METAL ION INTERACTIONS WITH IMMUNOGLOBULIN G (IgG) I. PRELIMINARY STUDIES WITH ELECTRON PARAMAGNETIC RESONANCE (EPR) SPECTROSCOPY AND ULTRAFILTRATION

Nyle Hedin, CPT, Chemical Corps
HQDA, MILPERCEN (DAPC-OPP-E)
200 Stovall Street
Alexandria, VA 22332

Final Report 12 December 1978.

Approved for public release; distribution unlimited.

A thesis submitted to the Graduate Division, South Dakota School of Mines and Technology, Rapid City, South Dakota, in partial fulfillment of the requirements for the degree of Master of Science in Chemistry.

(6)

METAL ION INTERACTIONS WITH IMMUNOGLOBULIN G (IgG). I. PRELIMINARY STUDIES WITH ELECTRON PARAMAGNETIC RESONANCE (EPR) SPECTROSCOPY AND ULTRAFILTRATION.

by

(10) Nyle Hedin

A thesis submitted to the Graduate Division
in partial fulfillment of the requirements
for the degree of
MASTER OF SCIENCE IN CHEMISTRY

SOUTH DAKOTA SCHOOL OF MINES AND TECHNOLOGY
RAPID CITY, SOUTH DAKOTA

(12) 99 p.

(11) 12 Dec 1978

(2) Master's Thesis

Approved by:

John J. Fitzgerald
Major Professor

Howard Doyle
Chairman, Department of Chemistry

Dean, Graduate Division

ABSTRACT

This report presents a systematic methodology to study the transition metal ions of Cu(II), Cr(III), Co(II), VO^{+2}, and Fe(III), and their binding to immunoglobulin G (IgG). Procedures include dialysis, DEAE-cellulose column chromatography, gel electrophoresis, immunoelectrophoresis, ultrafiltration, UV spectroscopy, atomic absorption spectroscopy, and electron paramagnetic resonance (EPR). IgG used is non specific, metal free, human γ-globulin. Conclusions show that Cu(II), Cr(III), Co(II), and Fe(III) do bind to IgG, while VO^{+2} does not under conditions used in this study. Ultrafiltration studies detail Cu(II) binding to IgG in mole ratios of .08 to 3.9. No end point was reached in this study.

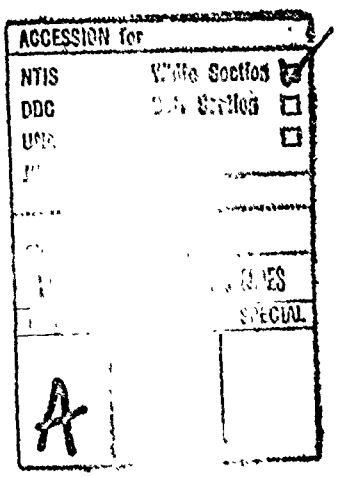

TABLE OF CONTENTS

LIST OF FIGURES

Note: Figures 1-7 taken from The Antibody Molecule[4]

LIST OF TABLES

INTRODUCTION

Some metals and their complexes pose a major environmental and industrial problem due to their reported toxicity effects. Occupational exposure to certain metal systems, such as Co, Ni, Zn, Cr, Be, have been linked to clinical hypersensitivity and especially contact hypersensitivity, the most common occupational disease. Metal ion systems, particularly chromium, arsenic, nickel, and beryllium have been related by epidemiological studies to an increased incidence of occupational cancer and, through experimental exposure in animals, a wide range of metal ion systems have been identified as cancer-producing agents.

This research effort is directed toward the molecular investigations of antibody-metal interactions as a potential mechanism of metal toxicological phenomenon. This report documents for the first time the initiation of systematic investigations of metal ion-immunoglobulin interactions. The procedures used to purify and isolate non-specific immunoglobulin G (IgG) from pooled human γ-globulin by column chromatography with DEAE-cellulose are described. Both gel electrophoresis and immunoelectrophoresis are used to verify protein purity of the separated IgG antibodies.

Preliminary Electron Paramagnetic Resonance (EPR) and ultrafiltration data are reported which establish the feasibility of systematic metal ion-IgG studies.

As background for this research effort, a brief review of pertinent toxicological literature is given.

METAL TOXICITY

Goyer and Mehlman[1] have stated that metals are unique environmental and industrial pollutants. Metals are neither created or destroyed, but are merely transported and transformed into various metabolic products. It is these various products that cause concern, especially when the biometabolism of metal complexes or metallic elements produces a new chemical of unknown form.

This research effort chose to concentrate upon the transition metals, and for good reason. Compounds of eight metals, beryllium, cadmium, chromium, cobalt, nickel, zinc, and iron-carbohydrate complexes have been shown to have deleterious effects following chronic, low level exposure to both humans and experimental animals. Examples include vanadium, arsenic, chromium, and nickel. Inhalation of vanadium dust causes severe irritation of the respiratory tract and may produce bronchitis and bronchospasm. Arsenic, besides being acutely toxic, has been linked to carcinogenesis. Chromium has been linked to respiratory cancer, while chronic exposure to nickel carbonyl can cause cancer.

METAL IMMUNE SYSTEM

Metals have been shown to have an effect on the immune response. The effects upon the immune response are as varied as the metals. An excellent review by Treagan[2] and a specific report by Wilde[3] exemplify the present state of clinical understanding of metal-immune system interactions.

Treagan's review provided insight for this research effort. The report was directed towards a summary of the reported effects of metals on immunoglobulin synthesis and on the cell-mediated immune reactions. Treagan writes in her article: "Antigenicity of metals is represented by numerous instances of hypersensitivity to metals in industrial workers, especially contact hypersensitivity. This very common condition provides evidence of the ability of metals to elicit an immune reaction. Contact hypersensitivity is caused by the direct effect of the metal-hapten on the skin, and, thus, depends on the ability of the hapten to penetrate into the skin, on the solubility of the hapten and on its ability to form covalent bonds with skin proteins."

Studies of the mechanism of metal hypersensitivity have indicated that the immunological nature is of the delayed type and generally includes contact as well as other types of hypersensitivity.

The immune reactions in this type of response are not only hapten but carrier specific. Both in vivo and in vitro studies have been reported for many different metals using diverse investigative methods. A major conclusion of present data is the general paucity of clearly understood mechanisms of metal-immune reactions. Some basic conclusions regarding previously reported results can be stated as follows:

a) The ability of metals to induce an immune response is supported by numerous instances of clinical

hypersensitivity to metals, including chromium, zirconium, beryllium, platinum, gold, and nickel.

b) The immunological mechanism of clinical and experimental hypersensitivities appears to be, generally, but not exclusively, of the cell-mediated type.

c) Nickel chloride shows a consistently suppressive effect on the interferon system (a protein component of the immune system), while salts of iron, chromium, cadmium, thorium, or lead do not.

d) Metals such as platinum, gallium, thorium, and gold have an immunosuppressive effect on cellular immunity.

e) Metal-deficient diets have an adverse effect on immunocompetence, metals in trace amounts have an opposite effect. Addition of metals in toxic quantities have a depressing effect on the immune system

f) It is probable that more than one mechanism is responsible for the effect of metals on the immune system.

Wilde has likewise reported experimental data concerning the effect of heavy metals on immune mechanism and on immune tolerance levels. Results of this study demonstrated that chronic, low level exposure to lead and cadmium suppressed the hemolytic activity of serum complement, serum albumin levels, secondary antibody levels, serum leucine aminopeptidase activity, and white blood cell counts. Mercury exhibited the same effects as lead and

cadmium except that mercury did not suppress the secondary response nor did it induce decreased production of white blood cells. Exposure to these metals enhanced the primary response and elevated serum γ-globulin levels.

Chronic exposure to these metals also induced anemia, increased blood-lead levels, and exerted various effects on liver, spleen, and kidneys.

In addition to the three heavy metals, mercury, cadmium, and lead being acute toxins, chronic exposure to lead and cadmium have been linked to carcinogenesis in animals.

DEFINITION OF RESEARCH PROBLEM

As concluded by Treagan and Wilde, metal ions have been shown to interfere with the biological immune response, both the primary and secondary responses.

Based on the premise that only certain metals exhibit specific and varied degrees of toxicological effects and the fact that metal ions have been shown to evoke an immune response, we chose to direct this research effort toward the transition metals and their molecular interactions with the major protein class of the humoral immune system, the immunoglobulins of human serum. The major thrust of this work is to investigate the possibility of metal ion binding to immunoglobulins. The specific work reported herein has focused on IgG and the aquated metal ions, copper (II), chromium (III), and cobalt (II). IgG was selected as the immunoprotein (γ-globulin) of choice for several reasons:

a) IgG makes up the largest class of γ-globulins, approximately 75 % of serum immunoglobulins;[4]

b) of all the γ-globulins, IgG has been most extensively studied and is the most well understood structurally and functionally;[5]

c) IgG has a rather simple and well defined secondary, tertiary, and quaternary structure and composition;

d) IgG has been extensively studied with respect to enzymatic proteolysis fragmentation and amino acid sequencing [6]

Several metals were considered for investigation. The metals under initial consideration included the non-toxic metal ions, iron (III), manganese (II), gadolinium (III), copper (II), and the toxic metal ions, chromium (III), cobalt (II), nickel (II), vanadyl (IV), VO^{+2}, and zinc (II). As a result of our preliminary EPR studies, this research effort has concentrated on five metal ions, copper (II), chromium (III), cobalt (II), iron (III), and vanadyl (IV) ions, and reports on four major areas:

a) Isolation of IgG from γ-globulin,

b) Purification, to insure metal free, immunologically pure, IgG for these studies without contaminating proteins or protein fragments,

c) Screening of potential metal ion binding to IgG, and

d) Development of applicable, systematic methods of investigation using ultrafiltration and EPR.

