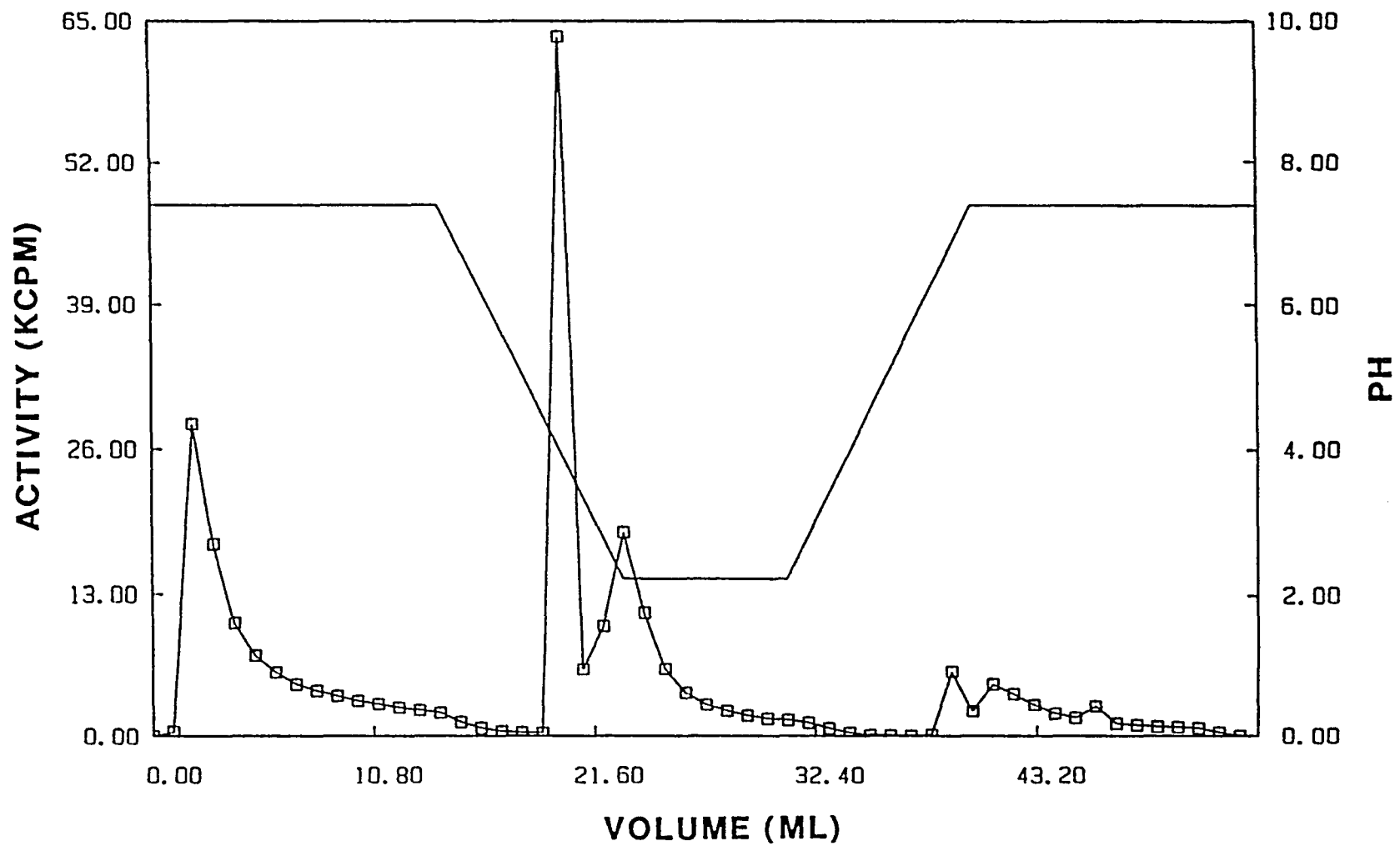


Figure 7. Effect of pH Gradient upon Antibody Elution.

necessitates adjusting the number of binding sites in the analytical system. Dilution of the stationary phase with a support material which has minimal interaction with the analyte would be desirable. Table 1 gives examples of supports which might be used to dilute the specific antigen immunosorbent, bovine IgG-Reactigel. An initial injection of affinity purified radioisotope labeled antibody was introduced via the mobile phase to each immunosorbent. The first exposure of the antibody to this stationary phase results in significant (4 percent) irreversible adsorption. Irreversibly adsorbed material is defined in Table 1 as that material which remains on the column after repeated washing under normally dissociating conditions. The bIgG-Reactigel (the specific antigen) attracted considerable radioisotope labeled antibody compared to the hIgG-Reactigel or any of the other supports. This suggests that irreversible adsorption was not due to nonspecific interactions alone. The specific reaction of anti-bovine IgG with bIgG may permit the anti-bovine IgG to approach the stationary phase more closely, thereby enhancing the nonspecific adsorption processes. Using the data in Table 1, the enhancement ratio of the binding of anti-bovine IgG binding to bIgG- and hIgG-Reactigel, respectively, was estimated as approximately 370. By using a radioisotope label, it was possible to account for essentially all of the antibody injected. Indeed less than 2 percent of the

Table 1. Binding Properties of Immunosorbent Columns.

One injection of affinity purified radioisotope labeled anti-bovine IgG was introduced into the mobile phase of each Reactigel based immunosorbent (HPLC immunosorbents, Chapter 2, column 0.2 cm X 4 cm, flow rate 0.5 ml/min).

Table 1. Binding Properties of Immunosorbent Columns.

Ligand	Picomoles(¹²⁵ I labeled anti-bovine IgG)		
	Free	Bound	Column
bIgG	5.4	98.0	4.3
hIgG	102.8	4.3	0.9
BSA	105.8	2.0	0.2
UN	106.1	1.6	0.2

radioisotope labeled protein initially injected was unaccounted for. Within experimental error, all of the modified supports(excluding bIgG-Reactigel), had minimal interaction with the particular monoclonal antibody studied. Under these experimental conditions there does not appear to be any advantage to using one of these stationary phases for dilution purposes in preference to the others.

Figure 8 shows the effect of repeated injection-elution cycles on the reaction of anti-bovine IgG with the immunosorbent. Although the first 5 injections(only 5 are shown for simplicity) gave quite varying ratios of bound to free fractions, the relative amounts of bound and free protein were stable beyond this stage. The shape of the curve traced by the column(irreversibly adsorbed) fractions leads to a plateau of about 5 picomoles after 20 injections. This quantity appears to be large compared to the bound and free fractions. However, this represents a small fraction(5 percent) of sites available in the column. One might reason that this is due to free ^{125}I alone binding to the column. If this were true one would expect the same elevated irreversible binding for all stationary phases since the same labeled antibody was used in all cases. However, as Table 1 indicates, this is not the case. Column saturation experiments have shown that as many as 100 picomoles of antibody can bind to this column. The initial wide variation in behavior of affinity chromatography observed

Figure 8. Affinity Chromatography Histogram.

Approximately 11 picomoles of affinity purified ^{125}I labeled antibovine IgG was repeatedly introduced into the mobile phase (pH 7.4 PBS). The column (0.2 cm X 4 cm, undiluted bovine IgG Reactigel, flow rate 0.5 ml/min) was treated with desorption buffer (pH 2.2 PBS) between injections. Column fractions were cumulative. Bar code: free (white), bound (hatched), and column (black).

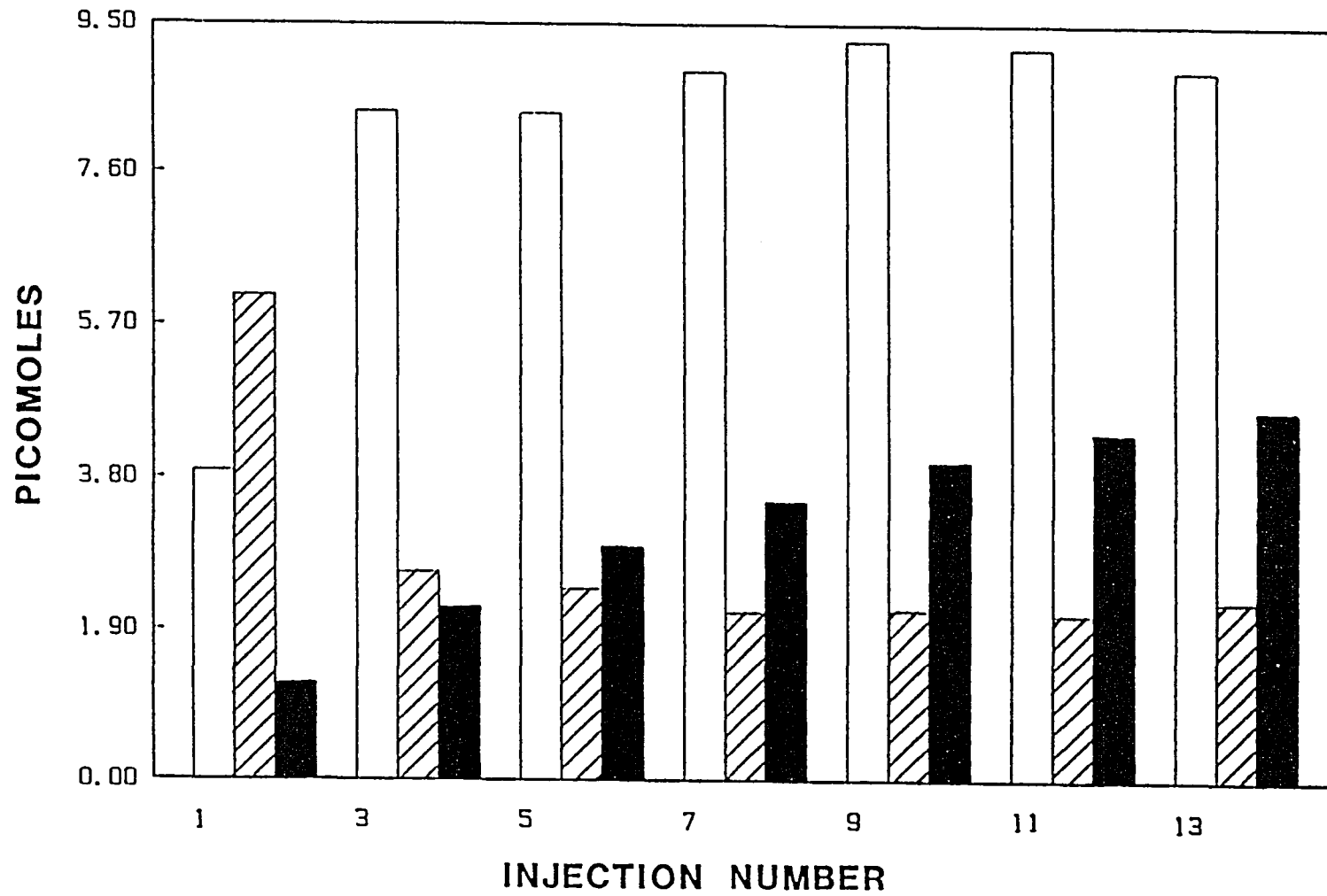


Figure 8. Affinity Chromatography Histogram.

here becomes very reproducible beyond several injections. Thus the column can be readily employed in analytical or preparative experiments even though some of the sites are apparently blocked.

The explanation for the heterogeneous elution(Figure 7) and irreversible binding(Figure 8 and and Table 1) of ^{125}I -anti-bovine IgG to bovine IgG-Reactigel may be related to the orientation through covalent attachment of a large antigen(bovine IgG) on an insoluble support. Several possibilities exist. 1) The point of attachment could coincide with the epitope(antigenic) leading to complete loss of biological activity. 2) Orientation(antigen) on the surface may allow covalent binding of an antibody molecule to two antigen molecules on the surface, a condition which could not normally occur in solution. Multiple binding can lead to at least a 100 fold enhancement in the binding constant. 3) The antibody may not be monospecific and may bind to multiple sites on the same antigen or adjacent molecules, again leading to an enhancement in binding.

A review by Ghosh and Campbell(1986) discussed how monoclonal antibodies may behave in a multispecific fashion. Support for this concept was taken from literature examples involving structural proteins, cell surface proteins(including immunoglobulins), DNA, and haptens. Milgrom and Swierczynska(1986) have performed experiments which raise the question, "Are cross-reacting natural

antibodies multispecific?". Antisera were raised which reacted with antigens from two different bacteria, Escherichia coli(Ea) and Serratia marcescens(Sa). Soluble Ea interfered with the reaction of antiserum with red blood cells(RBC) coated with Ea but not RBC coated with Sa. The opposite result was observed with soluble Sa. This apparent multispecificity of antibodies was attributed to the presence of two subsites within the confines of one combining site.

There are a number of factors which contribute to the performance of an immunoaffinity column whether used for preparative or analytical purposes. The present study has emphasized evaluation of column performance using affinity purified radioisotope labeled antibodies. Using this approach it has been possible to demonstrate that essentially all of the material applied to a column can be accounted for. Moreover after proper column conditioning, picomole levels of material can be recovered essentially quantitatively and the recovered material is practically fully immunologically active. Even the material found in the "wash peak" can be recovered and this material can be shown to possess high biological activity.

CHAPTER 4

CHARACTERIZATION OF MONOCLONAL ANTIBODY

BINDING BEHAVIOR

Clinical chemistry today relies heavily on the use of diagnostic kits which contain antibody reagents for analysis(Edwards, 1983). While most of the early immunoassays were based on antibody reagents derived from polyclonal antisera, many are beginning to be replaced by monoclonal antibodies(Sevier, et al., 1981). Due to particular characteristics of polyclonal and monoclonal antibodies(Collins, 1985), both will continue to be used in the future, often as consecutive reagents in the same assay(Portsmann, et al., 1983). Many tests in the clinical laboratory including those using antibodies are relied upon because they work, even though their reaction mechanisms may be poorly understood(Byers, 1986).

The transition from using radioisotope labels to stable and less dangerous fluorescent molecules or enzymes in immunoassays will be realized more easily when the basis for interpretation of reaction behavior is better developed. The data in Figure 9 suggest that equilibrium immunochemical reactions monitored with radioisotope labels are complicated by themselves.

Figure 9. Unusual Competitive Radioimmunoassay.

Plot of B/F versus T(dose) for binding of bovine IgG and ^{125}I -bovine IgG to polystyrene Removawells coated with affinity purified monoclonal anti-bovine(10 ug/ml). Each point represents an average of five experimental points.

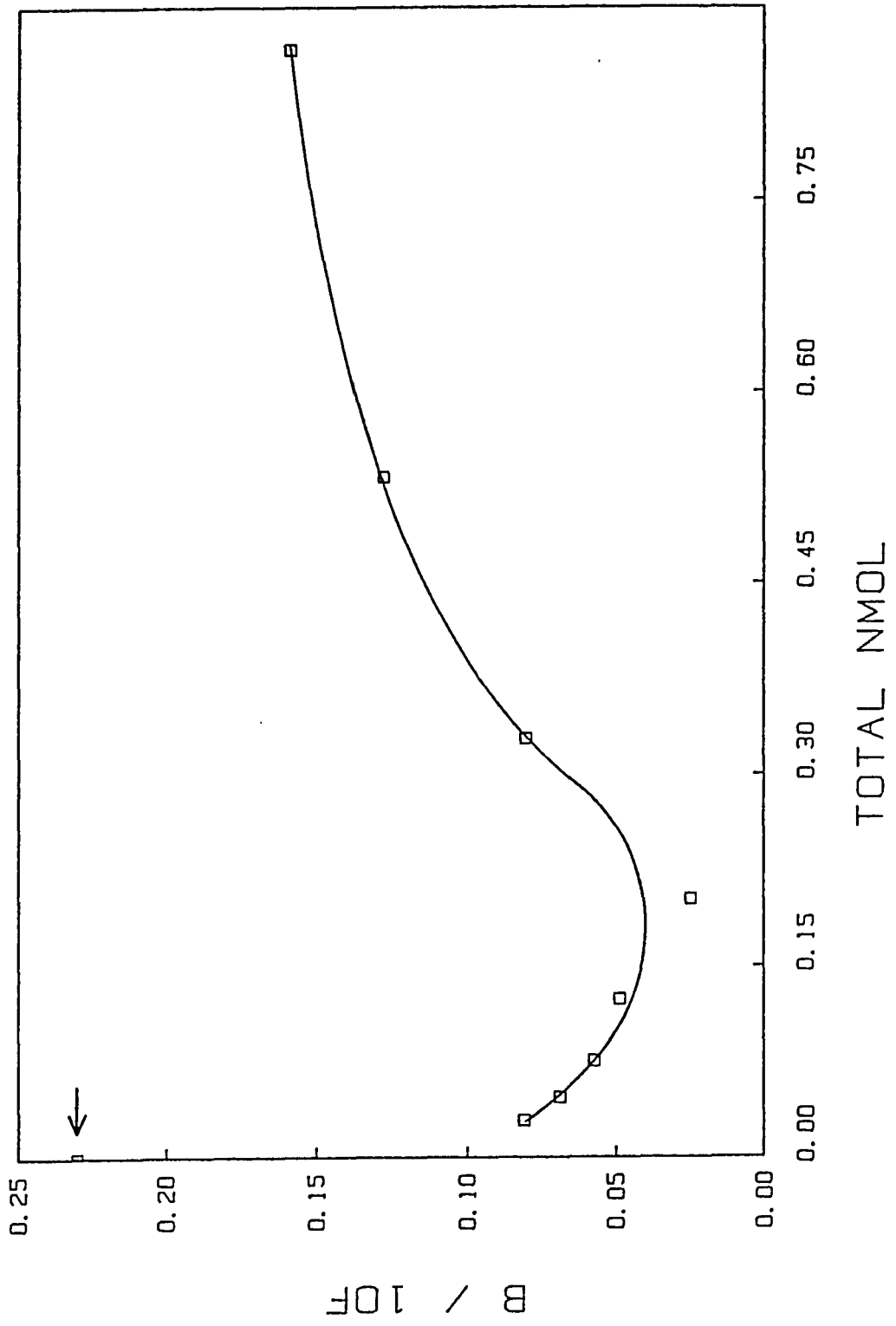


Figure 9. Unusual Competitive Radioimmunoassay.

The experiment from which Figure 9 was taken involved the reaction of ^{125}I -bovine IgG with monoclonal anti-bovine IgG (previously affinity purified) adsorbed on a polystyrene well (Removawell, see Methods Section). The results were displayed in the manner commonly used for radioimmunoassays (RIA). The ratio of labeled antigen complexed (B) and uncomplexed (F) with antibody was plotted as a function of dose or unlabeled antigen (T). For a homogeneous interaction, theory would predict a negatively sloping line. Searching for an explanation, the behavior of the B and F fractions were examined separately (Figure 10). The minimum in the plot of B/F (Figure 9) corresponds to the change in slope of the B fraction (Figure 10) at a value of 1.0-1.3 picomoles (bound) for the dose. This can be related to the number of antibody sites available. There were approximately 20 percent or 1.2 picomoles ^{125}I -bovine IgG adsorbed when the surface was saturated (Figure 11). Therefore, the minimum in Figure 9 corresponds to an amount of antigen adsorbed which exceeds the number of available binding sites. Repeating the equilibrium experiment over a lower dose range results in a more useful RIA (Figure 12). Unfortunately, the use of this technique was not very efficient with respect to the use of immunochemicals due to the low surface area available. Very low B/F ratios suggest most of the reagents were washed off the wells.

Figure 10. Plot of B(bound) and F(free) versus T(dose) for Radioimmunoassay.

The ratio of the B and F versus T was presented in Figure 9. Note the different scales for B and F.

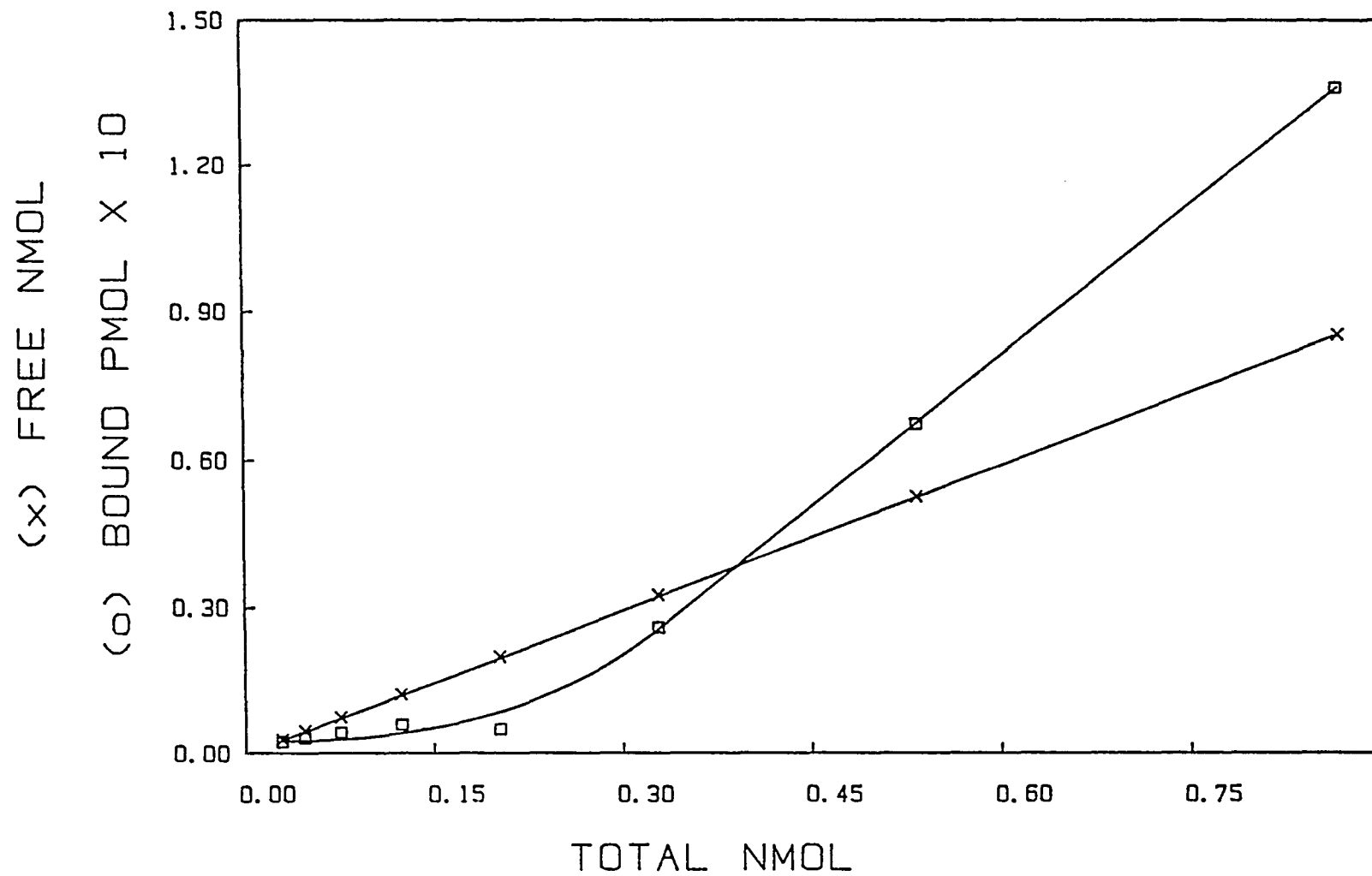


Figure 10. Plot of B(bound) and F(free) versus T(dose) for Radioimmunoassay.

Figure 11. Saturation of Removawells.

Bovine IgG labeled with ^{125}I was coated directly onto Removawells. The bound represents the protein which remained following washing. The data represent an average of five experimental measurements \pm one standard deviation.