Relatively few literature references were found

that dealt specifically with metal binding phenomenon to γ-globulins or to IgG. Although enormous effort has been made in the study of metal binding to numerous proteins of serum as well as other important biological proteins and enzymes, none of the reported work appears to have included a systematic approach to metal-binding studies to any immunoglobulin or to IgG particularly.

This research effort's intention was to develop systematic methods to study metal binding to immunoglobulins and, especially, the binding of chromium, cobalt, copper, iron, and vanadyl ions to IgG.

THEORETICAL DISCUSSION

IMMUNOCHEMISTRY

General

Immunoglobulins are protein molecules having the capability to react in an immune response to foreign bodies introduced or inoculated into an organism. The foreign bodies include chemicals, foreign organisms and other antigens. Immunoglobulin proteins are synonomous with antibodies. Immunoglobulin (Ig) is used as a general term in the literature while antibody is used interchangeably and especially when discussing a specific clinical immune response.

According to The Condensed Chemical Dictionary, 9th Ed., an antibody is a blood serum protein of the globular fraction which is formed in response to the introduction of an antigen. An antigen is defined as an infective organism (protein) able to induce formation of an antibody in an organism in which the antigen is introduced. One other term requiring definition is hapten. A hapten is an incomplete antigen. It is incapable of provoking an immune response by itself, but able to function as a partial immunogen when bound to another substance denoted as a carrier molecule. According to Landsteiner and Pauling, metal ions are included in their definition of hapten.

It would appear that antibodies are very specific toward an antigen. In other words, an antigen provokes not a general response by antibodies, but rather a specific re-

sponse by a specific antibody. This specificity is the subject of a good deal of study, and several theories have been proposed to explain this phenomenon. No one theory deals with the subject in an entirely satisfactory manner, although these theories relate specificity of antigen-antibody reactions to the size, stereochemistry and composition of the active sites on IgG as well as analogous properties of the antigen.

Major Immunoglobulin Classes

There are several classes of Ig. A comparison of their various properties is listed in the following table:

TABLE I IMMUNOGLOBULIN CLASSES

TYPE	FUNCTION	MOL WT	BINDING	REMARKS
IgG	Simple Reactions General Antibody/ Antigen Response	150,000	1 or 2	75 % of Ig in Human Serum
IgD	Reaction w/leucocyte Membrane (?)	160,000	1	Small Quantity on Leucocyte
IgE	Allergic Responses	190,000	2	Very Small Quantity in Serum. 15 % of Ig in Human Serum.
IgA	Neutralize Virus Respiratory/Ingestion	300,000	(+)4	Body Secretions
IgM	Large Organisms (Bacterial), Antibody to Blood Group Substances	950,000	5 or 10	Polyvalent 10 % of Ig in Human Serum

General Description of IgG

Of particular interest in this research effort is

Immunoglobulin G (IgG). An IgG molecule is an elongated, flexible molecule with two active combining sites. These active sites are defined as the amino acid region providing the interaction with an antigen. The terms active site, combining site, and binding site are considered to be interchangeable. A schematic diagram of IgG may be represented as shown in Figure 1.

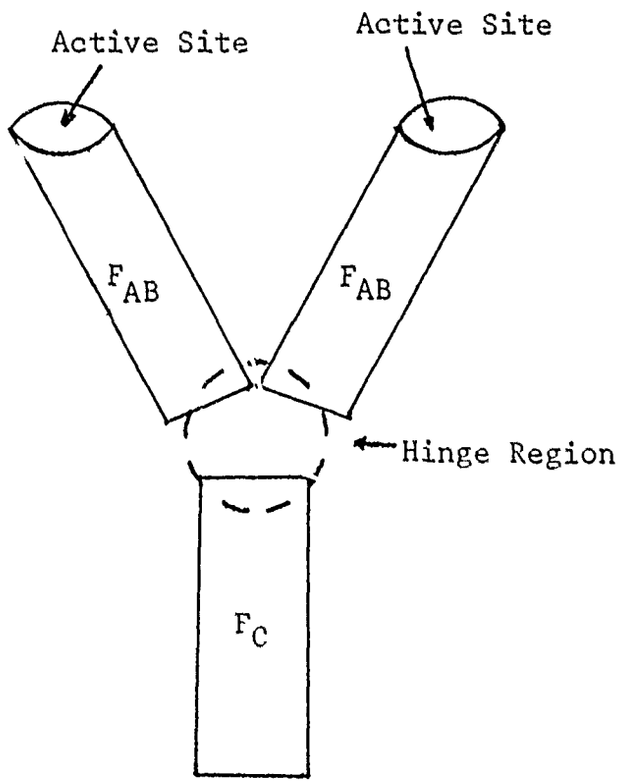

Figure 1 The IgG Molecule

Note that there are three basic fragments to an IgG molecule. These amino acid chains include two F_{AB} units (fragment-antigen binding) and one of F_C unit (fragment-crystallizable). The active sites of IgG are located at the N-terminus of the F_{AB} unit. The F_C unit participates in complement binding and other agglutination processes.

The IgG molecule is made up of chains of amino acid residue sequences. The chains are designated heavy (H) and light (L), and are covalently linked by interchain disulfide bonds.

Enzymatic Digestion and Protein Chain Characteristics

The IgG and other immunoglobulins molecules can be fragmented enzymatically in several ways. A more detailed diagram of an IgG molecule is given in Figure 2.

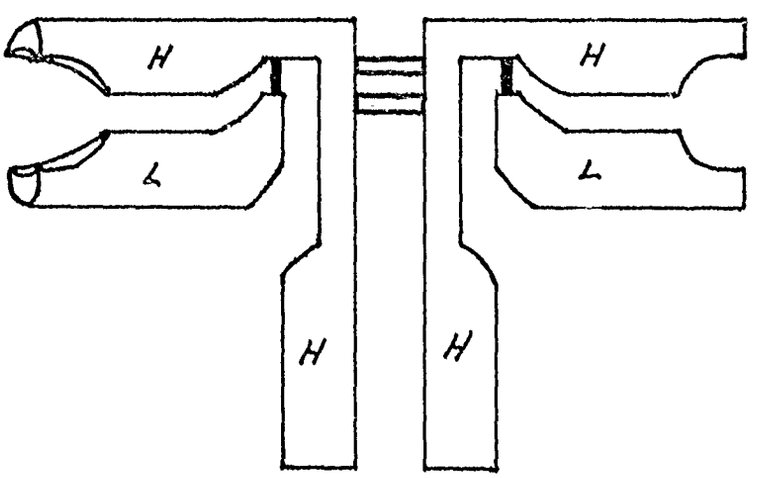

Figure 2 The IgG Molecule

Using papain proteolysis[7,8,9,10] the IgG may be broken into three segments, two F_{AB} segments and one F_C segment. Papain attacks the N-terminal peptide linkage of the heavy (H) chain, leaving the disulfide bonds intact between the H and L chain. See Figure 3.

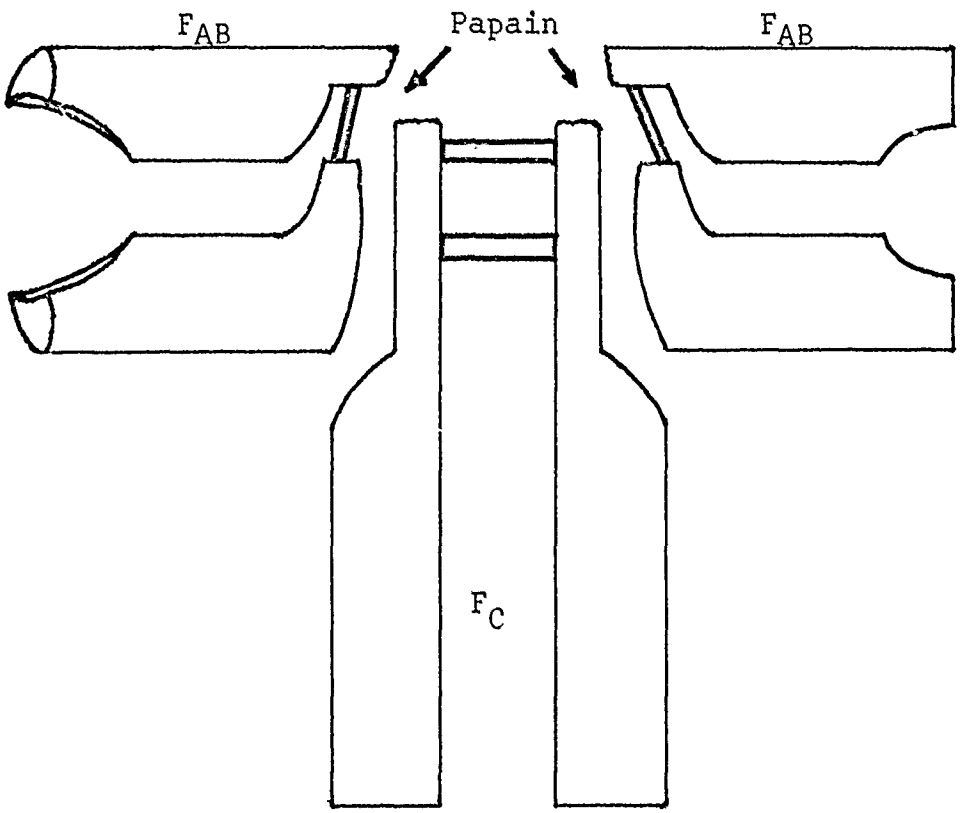

Figure 3 Papain Digestion of IgG Molecule

Using pepsin digestion under specific hydrolysis conditions,[11,12,13] the molecule is fragmented by attack at the C-terminal of the H-chain, causing further fragmentation of the F_C fragment, but leaving the two F_{AB} segments intact and attached by the two disulfide linkages. Further chemical reduction of the disulfide bonds creates two F_{AB}' segments, slightly larger than the F_{AB} segments from the papain digestion[14,15,16,17]. See Figure 4.