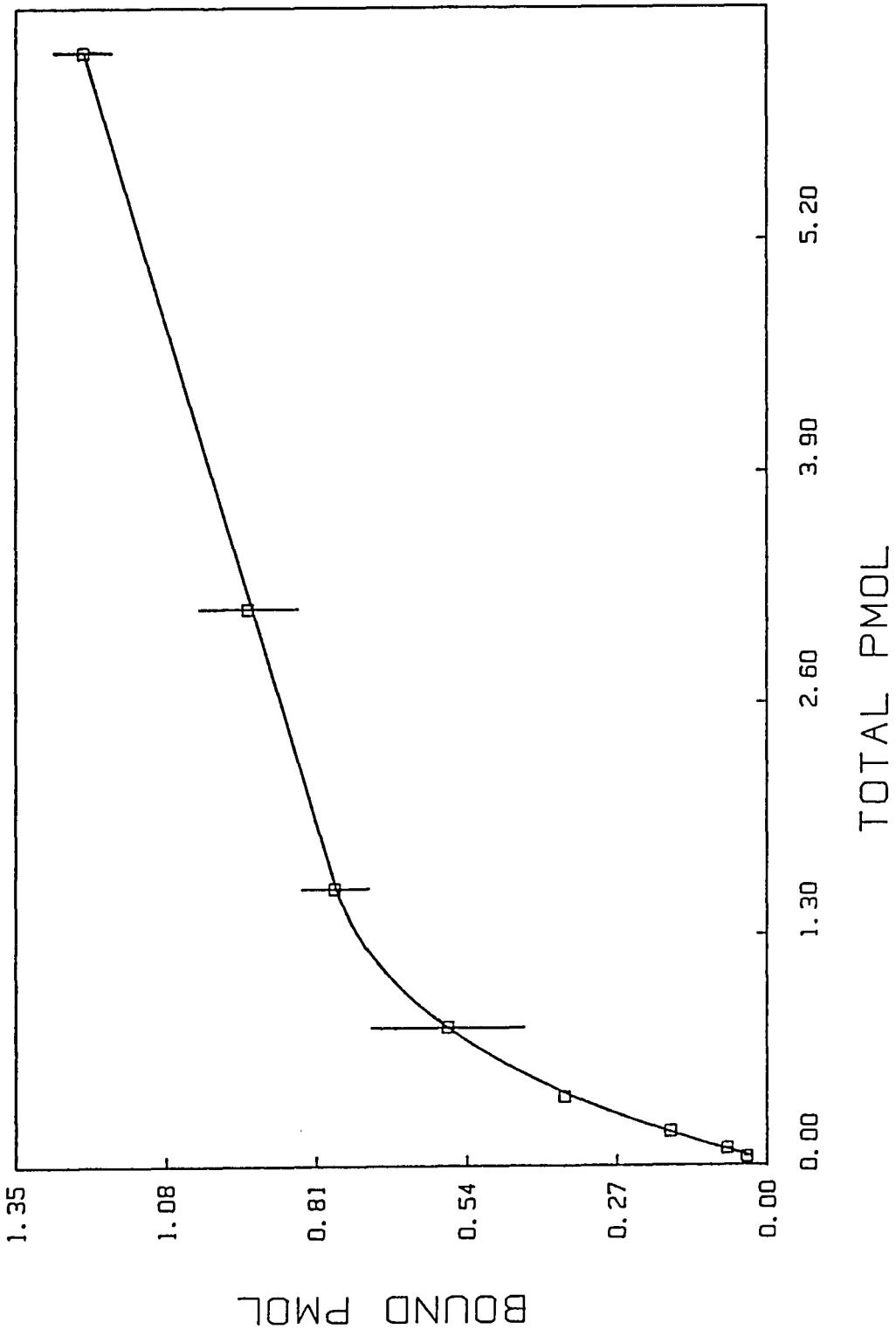


Figure 11. Saturation of Removavells.

Figure 12. Competitive Radioimmunoassay on Removawells.

Plot of B/P versus T(dose) for binding of bovine IgG and ^{125}I -bovine IgG to polystyrene Removawells coated with affinity purified monoclonal anti-bovine IgG(10 ug/ml). Error bars represent the range of +/- one standard deviation.

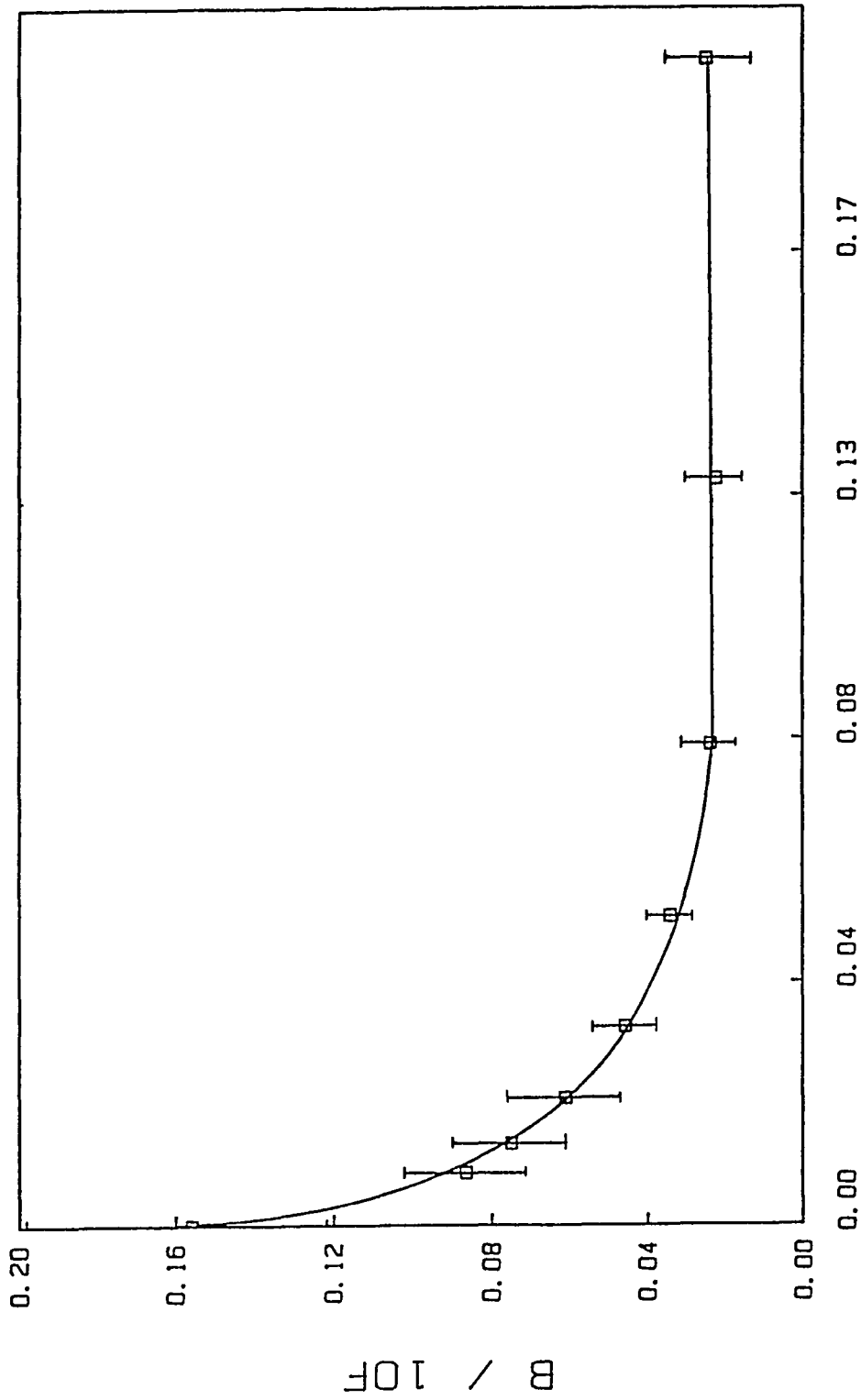
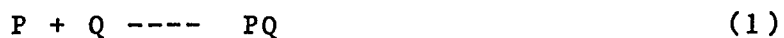


Figure 12. Competitive Radioimmunoassay on Removawells.

The reduced sensitivity of fluorescent molecules as compared to radioisotopes as protein labels has made their use in immunoassays more difficult to optimize. Most of the commonly used enzyme labels on the other hand, capable of converting 10^6 molecules of substrate into products within one minute by one enzyme molecule, are very sensitive (Ngo, 1985). However, the enzyme label and to a lesser degree the fluorescent molecule covalently attached to an antibody or antigen would be expected to sterically hinder their native chemical and immunochemical behavior.

In preparation for the interpretation of data from several experiments designed to study the effect of using a fluorescent molecule instead of a radioisotope as an antibody label some background for binding equilibria will be presented.

Competitive RIA can be described with the following reactions



where P and P* represent nonradioactive and radioactive analyte, respectively and Q is a binding site. PQ and P*Q represent nonradioactive and radioactive complexes, respectively. Several assumptions form the basis for describing ideal RIA behavior (Chase, 1983).

1. The nonradioactive and radioactive ligands, P and P*, are chemically and immunologically indistinguishable.
2. Both reactions go to completion. That is, both K and K* are large.
3. Ligand and binding sites react in a one-to-one ratio.
4. The binder is specific for a single analyte.
5. There is an excess of ligand(P + P*) over binder(Q).
6. The binding sites are independent of one another.

In order to model ligand binding to macromolecules, Scatchard(1949) introduced the following equilibrium relationship

$$R = B/F = K (q - B) \quad (3)$$

where B/F is the bound-over-free radioactivity ratio. K is the equilibrium constant, q is the concentration of binding sites and B is the concentration of bound ligand. Although this relationship is simple in form, computer simulations based on more elaborate equations may be more instructive.

For example, the quadratic equation below expresses the bound-to-free ratio(R) in terms of the input variables p, p*, q and K.

$$R^2 + R (1 + Kp + Kp* - Kq) - Kq = 0 \quad (4)$$

Ekins, Newman, and O'Riordan(1968) derived this relationship while Feldman and Rodbard(1971) generated many different

dose-response curves, showing the effects of varying one of the above variables at a time.

If one now wants to consider that P and P* might be chemically and immunologically different (Assumption 1 above) a relationship which considers both K and K* must be employed. This is a cubic equation (5) which also has been partially derived by Ekins et al.

$$R^3(K/K^*) + R^2[K/K^* + K(p^* + p - q) + 1] + R[1 + K^*(p^* - q) + K(p - q)] - K^*q = 0 \quad (5)$$

Modeling of experimental immunochemical reaction behavior should be an important predictive and interpretive practice. To date, however, theoretical and experimental work on an immunochemical binding experiment conducted by the same research group has yet to be found. Experimental data will be presented along with theoretical computer simulations of expected binding behavior. These simulations were developed by Amarasiri Fernando using the equations above. The software has been listed in Appendix B along with a description of its use.

A model for antibody-antigen reactions taking place in affinity chromatography has been developed. Sportsman and Wilson (1980) and Sportsman, Liddel, and Wilson (1983) showed that binding constants determined under flow conditions could be extrapolated to zero flow rate

yielding the same binding constants determined in the batch mode (equilibrium).

Experiments performed under flow conditions will be presented next. Figure 13 displays results from reactions between fluorescein labeled monoclonal anti-bovine IgG antibody and immobilized bovine IgG. B/F represents the ratio between bound and free antibody fractions eluting from the antigen column. [Total] represents the total concentration of antibody introduced into the mobile phase. The molar ratio of the unlabeled to labeled antibody fractions was 1 (curve A) and 0 (curves C and D). In curve B the labeled fraction was constant in the presence of an excess range of unlabeled fraction ($U/L = 25-500$). The reaction buffer for curve D was 0.5 M sodium chloride instead of 0.15 M as in the other experiments. A stainless steel column was packed with undiluted bovine IgG-Reactigel. The only difference in experiments A, B, and C was the ratio of unlabeled to labeled antibody fractions introduced into the mobile phase. The only difference between experiments C and D was the use of a higher ionic strength mobile phase.

Curve C should be the easiest to simulate using a program which describes the binding of two antibody populations to antigen. Experimental data and theoretical behavior are displayed in Figure 14. With the range of concentration of labeled antibody standards fixed, there is good agreement between the experimental data and theoretical

Figure 13. Comparison of Binding Behavior.

Only FITC-labeled antibody was used(C,D); the ratio of FITC-labeled to unlabeled antibody was always one(A); FITC-labeled antibody was used in the presence of an excess range of unlabeled antibody(B). The antibody was introduced into a mobile phase of pH 7.4 PBS(A,B,C) or 0.50 M sodium chloride, pH 7.4, phosphate buffer(D). U/L = unlabeled/labeled ratio. Column(0.2 cm X 4 cm, flow rate 0.5 ml/min) consists of undiluted bovine IgG Reactigel.

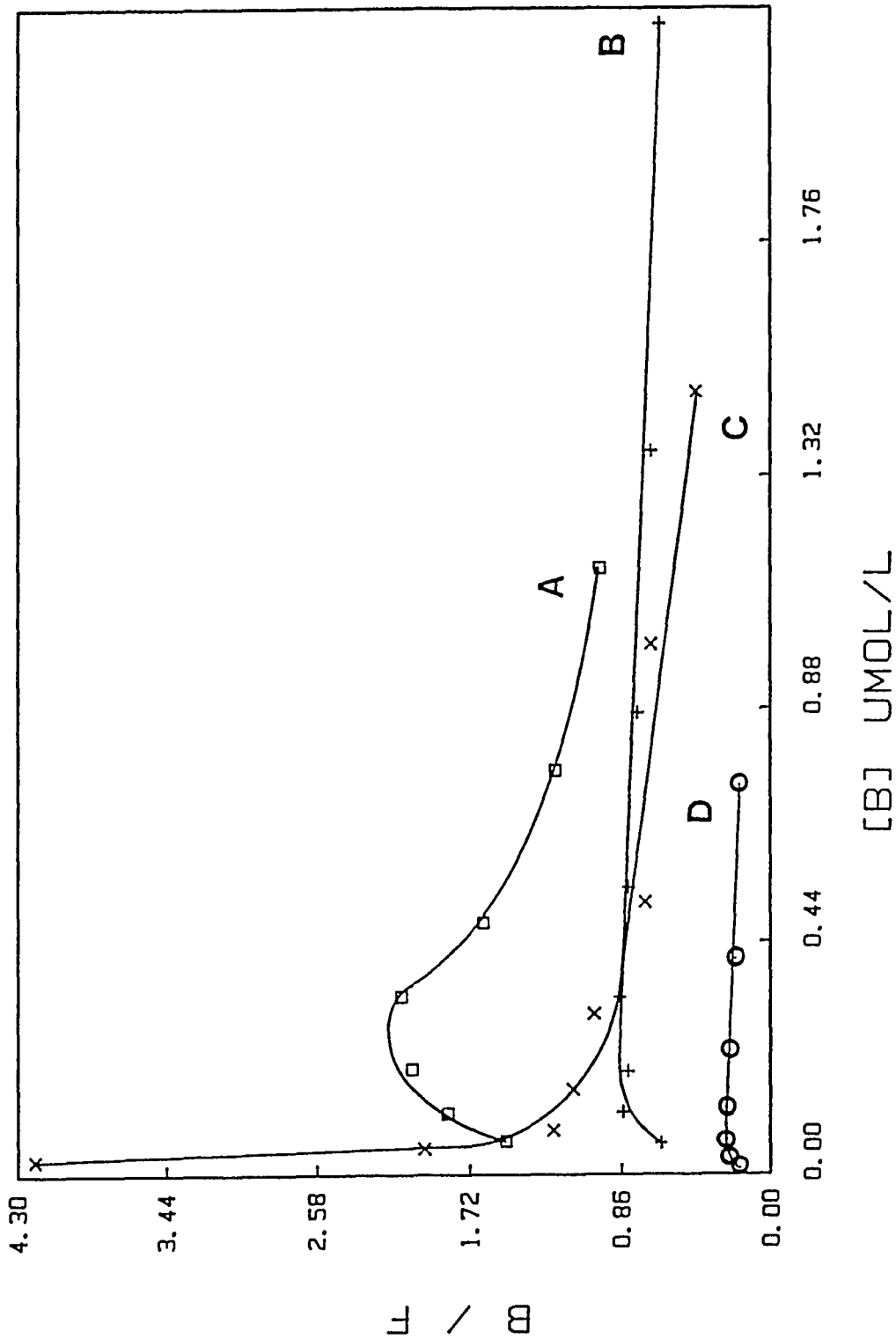


Figure 13. Comparison of Binding Behavior.

Figure 14. Binding Data Compared to Computer Simulation.

Computer program VERB1(Appendix B) was used with the following parameters: $K_1 = 2 \times 10^8$ L/mole; $K_2 = 6 \times 10^4$ L/mole; $q_1 = 5 \times 10^{-8}$ M; $q_2 = 10^{-5}$ M, and $[F] = 8.9 \times 10^{-9}$ to 3.4×10^{-6} M. The experiment was performed with only FITC-labeled monoclonal antibody to bovine IgG(Figure 13, curve C).

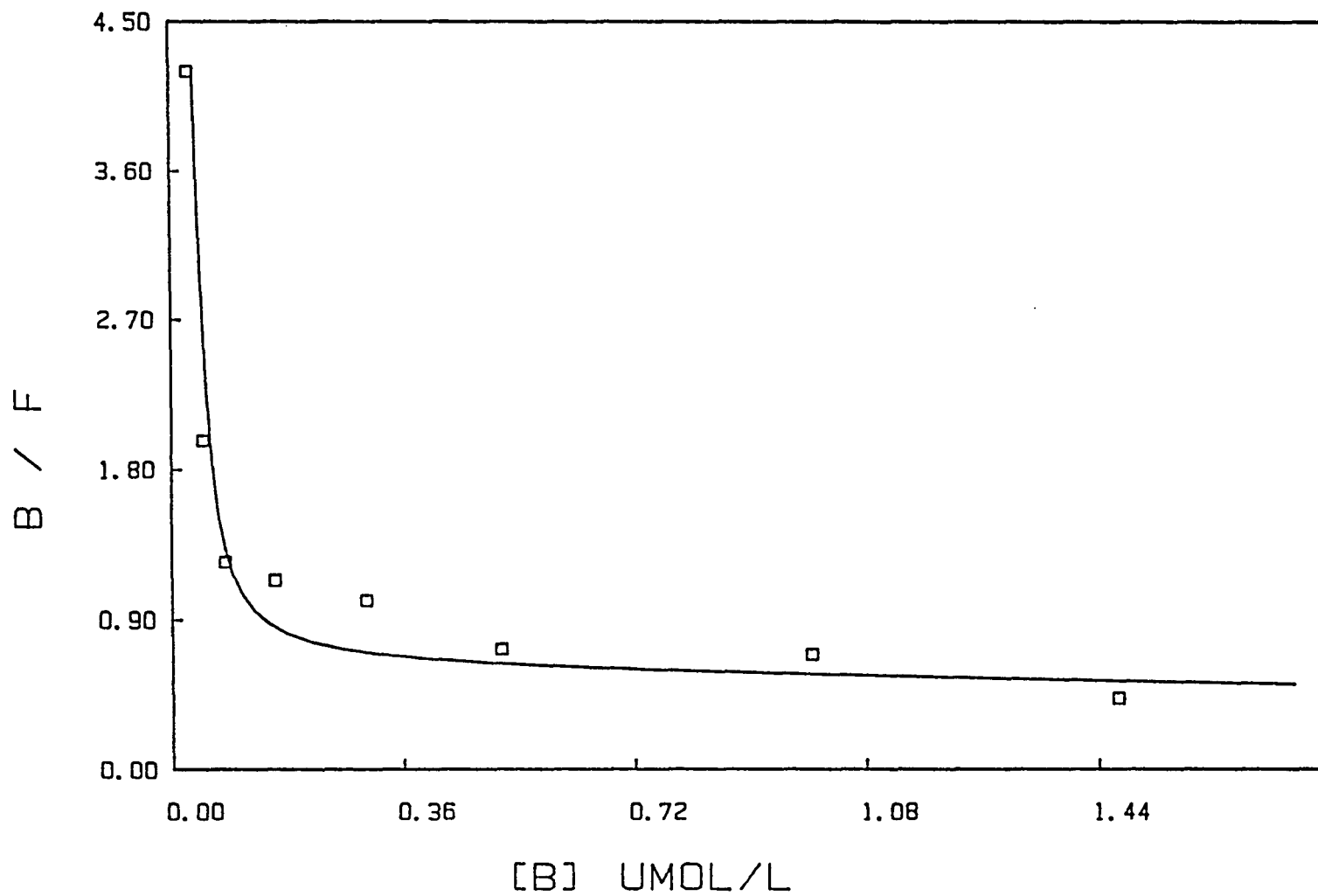


Figure 14. Binding Data Compared to Computer Simulation.

simulation for values of K_1 , K_2 , q_1 , and q_2 of 2×10^8 L/mole, 6×10^4 L/mole, 5×10^{-8} M, and 10^{-5} M, respectively.

Curve A(Figure 13) was generated by injecting a range of concentrations of antibody into the column always keeping the moles of labeled antibody equal to the moles of unlabeled antibody. Data and computer simulations were plotted in the form B/F versus T rather than B/F versus B to allow easier interpretation. One would expect behavior as exhibited in curve C, where a simple dilution series was introduced. Rather than trying to fit the curve directly, plots of experimentally determined B and F versus T(Figure 15) were compared to simulations. The simulated curves in Figure 16 were provided to show the relationship between the data in Figure 15 and expected overall behavior. However, when the two were superimposed as in Figure 17, it still appeared to be an incomplete fit. This simulation using program SCA(Appendix B) treats P and P* as chemically and immunologically equivalent. Without changing the known experimental concentration range, a better fit was not possible with this program. The ratio of the B and F simulated curves generated a negatively sloping curve without a maximum(Figure 18).

What about the possibility of K not being equal to K^* ?. This becomes conceivable if one considers the illustration in Figure 19. When P and P* are equivalent the

Figure 15. Plot of B and F versus T.

FITC-labeled and unlabeled monoclonal anti-bovine IgG mixed in a ratio of 1 and diluted together (experimental detail given in Figure 13, curve A).