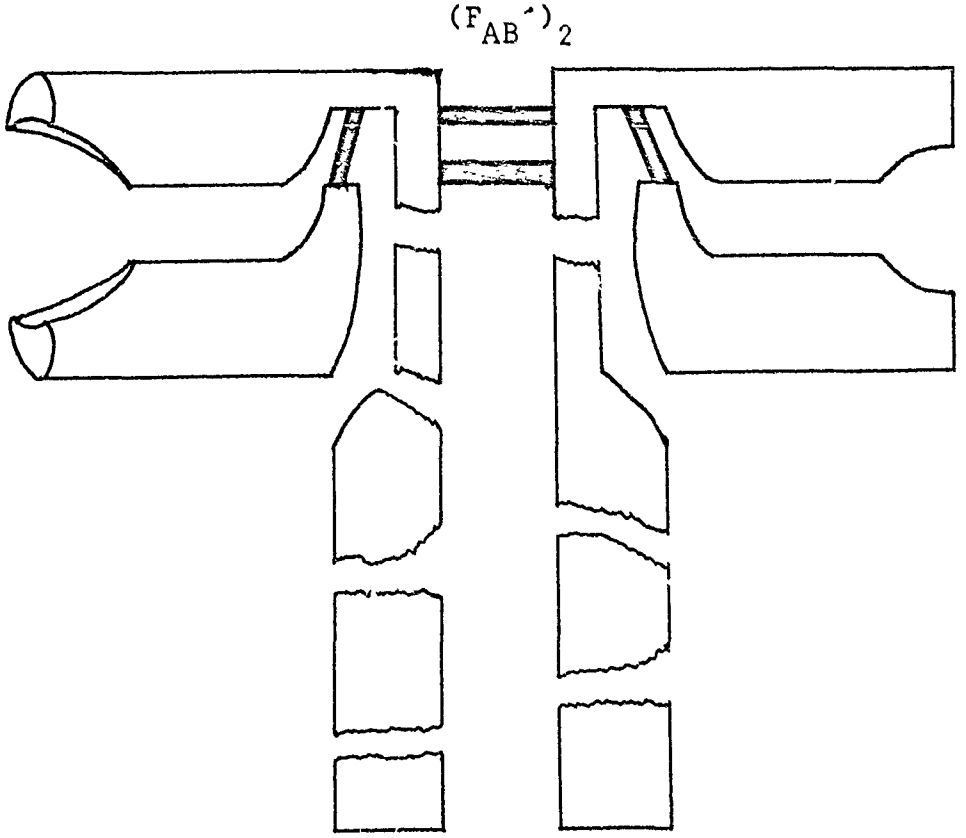

Figure 4 Pepsin Digestion of IgG Molecule

The product peptide chains from these digestions is two monoclonal fragments of IgG (one binding site on each F_{AB} fragment). Through these hydrolytic processes, it has been demonstrated that the F_{AB} fragments have both H and L chains while the F_C fragment is totally composed of H chains, no L chain present. The H and L chains of IgG proteins have both variable and constant regions. A constant region is defined as that region where the amino acid residue sequence is relatively invariant for numerous specific antibody molecules of the same class. The variable region is defined as that region where the amino acid sequence may not be constant. It has been shown that the variable portion of the heavy chain (V_H) is associated with the F_{AB} fragment and the constant portion of the heavy chain (C_H) is associated with the F_C fragment.[18,19] Conclusively, the variable positions of the H chain and the L chains are associated with the antigen combining sites.

Discussion of H and L Chains

The H chain of IgG has a molecular weight of roughly 50,000 daltons, two chains per IgG molecule; the L chain has a molecular weight of roughly 20,000 daltons, also two chains per molecule. The total molecular weight of IgG approximates 150,000 daltons.

The L chains are of two major types κ (kappa) and λ (lambda). The two types appear to have had a common evolutionary origin. The chains are of approximately equal length, each chain having two or three intrachain disulfide

loops and a half cystine residue through which the L chain is bonded to the H chain with disulfide linkages. The half cystine residue is generally the C-terminal residue in a κ chain and the penultimate residue in a λ chain.

The relative amounts of κ chain and λ chain vary greatly between species, human IgG being approximately 60 % κ chain. Both κ and λ chains are found incorporated in all classes of H chain.

The structure of the typical human L chain consists of about 214 amino acid residues. The second half of the chain, the C-terminal end, is approximately 107 residues and has been found to be relatively invariant. It is described as the constant portion of the L chain, or the C_L region. This end is normally not found associated with the active site. The first half of the chain, the N-terminal end, is widely variable and helps to account for the specificity of antigen-antibody responses. The L chain facilitates antibody specificity by governing molecular orientation necessary for the antigen to interact with specific locations on the H chain. See Figure 5.

The H chain portion of IgG consists of four amino acid sequence domains. These include the V_H region of approximately 115 aminio acid residues at the N-terminal end; and three domains of invariant sequence, C_H1, C_H2, and C_H3. All domains are approximately equal in size and each domain has the characteristic intrachain disulfide loop. The total amino acid residues approximate 446, the sequences of domains

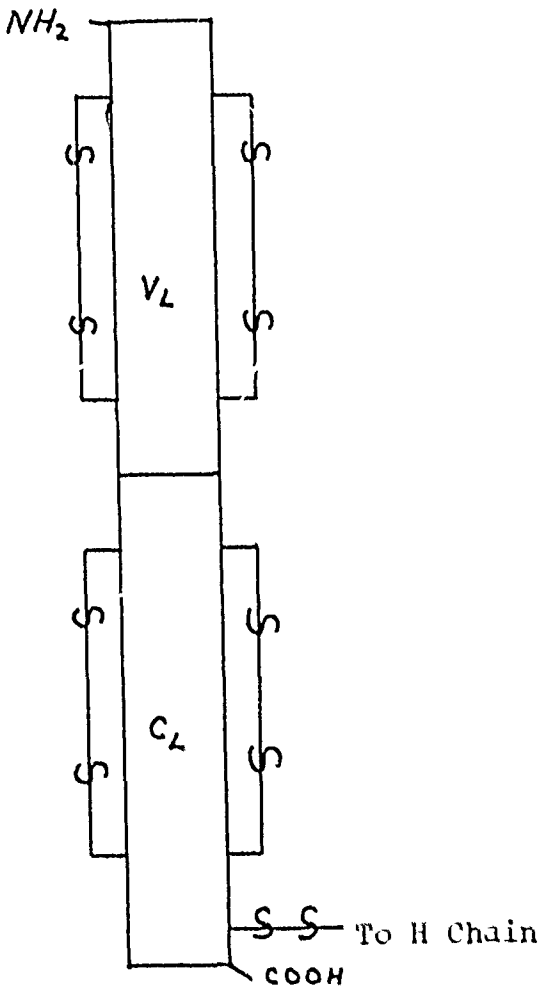

Figure 5 C and V Regions on Light Chain of IgG

and the disulfide bonds being shown in Figure 6.

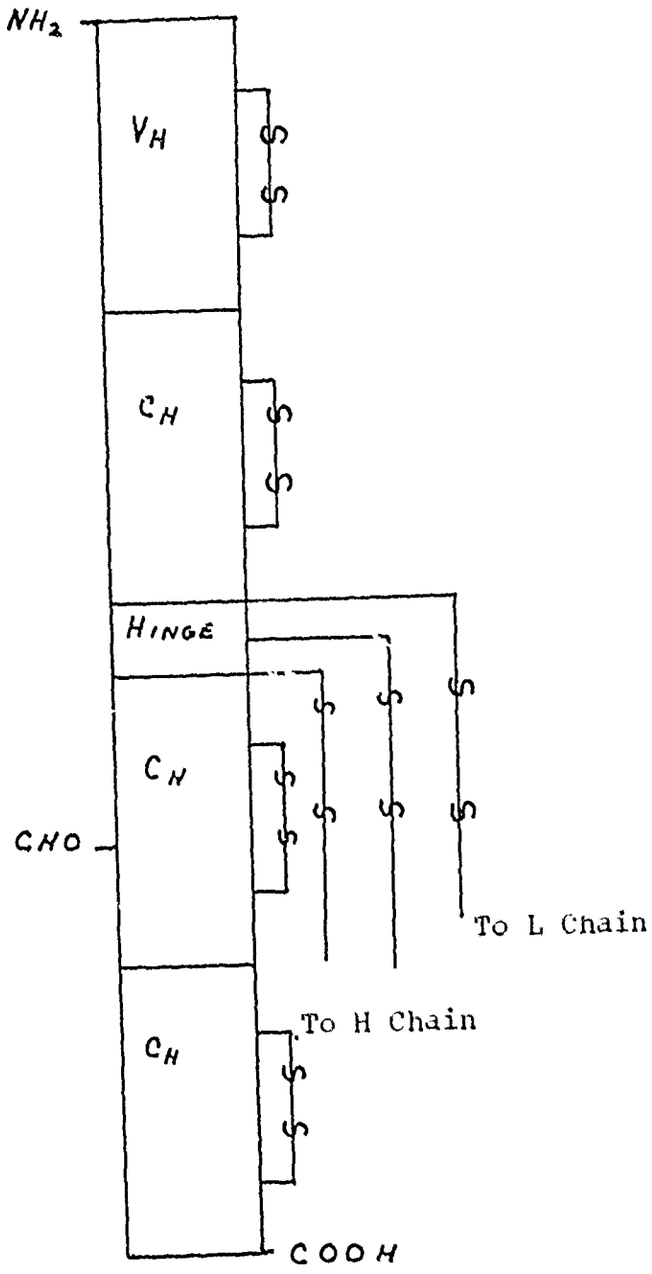

Figure 6 C and V Regions on Heavy Chain of IgG

Based upon the discussion and description above, the orientations of the H and L chains showing the V and C regions/domains can now be diagrammed, as shown in Figure 7.

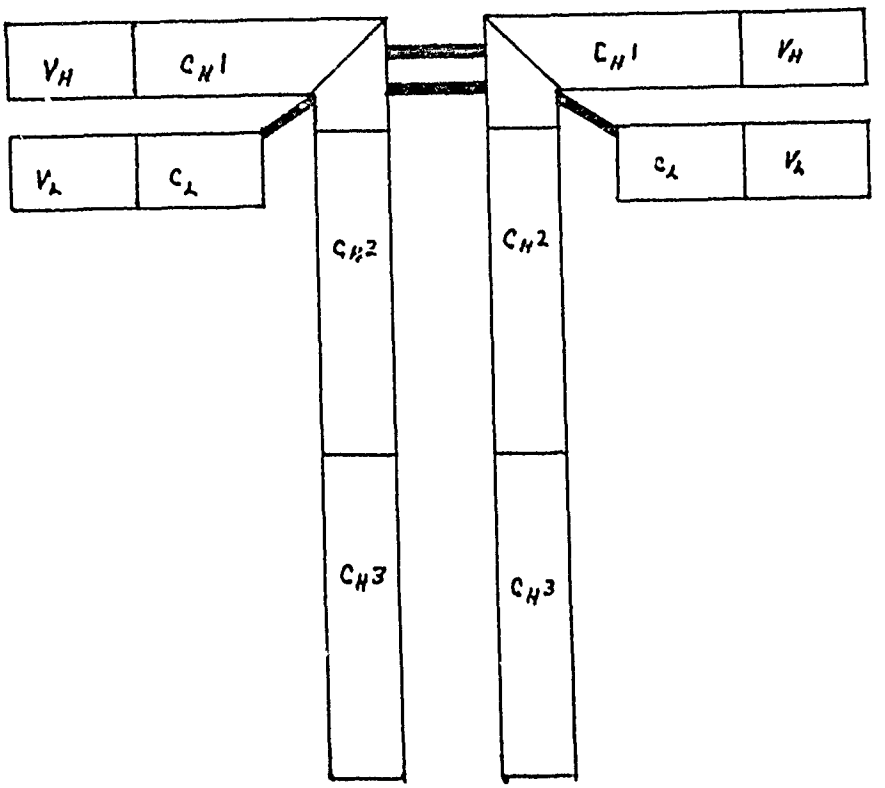

Figure 7 C and H Regions on IgG Molecule

The amino acid residues 220 to 234 on the H chain are designated as the hinge region of the IgG, allowing flexibility in the molecule. The hinge region lies between C_H1

and C_H2. This is also the region wherein the disulfide bonds lie between the L chain and H chain. The flexibility of the molecule due to the hinge region adds to the specificity of the antibody by allowing the antibody active sites to line up with the proper antigens, as required. There are four subclasses of human IgG molecule, termed IgG_1, IgG_2, IgG_3, and IgG_4, each with its own C_H sequence κ and λ variable specificity.

Active Sites of IgG

The antigen-binding activity of an IgG molecule is normally found associated with the variable region of the H and L chain. The variable regions surround the active site and help determine the antigen-antibody responses.

Any description of the variable regions (either V_L or V_H) is further complicated by several factors. Within the variable region are segments designated as the hypervariable regions. As an example, in V_H there are three such segments, of 2-7 residues each. These hypervariable regions seem to exhibit no sequencing relationship with other regions. Further, rather than being linear as depicted in the diagrams to this point, the IgG molecule is highly convoluted and folded. This folding is responsible for the hypervariable regions being in close proximity to each other, even though they are widely separated in the V-region sequencing.

Some research has been accomplished to attempt to define and accurately describe the important physical characteristics of the antigen binding site. Porter, et al.,[20] in

review of physical studies, sequence data, and affinity labeling of antibody combining sites concludes that the present evidence suggests that the combining sites are formed from a small number of amino acid residues which are scattered throughout both the heavy and light chains. Porter also describes the combining sites as a cleft in the molecule comparable to the catalytic sites in enzymes. This allows a wide range of specific binding affinities to be detected by changes in some fifteen to twenty amino acid residues.

Padlec, et al.[21] reported on a three dimensional structural study done of the F_{AB} fragment of McPC603 protein, a homogeneous protein from the serum of myeloma patients. The technique used was crystallographic analysis at a resolution of 3.1Å. The binding site of a specific antigen, phosphoryl choline, was a small portion of a cleft formed by three V_H hypervariable loops and the first and third hypervariable regions of the V_L chain. The hapten was in Van der Waals contact with various portions of the hypervariable loops. Hsia and colleagues[22] used EPR spectroscopy and fluorescence quenching to study rabbit antibodies specific for the 2,4,6-trinitrophenyl determinant group. They concluded that antibodies produced during primary and secondary immune responses contained different antigen combining sites. The primary response antibody sites were less tolerant to steric perturbations and formed less rigid complexes with haptens, and were more susceptible to organic solvent

denaturation. The secondary response antibody sites were parallel to the long axis of the spin-labeled ligand and had a size of about 10 Å.

R. A. Dwek, et al.[23] using EPR techniques to study myeloma protein MOPC 315, reports that its active site had considerable rigidity and had a cleft with dimensions of 11 Å by 9 Å by 6 Å. In an EPR study by J. C. Hsia and Lawrence H. Piette[24], the estimated average "depth" of the binding site was 10 Å.

In summary, a review of the literature shows that the binding site of an antibody has at least the following characteristics:

a) The site is formed by both the L and H chain, although the H chain may contain the bulk of the activity.

b) The site is dependent upon the variable regions and hypervariable regions of the L and H chains. These variable and hypervariable regions are comprised of different combinations of amino acid residues.

c) In addition to the amino acid sequence, the active site is dependent upon the physical conformation of the entire molecule.

d) The specificity of the antibody is governed by at least the characteristics listed above.

A further comment to more clearly define the research direction seems appropriate. As discussed earlier, the transition metal ions are haptens. This research will con-

sider these haptens and their binding properties with IgG. Of interest also is the association by the hapten with small conjugates or carrier molecules, and the effect of these associations upon the hapten IgG binding interactions.

METALS AND IMMUNOPROTEINS

General Metal-Ion Immunoprotein Interactions

Numerous serum proteins, enzymes and other biological components such as nucleic acids and carbohydrates have been studied to provide understanding and insight into their molecular interactions with metal-ions. Specific metal binding studies to immunoproteins, related proteins, or IgG are rather limited, although three reports deserve notation.

Glickson, et al.[25,26] have reported on the in vitro binding of gallium-67 to L1210 leukemia cells. The thrust of the study was the inhibition by human serum and various buffering compounds of gallium-67 uptake by the leukemia cells. Using radioisotopic determinations and cell fractionation methods such as gel filtration on Sephadex G-200, the capability of serum proteins to inhibit cellular uptake of gallium-67 was described. The high molecular weight components of serum were separated into three peaks.

Peak 1 contained substances whose molecular weight is in excess of 200,000 such as IgM. Peak 2 contained proteins with a molecular weight of approximately 160,000 like IgG. Peak 3 contained proteins in molecular weight range 75,000 to 100,000. Transferrin, albumin, and haptoglobin were

included within this peak. Each of the pooled and concentrated peaks inhibited gallium-67 uptake. Peak 2 exhibited the highest inhibiting capacity of 84 %, followed by Peak 3 at 59 % and Peak 1 at 39 %. This experimental data was used to formulate the following conclusions:

a) Both high and low molecular weight serum components have the capacity to inhibit the cellular uptake of gallium-67.

b) Reasons for the inhibition appears to be formation of tumor impermeable gallium complexes although interactions will cell surface components or modification of cellular metabolism cannot be ruled out.

c) No one inhibiting component of serum can account for all inhibition, but rather the presence of several or many components are necessary. It is very difficult to identify fully all the components.

d) Apotransferrin aids in the inhibition process, but only accounts for 10 % or less of the inhibiting capacity of the high molecular weight serum fraction.

Sigler and Blow[27] have used platinum and mercury in a heavy atom isomorphous replacement to study antibody crystal structure, but it would appear that the heavy metal ions are found in the interstitial spaces of the crystal lattice, rather than actually being bound to the protein amino acid residues.

Hardman[28] reports studies of a similar nature, including

information on a binding site for calcium and manganese on an extensively studied lectin known as Concanvolin A (CON A). A lectin is a protein that can bind carbohydrates. Lectins are particularly pertinent since they are classified as glycoproteins, as are immunoglobulins. CON A has also been shown to inhibit the growth of various tumors in vivo.