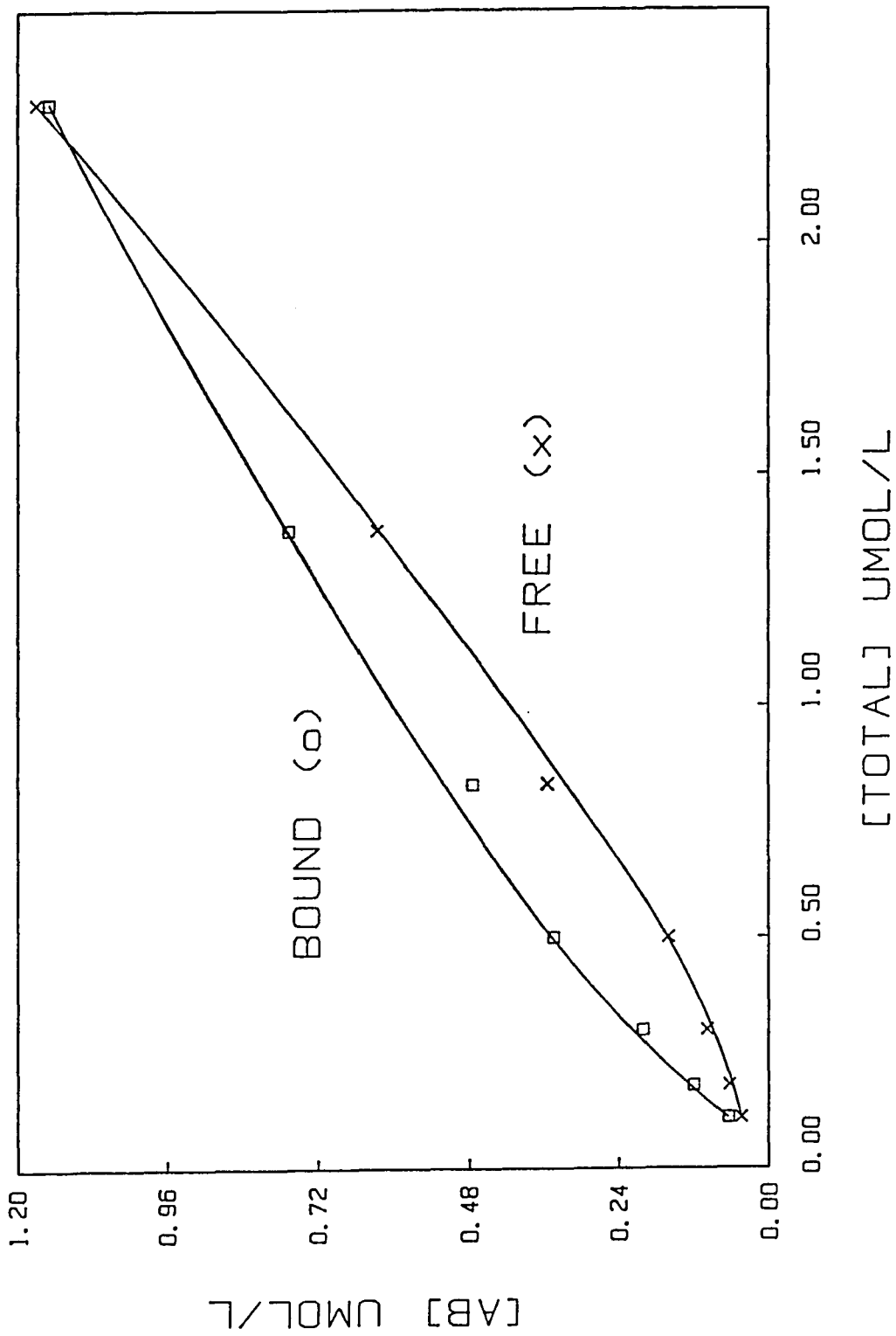


Figure 15. Plot of B and F versus T.

Figure 16. Computer Simulation of B and F versus T.

Computer program SCA3(Appendix B) was used with the following parameters: $K = 10^7$ L/Mole; $q = 10^{-6}$ M, and $p = 10^{-7}$ to 10^{-5} M. This simulation shows the behavior of B and F depending upon the region studied.

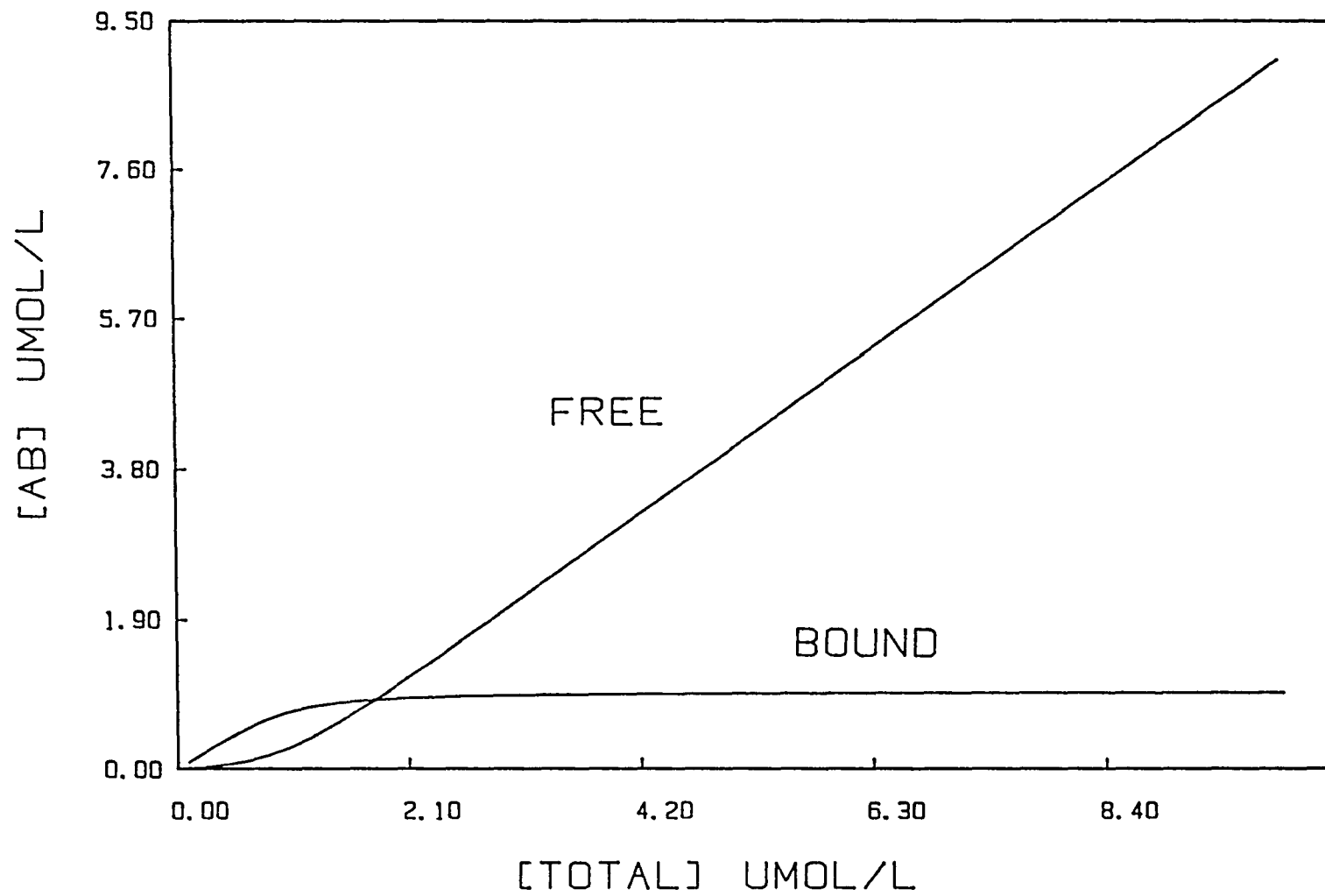


Figure 16. Computer Simulation of B and F versus T.

Figure 17. Comparison of Experimental and Simulated Behavior for B and F versus T.

The simulation was described in Figure 16 and the experiment was described in Figure 13(curve A).

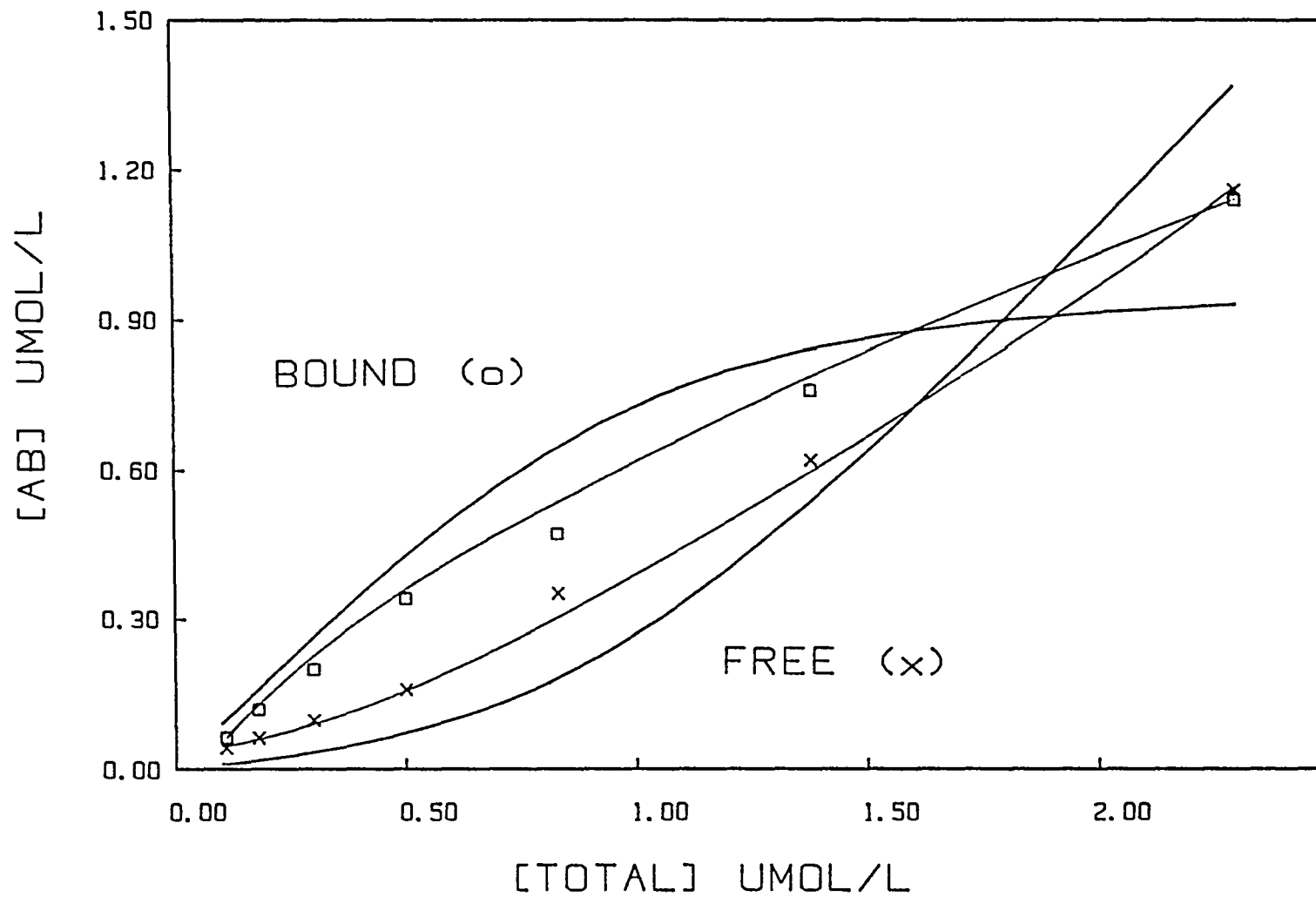


Figure 17. Comparison of Experimental and Simulated Behavior for B and F versus T.

Figure 18. Theoretical B/F versus T Behavior.

The simulations for B and F versus T were described in Figure 16. This does not resemble curve A in Figure 13.

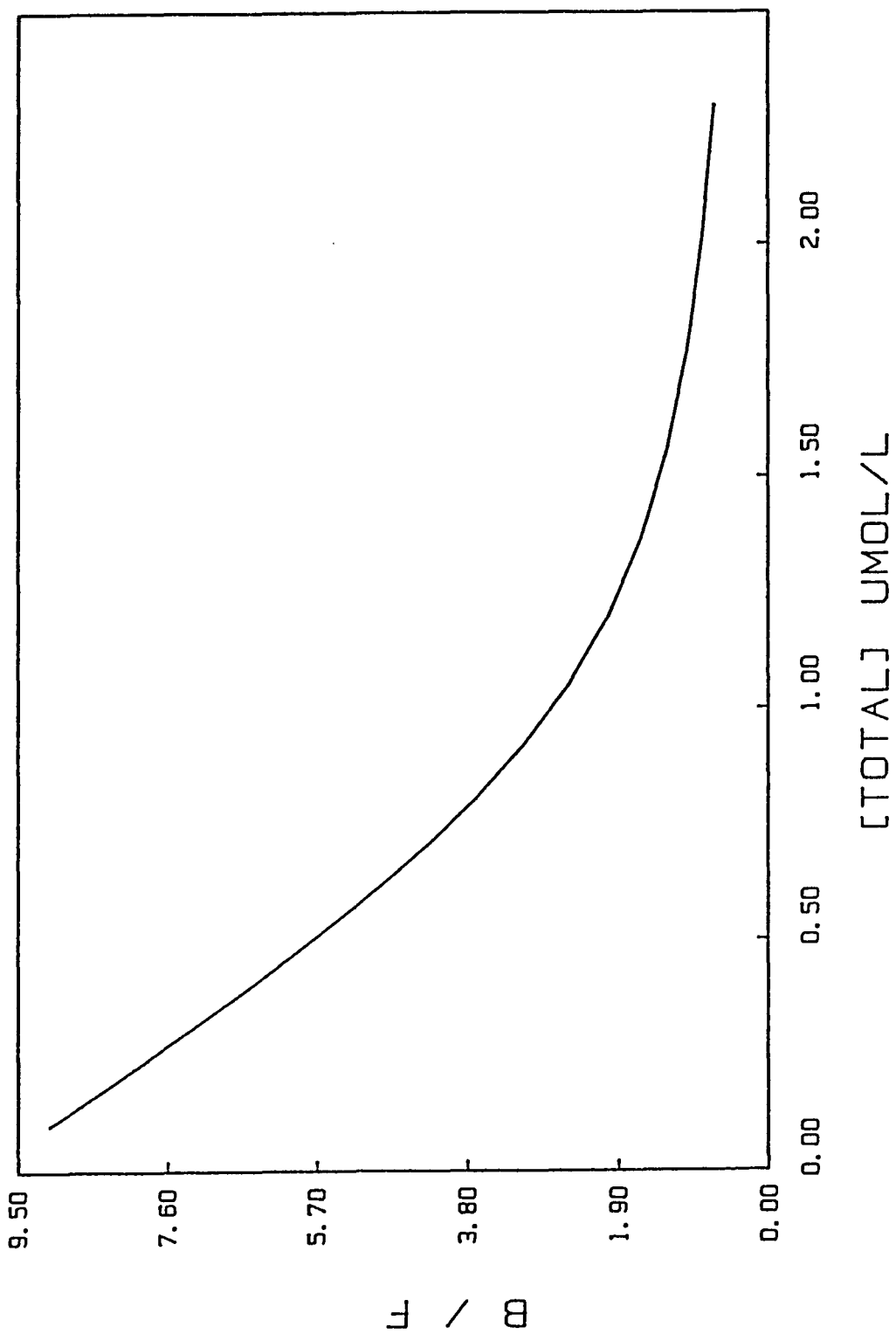


Figure 18. Theoretical B/F versus T Behavior.

Figure 19. Effect of Labeling on Binding Behavior.

Step 1 represents an immunosorbent with available binding sites. In step 2 an equilibrium has been established between free and bound unlabeled antigen. Labeled (hatched circles) and unlabeled analyte behave alike in step 3. Labeling lowers the affinity of the ligand resulting in a lower ratio of B/F (step 4).

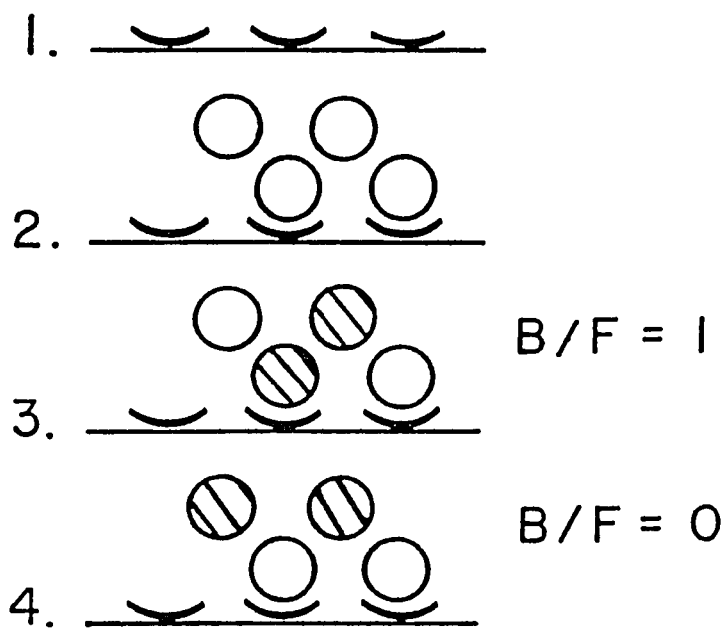


Figure 19. Effect of Labeling on Binding Behavior.

measured(observed) behavior of P^* accurately describes the behavior of unlabeled P (compare steps 2 and 3, Figure 19). However, one would predict that labeled antibody binds less strongly than unlabeled antibody to immobilized antigen sites resulting in dramatic changes in the observed behavior(step 4, Figure 19). Step 3(Figure 19) would be favored at higher antibody concentrations while step 4 would be more pronounced at lower antibody concentrations. The simulated curves in Figure 20 illustrate this point. Only the magnitude of K^* relative to K was varied. It is interesting to observe that for the case where K^* is equal to K the plot resembles what is commonly attributed to an experiment involving at least two antibody populations. This simulation illustrates how a homogeneous(antigen-antibody interaction) system performed in the range of excess antibody(compared to the number of sites) can be misinterpreted.

Assuming at least two populations of antibody, one might explain the observed behavior(Figure 13, curve A) in the following manner. At high concentrations of antibody introduced into the mobile phase the B/F ratio(actual) is lower than it should be because of the lower affinity of the labeled material. As the concentrations of labeled and unlabeled antibody are decreased, appreciable labeled antibody binds weakly to nonantigen sites present in enormous excess of the specific sites(higher B/F).

Figure 20. Effect of K Not Equal to K*.

Computer program SCA(Appendix B) used with the following binding parameters: $q = 10^{-8}$ M; $p^* = 5 \times 10^{-8}$ M; $K = 10^9$ L/mole. $K^* = 10^9$ L/mole (x), $K^* = 5 \times 10^8$ L/mole (o), and $K^* = 5 \times 10^7$ L/mole (+).

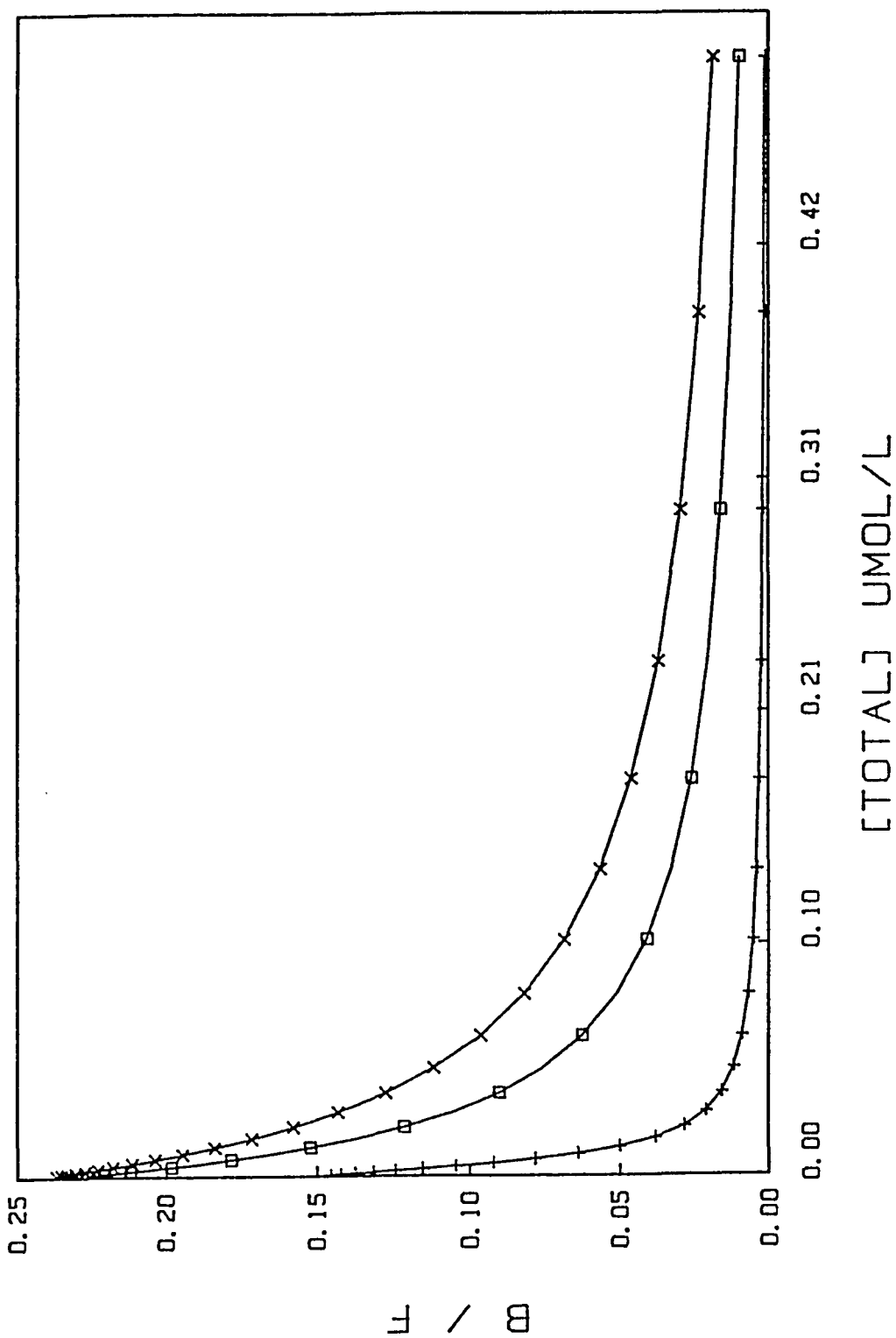


Figure 20. Effect of K Not Equal to K*.

Proceeding to still lower concentrations of mobile phase antibody, there is less opportunity for the labeled antibody to interact weakly with non-antigen sites and the B/F ratio decreases. An alternative explanation consistent with the observed results is that the unlabeled antibody competes more effectively than labeled antibody for the limited number of binding sites, also resulting in a decrease in the B/F ratio. The weaker interaction could be due either to a modified antibody with a low binding constant or to non-specific interactions. Curve B reaches a gradual maximum probably due to the same mechanism described for curve A, there being a much smaller amount of labeled antibody present in proportion to unlabeled antibody. Curve D also resembles curve B. One would expect that curves C and D would be similar but offset due to the difference in ionic strength. The increase in ionic strength appears to interfere or reduce the amount of antibody-antigen complex formation. The increased ionic strength probably exaggerates the differences in the lightly labeled and highly labeled antibody, producing a situation as described above. Further studies were needed to probe the possible effect of differences in labeling on this antibody population.

Two dimensional gel electrophoresis was performed to assess the heterogeneity of affinity purified monoclonal antibody reagents. Each antibody contains two identical heavy chains and two identical light chains, and under

Figure 21. Reductive Denaturation of IgG.

Solution conditions of 5 percent beta-mercaptoethanol and 9.5 M urea cause dissociation of disulfide bonds between heavy(H) and light(L) antibody chains.

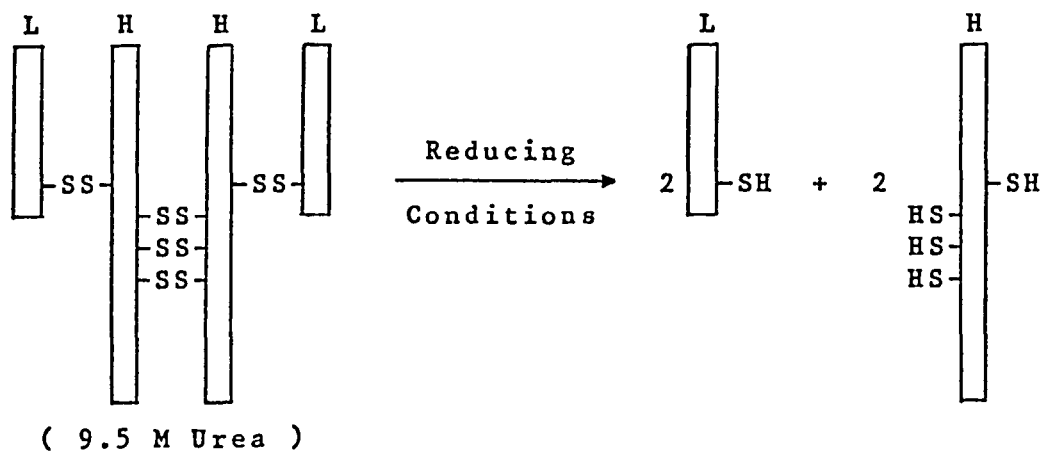


Figure 21. Reductive Denaturation of IgG.