Metal Ion-IgG Binding Studies

As stated earlier, very little direct, systematic work has been accomplished in the study of metal binding to IgG or any immunoprotein. R. A. Dwek and his colleagues have produced some definitive work that will be discussed in detail.

Baker and Hultquist[29] have also reported some data that bear directly on this research effort: the study of a copper binding immunoglobulin from a hypercupric myeloma patient. One characteristic of copper is that copper is not normally toxic unless present in excessive amounts, even though copper ion is highly reactive.[30] Baker and Hultquist defined the purification of a copper-protein and demonstrated that the protein is an IgG_1 myeloma protein that specifically binds copper. Of great significance was their report that the copper was in an indeterminable valence state for detection by EPR, as well as the use of some of the same techniques used in this research effort. The isolated Cu-IgG protein was identified as a normal molecular weight

immunoprotein, with λ light chains. The carbohydrate protein appeared to be abnormal, since it interacts with CON A whereas most Igs of the γ-globulin type do not interact. The report suggests that the copper-Ig complex may possess either additional carbohydrate residues or an altered carbohydrate structure.

By using isoelectric focusing, Baker and Hultquist demonstrated a separation of the copper-immunoglobulin into three bands, and give two possible hypotheses:

a) the bands are due to some microheterogeneity resulting after synthetic hydrolysis of amide bands or varying amounts of sialic acid, or
b) the three bands could correspond to the binding of 2, 1 and 0 atoms of copper by the immunoglobulin.

Hypothesis b is consistent with the analysis of the sample showing that 1.8 atoms of copper are found per 150,000 daltons of protein.

R. A. Dwek, et al.[31,32,33] have reported considerable research concerning the binding of the lanthanide metals to IgG and IgG fragments, as well as detailed reports on the size and shape of the combining site. The IgG used was nonimmune IgG isolated from rabbit serum.

The lanthanides, a series of fifteen elements, make up a series of plus three valence cations having similar chemical properties such that they are expected to bind the same site

or sites. The subtle graded characteristics of this metal series allow detailed inspection of some properties of the antibody site by various spectroscopic techniques. Lanthanide solutions are stable only up to pH7, due to formation and precipitation of hydroxide complexes. Even at pH6, extensive complex formation occurs.

Dwek has been especially active in investigating the use of Gd (III) as a probe to study the combining sites of the immunoglobulins. Gd (III) has been found to be the most applicable probe for the F_C fragment since the binding of Gd (III) to the F_C fragment is much tighter than to the F_{AB} fragment.

Affinity labeling[34] and spin-label mapping[35] have been used to determine size and conformation of the active site, as well as its amino acid sequencing.

It is expected that lanthanides will bind to carboxyl groups on the antibodies and antibody fragments. Such groups generally have pKa values in the range of pH4-6. Dwek made careful measurements over the pH range 3.5 to 6.5 to optimize the conditions for observation of metal binding. They have shown that IgG has six specific Gd (III) binding sites at pH 5.5. The sites are divided into two classes: two very tight binding sites on the F_C fragment (Kd approx. 6 μm) and two weaker sites on each of the F_{AB} fragments (Kd approx. 140 μm). The metal binding parameters for IgG can be explained as the sum of the metal binding to the F_C and to the F_{AB} fragments. This suggests that no apparent inter-

action occurs between the F_{AB} and F_C regions in the IgG molecules under consideration. Both Protein Relaxation studies and EPR spectroscopy results are in agreement with respect to the regional distribution of the six binding sites.

The screening of the antibody has been done to identify lanthanide binding sites. This screening may be useful in studying antibody-antigen complexes, as well as metal ion interactions with the complement system of proteins.

Metal Ion-IgA Binding Studies

In addition to their detailed investigation of lanthanide IgG interactions, Dwek and colleagues[36,37,38,39,40] have reported a great deal of study on the immunoglobulin IgA and its metal binding properties.

The IgA used was obtained from mouse myeloma protein MOPC 315. Using this antibody, intensive research has been reported concerning the combining site, its dimensions, conformation and composition.

The approach to the problem has resulted in the discovery of a new fragment of the molecule, termed the F_V fragment. The F_V fragment is prepared by peptic digestion of the F_{AB} fragment at pH 3.7. The F_V fragment has a molecular weight of approximately 250,000 and contains the variable regions of the heavy and light chains at the N-terminal ends. The F_V fragment is the smallest antibody fragment that retains the integrity of the combining site. Using NMR and spin-labeling, the amino acids around the site have been identified and the combining site mapped.

The combining site is hydrophobic and is reported to be a cleft with the dimensions of 11 Å by 9 Å by 6 Å, demonstrating considerable structural rigidity.

The three histidine residues of the active site are altered slightly in the presence of the hapten and therefore must be in or near the combining site. Metal binding studies show that these residues are near an intrinsic binding site for Gd (III) and therefore presumably for the other lanthanide metals.

Further study with the lanthanide metals and spin-label haptens indicates that the spin-label hapten and Gd (III) do not compete directly for one site, but have distinct binding sites. The interaction between the two distinct binding sites could be a direct interaction between two adjacent sites, such as site overlap, or indirect interaction, mediated by the protein, between two widely separated sites.

The F_V fragment appears to exist in at least two different conformers, each with different affinities for hapten and metals.

In the absence of either hapten or metal, the F_V fragment exists predominately in the preferential form for hapten binding, while the metal binds preferentially to the other form.

An observation concerning antigen-antibody specificity can be made from this study. An antibody directed against an antigenic determinant of a given molecule will react only with this determinant on a very similar determinant. This

specificity must result from the three-dimensional complementarity of the antibody combining site and the antigenic determinant.

EXPERIMENTAL DISCUSSION

MATERIALS

Buffer Solutions

a) .01 M acetate, from 1:10 dilution of .1 M acetate, pH 5.5 (12 g sodium acetate, $NaC_2H_3O_2 \cdot 3H_2O$ and .66 ml glacial acetic acid, distilled water to 1L).

b) .01 M HEPES, pH 7.0 (2.3831 g, N-2-hydroxyethyl piperazine-N^1-2-ethanesulfonic acid, diluted to 1L)

c) .01 M phosphate (.23 g $NaH_2PO_4 \cdot H_2O$, 2.268 g, $NaHPO_4 \cdot 7H_2O$, diluted to 1L distilled H_2O, degassed when used as eluent on column)

d) .01 M Tris-HCl, pH 8.9 (3.07 g Trisma/HCl, 3.70 g Trisma base, diluted to 1 L)

Chelex 100, 100-200 mesh, sodium form, Bio-Rad Laboratories, Richmond, Calif.

DEAE Cellulose, Lot Number 7081
ICN Pharmaceuticals, Inc.
Life Sciences Group, Cleveland, Ohio

Dithizone (Diphenylthiocarbazone), .001 % (.0256 g/ L distilled water)

γ-Globulin, Conn Fraction II, Stock #HG, II, Lot Number 77C-0071
Sigma Chemical Company, St. Louis, Mo.

Metal stock solutions prepared from reagent grade chloride or sulfates in range of $1x10^{-1}$ to $2x10^{-2}$ M/l

$CuCl_2 \cdot 2H_2O$ $FeCl_3 \cdot 6H_2O$

$CoCl_2 \cdot 6H_2O$ $VOSO_4 \cdot xH_2O$

$CrCl_3 \cdot 6H_2O$ $GdCl_3 \cdot xH_2O$

$NiCl_2 \cdot 6H_2O$

1,10 - Phenanthroline (Monohydrate) Lot Number 775114, ACS Fisher Scientific Co., Fairlawn, N.J. (.01 M - .1802 g/100 ml double distilled H_2O)

Standards for atomic absorption spectroscopy

Standards were made up in accordance with the Varian Techtron AA-6 manual and included standards for copper, cobalt, chromium, iron, and zinc.

Wash solution for recharging the column

2 M NaCl in 0.4 M NaH_2PO_4 (55.2 g $NaH_2PO_4 \cdot H_2O$, 116.9 g NaCl, diluted to 1 liter with double distilled H_2O)

EQUIPMENT/INSTRUMENTATION

Column Chromatography (Ace Glassware, Vineland, N.J.)
60 cm x 1.5 cm and 60 cm x 2.5 cm)

Dialysis Bags, Stock Number 250 -11, 15,000 MW cutoff (Sigma Chemical Co., St. Louis, Mo.)

Ultrafiltration Equipment

a) Amicon 50 ml cell, Model 52, with UM 10, PM 10, and XM50 membranes (Amicon Corp., Lexington, Mass.)

b) MRA cell, Model M129, with PM 10 Amicon membranes (MRA Corp., Boston, Mass.)

Atomic Absorption Spectrometer, Varian Techtron, Model AA-6 (Varian Techtron, Melbourne, Australia)

Electron Paramagnetic Resonance Spectrometer (Varian, Sunnyvale, Calif.) Varian E-9 dual cavity, X band frequencies of ca. 9.54 and 9.05 GHz, at both room temperature and at liquid nitrogen temperature, respectively.

Refractometer, LDC Model L1107L (Laboratory Data Co., Riviera Beach, Calif.)