Figure 22. Map of Standards for Electrophoresis.

The location of 3 protein standards and the heavy(H) and light(L) antibody chains for two dimensional gel electrophoresis(Figures 23 and 24) are given. Molecular weights were obtained from Cohn, Hughes, and Weave(1947) and Righetti, Tador, and Ek(1981). Isoelectric points were obtained from Nagle(1986).

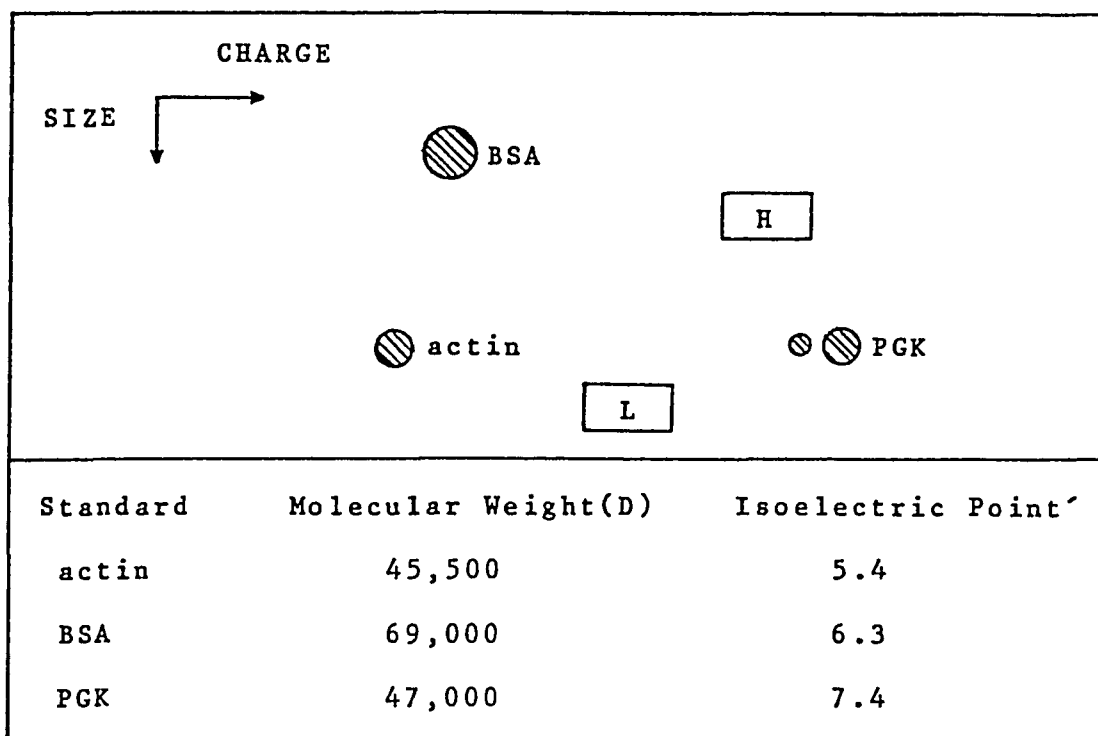


Figure 22. Map of Standards for Electrophoresis.

Figure 23. Two Dimensional Gel Electrophoresis,
Unlabeled Antibody.

Affinity purified monoclonal anti-bovine IgG(25 micrograms) was electrophoresed and stained with Coomassie Blue(see methods section).

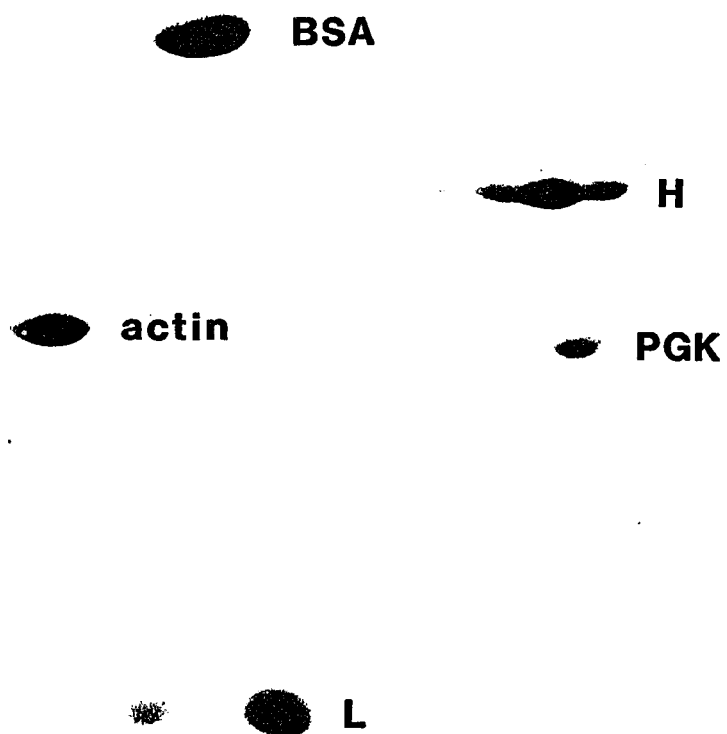


Figure 23. Two Dimensional Gel Electrophoresis, Unlabeled Antibody.

Figure 24. Two Dimensional Gel Electrophoresis,
Labeled Antibody.

Affinity purified FITC-labeled monoclonal anti-bovine IgG(25 micrograms) was electrophoresed and stained with Coomassie Blue(see methods section).

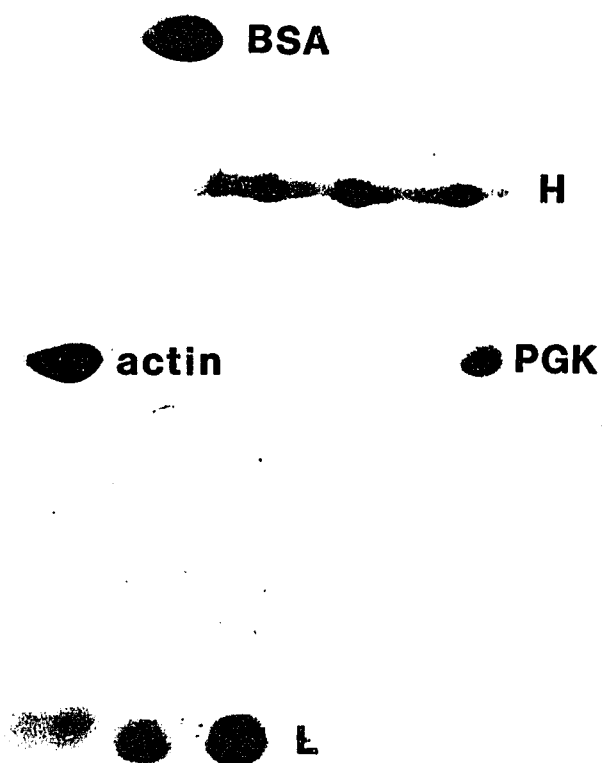


Figure 24. Two Dimensional Gel Electrophoresis, Labeled Antibody.

reducing conditions(Figure 21), one would expect a single high molecular weight spot(51,000 daltons) and a single low molecular weight spot(22,000 daltons). The intrachain disulfide bonds were omitted, but their cleavage would contribute to further fragment heterogeneity. Protein standards bovine serum albumin(BSA), phosphoglycerate kinase(PGK), and actin(see Figure 22 for location) were included for calibration purposes. This diffuse heavy chain band consists of at least 4 different fragment species. The light chain region consists of two spots or two different molecules. In Figure 24, the heavy and light chain fluorescein labeled antibody fragments are found in similar positions as the gel in Figure 23. A noticeable difference was the spreading out of the heavy chain fragments further in the acidic direction. A similar trend was observed with the formation of a third spot in the light chain region. The attachment of fluorescein molecules to the antibody introduces a carboxylic acid group which renders the antibody more acidic.

This heterogeneous mixture of affinity purified antibody may be related to the method of hybridoma production. It is common practice to fuse myeloma cells with spleen cells producing the desired antibody. The widely used myeloma cell line, NS-1, which produces light chain antibody fragments but not the intact antibody(Goding, 1980) was used in the development of this anti-bovine IgG

antibody(Fleenor et al., 1984). As many as 75 percent of the antibodies produced by hybridomas made with NS-1 cells may contain light chains derived from the NS-1 myeloma cell line(Goding, 1980). Affinity chromatography using the immobilized antigen would isolate the desired antibodies in addition to those which contain a light chain derived from each of the cell lines used in the fusion.

The observed heterogeneity of monoclonal antibodies evaluated by electrophoresis was not unique(Hoffman, 1977, Manil et al., 1986, and Pearson and Anderson, 1983). Of the many possible causes of observed isoelectric microheterogeneity of immunoglobulins, Hoffman reported in 1977 that much evidence had accumulated in support of carbohydrate heterogeneity. Carbohydrates were believed to be primarily attached to the heavy chains. Deamidation and sequence variability were also believed to contribute, but little evidence existed at that time.

The two dimensional gel electrophoresis patterns reported here compare well with the least heterogeneous samples of protein A purified mouse monoclonal antibodies examined by two dimensional gel electrophoresis in the literature(Manil et al., 1986; Pearson and Anderson, 1983). The evaluation of heterogeneity of monoclonal antibodies purified against their antigen using two dimensional gel electrophoresis has not been found in the literature. In addition, the covalent attachment of certain labels to

proteins can certainly affect their physical properties. Light chain heterogeneity due to mutation and allelic exclusion has been explained in two recent reviews (Honjo, 1983; Storb et al., 1986). Allelic exclusion was described as the failure of expression of immunoglobulin genes from more than one chromosome. Isotyping of monoclonal anti-bovine IgG (also known as IID12-5), previously performed by Fleenor et al. (1984) did not support the possibility that kappa light chains might have been accompanied by lambda light chains. The presence of carbohydrates known as sialic acids (N-acetylneuraminic acid) on the light chain could also have been responsible for heterogeneity. The complete amino acid and oligosaccharide structure determined for a human lambda type immunoglobulin (Chandrasekaran et al., 1981) supports the possibility of this occurring in mouse myeloma proteins. The number of light chains has often been suggested to be an indication of the number of different types of antibody molecules present. Regardless of the explanation behind the observed multiple spots, these data show that monoclonal antibodies purified by affinity chromatography exhibit structural heterogeneity. The definition of "monoclonal" antibody was traditionally applied to the homogeneous binding properties exhibited by these antibodies. Although few chromatographic methods capable of resolving monoclonal antibody populations into subpopulations have been developed, the traditional use of

the term monoclonal should be kept in mind when referring to hybridoma derived antibodies. Thus "monoclonal" may not imply either homogeneous or monospecific.

The binding of ^{125}I labeled anti-bovine IgG to bovine IgG-Reactigel was studied in the batch mode (equilibrium, Figure 25). These data were fit with a computer simulation with the binding parameters, K_1 , K_2 , q_1 , q_2 , equal to 3×10^8 L/mole, 1×10^5 L/mole, 1.8×10^{-8} M, and 2×10^{-6} M, respectively. The higher binding constant was sufficient to affinity purify reagents and perform a sandwich assay for anti-bovine IgG in the femtomole range (De Alwis and Wilson, 1985).

The explanation for the observed binding heterogeneity has not been firmly established. It has been assumed that the radioisotope labeled, affinity purified antibody would be more homogeneous than that labeled with multiple larger and charged fluorescein labels. Continuing with this line of reasoning, an explanation for the low binding constants for fluorescein labeled antibodies compared to ^{125}I labeled IgG was sought. Hymes, Mullinax and Mullinax, 1979 studied the immunoglobulin requirement of carbohydrate for the formation of IgG-antiIgG complexes. The removal of sialic acids (an amino carbohydrate, N-acetylneuraminic acid) by neuraminidase from the light chain of $\text{F}(\text{ab}')_2$ fragments eliminated complex formation between these $\text{F}(\text{ab}')_2$ fragments and an antiglobulin.

Figure 25. Competitive Equilibrium Radioimmunoassay.

Labeled(^{125}I) and unlabeled affinity purified anti-bovine IgG reacted with bovine IgG-Reactigel. Simulation was performed using VERB1(Appendix B) with the following parameters: $K_1 = 3 \times 10^8$ L/mole; $K_2 = 10^5$ L/mole; $q_1 = 1.8 \times 10^{-8}$ M; $q_2 = 2 \times 10^{-6}$ M and $[F] = 9.25 \times 10^{-7}$ to 7.3×10^{-10} .

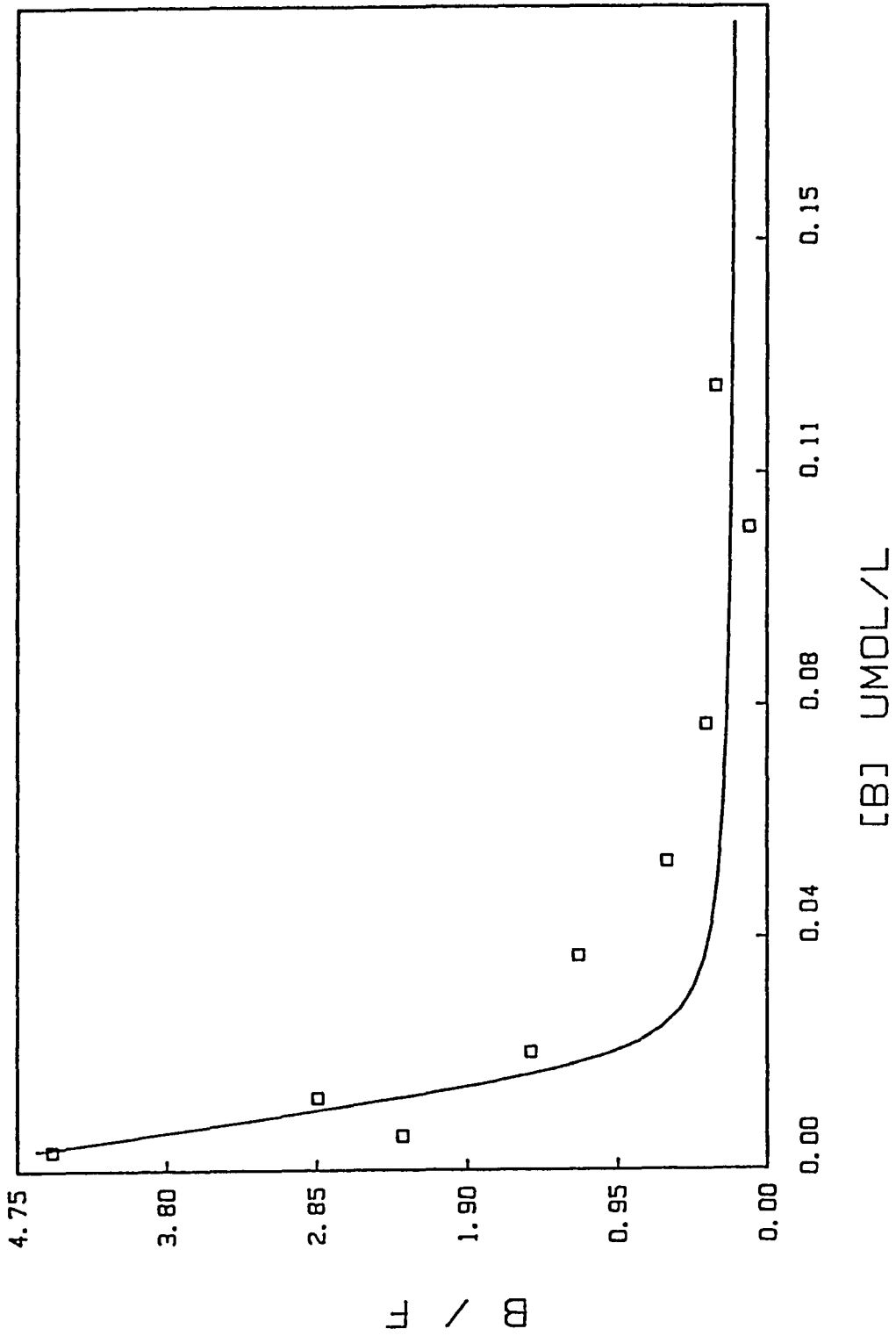


Figure 25. Competitive Equilibrium Radioimmunoassay.

A less likely possibility is based on the assumption that the FITC molecule may attach to the antibody sialic acid in the experiments reported here. This would probably interfere with carbohydrate moieties on reacting antibodies if carbohydrate-carbohydrate interaction were involved in the reaction between these two IgG molecules.

CHAPTER 5

CONCLUDING WORK AND SUGGESTIONS FOR TOMORROW

The use of immunochemical reagents in clinical analysis has taken many avenues since the pioneering work of Berson and Yalow(1959). This chapter will begin with the presentation of two nonisotopic immunoassays performed under flow conditions. Following this suggestions will be described for related investigations.

A sandwich assay using FITC as a protein label has been demonstrated. The typical sandwich assay involves the use of two antibodies which recognize different epitopes on the analyte molecule. In this experiment, the analyte is also an antibody. The stationary phase consists of bovine IgG-Reactigel. The analyte is a monoclonal antibody to bovine IgG, and this is recognized by an antimouse IgG(rabbit) labeled with FITC. The advantage of using a sandwich assay comes from ease of optimization. The response of the assay should vary directly with the analyte concentration. Fluorescence emission due to the free(unbound) antibody decreases and due to the bound peak increases as the analyte concentration increases. There must be an excess number of sites and molecules for the immunosorbent and labeled antibody, respectively over the

range of analyte concentrations studied. The assay(Figure 26) was performed with and without a 6 percent concentration of BSA. The high concentration of BSA, used to mimic a serum solution, made little difference in response to analyte present. The detection limit was 0.75 picomoles of anti-bovine IgG with a S/N of 2. While the analyte and labeled antibody were both previously affinity purified(after labeling the conjugate) before use, a similar assay could be employed to monitor the growth of monoclonal antibodies, using various immunosorbents and purified standards.

An enzyme labeled assay for insulin will be described. The enzyme converts a nonfluorescent substrate into a fluorescent product. The enzyme labeled assay for insulin was first attempted following the procedure of Kitigawa and Aikawa(1976) using beta-galactosidase as a label. Their procedure described the acylation of porcine insulin at a high concentration(6 mg/ml) with meta-maleimidobenzyl N-hydroxysuccinimide ester(MBS) at pH 7.0. This resulted in a saturated cloudy solution. This agreed with the information that insulin is practically insoluble between pH 4 - 7 and has an isoelectric point between 5.3 and 5.35(Merck Index, 1976). Bovine insulin(donated by Lilly Research Laboratories) had to be dissolved in dilute acid(10 mM HCl) before adjustment to a more dilute concentration at a slightly basic pH. Following the

Figure 26. Sandwich Assay for Anti-bovine IgG.

Affinity purified monoclonal anti-bovine IgG was determined using affinity purified FITC anti-mouse IgG (rabbit, 5.0×10^{-8} M), a 20 μ l sample loop, and a column (0.2 cm X 4 cm, flow rate 0.5 ml/min) packed with bovine IgG-Reactigel. Error bars indicate absolute average deviation.

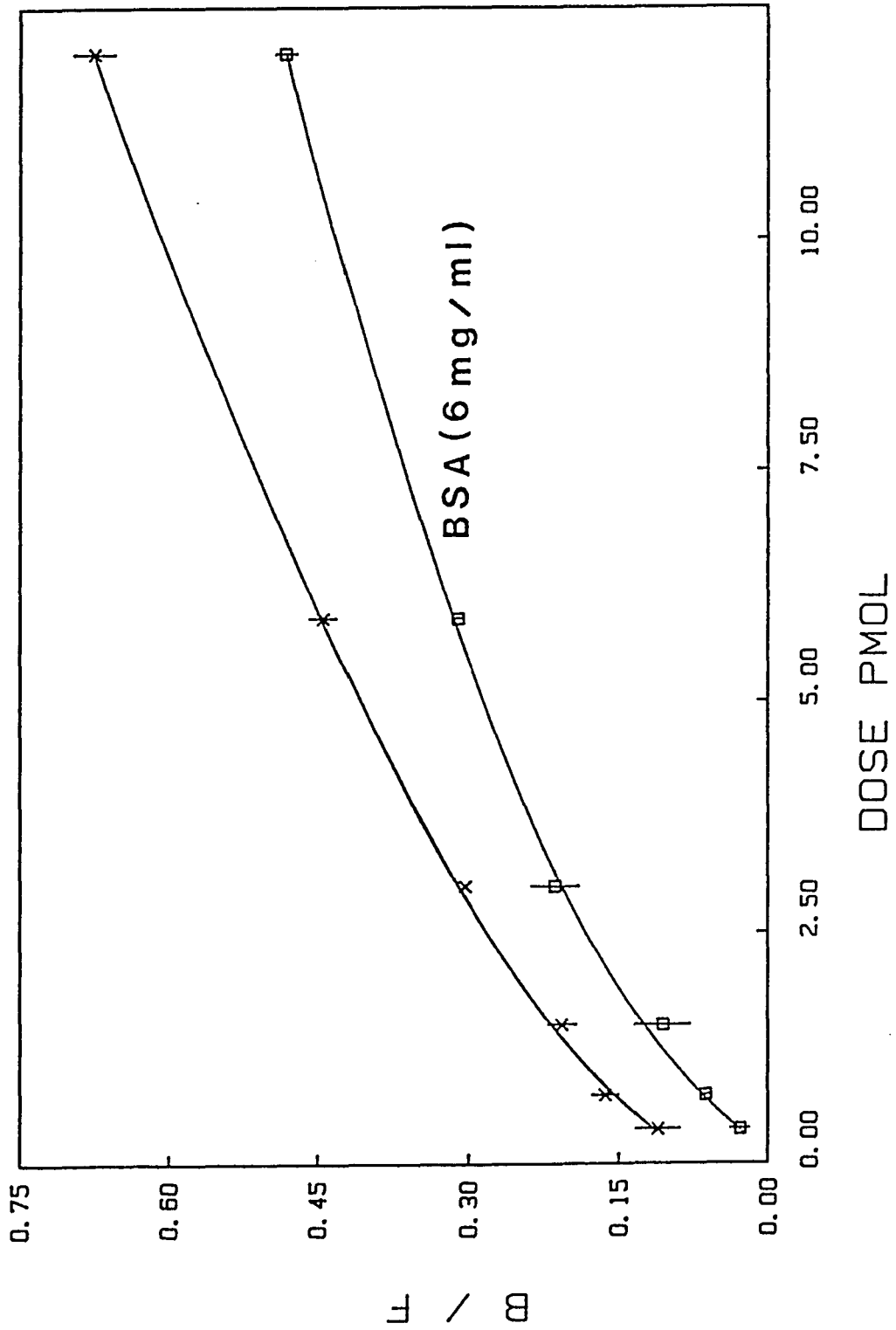


Figure 26. Sandwich Assay for Anti-bovine IgG.

coupling of the MBS acylated insulin to beta-galactosidase the reaction was stopped by addition of 2 M mercaptoethanol to a concentration of 0.01 M as directed by O'Sullivan et al.(1978) for the production of enzyme-antibody conjugates. The excess insulin was removed by size exclusion chromatography. Unfortunately, the resulting conjugate did not appear to bind to anti-insulin immunosorbents to any greater extent than to unmodified Reactigel. It was concluded that the large enzyme(540000 daltons) with a history of binding to various modified Sepharose stationary phases(Morrow, Carbonelli, and McCoy, 1975) was a bad choice as a label for flow through experiments.

Later, another possible problem was considered. Sportsman(1986) expressed concern over the stability of the insulin molecule in the presence of mercaptoethanol. Homogeneous PAGE(5 percent crosslinked gel) without sodium dodecyl sulfate, was performed with and without 0.01 M mercaptoethanol in the sample. The gel showed a dramatic difference in the two insulin samples. The problems observed earlier may have been due in part to denatured insulin, because the electrophoresis showed two well separated protein bands, probably due to separate insulin chains A and B.

Insulin was labeled with horseradish peroxidase following the method of Nakane and Wilson(1978). The insulin-conjugate was isolated on a Sephadex G-75 column(2

cm X 32 cm, 0.25 ml/min). The first two protein peaks to elute were both enzymatically and immunologically active. The immunoreactivity was evaluated using a minicolumn containing anti-insulin immunosorbent. Unfortunately, the affinity purified conjugate was no longer enzymatically active. The loss of the heme group from the enzyme was confirmed by a severe reduction in absorbance of the protein solution at 403 nm. According to Whitaker(1972) the ferriprotoporphyrin III may be replaced to recover most of the original activity.

The molar ratio of horseradish peroxidase to insulin was 1.07 and 1.22 for protein peaks eluting first and second, respectively, from the size exclusion column. These ratios were determined by measuring the amount of ¹²⁵I labeled insulin tracer and the moles of horseradish peroxidase by absorption of the heme group. The first peak eluting contained some enzyme-enzyme conjugates as well as multiples of the 1:1 insulin-enzyme conjugate. The exclusion limit of the G-75 column was approximately 70000 daltons. The second peak, was chosen for use in a competitive assay. Later, the procedure was modified as described in Chapter 2 to improve the amount of heterodimer, insulin-peroxidase formed. The development of this competitive assay is still in progress. The enzyme label, horseradish peroxidase bound to the antiinsulin affinity column to the same degree as the insulin-enzyme

conjugate(based on the concentration of peroxidase). Later, it was observed that washing the column with pH 7.4 phosphate buffered saline(PBS) containing 0.5 percent Tween 20, removed the enzyme(not conjugated) but not the insulin enzyme conjugate. The conjugate was eluted from the column with pH 2.2 PBS. Under these conditions it should be possible to optimize a dose response curve.

As discussed in Chapter 3, the transition from using radioisotopic to nonradioisotopic methods will proceed more readily when a better understanding of this optimization process exists.

The future work to be considered has to do with developing methods. A continuing area of interest in the production of antibodies is the rapid and reliable determination of antibody affinity and concentration. The overall process could be completed within 4 hours, assuming appropriate manual or automatic operation.

One experiment which comes to mind involves the use of a stationary phase which can quickly(15 seconds) bind proteins through covalent linkage. The activated stationary phase could be packed into an HPLC column followed by introduction of a sample of antiserum or ascites fluid into the mobile phase. The second step would be to deactivate uncoupled sites with a noninteracting protein such as BSA. The immunosorbent could next be washed with alternate low and high pH buffer mobile phases suitable for affinity

purification. The immunosorbent would be saturated with labeled antigen(repeated injections without washing) and then a competitive binding experiment performed.

While this stationary phase is not commercially available, an activated porous membrane called immobilon has been introduced by the Millipore Corporation(Blankstein and Dohrman, 1985). The polymer base is polyvinylidene difluoride modified with a proprietary coupling reagent. The membrane might be sandwiched into a channel made between two pieces of plexiglass with appropriate input and output ports for the mobile phase. A conventional beaded or silica based support activated with the appropriate chemical functionality would be expected to outperform any membrane with respect to the available surface area and the amount of peak band broadening.

Another area for further investigation involves improving the procedure for assay development using the flow through method. Up to now, optimization has been based on titer experiments performed in the batch mode(equilibrium). The titer experiment as described by Sportsman(1982) allows one to determine the correct dilution of immunosorbent for a given range of antigen concentration over which a dose response curve is to be generated. The procedure consists of the reaction of a series of immunosorbent dilutions with 1) radioisotope labeled antigen alone and 2) a fixed ratio of labeled and unlabeled antigen. The difference in bound

labeled antigen for immunosorbent dilutions in steps 1 and 2 defines the conditions (immunosorbent dilution appropriate to analyte concentration range) to be used for the assay.

It should be possible to perform a similar experiment without radioisotopes using the flow through method. Starting with the immunosorbent, an arbitrary dilution should be made first on the basis of an educated guess. Knowing the desired antigen concentration range for the assay to be developed, the column dilution should be made so that not all of the analyte molecules at the minimum dose concentration may bind to the column. A dilution series of antigen standards containing a fixed ratio of labeled to unlabeled molecules should be introduced into this column. Choose the starting concentration (number one in dilution series) with an unlabeled analyte concentration in the middle of the range for which the assay is to be developed. Choose the labeled analyte concentration such that sensitivity requirements are met and the rate of usage allows economical consumption of this reagent. The result should appear similar to curve A in Figure 13(B/F vs B) of Chapter 4. If no maximum appears then a higher or lower dilution of immunosorbent should be attempted. Ideally the maximum will occur close to the initial dilution of antigen (labeled and unlabeled). Based on previous experience the maximum is related in some way to finding the optimum antigen concentration range for a particular

dilution of immunosorbent. If the maximum should move to lower concentration(dose concentrations) as the immunosorbent is diluted.

Immunological reagents and development time are both costly. With the available software(Appendix B) it should be possible to consume less of each, performing batch or flow through experiments. One may prepare as few as 3 samples for evaluation where sample 1 contains no unlabeled analyte(antigen or antibody), sample 2 contains the maximum concentration of unlabeled analyte of interest, and sample 3 contains some concentration of unlabeled analyte intermediate between samples 1 and 2. A fixed concentration of labeled analyte is added to each sample. The difference in the average responses of samples 1 and 2 above define the maximum change in signal over that concentration range for those experimental conditions. Together, the three data points allow one to make predictions of the over all picture using the available software(Appendix B). The more rigorous evaluation of many standards for a competitive binding assay may then be performed.

The detailed investigation and characterization of sandwich immunoassays would be an interesting pursuit. Labeling the middle and last components in the sandwich scheme with different radioisotopic tags(^{14}C and ^{125}I for example) would allow one to follow both steps of sandwich formation independently. This might be performed using

Removawells as described in Chapter 2. An alternative would be to prepare an immunosorbent and pack this into a glass column. Placing the glass column in the gamma well of a counter(measure ^{125}I) followed by postcolumn mixing with a scintillation cocktail and an absorbance flowcell(measure ^{14}C) may be one possible monitoring scheme. The free and bound fractions for each step of the sandwich should be measured(directly or indirectly) for each component of the sandwich.

Soluble affinity chromatography provides another avenue for possible investigation. In view of the many advantages of this technique over solid phase affinity chromatography made by Warden and Giese(1984), it might be possible in some cases to determine binding parameters in addition to making analytical measurements. The rapid determination of human IgG was carried out by Hosotsubo, Arai, and Iwamura(1985) using FITC-labeled antibody and HPLC size exclusion chromatography. Binding parameters obtained through evaluation of an immunochemical reaction with the appropriate HPLC size exclusion or ion exchange column would need to be validated using more conventional methods.

As the demand for simple and reliable immunoassays requiring little or no instrumentation increases, a greater burden is placed on the manufacturer. This burden involves research to develop a thorough understanding of the specific reaction behavior for each particular immunochemical system.

Custom reagents produced through hybridoma and genetic engineering technologies are expensive and must be used effectively to reduce the cost of assays. Long term storage at room temperature or in dry form further complicate assay development.

Proteins immobilized on insoluble supports through covalent attachment combined with conventional HPLC instrumentation will continue to be a good starting place for the characterization of binding interactions under controlled experimental conditions. While monoclonal antibodies are much less heterogeneous than polyclonal antibodies in terms of structure and binding behavior, the term "monoclonal" is still misleading. Perhaps it would be more appropriate to use the terms monoclonal and polyclonal more loosely to describe only the method of antibody production. Although antibodies are often associated with high binding constants, the interaction with antigens is a dynamic equilibrium dependent upon concentration, pH, solvent polarity, ionic strength, temperature, and covalent modification (labeling, fragmentation, and immobilization). Obtaining information on the behavior of a particular antibody-antigen couple from the line shape of binding data using a particular graphical method is a very complicated task. Fitting the raw binding data using various computer simulations has shown how multiple interpretations may easily be obtained. Complementary experiments and data

manipulations will enable one to make a more accurate assessment of reaction behavior.

APPENDIX A

The following information has been provided in order to facilitate the use of several instruments comprising a specific chromatographic system. This information does not in any way take the place of reading appropriate instrument manuals.

HPLC Pump Care / Use

Washing

Concentrated salt solutions are very corrosive to stainless steel pump heads and associated tubing. When using concentrated salt solutions, wash the pump thoroughly after each use. Rinse with doubly distilled water for at least 15 min at 0.5 ml/min. While washing, also rinse the pistons externally with copious amounts of deionized water. Store the pump in water or methanol when not in use. Water is preferred in dry environments as methanol evaporates more readily.

Routine Maintenance

The performance of the pump unit may be checked using the built-in diagnostics (Spectra Physics Model 8700 Operation Manual). Often poor performance readings on the check valve test will be observed if they need to be cleaned or the mobile phase contains particulate matter. Remove and

wash the check valves(use an ultrasonicator and filtered solvents) before 1) refiltering the mobile phase solvents and cleaning the inlet spargers and filters and 2) replacing the piston seals. The procedure for replacing the seals is described in the manual. When replacing the seals(yellow with spring), the washers(black) do not need to be replaced every time. Whenever repairs of injectors or pump seals are undertaken, do not use paper towels or any fibrous cloth material. Instead use only filtered solvents such as methanol or water.

Operation

After sitting idle for a period of time the Spectra Physics 8700 pump often will not start pumping at a requested rate. One must tell the pump to purge for a few seconds followed by normal programmed flow rate operation. In addition, the flow rate should be checked once during every experiment. During operation the pump pressure should be displayed on the control panel to indicate malfunctions(rapid changes in pump pressure - greater than ± 10 psi after the appearance of the flow steady light).

The proper function of a feedback stabilized(computer controlled) pump will only be achieved with some backpressure resistance. Low pressure stationary phases such as Reactigel 6X do not provide sufficient backpressure. A small backpressure column may be prepared

with silica after the fines are removed as described below.

1. Suspend 10 u silica in isopropyl alcohol with an ultrasonicator.
2. Allow settling for 15 - 25 min then discard the supernatant.
3. Repeat steps 1 and 2 twice.

Stainless steel column frits may be cleaned in dilute HNO_3 (25 percent concentrated HNO_3 in deionized water). Ultrasonicate the frits for 30 min followed by washing away the HNO_3 solution. Store the frits dry or in methanol.

Manual Injector Valves

These must be rinsed of all salt solutions following use. Most syringe needles must be cut short before using with these injectors (make blunt cut as when cutting stainless steel HPLC tubing). A syringe needle of the wrong length may reach the injector rotor scarring the hard rubber surface, thereby destroying the effectiveness of the injector.

Autosampler Care / Use

When using teflon cap liners in the sample vials, the Pharmacia autosampler (Act 100) injector needle must be sharpened after each experiment. Whenever new pump tubing (use only 1 mm id tubing) is installed on the Act 100 peristaltic pump, recalibrate the pump and measure the

volume(weigh samples before and after) taken up by the pump rather than delivered as suggested in the operation manual. Set the flow rate under 200 ul/min. Whenever the needle height is adjusted(Operation Mode 8), preset parameters including pump calibration are loaded.

While the manufacturer suggests a delay volume of 0.08 ml, 0.11 to 0.13 ml was found to be more appropriate. Visually follow the progression of the sample through the transparent tubing and injection loop for optimization.

In operation mode 1 with a 25 ul sample loop in place, the following volumes were used: 0.05 ml(wash); 0.08 ml(load); and 0.13 ml(delay). After each use the injector needle was washed with several milliliters of filtered doubly distilled water to remove salt and protein residues.

When samples were dissolved in PBS buffer containing 0.5% Tween 20(a non ionic surfactant) small bubbles collected along autosampler tubing which severely interfered with operation.

Automation

With appropriate time sequencing the Pharmacia autosampler(Act 100) can control the operation of the Spectra Physics 8700 pump and the Hewlett Packard 3390A integrator using TTL logic compatible signals(Figure 27). The analysis time(Act 100), gradient time(SP 8700), and collection time(HP 3390A) must each be carefully set.

Figure 27. Schematic for Automatic Control of Chromatographic Analysis.

The relay chip is located inside the integrator. Wires from pins 2 and 3 of the relay chip may be interchanged with the +5 V, 20 mA or run/grad posts on the pump barrier strip.

AUTOSAMPLER	PUMP	RELAY CHIP	INTEGRATOR
Pharmacia Act-100	Spectra Physics 8700	Magnecraft W117 DIP-68	Hewlett Packard 3390A
Remote Connector	Barrier Strip	Chip (Top view)	Connector J-104

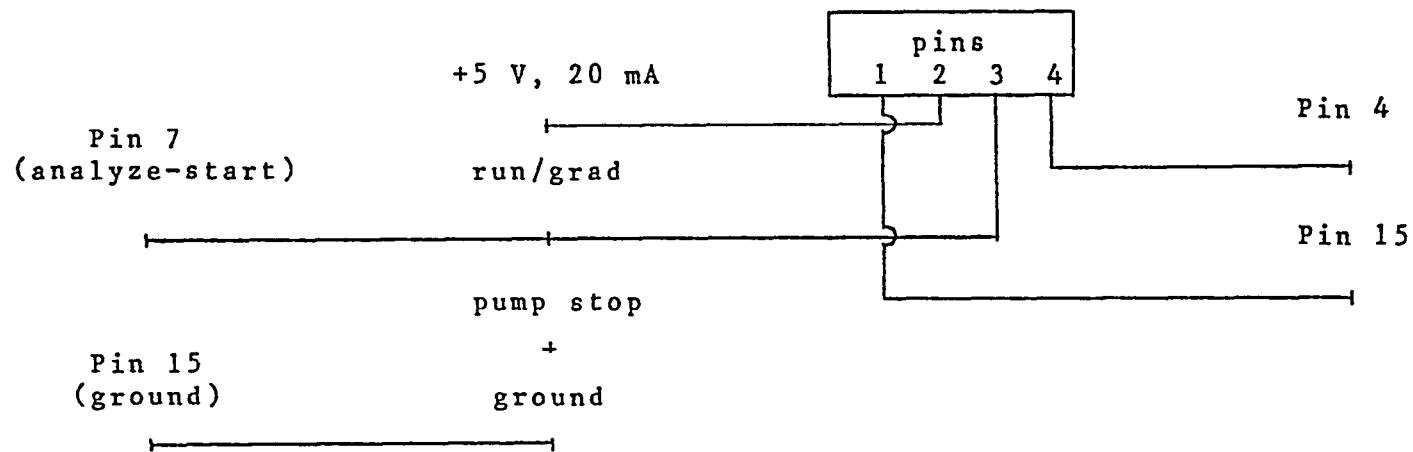


Figure 27. Schematic for automatic control of chromatographic analysis.

The SP 8700 pump has a barrier strip on the back cover with the following electrical contacts: 1) +5 volts at 20 mA; 2) run/grad; 3) stop pump; and 4) ground. The pump gradient can be started by bringing the run/grad contact briefly to ground.

The Act 100 autosampler brings pin 7(analyze-start) of the remote socket to ground at the start of injection(otherwise it floats - open collector). Pin 15(autosampler ground) was connected to the ground contact on the SP 8700 pump. Pin 7 of the Act 100 was connected to the run/grad contact on the SP 8700 pump. In this configuration the SP 8700 begins a gradient when the autosampler injects the sample into the mobile phase.

The start button(pins 4 and 15 on connector J104) on the HP 3390A integrator was bypassed through the use of a 5 volt relay chip located under the integrator cover. The black lead from the relay chip was connected to the run/grad contact on the SP 8700 pump, and the white lead was connected to the +5 volt contact on the pump.

APPENDIX B

Computer programs reported here have made possible the interpretation and prediction of immunochemical reaction behavior. These FORTRAN programs were developed by Amarasiri Fernando of this laboratory. The basic assumptions and relationships used were described in Chapter 4. Some common terms will be defined below.

NPTS = number of data points desired

K = binding constant

BS = fixed concentration(moles/liter) of antibody binding sites(q)

UAN = concentration(moles/liter) of unlabeled antigen(p)

LAN = concentration(moles/liter) of labeled antigen(p*)

B = concentration(moles/liter) of antigen complexed with antibody

F = concentration(moles/liter) of free antigen

T = total concentration(moles/liter) of antigen; $T = F + B$

BO = response or bound concentration(moles/liter) at zero dose

[Ag] = free antigen concentration

n = number of binding sites

r = [bound antigen] / [total antibody]; an expression of the extent of reaction

Program SCA3

This program considers the situation in which only labeled antigen is present. The following variables are included: NPTS; K; BS; and LAN(N) to LAN(1). Plots available are listed below.

1. free LAN versus total LAN
2. bound LAN versus total LAN
3. free BS versus total LAN
4. bound BS versus total LAN
5. B / F versus bound LAN
6. B / F versus total LAN

Scatchard Plots

7. $r / [Ag]$ versus r for $r/[Ag] = nK - rK$
 8. $1 / r$ versus $1 / [Ag]$ for $1/r = n[Ag]K + 1/n$
- Sips Plot for $\text{Log } [r/(n-r)] = a \text{Log } [Ag] + a \text{log } K$
9. $\text{Log } [r/(n-r)]$ versus $\text{Log } [Ag]$

```

c   program sca3; binding assay; under equil conditions
c
COMMON/HEADER/NPTS,KEY,PRAMS(10,6),ICMNT(40),IFILE(8)
VIRTUAL BLAN(2100,10),R(2100,10)
VIRTUAL TLAN(2100,10),FLAN(2100,10)
VIRTUAL FBS(2100,10)
VIRTUAL BBS(2100,10)
VIRTUAL BLABS(2100,10),RBS(2100,10)
VIRTUAL DFLAN(2100,10),DBLABS(2100,10)
VIRTUAL LFLAN(2100,10),LBLABS(2100,10)
DIMENSION LAN(101),B(100),D(100)
REAL LAN,INCR,K,LFLAN,LBLABS
INTEGER ACTION,SIZE,NUM
ACTION = 'WR'
SIZE = 'WH'
KEY = 1
NUM = 1
TYPE*, 'PROGRAM SCA3'
TYPE*, 'PROGRAMMER: S.A.FERNANDO'

```

```

TYPE*, '*****'
TYPE*, '          BINDING ASSAY'
TYPE*, '*****'
TYPE*, 'THIS PROGRAM COMPUTES BINDING PARAMETERS FOR'
TYPE*, '          BINDING ASSAYS'
TYPE*, 'ONLY LABELED ANTIBODY OR ANTIGEN IS USED'
TYPE*, 'PLEASE ENTER YOUR DATA IN THE FOLLOWING ORDER'
TYPE*, 'N,K,BS,LAN(I)'

c
c   number of data points
c
TYPE*, 'PLEASE ENTER NUMBER OF DATA POINTS, UP TO 100'
READ(5,5000) N
WRITE(6,5100) N
NPTS = N

c
c   binding constant
c
TYPE*, 'PLEASE ENTER THE BINDING CONSTANT (K)'
READ(5,5200) K
WRITE(6,5300) K

c
c   binding sites (BS) or antibody concentration
c
TYPE*, 'PLEASE ENTER THE FIXED CONCENTRATION OF'
TYPE*, 'ANTIBODY (BS)'
READ(5,5200) BS
WRITE(6,5400) BS

c
c   labeled antigen (LAN)
c
TYPE*, 'LABELED ANTIGEN CONCENTRATION (LAN)'
TYPE*, 'DO YOU WISH TO HAVE CONCENTRATION RANGE'
TYPE*, 'FOR LAN?'
TYPE*, 'WHAT IS THE RANGE FOR LAN CONCENTRATION?'
TYPE*, '
TYPE*, 'HIGHEST LAN ='
READ(5,5200) LAN(N)
TYPE*, 'LOWEST LAN ='
READ(5,5200) LAN(1)
WRITE(6,5500)
WRITE(6,5600) LAN(N),LAN(1)

c
c   calculation of increment
c
INCR = (ALOG10(LAN(N)) - ALOG10(LAN(1)))/(N - 1)
WRITE(6,5700) INCR

c
c   calculation of B/F ratio
c
Z = 10.0

```

```

DO 100 I = 1,10
B(I) = K*LAN(I) - K*BS + 1.0
C = -K*BS
D(I) = SQRT((B(I))**2 - 4.0*C)
R(I,10) = (-B(I) + D(I))/2.0
FLAN(I,10) = LAN(I)/(R(I,10) + 1.0)
BLAN(I,10) = (LAN(I))*(R(I,10))/(R(I,10) + 1.0)
TLAN(I,10) = FLAN(I,10) + BLAN(I,10)
FBS(I,10) = BS - BLAN(I,10)
BBS(I,10) = BS - FBS(I,10)
LAN(I+1) = Z**(ALOG10(LAN(I)) + INCR)
100 CONTINUE
c
TYPE*, 'LABELED ANTIGEN CONCENTRATION (LAN)'
TYPE*, 'DO YOU WISH TO HAVE A PRINTOUT (Y/N)?'
READ(5,5800) ANS
WRITE(7,600)
WRITE(7,6100) (LAN(I),I=1,N)
IF(ANS.EQ.'N') GO TO 200
WRITE(6,6000)
WRITE(6,6100) (LAN(I),I=1,N)
c
200 TYPE*, '
TYPE*, 'PLOT: FREE Ag CONC. VS TOTAL Ag CONC.'
TYPE*, 'DO YOU WISH TO HAVE A PRINTOUT (Y/N)?'
READ(5,5800) ANS
WRITE(7,6200)
WRITE(7,5600) ((TLAN(I,10),FLAN(I,10)),I=1,N)
IF(ANS.EQ.'N') GO TO 300
WRITE(6,6200)
WRITE(6,5600) ((TLAN(I,10),FLAN(I,10)),I=1,N)
300 TYPE*, 'DO YOU WISH TO WRITE VALUES TO THE DISC (Y/N)?'
READ(5,5800) ANS
IF(ANS.EQ.'N') GO TO 400
CALL CAL(IFILE)
CALL DISC10(ACTION,SIZE,TLAN,FLAN,NUM)
c
400 TYPE*, '
TYPE*, 'PLOT: BOUND Ag CONC. VS TOTAL Ag CONC.'
TYPE*, 'DO YOU WISH TO HAVE A PRINTOUT (Y/N)?'
READ(5,5800) ANS
WRITE(7,6300)
WRITE(7,5600) ((TLAN(I,10),BLAN(I,10)),I=1,N)
IF(ANS.EQ.'N') GO TO 500
WRITE(6,6300)
WRITE(6,5600) ((TLAN(I,10),BLAN(I,10)),I=1,N)
500 TYPE*, 'DO YOU WISH TO WRITE VALUES TO THE DISC (Y/N)?'
READ(5,5800) ANS
IF(ANS.EQ.'N') GO TO 600
CALL CAL(IFILE)
CALL DISC10(ACTION,SIZE,TLAN,BLAN,NUM)