Ultra Violet Spectrometers a) Bechman Model DB, with matched quartz cells, (Fullerton, Calif.)

b) ISCO Model UA-5 Monitor, with type 6 optical flow cell unit with 280 nm filter (Lincoln, Nebraska) absorbance range: 2, chart speed: 2 ÷ 60 inches/minute (2 inches/hour)

GENERAL PROCEDURES

Procedures used in this research effort include protein dissolution, dialysis, and column chromotography to obtain isolated IgG; gel electrophoresis and immunoelectrophoresis for protein purity checks; atomic absorption and ultra violet absorption spectroscopy for metal and protein concentration determination; ultrafiltration and EPR for metal-protein binding studies.

Protein Purification and Separation Techniques

Dissolution - The initial procedure used was dissolving 400 mg in 100 ml of .01 M phosphate buffer. This procedure resulted in too much loss of protein in dialysis due to aggregation. The procedure was modified by dissolving 200 mg in 100 ml of .01 M phosphate buffer. One additional procedure was used, and was adopted as the procedure of choice. This procedure involved dissolving 800 mg of γ-globulin in 100 ml of .01M acetate buffer. All protein appeared to go into solution readily within 45 minutes and indicates that more protein could be used, if desired. This compares with 800 mg-1800 mg of IgG in 100 ml of normal serum.

Dialysis - The 100 ml of γ-globulin solution was placed in three dialysis bags, each holding 30-40 ml of protein solution and dialysed against 4 l. of .01 M phosphate buffer for 48 hours, with one buffer change after 24 hours. A white precipitate formed during the dialysis, which after immunoelectrophoresis checks, was determined to be IgG, possibly

an aggregate of IgG. The bags were used for several dialyses, the white precipitate being rinsed out prior to use. The white precipitate was either discarded or retained for study, as required.

Column Chromatography - Following dialysis, the γ-globulin solution is ready for the final purification process, column chromatography with DEAE cellulose.

Initially, an Ace column, 60.0 cm x 1.5 cm, was used. Due to the volume with this column, the dialysed protein solution had to be concentrated down to 30 ml, using an Amicon ultrafiltration cell. An Ace column, 60.0 cm x 2.5 cm, was purchased and used to separate the protein. With the new column, no concentrating was required and the 100 ml aliquot of sample could be placed on the column at one time.

The column capacity was calculated using the capacity of DEAE cellulose as listed in Methods in Immunology.[41] As stated, no more than 30-50 mg of protein per 1g of dry cellulose is recommended. It was determined experimentally that 5 ml of wet cellulose weighed .298 g when dried. The smaller column contained 140 ml of wet cellulose, or 8.344 g of dry cellulose. The larger column contained 700 ml of wet cellulose or 41.72g of dry cellulose. The smaller column capacity was calculated to be 250-414 mg of protein, while the larger column capacity was calculated to be 1.252 g to 2.086 g of protein. To avoid overloading the smaller column, sample size was kept at 200 mg of protein or less. To date, the maximum amount of protein separated on the large

column was .8 g or less.

Initially, the smaller column separation was monitored with a refractometer and a UV spectrometer. Due to zeroing complications with the refractometer and the restriction of column flow rate, the refractometer monitoring was discontinued and UV monitoring was used exclusively. The column separation procedures proved to be so predictable that several runs were made by monitoring by eluent volume alone. The last three runs were made with the large Ace column, all runs being monitored by UV spectroscopy.

To preclude any protein denaturing, cold temperatures were maintained during the disolution, through dialysis, to column separation. Dissolution was accomplished in an ice bath, dialysis was done in a refrigerator, regulated to 4°C, while the column chromatography was kept chilled by pumping ice water through the column condenser and placing the eluent reservoir bottle in an ice bath. Temperature range was 4°C to 12°C. See Figure 8 for diagram of equipment set up.

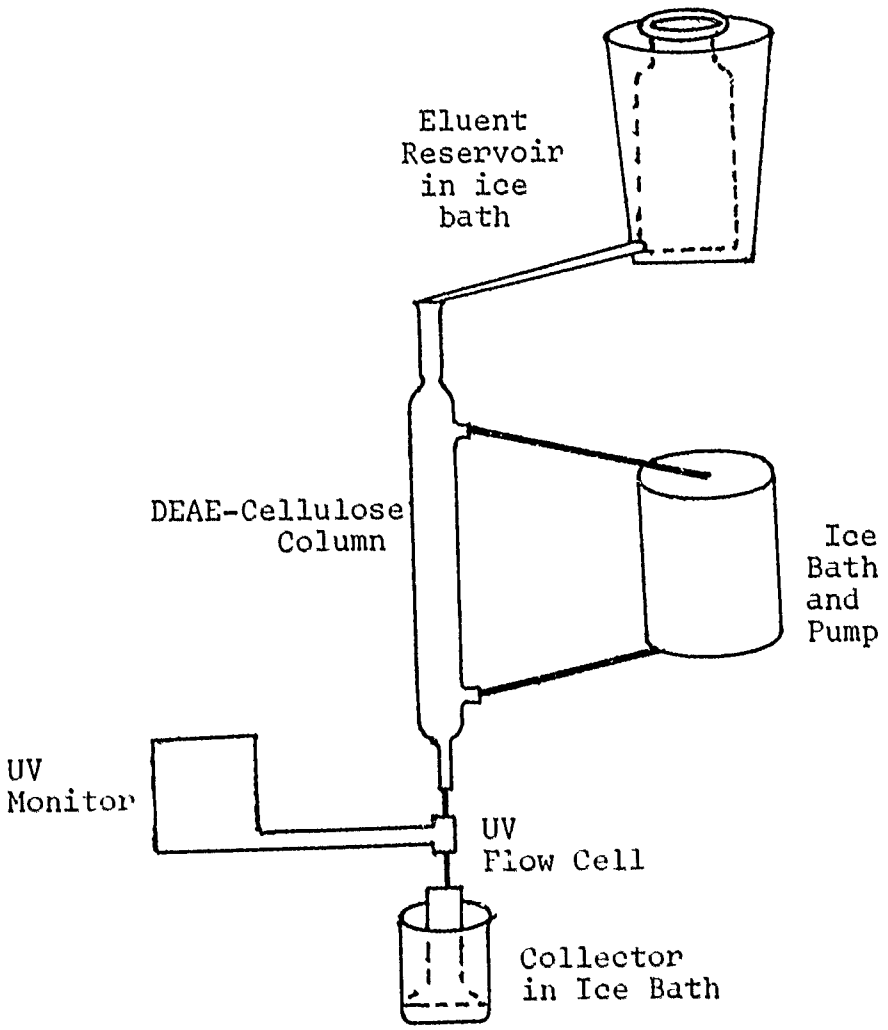

Figure 8 Equipment Diagram, Protein Isolation by Column Chromatography

Protein Purification of IgG

Gel Electrophoresis

Gel electrophoresis was used to check purity of the protein following column separations and to monitor the purification process. All gel electrophoresis work was done by a fellow graduate student, Mr. Duane Krueger. Discussion of this procedure depends heavily upon Mr. Krueger's notes and comments.

Apparatus used was an ISCO Gel Electrophoresis Rack, Model number 1270. The procedure and composition of the gel was taken from Methods in Immunology, 2nd Ed. pages 94-104, and also the ISCO instruction manual and allied pamphlets. The gel used was acrylamide, a synthetic polymer. This gel is nonionic and features other desirable properties such as chemical inertness, transparency, rigidity, and thermostability. Separation of components in a mixture occur both by size (molecular sieving) and by charge (electrophoresis). The upper chamber buffer was Tris-Glycine, pH 9.3. The lower chamber buffer was Tris-HCl, pH 8.1. The gel was stained with .01 % Amido Black in 3 % acetic acid.

Determination of an unknown protein sample was accomplished by comparing the unknown sample with a known plasma standard. By observing similarity in mobility the unknown component may be determined. Molecular weights may also be determined accurately.

During the runs made, one of the major problems was getting protein solutions in high enough concentration

for proper determination. This problem was not due to the gel electrophoresis, but to the quantity of protein provided for study. With later runs, the protein concentration was much more suited to this procedure and adequate determinations resulted.

The electrophoretic checks of protein after separation on the DEAE-cellulose column indicate that a single protein band consisting of pure IgG is being eluted from the column. This method of gel electrophoresis rapidly and conclusively confirms the purity of the IgG sample.

Immunoelectrophoresis

Following the column separation, .2 - 1 ml samples were retained to check the purification process.

Duplicate samples were provided to Rapid City Regional Hospital Lab and the Lab of Clinical Medicine, Sioux Falls, SD, for purity checks using immunoelectrophoresis. One additional sample was sent to LCM, Sioux Falls. The findings of each institution support each other and indicate the following: the bulk of the γ-globulin is IgG; no IgM is present in any of the duplicate samples. The last sample sent to LCM was γ-globulin dissolved in acetate buffer, no dialysis and column separation was performed. Immunoelectrophoresis checks indicated presence of IgG and a small amount of IgA. As a result of these findings, the final purified product is considered to be pure IgG, and suitable for metal-protein binding studies.

Immunoelectrophoresis by its nature requires antisera

specific to the sample. The possibility, however small, exists that the presence of an immunological inactive substance in the protein sample may not be detected.

Gel electrophoresis, on the other hand, depends upon electric mobility and not an immune response. The gel tube can be stained and all substances can be separated and detected. Quanitative work can be done with a gel scanner.

Protein Concentration and Buffer Change

An ultrafiltration cell, Model 52, was purchased from Amicon Corporation, Lexington, Mass. This cell had a capacity of 50 ml and, by exchanging filter sizes, had a capability of several different protein retention and filtration rates. The membranes used most often in this research effort were several, with the characteristics and capabilities listed below. Data has been obtained from the Amicon Bulletin #448 B.

TABLE II MEMBRANE CHARACTERISTICS

Membrane Number	IgG Retention Level	Flow Rate (Deionized Water) $ml/cm^2/min$	Nominal Molecular Weight cut off
UM10	> 98 %	0.1 - 0.3	10,000
PM10	> 98 %	1 - 4	10,000
XM50	> 98 %	.5 - 2.0	50,000

In practice, the XM50 membrane met our requirements most fully.

As noted earlier, the protein concentration obtained from the small Ace column was very dilute, approximately

8×10^{-7} M. Reasons for this include small initial quantities of protein dissolved in phosphate buffer, further reduced during dialysis by aggregation, and by elution gradient dilution effects on the column itself. Protein levels were required to be three orders of magnitudes higher. These levels were obtained by using the Amicon ultrafiltration cell, and by pressure removal of excess eluent. Pure IgG was obtained in concentration ranges 1×10^{-4} M and 5×10^{-5} M. The process became much easier after initiation of the large Ace column, much less concentrating by ultrafiltration being necessary.

Following the protein purification and isolation techniques and after identification as pure IgG, the next procedure was to conduct metal ion content checks and removal.

Protein and Metal Content Determination

Protein concentration was determined by ultra violet spectroscopy. Molar absorptivity coefficient used for calculating protein concentration was $\epsilon = 2.02 \times 10^5$ liters/mole-cm, from $E^{1\%}_{\substack{ICM \\ @\ 280\mu m}} = 13.5$.[42] Absorbance values were determined using a Bechman DB spectrometer. Metal concentrations (except VO^{+2}, determined by UV)[43] were determined by atomic absorbance spectroscopy, using a Varian Techtron AA-6 spectrometer. Standard curves were plotted and the unknown values read directly from the graph.

Atomic absorption spectroscopy is a relatively routine

procedure, and only a minimal discussion follows.

Atomic absorption was used to determine the concentration of the metal stock solutions, as well as to monitor the amount of metal binding to a sample of IgG, or the amount of metal contamination in the protein solution.

The procedure entailed first making up metal standard solutions, using, in most cases, purchased standards, and making dilutions as required for the concentration (in ppm) range desired. By making solutions of standard concentration the standard curve could be plotted and the unkown metal concentration determined by use of the standard curve.

Due to large and varied waters of hydration content in several cases, specifically chromium, iron, and nickel, the stock metal solutions were prepared by adding solid metal chloride to the solution until a proper atomic absorption reading was observed, indicating the desired concentration was reached. This procedure was also followed with VO^{+2}, the determination being made with UV spectroscopy.

Atomic absorption was used to detect metal binding to protein. Briefly, by measuring the metal concentration before and after addition to IgG it was possible to determine the amount of metal bound to the IgG. This procedure is discussed in greater detail in the section entitled Metal-IgG studies: use of MRA ultrafiltration cell.

Both multielement and single element lamps were used, depending upon the particular metal under observation.

Preparation of Metal Free IgG Solutions

To insure the pure IgG was also metal free, the IgG was added to .01 M 1,10-phenanthroline solution. The solution was stored at least overnight in the refrigerator. This allowed the 1,10-phenanthroline to complex and remove any free or bound metal that may have been associated with the IgG, as determined by atomic absorption analysis.

Following equilibration, the IgG - 1,10-phenanthroline solution was placed in the Amicon cell. The solution was concentrated down from 50 ml to 5 ml. Double distilled water was passed thru a chelex column to remove any metals. All other buffers, .01 M acetate, .01 M Tris-HCl, were also passed through the chelex column and presence of metal was checked by extracting the buffer with .001 % dithizone. The chelexed, double distilled water was then added to the cell, bringing the volume back up to 50 ml. The solution was again concentrated to 5 ml. The water addition, concentration procedure was repeated 5 times. This removed essentially all traces of 1,10-phenanthroline. After the fifth addition of water the solution was concentrated to approximately 16 ml, one ml was retained for ultra violet scan to determine concentration. The remaining 15 ml was divided into three 5 ml aliquots, ready for further buffer change as required. Buffers used included .01 M acetate, pH 5.5; .01 M Tris-HCl, pH 8.9, and later, .1 M HEPES, pH 7. The HEPES buffer was adopted following Dr. Fitzgerald's trip to New Hampshire, where it was determined that the acetate and Tris-HCl buffers

were unable to hold the pH as required due to the extreme acidity of the IgG. Changes from protein/double distilled water to buffer of choice were accomplished as described above, using two change cycles.

Following treatment of protein solution by 1,10-phenanthroline and buffer changes with the chelexed buffer of choice, the solution was again analysed using atomic absorption spectroscopy. All measureable amounts of metal were found to have been removed. Metals checked for included copper, manganese, zinc, and iron. Detection limits by atomic absorption were as follows:

TABLE III DETECTION LIMITS, ATOMIC ABSORPTION SPECTROSCOPY

Metal	Typical Detection Limits (µg/ml)	Wavelength (Nm)
Copper	.04	324.7
Iron	.062	248.3
Manganese	.024	279.5
Zinc	.009	213.9

METAL ION-IgG STUDIES: Use of MRA Ultrafiltration Cell

Although EPR studies were the method of choice for metal-IgG binding analysis, the MRA Ultrafiltration cell was used due to in-department EPR equipment breakdown. The MRA cell was used in conjunction with atomic absorption analysis and ultraviolet spectroscopy.

The MRA cell is an eight channel apparatus originally designed for rapid and direct analysis of binding by radioactive ligands to proteins. Eight samples can be processed by ultrafiltration within an hour. Membranes in each channel allow passage of the metal stock solutions, but retain protein and metal-protein complexes. Controls were used to monitor retention, if any, of the metal on the membrane. The amount of bound metal ion can be determined by knowing the concentration of the metal stock solution added to the IgG sample, and by determining

a) amount (if any) of metal retained on the membrane, and

b) amount of metal in eluent.

A simple calculation gives the amount of metal held by the IgG molecule:

[Bound Metal] = [Original Amount of Metal Added]

- [Amount of Metal Held by Membrane] - [Amount of Metal in Eluent]

By comparing the concentration of bound metal with the concentration of the IgG sample, a metal/IgG ratio can be established.

The study of various metal/IgG ratios can be determined by varying the metal/IgG ratios in each channel and comparing

results.

To preclude inordinate dilution effects, the metal stock solutions were .01 molar, thereby requiring very small quantities of metal solutions in comparison to protein solution size. The basic procedure used was to keep IgG concentration (volume) standard and vary the metal concentration as necessary to obtain the metal/IgG ratios under consideration.

The prepared metal/IgG solutions were mixed with a vortex stirrer and allowed to equilibrate under refrigeration overnight prior to ultrafiltration in the MRA cell.

Following the addition to the MRA cell, the metal/IgG solutions were pressurized with nitrogen gas and were ultrafiltrated. The eluent was collected and analyzed for metal content by atomic absorption analysis.

Membranes used to retain the protein in the cell were Diaflo, PM10, or UM10 from Amicon Corporation.

Due to time constraints and equipment breakdown, only one run with the MRA cell was made.

EPR Studies of Metal Ion-IgG Systems

Preliminary EPR measurements were performed using the Varian E-9 at Denver University, Denver, Colorado. These preliminary studies provided data used to select potentially applicable metal ions for consideration in the present study and to define techniques and conditions necessary for further investigation. Buffered IgG solutions included IgG separated and purified from six column separations. This was necessary to obtain sufficient IgG to provide protein concentrations in the range of 1×10^{-4}M to 1.7×10^{-5}M.

Buffers were processed to remove any metal contamination prior to contact with the IgG. Chelexed, doubled distilled water was the solvent used initially and all buffers were exchanged in the Amicon ultrafiltration cell. Due to a miscalculation, the protein solution from the initial three column separations was allowed to run dry during Amicon concentration procedures. The IgG was retained on an XM50 membrane. To preclude denatured protein contamination, the IgG and membrane were removed from the cell and placed in distilled water. The concentrating procedures were renewed again, using IgG from three additional column separations. The concentration and exchange procedures proceeded without incident to provide the final buffered and unbuffered IgG-buffer solutions, which are summarized in Table IV.

TABLE IV IgG-BUFFER COMBINATION, DENVER STUDIES

Buffer	IgG Concentration (moles/liters)
Chelexed Double Distilled water	1.01×10^{-4}
.01M acetate	7.13×10^{-5}
.01M Tris/HCl	6.34×10^{-5}
(IgG from XM50 membrane)	9.63×10^{-5}
Diluted XM50 IgG and Distilled water	1.71×10^{-5}

Metal Stock solutions used in the Denver EPR studies are listed in Table V.

TABLE V METAL STOCK SOLUTION, DENVER

Metal Ion	Concentration (moles/liters)
Co^{+2}	7.64×10^{-3}
Fe^{+3}	9.68×10^{-3}
Ni^{+2}	7.64×10^{-3}
Mn^{+2}	9.11×10^{-3}
Cu^{+2}	9.84×10^{-3}
Cr^{+3}	9.9×10^{-3}
VO^{+2}	1.05×10^{-2}

Due to time constraints, only spectra of apo-IgG and IgG with VO^{+2}, Cu^{+2}, Gd^{+2}, and Cr^{+3} were recorded.

Room temperature spectra were recorded as shown in Table VI.