```

```

c
600  TYPE*, '
      TYPE*, 'PLOT:  FREE ANTIBODY CONC. '
      TYPE*, '      VS TOTAL ANTIGEN CONC. '
      TYPE*, 'DO YOU WISH TO HAVE A PRINTOUT (Y/N)? '
      READ(5,5800) ANS
      WRITE(7,6400)
      WRITE(7,5600) ((TLAN(I,10),FBS(I,10)),I=1,N)
      IF(ANS.EQ.'N') GO TO 700
      WRITE(6,6400)
      WRITE(6,5600) ((TLAN(I,10),FBS(I,10)),I=1,N)
700  TYPE*, 'DO YOU WISH TO WRITE VALUES TO THE DISC (Y/N)? '
      READ(5,5800) AND
      IF(ANS.EQ.'N') GO TO 800
      CALL CAL(IFILE)
      CALL DISC10(ACTION,SIZE,TLAN,FBS,NUM)

c
800  TYPE*, '
      TYPE*, 'PLOT:  BOUND AB(BBS) VS TOTAL Ag '
      TYPE*, 'DO YOU WISH TO HAVE A PRINTOUT (Y/N)? '
      READ(5,5800) ANS
      WRITE(7,6500)
      WRITE(7,5600) ((TLAN(I,10),BBS(I,10)),I=1,N)
      IF(ANS.EQ.'N') GO TO 900
      WRITE(6,6500)
      WRITE(6,5600) ((TLAN(I,10),BBS(I,10)),I=1,N)
900  TYPE*, 'DO YOU WISH TO WRITE VALUES TO THE DISC (Y/N)? '
      READ(5,5800) ANS
      IF(ANS.EQ.'N') GO TO 1000
      CALL CAL(IFILE)
      CALL DISC10(ACTION,SIZE,TLAN,BBS,NUM)

c
1000 TYPE*, '
      TYPE*, 'PLOT:  B/F VS B '
      TYPE*, 'DO YOU WISH TO HAVE A PRINTOUT (Y/N)? '
      READ(5,5800) ANS
      WRITE(7,5900)
      WRITE(7,5600) ((BLAN(I,10),R(I,10)),I=1,N)
      IF(ANS.EQ.'N') GO TO 1100
      WRITE(6,5900)
      WRITE(6,5600) ((BLAN(I,10),R(I,10)),I=1,N)
1100 TYPE*, 'DO YOU WISH TO WRITE VALUES TO THE DISC (Y/N)? '
      READ(5,5800) ANS
      IF(ANS.EQ.'N') GO TO 1200
      CALL CAL(IFILE)
      CALL DISC10(ACTION,SIZE,BLAN,R,NUM)

c
1200 TYPE*, '
      TYPE*, 'PLOT:  B/F VS TOTAL '
      TYPE*, 'DO YOU WISH TO HAVE A PRINTOUT (Y/N)? '
      READ(5,5800) ANS

```

```

WRITE(7,6600)
WRITE(7,5600) ((TLAN(I,10),R(I,10)),I=1,N)
IF(ANS.EQ.'N') GO TO 1300
WRITE(6,6600)
WRITE(7,5600) ((TLAN(I,10),R(I,10)),I=1,N)
1300 TYPE*, 'DO YOU WISH TO WRITE VALUES TO THE DISC (Y/N)?'
READ(5,5800) ANS
IF(ANS.EQ.'N') GO TO 1400
CALL CAL(IFILE)
CALL DISC10(ACTION,SIZE,TLAN,R,NUM)
c
1400 TYPE*, '
TYPE*, 'SCATCHARD PLOT:   $r/[Ag] = -rK + nK$ 
TYPE*, '
TYPE*, 'PLOT:   $r/[Ag]$  VS  $r$ '
c
DO 1500 I = 1,N
BLABS(I,10) = BLAN(I,10)/BS
RBS(I,10) = R(I,10)/BS
1500 CONTINUE
c
TYPE*, 'DO YOU WISH TO HAVE A PRINTOUT (Y/N)?'
READ(5,5800) ANS
WRITE(7,6700)
WRITE(7,5600) ((BLABS(I,10),RBS(I,10)),I=1,N)
IF(ANS.EQ.'N') GO TO 1600
WRITE(6,6700)
WRITE(6,5600) ((BLABS(I,10),RBS(I,10)),I=1,N)
1600 TYPE*, 'DO YOU WISH TO WRITE VALUES TO THE DISC (Y/N)?'
READ(5,5800) ANS
IF(ANS.EQ.'N') GO TO 1700
CALL CAL(IFILE)
CALL DISC10(ACTION,SIZE,BLABS,RBS,NUM)
c
1700 TYPE*, '
TYPE*, 'PLOT:   $1/r$  VS  $1/[Ag]$  ;  $1/r = 1/n[Ag]K + 1/n$ '
c
DO 1800 I = 1,N
DBLABS(I,10) = 1/BLABS(I,10)
DFLAN(I,10) = 1/FLAN(I,10)
1800 CONTINUE
c
TYPE*, 'DO YOU WISH TO HAVE A PRINTOUT (Y/N)?'
READ(5,5800) ANS
WRITE(7,6800)
WRITE(7,5600) ((DFLAN(I,10),DBLABS(I,10)),I=1,N)
IF(ANS.EQ.'N') GO TO 1900
WRITE(6,6800)
WRITE(6,5600) ((DFLAN(I,10),DBLABS(I,10)),I=1,N)
1900 TYPE*, 'DO YOU WISH TO HAVE A PRINTOUT (Y/N)?'
READ(5,5800) ANS

```

```

IF(ANS.EQ.'N') GO TO 2000
CALL CAL(IFILE)
CALL DISC10(ACTION,SIZE,DFLAN,DBLABS,NUM)
c
2000 TYPE*,'
c
TYPE*, 'SIPS PLOT:  LOG[r/(n - r)] = aLOG[Ag] + aLOGK'
c
DO 2100 I = 1,N
LFLAN(I,10) = ALOG10(FLAN(I,10))
LBLABS(I,10) = ALOG(BLABS(I,10))/(1 - BLABS(I,10))
2100 CONTINUE
c
TYPE*, 'DO YOU WISH TO HAVE A PRINTOUT (Y/N)?'
READ(5,5800) ANS
WRITE(7,6900)
WRITE(7,5600) ((LFLAN(I,10),LBLABS(I,10)),I=1,N)
IF(ANS.EQ.'N') GO TO 2200
WRITE(6,6900)
WRITE(6,5600) ((LFLAN(I,10),LBLABS(I,10)),I=1,N)
2200 TYPE*, 'DO YOU WISH TO WRITE VALUES TO THE DISC (Y/N)?'
READ(5,5800) ANS
IF(ANS.EQ.'N') GO TO 2300
CALL CAL(IFILE)
CALL DISC10(ACTION,SIZE,LFLAN,LBLABS.NUM)
c
2300 TYPE*,'
5000 FORMAT(I3)
5100 FORMAT(5X,'NUMBER OF DATA POINTS',5X,I3)
5200 FORMAT(E15.4)
5300 FORMAT(5X,'AFFINITY CONSTANT (K)',E15.4)
5400 FORMAT(5X,'ANTIBODY CONCENTRATION',E15.4)
5500 FORMAT(5X,'HIGH LAB Ag CONC.',10X,'LOW LAB Ag CONC.')
5600 FORMAT(5X,E15.4,5X,E15.4)
5700 FORMAT(5X,' INCR ',E15.4)
5800 FORMAT(A1)
5900 FORMAT(5X,'BOUND CONC.', ' B/F ')
6000 FORMAT(5X,'CONC. RANGE FOR LABELED ANTIGEN')
6100 FORMAT(E15.4)
6200 FORMAT(5X,'TOTAL Ag',10X,'FREE Ag')
6300 FORMAT(5X,'TOTAL Ag',10X,'BOUND Ag')
6400 FORMAT(5X,'TOTAL Ag',10X,'FREE Ab')
6500 FORMAT(5X,'TOTAL Ag',10X,'BOUND Ab')
6600 FORMAT(5X,'TOTAL Ag',10X,' B/F ')
6700 FORMAT(5X,' r ',10X,' r/[Ag] ')
6800 FORMAT(5X,' 1/[Ag] ',10X,' 1/r ')
6900 FORMAT(5X,' LOG[Ag] ',10X,' LOG[r/(n - r)]')
STOP
END

```

```
      SUBROUTINE CAL(JFILE)
      DIMENSION JFILE(8)
      TYPE*, 'PLEASE INPUT THE NAME OF THE FILE'
      READ(5,9100) (JFILE(J),J=1,8)
      WRITE(6,9200) (JFILE(J),J=1,8)
      WRITE(7,9200) (JFILE(J),J=1,8)
9100  FORMAT(8A2)
9200  FORMAT(5X, 'THE NAME OF THE FILE ',8A2)
      RETURN
      END
```

Program VERB1

This program allows one to employ two binding constants to describe immunochemical reaction behavior. The following parameters are required: NPTS; K1; K2; BS1; BS2; and the range of free UAN(not total UAN). The plots available are listed below.

1. B / F versus [B]
2. [B] versus [T]
3. [F] versus [T]

```

c   program verb1
c
c   This program generates Scatchard Plots for
c   up to two different antibody populations, each with
c   its own concentration and value of K. A monovalent
c   interaction with a single type of antigenic
c   determinant is assumed for a reaction given by:
c   Ab + Ag ---- Ab-Ag, where K is defined as
c    $K = \frac{[Ab-Ag]}{[Ab][Ag]}$ . The user may select the range
c   of antigen concentrations to be studied, and the
c   number of points to be generated. The Scatchard
c   Equation is:  $\frac{[B]}{[F]} = \frac{NK}{1 + K[F]}$ , where B = bound
c   antigen concentration, [F] = free antigen
c   concentration, N = NBS = number of binding sites,
c   and K = binding constants.
c   The above equation can be written as
c    $[B] = \frac{NK[F]}{1 + K[F]}$ 
c   For two different Antibody populations
c    $[B] = [B1] + [B2] = \frac{N1K1[F]}{1 + K1[F]} + \frac{N2K2[F]}{1 + K2[F]}$ 
c
COMMON/HEADER/NPTS,KEY,PRAMS(10,6),ICMNT(40),IFILE(8)
VIRTUAL B(2100,10),BF(2100,10)
VIRTUAL T(2100,10),ADUM(2100,10)
DIMENSION A(2100),C(200),K(2),NBS(2)
REAL K,NBS,INCR
INTEGER ACTION,SIZE,NUM
ACTION = 'WR'
SIZE = 'WH'
KEY = 1
NUM = 1
1  CONTINUE
TYPE*, 'PROGRAM VERB1'

```

```

TYPE*, 'PROGRAMMER : S.A.FERNANDO'
TYPE*, 'THIS PROGRAM COMPUTES BINDING PARAMETERS FOR'
TYPE*, 'SCATCHARD TYPE PLOTS:TWO DIFFERENT'
TYPE*, 'BINDING SITES'
TYPE*, 'DATA REQUIRED TO USE THIS PROGRAM'
TYPE*, 'N,NBS(I),K(I),A(I)'
TYPE*, '
TYPE*, '*****'
TYPE*, 'SCATCHARD TYPE PLOTS: ANTIBODY-ANTIGEN'
TYPE*, 'INTERACTIONS'
TYPE*, '*****'
c
c   number of binding sites(NBS)
c
TYPE*, '
TYPE*, 'PLEASE ENTER NUMBER OF BINDING SITES(AB CONC)'
TYPE*, 'ORDER OF ENTERING:NBS(1),RETURN,THEN NBS(2)'
DO 100 I=1,2
READ(5,5900) NBS(I)
WRITE(6,6000) NBS(I)
WRITE(7,6000) NBS(I)
100 CONTINUE
c
c   number of data points wanted
c
TYPE*, 'NUMBER OF DATA POINTS WANTED (N)'
READ(5,6100) N
WRITE(6,6200) N
WRITE(7,6200) N
NPTS = N
c
c   binding constants(K)
c
TYPE*, 'PLEASE ENTER BINDING CONSTANTS :K1,K2'
TYPE*, 'THE ORDER OF ENTERING:K1,HIT RETURN,THEN K2'
READ(5,5900) (K(I),I=1,2)
WRITE(6,6300) (K(I),I=1,2)
WRITE(7,6300) (K(I),I=1,2)
c
c   assume [AG] standard = [F] free antigen concentration
c   where [F] = total AG - bound AG(AG-AB)
c
TYPE*, 'WHAT IS THE RANGE OF FREE ANTIGEN CONC'
TYPE*, 'HIGHEST [AG](FREE ANTIGEN) = '
READ(5,5900) A(N)
TYPE*, 'LOWEST [AG](FREE ANTIGEN) = '
READ(5,5900) A(1)
WRITE(6,6400)
WRITE(7,6400)
WRITE(6,5200) A(N),A(1)
WRITE(7,5200) A(N),A(1)

```

```

c
c   calculation of increment(INCR)
c
      INCR=(ALOG10(A(N))-ALOG10(A(1)))/(N-1)
      WRITE(6,6500) INCR
      WRITE(7,6500) INCR
c
c   calculation of bound concentration
c
      Z=10.0
      TEMP=NBS(1)*K(1)
      TOTE=NBS(2)*K(2)
      DO 200 I=1,N
      C(I)=A(I)*(TEMP*(1.0+K(2)*A(I))+TOTE*(1.0+K(1)*A(I)))
      B(I,10)= C(I)/(((1.0+K(1)*A(I))*(1.0+K(2)*A(I)))
c
c   calculation of bound/free(B/F) ratio
c
      BF(I,10)=B(I,10)/A(I)
      A(I+1)=Z**(ALOG10(A(I))+INCR)
200  CONTINUE
c
c   data for the plot of B/F VS B
c
      TYPE*, ' PLOT: B/F VS B '
      TYPE*, '
      TYPE*, 'DO YOU WISH TO HAVE A PRINT OUT?'
      READ(5,5000) ANS
      WRITE(7,5100)
      WRITE(7,5200) ((B(I,10),BF(I,10)),I=1,N)
      IF(ANS.EQ.'N') GO TO 300
      WRITE(6,5100)
      WRITE(6,5200) ((B(I,10),BF(I,10)),I=1,N)
300  TYPE*, 'DO YOU WISH TO WRITE VALUES TO THE DISK?'
      READ(5,5000) ANS
      IF(ANS.EQ.'N') GO TO 400
      TYPE*, 'PLEASE INPUT THE NAME OF THE FILE'
      READ(5,5300) (IFILE(I),I=1,8)
      WRITE(6,5400) (IFILE(I),I=1,8)
      WRITE(7,5400) (IFILE(I),I=1,8)
      CALL DISC10(ACTION,SIZE,B,BF,NUM)
c
c   data for the plot for B vs TOTAL
c
400  DO 500 I=1,N
      T(I,10) = B(I,10) + A(I)
500  CONTINUE
      TYPE*, '
      TYPE*, 'PLOT: [B] VS TOTAL'
      TYPE*, '
      TYPE*, 'DO YOU WISH TO HAVE A PRINT OUT '

```

```

READ(5,5000) ANS
WRITE(7,5500)
WRITE(7,5200) ((T(I,10),B(I,10)),I=1,N)
IF(ANS.EQ.'N') GO TO 600
WRITE(6,5500)
WRITE(6,5200) ((T(I,10),B(I,10)),I=1,N)
600  TYPE*, 'DO YOU WISH TO WRITE VALUES TO THE DISK?'
READ(5,5000) ANS
IF(ANS.EQ.'N') GO TO 700
TYPE*, 'PLEASE INPUT THE NAME OF THE FILE'
READ(5,5300) (IFILE(I),I=1,8)
WRITE(6,5600) (IFILE(I),I=1,8)
WRITE(7,5600) (IFILE(I),I=1,8)
CALL DISC10(ACTION,SIZE,T,B,NUM)

c
c   data for the plot [F] vs TOTAL
c
700  TYPE*, '
TYPE*, ' PLOT: [AG](FREE) VS TOTAL'
TYPE*, '
TYPE*, 'DO YOU WISH TO HAVE A PRINT OUT'
READ(5,5000) ANS
WRITE(7,5700)
WRITE(7,5200) ((T(I,10),A(I)),I=1,N)
IF(ANS.EQ.'N') GO TO 800
WRITE(6,5700)
WRITE(6,5200) ((T(I,10),A(I)),I=1,N)
800  TYPE*, 'DO YOU WISH TO WRITE VALUES TO THE DISK?'
READ(5,5000) ANS
IF(ANS.EQ.'N') GO TO 900
TYPE*, 'PLEASE INPUT THE NAME OF THE FILE'
READ(5,5300) (IFILE(I),I=1,N)
WRITE(6,5800) (IFILE(I),I=1,N)
WRITE(7,5800) (IFILE(I),I=1,N)
900  DO 1000 I=1,N
ADUM(I,10)= A(I)
1000 CONTINUE
CALL DISC10(ACTION,SIZE,T,ADUM,NUM)
STOP

5000 FORMAT(A1)
5100 FORMAT(12X,'BOUND[AG]',17X,'B/F RATIO')
5200 FORMAT(8X,E15.3,8X,E15.3)
5300 FORMAT(8A2)
5400 FORMAT(5X,'NAME OF THE FILE B/F VS B',10X,8A2)
5500 FORMAT(12X,'TOTAL',17X,'BOUND[AG]')
5600 FORMAT(5X,'NAME OF THE FILE B VS T',10X,8A2)
5700 FORMAT(12X,'TOTAL',17X,'FREE[AG]')
5800 FORMAT(5X,'NAME OF THE FILE FREE VS TOTAL',10X,8A2)
5900 FORMAT(E15.4)
6000 FORMAT(5X,'CONCENTRATION OF BINDING SITES',5X,E15.8)
6100 FORMAT(I3)

```

```
6200  FORMAT(5X,'NUMBER OF DATA POINTS',5X,I3)
6300  FORMAT(5X,'BINDING CONSTANTS',E15.4)
6400  FORMAT(5X,'HIGHEST [Ag]',10X,'LOWEST [Ag]')
6500  FORMAT(5X,' INCR = ',E15.4)
      END
```

Program SCA1

Program SCA1 allows one to vary the concentration of tracer(p^*). The following parameters are required: NPTS; K; BS; LAN; UAN(N) to UAN(1); and B0. B0 represents the bound labeled antigen concentration at zero dose(UAN = 0). B0 must be calculated using program SCA4(below) before using SCA1. The plots available are listed below.

1. bound LAN versus dose(UAN)
2. free LAN versus dose(UAN)
3. free LAN versus total(LAN + UAN)
4. B / F of LAN versus total(LAN + UAN)
5. B / F of LAN versus total(LAN + UAN)
6. B / F of LAN versus dose(UAN)
7. F / B of LAN versus dose(UAN)

8. B / B0 versus log(dose)

Logit Transformation $\text{Logit } B/B0 = \ln [(B/B0)/(1 + B/B0)]$

9. Logit B/B0 versus log(dose)

Scatchard Plots

10. B / F versus [B]
11. r / [Ag] versus r

Sips Plots

12. Log [r/(n-r)] versus Log [Ag]
13. 1 / r versus 1 / [Ag]

c program scal

c

c computer program for competitive binding assays

c

COMMON/HEADER/NPTS,KEY,PRAMS(10,6),ICMNT(40),IFILE(8)


```

c      binding constant(K)
c
TYPE*, 'PLEASE ENTER THE BINDING CONSTANT(K)'
READ(5,5200) K
WRITE(6,5300) K
WRITE(7,5300) K

c
      binding sites(NBS): Antibody concentration
c
TYPE*, 'PLEASE ENTER THE(FIXED) CONC OF ANTIBODY (BS)'
READ(5,5200) BS
WRITE(6,5400) BS
WRITE(7,5400) BS

c
      labeled antigen(LA)
c
TYPE*, 'PLEASE ENTER THE (FIXED) CONCENTRATION OF LAN'
READ(5,5200) LA
WRITE(6,5500) LA
WRITE(7,5500) LA

c
TYPE*, 'ANTIBODY/LABELED ANTIGEN RATIO'
TYPE*, '
BSLA = BS/LA
WRITE(6,5200) BSLA
WRITE(7,5200) BSLA

c
      Unlabeled antigen concentration
c
TYPE*, 'DO YOU WANT TO HAVE A CONC RANGE FOR UAN?'
TYPE*, 'WHAT IS THE RANGE OF UAN CONCENTRATION?'
TYPE*, 'HIGHEST UAN = '
READ(5,5200) UAN(N)
TYPE*, 'LOWEST UAN = '
READ(5,5200) UAN(1)
WRITE(6,5600)
WRITE(7,5600)
WRITE(6,5700) UAN(N),UAN(1)
WRITE(7,5700) UAN(N),UAN(1)

c
      calculation of increment
c
INCR = (ALOG10(UAN(N))-ALOG10(UAN(1)))/(N-1)
WRITE(6,5800) INCR
WRITE(7,5800) INCR

c
      calculation of B/F ratio
c
Z=10.0
DO 100 I=1,N

```

```

B(I) = K*(UAN(I))+K*LA-K*BS+1.0
C = -K*BS
D(I) = SQRT((B(I))**2-4.0*C)
R(I) = (-B(I) + D(I))/2.0
BLA(I) = LA*(R(I))/(R(I)+1.0)
FLA(I) = LA/(R(I)+1.0)
TLA(I) = BLA(I)+FLA(I)
FBLA(I) = FLA(I)/BLA(I)
BFLA(I) = BLA(I)/FLA(I)
FBS(I) = R(I)/K
BBS(I) = BS - FBS(I)
BUAN(I) = BS - BLA(I) - R(I)/K
FUAN(I) = UAN(I) - BUAN(I)
TUAN(I) = BUAN(I) + FUAN(I)
TLAUN(I) = TLA(I) + TUAN(I)
BFUAN(I) = BUAN(I)/FUAN(I)
FBUAN(I) = FUAN(I)/BUAN(I)
BTUAN(I) = BUAN(I)/TUAN(I)
UAN(I+1) = Z**(ALOG10(UAN(I))+INCR)
100 CONTINUE
c
c write the value of B(I)
c
TYPE*,
TYPE*, B(I) = K*UAN(I) + K*LA - K*BS + 1
TYPE*, DO YOU WISH TO HAVE A PRINT OUT (Y/N)?
READ(5,5900) ANS
WRITE(7,8400)
WRITE(7,6000) (B(I),I=1,N)
IF(ANS.EQ.'N') GO TO 150
WRITE(6,8400)
WRITE(6,6000) (B(I),I=1,N)
c
c labeled antigen concentration range
c
150 TYPE*,
TYPE*, CONC RANGE USED FOR UNLABELED ANTIGEN
TYPE*, (OR DOSE)
TYPE*, DO YOU WANT TO HAVE A PRINT OUT?
READ(5,5900) ANS
WRITE(7,6100)
WRITE(7,6000) (UAN(I),I=1,N)
IF(ANS.EQ.'N') GO TO 200
WRITE(6,6100)
WRITE(6,6000) (UAN(I),I=1,N)
c
c antibody/unlabeled antigen ratio
c
200 TYPE*,
TYPE*, ANTIBODY/UNLABELED ANTIGEN RATIO
TYPE*,

```

```

DO 250 I=1,N
BSUAN(I) = BS/(UAN(I))
250 CONTINUE
TYPE*, 'DO YOU WISH TO HAVE A PRINT OUT?'
READ(5,5900) ANS
WRITE(7,5200) (BSUAN(I),I=1,N)
IF(ANS.EQ.'N') GO TO 300
WRITE(6,5200) (BSUAN(I),I=1,N)

c
c unlabeled antigen/labeled antigen ratio
c
300 TYPE*, '
TYPE*, 'UNLABELED/LABELED ANTIGEN RATIO'
TYPE*, '
DO 325 I=1,N
UANL(I) = UAN(I)/LA
325 CONTINUE
TYPE*, 'DO YOU WISH TO HAVE A PRINT OUT?'
READ(5,5900) ANS
WRITE(7,5200) (UANL(I),I=1,N)
IF(ANS.EQ.'N') GO TO 350
WRITE(6,5200) (UANL(I),I=1,N)

c
c total labeled antigen concentration
c
350 TYPE*, '
TYPE*, 'TOTAL LABELED ANTIGEN (BOUND+FREE)'
TYPE*, 'DO YOU WANT TO HAVE A PRINT OUT'
READ(5,5900) ANS
WRITE(7,6200)
WRITE(7,6000) (TLA(I),I=1,N)
IF(ANS.EQ.'N') GO TO 375
WRITE(6,6200)
WRITE(6,6000) (TLA(I),I=1,N)

c
c total unlabeled antigen or dose
c
375 TYPE*, '
TYPE*, 'TOTAL UNLABELED ANTIGEN (BOUND+FREE) OR DOSE'
TYPE*, 'DO YOU WANT TO HAVE A PRINT OUT?'
READ(5,5900) ANS
WRITE(7,6300)
WRITE(7,6000) (TUAN(I),I=1,N)
IF(ANS.EQ.'N') GO TO 400
WRITE(6,6300)
WRITE(6,6000) (TUAN(I),I=1,N)

c
c antibody unbound concentration
c
400 TYPE*, '
TYPE*, 'ANTIBODY CONCENTRATION (BINDING SITES):UNBOUND'

```

```

TYPE*, 'DO YOU WANT TO HAVE A PRINT OUT'
READ(5,5900) ANS
WRITE(7,6400)
WRITE(7,6000) (FBS(I),I=1,N)
IF(ANS.EQ.'N') GO TO 500
WRITE(6,6400)
WRITE(6,6000) (FBS(I),I=1,N)
c
c   bound antibody concentration
c
500 TYPE*, '
TYPE*, ' ANTIBODY CONC OR BINDING SITES: BOUND'
TYPE*, 'DO YOU WANT TO HAVE A PRINT OUT'
READ(7,5900) ANS
WRITE(7,6500)
WRITE(7,6000) (BBS(I),I=1,N)
IF(ANS.EQ.'N') GO TO 600
WRITE(6,6500)
WRITE(6,6000) (BBS(I),I=1,N)
c
c   plot : bound antigen vs total
c
600 TYPE*, '
TYPE*, ' PLOT : BOUND (LABELED) ANTIGEN CONC VS'
TYPE*, ' DOSE(UNLABELED ANTIGEN CONC): BLA VS P'
TYPE*, '
TYPE*, 'DO YOU WISH TO HAVE A PRINT OUT(Y/N)?'
READ(5,5900) ANS
WRITE(7,6600)
WRITE(7,5700) ((TUAN(I),BLA(I)),I=1,N)
IF(ANS.EQ.'N') GO TO 625
WRITE(6,6600)
WRITE(6,5700) ((TUAN(I),BLA(I)),I=1,N)
625 TYPE*, 'DO YOU WISH TO WRITE VALUES TO THE DISC?'
READ(5,5900) ANS
IF(ANS.EQ.'N') GO TO 700
DO 650 I=1,2100
DO 650 J=1,10
      X(I,J) = 0.0
      Y(I,J) = 0.0
650 CONTINUE
DO 675 I=1,N
      X(I,10) = TUAN(I)
      Y(I,10) = BLA(I)
675 CONTINUE
CALL CAL
CALL DISC10(ACTION,SIZE,X,Y,NUM)
c
c   plot : free antigen concentration vs total(dose)
c
700 TYPE*, '

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```

TYPE*, ' PLOT : FREE (LABELED) ANTIGEN CONC VS '
TYPE*, ' DOSE(UNLABELED ANTIGEN CONC): FLA VS P '
TYPE*, ' DO YOU WISH TO HAVE A PRINT OUT(Y/N)? '
READ(5,5900) ANS
WRITE(7,6700)
WRITE(7,5700) ((TUAN(I),FLA(I)),I=1,N)
IF(ANS.EQ.'N') GO TO 725
WRITE(6,6700)
WRITE(6,5700) ((TUAN(I),FLA(I)),I=1,N)
725 TYPE*, ' DO YOU WISH TO WRITE VALUES TO THE DISC? '
READ(5,5900) ANS
IF(ANS.EQ.'N') GO TO 800
DO 750 I=1,2100
DO 750 J=1,10
      X(I,J) = 0.0
      Y(I,J) = 0.0
750 CONTINUE
DO 775 I=1,N
      X(I,10) = TUAN(I)
      Y(I,10) = FLA(I)
775 CONTINUE
CALL CAL
CALL DISC10(ACTION,SIZE,X,Y,NUM)

c
c   total concentration of antigen : labeled   unlabeled
c
800 TYPE*, '
TYPE*, ' TOTAL CONC OF Ag: LABELED AND UNLABELED '
TYPE*, '      (P + P*) '
TYPE*, ' DO YOU WANT TO HAVE A PRINT OUT(Y/N)? '
READ(5,5900) ANS
WRITE(7,6800)
WRITE(7,6000) (TLAUN(I),I=1,N)
IF(ANS.EQ.'N') GO TO 825
WRITE(6,6800)
WRITE(6,6000) (TLAUN(I),I=1,N)

c
c   plot : bound Ag conc. vs total Ag
c
825 TYPE*, '
TYPE*, ' PLOT : BOUND LABELED Ag VS TOTAL Ag CONC '
TYPE*, ' DO YOU WISH TO HAVE A PRINT OUT(Y/N)? '
READ(5,5900) ANS
WRITE(7,6900)
WRITE(7,5700) ((TLAUN(I),BLA(I)),I=1,N)
IF(ANS.EQ.'N') GO TO 850
WRITE(6,6900)
WRITE(6,5700) ((TLAUN(I),BLA(I)),I=1,N)
850 TYPE*, ' DO YOU WISH TO WRITE VALUES TO THE DISC? '
READ(5,5900) ANS
IF(ANS.EQ.'N') GO TO 925

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IF(ANS.EQ.'N') GO TO 1225
DO 1075 I=1,2100
DO 1075 J=1,10
      X(I,J) = 0.0
      Y(I,J) = 0.0
1075 CONTINUE
DO 1100 I=1,N
      X(I,10) = TLAUN(I)
      Y(I,10) = BFLA(I)
1100 CONTINUE
CALL CAL
CALL DISC10(ACTION,SIZE,X,Y,NUM)
c
c   plot : F/B vs total Ag concentration
c
1225 TYPE*,
TYPE*, PLOT : F/B (LABELED Ag) VS TOTAL Ag CONC
TYPE*, DO YOU WISH TO HAVE A PRINT OUT(Y/N)?
READ(5,5900) ANS
WRITE(7,7200)
WRITE(7,5700) ((TLAUN(I),BFLA(I)),I=1,N)
IF(ANS.EQ.'N') GO TO 1250
WRITE(6,7200)
WRITE(6,5700) ((TLAUN(I),BFLA(I)),I=1,N)
1250 TYPE*, DO YOU WISH TO WRITE VALUES TO THE DISC?
READ(5,5900) ANS
IF(ANS.EQ.'N') GO TO 1305
DO 1275 I=1,2100
DO 1275 J=1,10
      X(I,J) = 0.0
      Y(I,J) = 0.0
1275 CONTINUE
DO 1300 I = 1,N
      X(I,10) = TLAUN(I)
      Y(I,10) = BFLA(I)
1300 CONTINUE
CALL CAL
CALL DISC10(ACTION,SIZE,X,Y,NUM)
c
c   B/F vs unlabeled Ag concentration (or dose)
c
1305 TYPE*,
TYPE*, PLOT : B/F (LABELED Ag) VS UNLABELED Ag CONC.
TYPE*, OR DOSE
TYPE*, DO YOU WANT TO HAVE A PRINT OUT (Y/N)?
READ(5,5900) ANS
WRITE(7,7300)
WRITE(7,5700) ((TUAN(I),BFLA(I)),I=1,N)
IF(ANS.EQ.'N') GO TO 1325
WRITE(6,7300)
WRITE(6,5700) ((TUAN(I),BFLA(I)),I=1,N)

```

```

1325  TYPE*, 'DO YOU WISH TO WRITE VALUES TO THE DISC?'
      READ(5,5900) ANS
      IF(ANS.EQ.'N') GO TO 1400
      DO 1350 I=1,2100
      DO 1350 J=1,10
          X(I,J) = 0.0
          Y(I,J) = 0.0
1350  CONTINUE
      DO 1375 I=1,N
          X(I,10) = TUAN(I)
          Y(I,10) = BFLA(I)
1375  CONTINUE
      CALL CAL
      CALL DISC10(ACTION,SIZE,X,Y,NUM)
c
c      plot : F/B vs unlabeled Ag conc. or dose
c
1400  TYPE*, '
      TYPE*, ' PLOT : F/B (LABELED Ag) VS UNLABELED Ag CONC.'
      TYPE*, ' OR DOSE'
      TYPE*, '
      TYPE*, 'DO YOU WISH TO HAVE A PRINT OUT (Y/N)?'
      READ(5,5900) ANS
      WRITE(7,7400)
      WRITE(7,5700) ((TUAN(I),FBLA(I)),I=1,N)
      IF(ANS.EQ.'N') GO TO 1425
      WRITE(6,7400)
      WRITE(6,5700) ((TUAN(I),FBLA(I)),I=1,N)
1425  TYPE*, 'DO YOU WISH TO WRITE VALUES TO THE DISC?'
      READ(5,5900) ANS
      IF(ANS.EQ.'N') GO TO 1500
      DO 1450 I=1,2100
      DO 1450 J=1,10
          X(I,J) = 0.0
          Y(I,J) = 0.0
1450  CONTINUE
      DO 1475 I=1,N
          X(I,10) = TUAN(I)
          Y(I,10) = FBLA(I)
1475  CONTINUE
      CALL CAL
      CALL DISC10(ACTION,SIZE,X,Y,NUM)
c
c      calculation of bound Ag concentration (B0)
c
1500  TYPE*, '
      TYPE*, 'CALCULATION OF BOUND ANTIGEN CONC (B0)
      TYPE*, ' AT ZERO DOSE
      TYPE*, 'PLEASE USE PROGRAM SCA4 TO CALCULATE B0
      TYPE*, '
      TYPE*, 'PLEASE ENTER B0'

```

```

c
  READ(5,5200) B0
  DO 1550 I=1,N
  BBLA(I) = BLA(I)/B0
1550 CONTINUE
c
c   plot : B/B0 unlabeled Ag conc. or dose
c
  TYPE*, ' PLOT : B/B0 VS DOSE OR UNLABELED Ag '
  TYPE*, 'DO YOU WISH TO HAVE A PRINT OUT (Y/N)?'
  READ(5,5900)
  WRITE(7,7500)
  WRITE(7,5700) ((TUAN(I),BBLA(I)),I=1,N)
  IF(ANS.EQ.'N') GO TO 1575
  WRITE(6,7500)
  WRITE(6,5700) ((TUAN(I),BBLA(I)),I=1,N)
1575 TYPE*, 'DO YOU WISH TO WRITE VALUES TO THE DISC?'
  READ(5,5900)
  IF(ANS.EQ.'N') GO TO 1650
  DO 1600 I=1,2100
  DO 1600 J=1,10
    X(I,J) = 0.0
    Y(I,J) = 0.0
1600 CONTINUE
  DO 1625 I=1,N
    X(I,10) = TUAN(I)
    Y(I,10) = BBLA(I)
1625 CONTINUE
  CALL CAL
  CALL DISC10(ACTION,SIZE,X,Y,NUM)
c
c   the logit transformation
c
1650 TYPE*, '
  TYPE*, 'THE LOGIT TRANSFORMATION '
  TYPE*, '---- LOGIT(B/B0) = LOGe[(B/B0)/(1-B/B0)]----'
  DO 1675 I=1,N
  LTUAN(I) = ALOG10(TUAN(I))
1675 CONTINUE
  DO 1700 I=1,N
  LBBLA(I) = 2.303*ALOG10((BBLA(I))/(1-BBLA(I)))
1700 CONTINUE
c
c   plot : B/B0 vs log(dose)
c
  TYPE*, '
  TYPE*, ' PLOT : B/B0 VS LOG DOSE '
  TYPE*, 'DO YOU WISH TO HAVE A PRINT OUT(Y/N)?'
  READ(5,5900) ANS
  WRITE(7,7600)
  WRITE(7,5700) ((LTUAN(I),BBLA(I)),I=1,N)

```

```

IF(ANS.EQ.'N') GO TO 1725
WRITE(6,7600)
WRITE(6,5700) ((LTUAN(I),BBLA(I)),I=1,N)
1725 TYPE*, 'DO YOU WISH TO WRITE VALUES TO THE DISC?'
READ(5,5900) ANS
IF(ANS.EQ.'N') GO TO 1800
DO 1750 I=1,2100
DO 1750 J=1,10
      X(I,J) = 0.0
      Y(I,J) = 0.0
1750 CONTINUE
DO 1775 I=1,N
      X(I,10) = LTUAN(I)
      Y(I,10) = BBLA(I)
1775 CONTINUE
CALL CAL
CALL DISC10(ACTION,SIZE,X,Y,NUM)

c
c   plot : logit(B/B0) vs log(dose)
c
1800 TYPE*, '
TYPE*, ' PLOT : LOGIT(B/B0) VS LOG DOSE'
TYPE*, 'DO YOU WISH TO HAVE A PRINT OUT(Y/N)?'
READ(5,5900) ANS
WRITE(7,7700)
WRITE(7,5700) ((LTUAN(I),LBBLA(I)),I=1,N)
IF(ANS.EQ.'N') GO TO 1825
WRITE(6,7700)
WRITE(6,5700) ((LTUAN(I),LBBLA(I)),I=1,N)
1825 TYPE*, 'DO YOU WISH TO WRITE VALUES TO THE DISC?'
READ(5,5900) ANS
IF(ANS.EQ.'N') GO TO 1900
DO 1850 I=1,2100
DO 1850 J=1,10
      X(I,J) = 0.0
      Y(I,J) = 0.0
1850 CONTINUE
DO 1875 I=1,N
      X(I,10) = LTUAN(I)
      Y(I,10) = LBBLA(I)
1875 CONTINUE
CALL CAL
CALL DISC10(ACTION,SIZE,X,Y,NUM)

c
c   scatchard plots : B/F VS B
c
1900 TYPE*, '
TYPE*, '-----SCATCHARD PLOTS -----'
TYPE*, ' PLOT : B/F VS B'
TYPE*, 'DO YOU WISH TO HAVE A PRINT OUT(Y/N)?'
READ(5,5900) ANS

```

```

WRITE(7,8000)
WRITE(7,5700) ((BUAN(I),R(I)),I=1,N)
IF(ANS.EQ.'N') GO TO 1925
WRITE(6,8000)
WRITE(6,5700) ((BUAN(I),R(I)),I=1,N)
1925 TYPE*, 'DO YOU WISH TO WRITE VALUES TO THE DISC?'
READ(5,5900) ANS
IF(ANS.EQ.'N') GO TO 2000
DO 1950 I=1,2100
DO 1950 J=1,10
      X(I,J)=0.0
      Y(I,J)=0.0
1950 CONTINUE
DO 1975 I=1,N
      X(I,10)=BUAN(I)
      Y(I,10)=R(I)
1975 CONTINUE
CALL CAL
CALL DISC10(ACTION,SIZE,X,Y,NUM)
c
c   plot : r/[Ag] vs r
c
2000 TYPE*, '
TYPE*, '           r/[Ag] = nK - rK
TYPE*, ' PLOT : r/[Ag] VS r
TYPE*, '
c
DO 2025 I = 1,N
RBS(I) = R(I)/BS
BLBS(I) = BUAN(I)/BS
2025 CONTINUE
c
TYPE*, 'DO YOU WISH TO HAVE A PRINT OUT'
READ(5,5900) ANS
WRITE(7,8100)
WRITE(7,5700) ((BLBS(I),RBS(I)),I=1,N)
IF(ANS.EQ.'N') GO TO 2050
WRITE(6,8100)
WRITE(6,5700) ((BLBS(I),RBS(I)),I=1,N)
2050 TYPE*, 'DO YOU WISH TO WRITE VALUES TO THS DISC?'
READ(5,5900) ANS
IF(ANS.EQ.'N') GO TO 2125
DO 2075 I=1,2100
DO 2075 J=1,10
      X(I,J)=0.0
      Y(I,J)=0.0
2075 CONTINUE
DO 2100 I=1,N
      X(I,10)=BLBS(I)
      Y(I,10)=RBS(I)
2100 CONTINUE

```

```

CALL CAL
CALL DISC10(ACTION,SIZE,X,Y,NUM)
c
c   sips plot
c
2125 TYPE*, '
TYPE*, ' ----- SIPS PLOT ----- '
TYPE*, '
TYPE*, '           Log[r/(n-r)] = aLog[Ag] + aLog(K) '
c
DO 2150 I=1,N
SIP(I) = ALOG10(BLBS(I)/(1.0-BLBS(I)))
LFLA(I) = ALOG10(FLA(I))
2150 CONTINUE
c
TYPE*, '
TYPE*, ' PLOT : Log[r/(n-r)] vs Log[Ag] '
TYPE*, '
TYPE*, ' DO YOU WISH TO HAVE A PRINT OUT(Y/N)? '
READ(5,5900) ANS
WRITE(7,8200)
WRITE(7,5700) ((LFLA(I),SIP(I)),I=1,N)
IF(ANS.EQ.'N') GO TO 2175
WRITE(6,8200)
WRITE(6,5700) ((LFLA(I),SIP(I)),I=1,N)
2175 TYPE*, ' DO YOU WISH TO WRITE VALUES TO THE DISC? '
READ(5,5900) ANS
IF(ANS.EQ.'N') GO TO 2250
DO 2200 I=1,2100
DO 2200 J=1,10
X(I,J)=0.0
Y(I,J)=0.0
2200 CONTINUE
DO 2225 I=1,N
X(I,10)=LFLA(I)
Y(I,10)=SIP(I)
2225 CONTINUE
CALL CAL
CALL DISC10(ACTION,SIZE,X,Y,NUM)
c
c   plot : 1/r vs 1/[Ag]
c
2250 TYPE*, '
TYPE*, '           1/r = 1/n[Ag]K + 1/n '
TYPE*, ' PLOT : 1/r VS 1/[Ag] '
TYPE*, '
c
DO 2275 I=1,N
RBLBS(I) = 1.0/BLBS(I)
RFLA(I) = 1.0/FLA(I)
2275 CONTINUE

```

```

c
TYPE*, 'DO YOU WISH TO HAVE A PRINT OUT(Y/N)?'
READ(5,5900) ANS
WRITE(7,8300)
WRITE(7,5700) ((RFLA(I),RBLBS(I)),I=1,N)
IF(ANS.EQ.'N') GO TO 2300
WRITE(6,8300)
WRITE(6,5700) ((RFLA(I),RBLBS(I)),I=1,N)
2300 TYPE*, 'DO YOU WISH TO WRITE VALUES TO THE DISC?'
READ(5,5900) ANS
IF(ANS.EQ.'N') GO TO 2375
DO 2325 I=1,10
DO 2325 J=1,10
      X(I,J) = 0.0
      Y(I,J) = 0.0
2325 CONTINUE
DO 2350 I=1,N
      X(I,10) = RFLA(I)
      Y(I,10) = RBLBS(I)
2350 CONTINUE
CALL CAL
CALL DISC10(ACTION,SIZE,X,Y,NUM)
2375 TYPE*, '
c
c   format statements
c
5000 FORMAT(I3)
5100 FORMAT(5X,'NUMBER OF DATA POINTS',5X,I3)
5200 FORMAT(F10.5)
5300 FORMAT(5X,'BINDING CONSTANT',E15.4)
5400 FORMAT(5X,'ANTIBODY CONCENTRATION',E15.4)
5500 FORMAT(5X,'LABELED ANTIBODY CONCENTRATION',E15.4)
5600 FORMAT(5X,'HIGHEST UAN',10X,'LOWEST UAN')
5700 FORMAT(5X,E15.4,5X,E15.4)
5800 FORMAT(5X,'INCR',F10.5)
5900 FORMAT(A1)
6000 FORMAT(5X,E15.4)
6100 FORMAT(5X,'CONCENTRATION RANGE FOR UNLABELED ANTIGEN (DOSE)')
6200 FORMAT(5X,'TOTAL LABELED ANTIGEN (B + F)')
6300 FORMAT(5X,'TOTAL UNLABELED ANTIGEN (B + F)')
6400 FORMAT(5X,'FREE AB CONC.(BINDING SITES,AB)')
6500 FORMAT(5X,'BOUND AB CONC.(BINDING SITES,BBS)')
6600 FORMAT(5X,'UNLABELED ANTIGEN(TOTAL)',5X,'BOUND
cLABELED Ag')
6700 FORMAT(5X,'UNLABELED ANTIGEN(TOTAL)',5X,'FREE
cLABELED Ag')
6800 FORMAT(5X,'TOTAL Ag CONC.(LAg+ULAg) (LABELED AND
cUNLABELED)')
6900 FORMAT(5X,'TOTAL Ag CONC.(LAg+ULAg)',5X,'BOUND
cLABELED Ag')
7000 FORMAT(5X,'TOTAL Ag CONC.(LAg+ULAg)',5X,'FREE

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```

cLABELED Ag )
7100  FORMAT(5X, 'TOTAL Ag CONC.(LAg+ULAg)',5X, 'B/F RATIO')
7200  FORMAT(5X, 'TOTAL Ag CONC.(LAg+ULAg)',5X, 'F/B RATIO')
7300  FORMAT(5X, 'UNLABELED Ag(TOTAL,B+F)',5X, 'B/F RATIO')
7400  FORMAT(5X, 'UNLABELED Ag(TOTAL,B+F)',5X, 'F/B RATIO')
7500  FORMAT(5X, 'UNLABELED Ag(DOSE)',5X, 'B/B0 ')
7600  FORMAT(5X, 'LOG DOSE ',5X, 'B/B0 ')
7700  FORMAT(8X, 'LOG DOSE ',8X, 'LOGIT(B/B0) ')
8000  FORMAT(5X, 'B ',8X, 'B/F ')
8100  FORMAT(5X, 'r ',8X, 'r/[Ag] ')
8200  FORMAT(5X, 'Log[Ag] ',8X, 'Log[r/(n-r)]')
8300  FORMAT(5X, '1/[Ag] ',8X, '1/r ')
8400  FORMAT(5X, 'B(I) ')

```

```

STOP
END

```

```

C
C
C
C
C

```

```

SUBROUTINE CAL
COMMON/HEADER/NPTS,KEY,PRAMS(10,6),ICMNT(40),IFILE(8)
TYPE*, 'PLEASE INPUT THE NAME OF THE FILE?'
READ(5,7800) (IFILE(J),J=1,8)
WRITE(6,7900) (IFILE(J),J=1,8)
WRITE(7,7900) (IFILE(J),J=1,8)
7800  FORMAT(8A2)
7900  FORMAT(5X, 'NAME OF THE DATA FILE',8X,8A2)
RETURN
END

```

Program SCA4

This program calculates single values of B / F and $[B]$ for a given set of parameters (K , BS , LAN , and UAN). This is especially important for the determination of the bound labeled antigen concentration at zero dose.

```

c   program sca4
c
  REAL K,LAN
  TYPE*, 'PROGRAM SCA4'
  TYPE*, 'PROGRAMMER : S.A.FERNANDO'
  TYPE*, 'THIS PROG COMPUTES PARAMETERS FOR COMPETITIVE'
  TYPE*, 'BINDING ASSAY: EQUILIBRIUM CONDITIONS'
  TYPE*, 'IT IS POSSIBLE TO CALCULATE B/F(R) USING THIS'
  TYPE*, 'PROGRAM'
  TYPE*, 'CONDITIONS REQUIRED FOR THIS PROGRAM'
  TYPE*, 'INITIAL CONC OF LABELED AND UNLABELED'
  TYPE*, 'ANTIBODY OR ANTIGEN'
  TYPE*, 'BINDING CONSTANT OF LABELED AND UNLABELED'
  TYPE*, 'ANTIGEN'
  TYPE*, 'OR ANTIBODY IS EQUAL'
  TYPE*, 'REQUIRED DATA: K,BS,LAN,UAN'
  TYPE*, 'K --- BINDING CONSTANT'
  TYPE*, 'BS --- BINDING SITES'
  TYPE*, 'LAN --- LABELED ANTIGEN'
  TYPE*, 'UAN --- UNLABELED ANTIGEN'
c
c   binding constant(K)
c
  TYPE*, 'PLEASE ENTER THE VALUE OF BINDING CONSTANT'
  READ(5,10) K
10  FORMAT(E15.4)
  WRITE(7,20) K
20  FORMAT(5X, 'BINDING CONSTANT', E15.4)
c
c   binding sites(BS)
c
  TYPE*, 'PLEASE ENTER THE CONCENTRATION OF BINDING'
  TYPE*, 'SITES'
  READ(5,30) BS
30  FORMAT(E15.4)
  WRITE(7,40) BS
40  FORMAT(5X, 'BINDING SITES', E15.4)
c
c   labeled antigen(LAN)
c
  TYPE*, 'PLEASE ENTER CONCENTRATION OF LABELED ANTIGEN'

```

```

READ(5,50) LAN
50  FORMAT(E15.4)
WRITE(7,60) LAN
60  FORMAT(5X,'LABELED ANTIGEN CONCENTRATION',E15.4)
c
c  unlabeled antigen(UAN)
c
69  TYPE*, 'PLEASE ENTER CONC OF UNLABELED ANTIGEN'
READ(5,70) UAN
70  FORMAT(E15.4)
WRITE(7,80) UAN
80  FORMAT(5X,'UNLABELED ANTIGEN CONCENTRATION',E15.4)
c
c  calculation of B/F ratio
c
B = K*UAN+K*LAN-K*BS+1.0
C = -K*BS
D = SQRT(B**2-4.0*C)
R1 = (-1.0*B+D)/2.0
R2 = (-1.0*B-D)/2.0
BAN1 = BS - R1/K
BAN2 = BS - R2/K
TYPE*, ' D=SQRT(B**2-4.0*A*C)'
WRITE(7,88)
88  FORMAT(5X,'VALUE OF D')
WRITE(7,*) D
WRITE(7,90)
90  FORMAT(5X,'BOUND/FREE RATIO')
WRITE(7,*) R1,R2
TYPE*, 'BOUND ANTIGEN CONC.(BAN1,R1),(BNA2,R2)'
WRITE(7,*) BAN1
WRITE(7,*) BAN2
TYPE*, 'DO YOU WANT TO CONTINUE(Y/N)'
TYPE*, 'IF YOU WANT TO CONTINUE PLEASE ENTER (Y)'
READ(5,100) GO
100 FORMAT(A1)
IF(GO.EQ.'Y') GO TO 69
STOP
END

```

Program SCA

This program allows one to vary the binding constants of the labeled and unlabeled antigen separately. The following parameters are required: LAN; UAN(N) to UAN(1); K1; K2; and BS. A cubic equation(see Chapter 4, equation 5) was solved using Newton's method(Szidarovsky and Yakowitz, 1979). Several plots are available.

1. bound LAN versus dose(UAN)
2. free LAN versus dose(UAN)
3. B / F of LAN versus bound UAN
4. B / F of LAN versus total bound antigen(LAN + UAN)

```

c      program sca
c
DIMENSION FLA(100),BLA(100),RBT(100),RFT(100),UAB(100)
REAL K1,K2,LAN,INCR
TYPE*, 'PROGRAM SCA : PROGAMMER :S.A.FERNANDO'
TYPE*, '*****'
TYPE*, ' *'
TYPE*, ' *'
TYPE*, ' *          COMPETITIVE BINDING ASSAY          *'
TYPE*, ' *'
TYPE*, ' *      HETEROGENITY IN BINDING OF LABELED      *'
TYPE*, ' *                      AND                      *'
TYPE*, ' *                      UNLABELED LIGAND          *'
TYPE*, ' *'
TYPE*, ' *'
TYPE*, ' *****'
TYPE*, '
TYPE*, '      P + Q ----- P-Q (K1)
TYPE*, '
TYPE*, '      *                      *
TYPE*, '      P + Q ----- P-Q (K2)
TYPE*, ' P - UNLABELED ANTIGEN , Q - ANTIBODY
TYPE*, ' P(*) - LABELED ANTIGEN

c
c      number of data points
c
TYPE*, 'PLEASE ENTER THE NUMBER OF DATA POINTS'
READ(5,100) N
WRITE(6,200) N

```

```

WRITE(7,200) N
NPTS = N
c
c   binding constant for unlabeled antigen
c
TYPE*, '
TYPE*, 'PLEASE ENTER THE BINDING CONSTANT: '
TYPE*, 'FOR UNLABELED ANTIGEN (K1) '
READ(5,300) K1
WRITE(6,400) K1
WRITE(7,400) K1
c
c   binding constant for labeled antigen
c
TYPE*, '
TYPE*, 'PLEASE ENTER THE BINDING CONSTANT: '
TYPE*, 'FOR LABELED ANTIGEN (K2) '
READ(5,300) K2
WRITE(6,500) K2
WRITE(7,500) K2
c
c   the ratio of the binding constants
c
ALPHA = K1/K2
WRITE(6,600) ALPHA
WRITE(7,600) ALPHA
c
c   concentration of antibody : Q
c
TYPE*, '
TYPE*, 'PLEASE ENTER THE FIXED CONC OF ANTIBODY: BS '
READ(5,300) BS
WRITE(6,700) BS
WRITE(7,700) BS
c
c   concentration of unlabeled antigen
c
TYPE*, '
TYPE*, 'PLEASE ENTER THE CONC OF UNLABELED ANTIGEN:UAN '
READ(5,300) UAN
WRITE(6,800) UAN
WRITE(7,800) UAN
c
c   concentration of labeled antigen : LAN
c
TYPE*, '
TYPE*, 'PLEASE ENTER THE CONC OF LABELED ANTIGEN:LAN '
READ(5,300) LAN
WRITE(6,900) LAN
WRITE(7,900) LAN
c

```

```

c      ratio between conc of labeled and unlabeled antigen
c
c

```

```

TYPE*, '
TYPE*, 'THE RATIO CONCENTRATIONS UAN/LAN'
C = UAN/LAN
WRITE(6,1000) C
WRITE(7,1000) C

```

```

c
c      concentration range for free labeled antigen
c

```

```

TYPE*, '
TYPE*, 'CONC RANGE FOR FREE LABELED ANTIGEN:FLA'
TYPE*, 'HIGHEST CONCENTRATION:FLA(N)'
READ(5,300) FLA(N)
TYPE*, 'LOWEST CONCENTRATION:FLA(1)'
READ(5,300) FLA(1)
WRITE(6,1100)
WRITE(7,1100)
WRITE(6,1200) FLA(N),FLA(1)
WRITE(7,1200) FLA(N),FLA(1)

```

```

c
c      calculation of the increment
c

```

```

INCR = (ALOG10(FLA(N)) - ALOG10(FLA(1)))/(N-1)
WRITE(6,1300) INCR
WRITE(7,1300) INCR

```

```

c
c      calculation of bound labeled antigen : BLA
c

```

```

DO 1400 I=1,N
BLA(I) = LAN - FLA(I)
1400 CONTINUE

```

```

c
TYPE*, '
TYPE*, 'BOUND LABELED ANTIGEN : BLA '
TYPE*, 'DO YOU WISH TO HAVE A PRINT OUT(Y/N)'
READ(5,1500) ANS
WRITE(7,1600)
WRITE(7,1700) (BLA(I),I=1,N)
IF(ANS.EQ.'N') GO TO 1800
WRITE(6,1600)
WRITE(6,1700) (BLA(I),I=1,N)

```

```

c
c      calculation of bound/total ratio of labeled conc
c

```

```

1800 TYPE*, '
DO 1900 I=1,N
RBT(I) = BLA(I)/LAN
1900 CONTINUE
c

```

```

TYPE*, 'RATIO BOUND/TOTAL LABELED ANTIGEN CONCS'
TYPE*, 'DO YOU WISH TO HAVE A PRINT OUT(Y/N)'
READ(5,1500) ANS
WRITE(7,2000)
WRITE(7,1700) (RBT(I),I=1,N)
IF(ANS.EQ.'N') GO TO 2100
WRITE(6,2000)
WRITE(6,1700) (RBT(I),I=1,N)

c
c calculation of free/total ratio of labeled concs
c
2100 TYPE*, '
DO 2200 I=1,N
RFT(I) = 1.0 - RBT(I)
2200 CONTINUE

c
TYPE*, 'RATIO FREE/TOTAL LABELED ANTIGEN CONC'
TYPE*, 'DO YOU WISH TO HAVE A PRINT OUT(Y/N)'
READ(5,1500) ANS
WRITE(7,2300)
WRITE(7,1700) (RFT(I),I=1,N)
IF(ANS.EQ.'N') GO TO 2400
WRITE(6,2300)
WRITE(6,1700) (RFT(I),I=1,N)

c
c calculation of unbound antibody concentration
c
2400 TYPE*, '
DO 2500 I=1,N
UAB(I) = RBT(I)/(RFT(I)*K2)
2500 CONTINUE

c
TYPE*, 'UNBOUND ANTIBODY CONCENTRATION '
TYPE*, 'DO YOU WISH TO HAVE A PRINT OUT(Y/N)'
READ(5,1500) ANS
WRITE(7,2600)
WRITE(7,1700) (UAB(I),I=1,N)
IF(ANS.EQ.'N') GO TO 2700
WRITE(6,2600)
WRITE(6,1700) (UAB(I),I=1,N)

c
c calculation of bound antibody concentration
c
2700 TYPE*, '
100 FORMAT(I3)
200 FORMAT(5X, 'NUMBER OF DATA POINTS', I3)
300 FORMAT(F15.4)
400 FORMAT(5X, 'BINDING CONSTANT: K1', E15.4)
500 FORMAT(5X, 'BINDING CONSTANT: K2', E15.4)
600 FORMAT(5X, 'THE RATIO K1/K2 ', F10.5)
700 FORMAT(5X, 'ANTIBODY CONCENTRATION', E15.4)

```

```
800  FORMAT(5X,'UNLABELED ANTIGEN CONCENTRATION',E15.4)
900  FORMAT(5X,'LABELED ANTIGEN CONCENTRATION',E15.4)
1000 FORMAT(5X,'LABELED,UNLABELED CONC. RATIO',F10.5)
1100 FORMAT(5X,'FREE LABELED CONC:HIGHEST',5X,'LOWEST')
1200 FORMAT(5X,E15.4,5X,E15.4)
1300 FORMAT(5X,' INCR ',F10.5)
1500 FORMAT(A1)
1600 FORMAT(5X,'BOUND LABELED ANTIGEN CONCENTRATION')
1700 FORMAT(5X,E15.4)
2000 FORMAT(5X,'B/T OF LABELED ANTIGEN CONC.')
2300 FORMAT(5X,'F/T OF LABELED ANTIGEN CONC.')
2600 FORMAT(5X,'UNBOUND ANTIBODY CONCENTRATION')
      STOP
      END
```

LIST OF REFERENCES

- Ackerman, S., Cool, B., and Furth, J.J. (1979) *Anal. Biochem.* 100, 174-178.
- Ackermans, F., Klein, J., Ogier, J., Bazin, H., Cormont, F., and Frank, R.M. (1985) *Biochem. J.* 228, 211-217.
- Bachus, L.G., Tsalta, C.D., and Meyerhoff, M.E. (1986) *Biotechniques* 4, 42-55.
- Benedek, K., Doug, S., and Karger, B.C. (1984) *J. Chromatogr.* 317, 227-243.
- Berson, S.A., and Yalow, R.S. (1959) *J. Clin. Invest.* 38, 1996-2016.
- Blankstein, L.A., and Dohrman, L. (1985) *Amer. Clin. Prod. Rev.*, November, 1-7.
- Bolton, A.E., and Hunter, W.M. (1973) *Biochem. J.* 133, 529-539.
- Borman, S., (1987) *Anal. Chem.*, 59, 969A-973A.
- Brighton, W.D., and Johnson, E.A. (1971) *Ann. N.Y. Acad. Sci.* 177, 501-505.
- Buckles, R.E., and Bader, J.M. (1967) *Inorg. Synth.* IX, 130.
- Byers, J.M. (1986) Pathologist, Arizona Health Sciences Center, University of Arizona, Tucson, AZ, personal communication.
- Campeau, J.D., Marrs, R.P., and Dizerega, G.S. (1983) *J. Chromatogr.* 262, 334-339.
- Chandrasekaran, E.V., Mendocino, A., Garver, F.A., and Mendocino, J. (1981) *J. Biol. Chem.* 256, 1549-1555.
- Chase, G.D. (1983) in Ashkar, F.S., editor, Radiobioassays, CRC Press, Boca Raton, FL, vol. I, 52.
- Clezardin, P., McGregor, J.L., Manach, M., Boukerche, H., and Dechavanne, M. (1985) *J. Chromatogr.* 319, 67-77.

- Cohn, E.J., Hughes, Jr., W.L., and Weare, J.H. (1947) J. Am. Chem. Soc. 69, 1753-1761.
- Collins, W.P., editor (1985) Alternative Immunoassays, John Wiley and Sons, LTD., N.Y., 20-35.
- Colowick, S.P., and Kaplan, N.O. (1976) Methods Enzymol. XLIV, 27.
- Contreras, M.A., Bale, W.F., and Spar, I.C. (1983) Methods Enzymol. 92(Part E), 277-292.
- Crean, E.V., and Rossomando, E.F. (1977) Biochem. Biophys. Res. Commun. 75, 488-495.
- De Alwis, U. (1984) unpublished results.
- De Alwis, W. U., and Wilson, G.S. (1985) Anal. Chem. 57, 2754-2756.
- Edwards, L. (1983) with American Association for Clinical Chemistry, Clin. Chem. 29, 892-986.
- Ekins, R.P., Newman, G.B., and O'Riordan, J.L.H. (1968) in Hayes, R.L., Goswitz, F.A., and Murphy, B.E.P., editors, Radioisotopes in Medicine: In Vitro Studies, U.S. Atomic Energy Commission, Oak Ridge, 63-64, 75-76.
- Feinstein, G., Hofstein, R., Koifmann, J., and Sokolovsky, M. (1974) Eur. J. Biochem. 43, 569-581.
- Feldman, H., and Rodbard, D. (1971) in Odell, W.D., and Daughaday, W.H., editors, Principles of Competitive Protein-Binding Assays, J.B. Lippincott Company, Philadelphia, 158-203.
- Fleenor, W.A., Lucas, D.O., Stott, G.H., and Guidry, A. (1984) Vet. Immunol. Immunopath. 6, 365-378.
- Forsgren, A., and Sjoquist, J. (1966) J. Immunol. 97, 822-827.
- Garlick, R.L., Mazer, J.S., Higgins, P.J., and Bunn, H.F. (1983) J. Clin. Invest. 71, 1062-1072.
- Ghosh, S., and Campbell, A.M. (1986) Immunol. Today 7, 217-222.
- Gmelins (1927) Handbuch der Anorganischen Chemie, Verlag Chemie G.m.b.H., Berlin, Chlorine, vol. 6, main text, 5-7.

- Goding, J.W. (1980) *J. Immunol. Meths.* 39, 285-308.
- Grasseti, D.R., and Murray, Jr., J.F. (1967) *Arch. of Biochem. and Biophys.* 119, 41-49.
- Hannestad, U., Lundquist, P., and Sorbo, B. (1982) *Anal. Biochem.* 126, 200-204.
- Henschen, A., Hupe, K., Cottspeich, F., and Voelter, W., editors (1985) High Performance Liquid Chromatography in Biochemistry, VCH Publishers, Deerfield Beach, FL, 166-216.
- Hjelm, H., Hjelm, K., and Sjoquist, J. (1972) *FEBS Letters* 28, 73-76.
- Hjerten, S., Liao, J., and Yao, K. (1987) *J. Chromatogr.* 387(1), 127-138.
- Hoffman, D.R. (1977) in Catsimpoolas, N., and Drysdale, J., editors, Biological and Biomedical Applications of Isoelectric Focusing, Plenum Press, N.Y., 121-153.
- Honjo, T. (1983) *Ann. Rev. Immunol.* 1, 499-528.
- Hosotsubo, H., Arai, K., and Iwamura, J. (1985) *J. Immunol. Meths.* 85, 115-119.
- Hsia, C.J.C., Er, S.S., Hronowski, L.J., Persand, K., and Ansari, M.R. (1986) *J. Chromatogr.* 381, 153-157.
- Hymes, A.J., Mullinax, G.L., and Mullinax, F. (1979) *J. Biol. Chem.* 254, 3148-3151.
- Imai, H., Tamai, G., and Sakura, S. (1986) *J. Chromatogr.* 371, 29-35.
- Ingraham, R.H., Lau, S.Y.M., Taneja, A.K., and Hodges, R.S. (1985) *J. Chromatogr.* 327, 77-92.
- Kato, Y., Kitamura, T., and Hashimoto, T. (1983) *J. Chromatogr.* 266, 49-54.
- Katzenstein, G.E., Vrona, S.A., Wechsler, R.J., Steadman, B.L., Lewis, R.V., and Middaugh, C.R. (1986) *Proc. Natl. Acad. Sci. USA* 83, 4268-4272.
- Kawamura, Jr., A., editor (1977) Fluorescent Antibody Techniques and Their Applications, University Park Press, Baltimore, MA, 37-43.

- Kennedy, J.F., and Barnes, J.A. (1983) *J. Chromatogr.* 281, 83-93.
- Kitigawa, T., and Aikawa, T. (1976) *J. Biochem.* 79, 233-236.
- Koch, G.L.E., and Smith, M.J. (1986) *Br. J. Cancer* 53, 13-22.
- Kohler, G., and Milstein, C. (1975) *Nature* 256, 495-497.
- Krieger, D.E., Erickson, B.W., and Merrifield, R.B. (1976) *Proc. Natl. Acad. Sci. USA* 73, 3160-3164.
- Kummel, G., Daus, H., and Mauch, H. (1979) *J. Chromatogr.* 172, 221-226.
- Lau, S.Y.M., Taneja, A.K., and Hodges, R.S. (1984) *J. Chromatogr.* 317, 129-140.
- Luiken, J., Van Der Zee, R., and Welling, G.W. (1984) *J. Chromatogr.* 284, 482-486.
- Manil, L., Motte, P., Pernas, P., Troalen, F., Bohuon, C., and Bellet, D.J. (1986) *J. Immunol. Meths.* 90, 25-37.
- McLeod, R., Lacko, A.G., Pritchard, P.H., and Frohlich, J. (1986) *J. Chromatogr.* 381, 271-283.
- Merck Index (1976) Merck and Co., N.J., 659.
- Mezdour, H., Clavey, V., Kora, I., Kattigan, M., Barkia, A., and Fruchart, J. (1987) *J. Chromatogr.* 414(1), 35-45.
- Milgrom, F., and Swierczynska, Z. (1986) *Int. Archs. Allergy Appl. Immun.* 80, 200-210.
- Morrow, R.M., Carbonell, R.G., and McCoy, B.J. (1975) *Biotech. Bioeng.* XVII, 895-914.
- Nagle, Raymond (1986) *Pathologist, University of Arizona Health Sciences Center, Tucson, Arizona, unpublished results.*
- Nairn, R.C. (1976) Fluorescent Protein Tracing, Longman Group Limited, N.Y., 27-46.
- Ngo, T.T. (1985) in Ngo, T.T., and Lenhoff, H.M. editors, Enzyme-Mediated Immunoassay, Plenum Press, N.Y., 5.

- O'Farrell, P.H. (1975) *J. Biol. Chem.* 250, 4007-4021.
- O'Farrell, P.Z., Goodman, H.M., and O'Farrell, P.H. (1977) *Cell* 12, 1133-1142.
- O'Sullivan, M.J., Gnemmi, E., Mowis, D., Chieriegatti, G., Simmons, M., Simmonds, A.D., Bridges, J.W., and Markes, V. (1978) *FEBS Letters* 95, 311-313.
- Parola, G.L. (1934) *Gazz. Chim. Ital.* 64, 913-931.
- Pearson, T.W., and Anderson, N.C. (1983) *Methods Enzymol.* 92, 196-220.
- Peterson, G.L. (1977) *Anal. Biochem.* 83, 346-356.
- Peterson, L.M., Sokolovsky, M., and Vallee, B.L. (1976) *Biochem.* 15, 2501-2508.
- Phillips, T.M., More, N.S., Queen, W.D., and Thompson, A.M. (1985a) *J. Chromatogr.* 327, 205-211.
- Phillips, T.M., Queen, W.D., More, N.S., and Thompson, A.M. (1985b) *J. Chromatogr.* 327, 213-219.
- Portsmann, T., Portsmann, B., Nugel, E., Micheel, B., Karsten, U., and Fiebach, H. (1983) *Clin. Chim. Acta* 135, 13-22.
- Righetti, P.G., Tador, G., and Ek, K. (1981) *J. Chromatogr.* 220, 115-194.
- Sadek, P.C., Carr, P.W., Bowers, L.D., and Haddad, L.C. (1986) *Anal. Biochem.* 153, 359-371.
- Sadler, A.J., Micanovic, R., Katzenstein, G.K., Lewis, R.V., and Middaugh, C.R. (1984) *J. Chromatogr.* 317, 93-101.
- Sadtler Research Laboratories (1977) Proton Magnetic Resonance Spectra, vol. 42, 25720M.
- Santa-Coloma, T.A., Garraffo, H.M., Gros, E.G., and Charreau, E.H. (1987) *J. Chromatogr.* 415, 297-304.
- Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* 51, 660-672.
- Searle, N.E. (1948) *U.S. Patent* 2,444,563.
- Sevier, E.D., David, G.S., Martinis, J., Desmond, W.J., Bartholomew, R.M., and Wang, R. (1981) *Clin. Chem.* 27, 1791-1806.

- Sittampalam, G. (1982) Ph.D. Dissertation, Department of Chemistry, University of Arizona.
- Sportsman, J.R. (1982) Ph.D. Dissertation, Department of Chemistry, University of Arizona.
- Sportsman, J.R. (1986) Biochemistry Department, Lilly Research Laboratories, Indianapolis, IN, personal communication.
- Sportsman, J.R., Liddil, J.D., and Wilson, G.S. (1983) *Anal. Chem.* 55, 771-775.
- Sportsman, J.R., and Wilson, G.S. (1980) *Anal. Chem.* 52, 2013-2018.
- Storb, U., Ritchie, K.A., O'Brien, R., Arp, B., and Brinster, R. (1986) *Immunol. Rev.* 89, 85-102.
- Szidarovsky, F., and Yakowitz, S. (1978) Principles and Procedures of Numerical Analysis, Plenum Press, N.Y., 149-154.
- Tigyi, G.J., Balazs, L., Monostori, E., and Ando, I. (1984) *Mol. Immunol.* 21, 889-894.
- Van Der Zee, R., and Welling, G.W. (1985) *J. Chromatogr.* 327, 377-380.
- Warden, B.A., and Giese, R.W. (1984) *J. Chromatogr.* 314, 295-302.
- Warren, D.C. (1984) *Anal. Chem.* 56, 1529A-1544A.
- Welling, G.W., Nijmeijder, J.R.J., Van Der Zee, R., Groen, G., Wilterdink, J.B., and Welling-Wester, S. (1984) *J. Chromatogr.* 297, 101-109.
- Whitaker, J.R. (1972) Principles of Enzymology for the Food Sciences, Marcel Dekker, Inc., N.Y., 592-600.
- Wilson, M.B., and Nakane, P.K. (1978) in Knapp, W., Holubar, K., and Wick, G., editors, Immunofluorescence and Related Staining Techniques, Elsevier/North Holland Press, N.Y., 215-224.
- Wingfield, P.T., Graber, P., Rose, K., Simona, M.G., and Hughes, G.J. (1987) *J. Chromatogr.* 387, 291-300.