TABLE VI ROOM TEMPERATURE SPECTRA, DENVER

Buffer	Metal ion	Metal ion-IgG ratio
.01 M acetate	VO^{+2}	1/1
.01 M acetate	Vo^{+2}	5/1
Tris/HCl	Cu^{+2}	1/1
Tris/HCl	Cu^{+2}	5/1
Tris/HCl	VO^{+2}	5/1
Distilled Water	VO^{+2}	1/1

Following room temperature scans, the instrument was set up for liquid nitrogen (77°K) temperature scans. Ig-G metal ion combinations included those run at room temperature (Table V), as well as Gd^{+3}/IgG in 1/1 mole ratio and Cr^{+3}/IgG in 1/1 mole ratio, both in acetate buffer. In addition to these metal ion/IgG combinations, several spectra were recorded of apo-IgG in distilled water, in the absence of any added metal ions.

Of the spectra recorded, no spectra clearly exhibited resonance features of IgG-bound metal ions. There was, however, sufficient deviation from spectra of unbound metal ion to suggest further study. These deviations were noted with copper (II) and chromium (III) ions. No change was noted with vanadyl, VO^{+2}, and gadolinium (III) ion systems.

The IgG from the XM50 membrane was retained in distilled water, and was presumed to contain native or contaminating trace metal ions. No equilibrium decontamination procedures were utilized with 1,10-phenanthroline. A scan of this IgG-

distilled water solution exhibited a weak EPR signal, indicating the presence of copper. An atomic absorption analysis conducted on this sample showed copper to be present in a mole ratio of .009 to one. The anisotropic spectrum observed indicated that the copper ion may have been bound to the IgG molecule. An EPR spectral comparison of metal-free IgG (equilibrated with 1,10-phenanthroline) with Cu^{+2} contaminated IgG indicated the absence of any EPR active metal ion and is denoted as apo-IgG.

As a result of analysis of the EPR spectra obtained at Denver University, several conclusions and recommendations of the EPR related studies were made:

a) no protein bound VO^{+2} spectra was observed, possibly due to conditions under which the spectra was recorded,

b) there was some indication of binding by IgG of the metal ions Cu(II) and Cr(III),

c) modification of experimental conditions for future studies were made and included pH adjustments of IgG-buffer solutions, metal ion-IgG ratios, as well as increases in modulation amplitude of the EPR instrument.

A new buffer, HEPES, was found to hold the pH at approximately 7.8. HEPES was adopted as the buffer of choice. These recommendations were adopted for use during the subsequent investigations at the University of New Hampshire. The pH of IgG-buffer solutions were found to be in the range of 3 to

5, and were titrated with NaOH to pH 6.3 to 9.3. Metal ions studied included Cu(II), Cr(III), Co(II), VO^{+2}(IV), and Fe (III) in metal ion-IgG ratios of 1.65, 3.2, 2.6, 10 and 7.3, respectively. Scans of metal ion-IgG solutions were made at room temperature as well as liquid nitrogen temperature, 77°K.

EPR Measurements

Two similar EPR spectrometer instruments were used to record the experimental EPR spectra. One instrument, located at the University of Denver, Denver, Colorado was provided for our use by Dr. Gareth Eaton. This instrument was used for our initial screening of metals and their possible interactions with IgG. The use of the EPR spectrometer at the University of New Hampshire, Department of Chemistry, Durham, New Hampshire, was generously provided by Dr. N. Dennis Chasteen.

Both spectrometers were Varion E-9's with dual cavities and used X-band frequencies of 9.54 and 9.05 GHz, respectively. Spectra were recorded at both room temperature and at liquid nitrogen (77°K) temperatures, and were calibrated using DPPH (2,2-diphenyl-1-picryl-hydrazyl).

During the Denver investigation, quartz tubes were used for all liquid nitrogen measurements to measure the metal-protein solutions. Quartz flat cells were tried but discontinued due to excessive foaming in the flat cell. The foaming was due to the procedures used to fill the cell. The cells required some modification on the ends to allow

a rubber septum to be mounted. The modification solved the foaming problem, but not in time to use at Denver.

The New Hampshire metal-protein samples were prepared in unbuffered and buffered media using microsyringe techniques in EPR capillary aqueous flat cells or microfiltration cells. The specific details are referenced in an article by Fitzgerald and Chasteen.[43]

EXPERIMENTAL RESULTS AND DISCUSSION

Protein Isolation

The source of the IgG used in this experimentation was pooled, non immune, human, γ-globulin. IgG was not separated from serum, as some researchers have done.

The final adopted procedure for protein dissolution included dissolving up to 800 mg of the pooled γ-globulin in 100 ml of .01 M acetate buffer, followed by dialysis over a forty-eight hour period against four liters of .01 M phosphate buffer. The phosphate buffer was changed after twenty-four hours.

The separation procedure involved a large Ace DEAE-cellulose column equilibrated with the phosphate buffer. The separation was accomplished at cold temperatures, 4°C-12°C, to preclude denaturing of the protein. The column separation process was monitored by ultra violet spectroscopy. Figure 9 presents a UV elution profile, absorbance at 280 nm versus elution volume and consists of a broad single eluent protein band. The recovered eluent contains total protein in the range of 200 to 500 milligrams of protein, as determined by ultra violet spectroscopy.

Protein Purification

One major concern of this research was to insure that the separation and isolation procedures produced purified IgG. Immunoelectrophoresis (IEP) and gel electrophoresis were used to check the purity of the protein product. As a

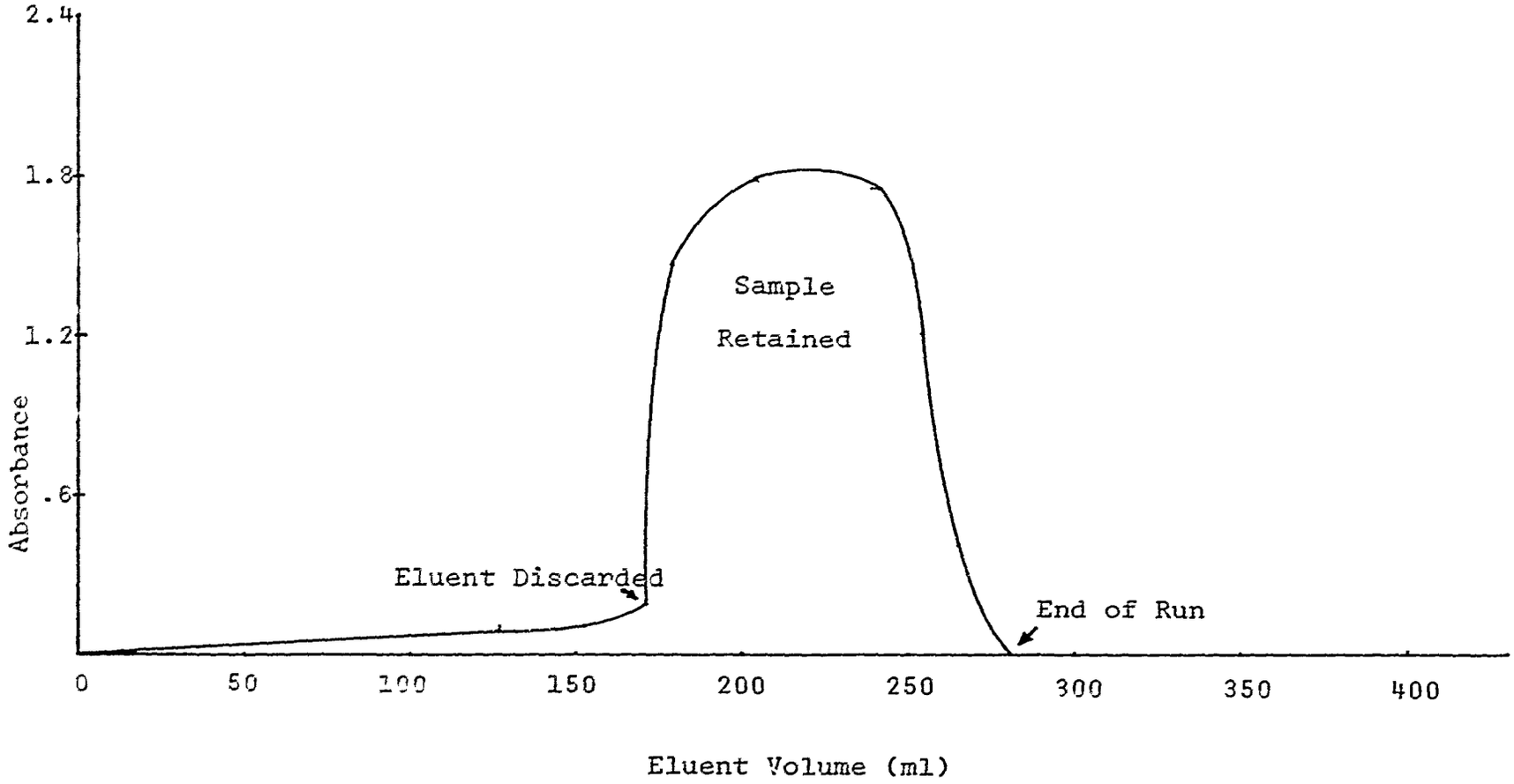

Figure 9 Typical UV Curve Column Separation

result of the immunoelectrophoresis and gel electrophoresis procedures, the product obtained after separation and isolation is pure IgG. An example of a typical immunoelectrophoresis pattern observed on immunoelectrophoresis (IEP) stained plates is presented in Figure 10. The samples sent to the LCM in Sioux Falls, South Dakota, were taken at different points during the purification process in order that the total process could be evaluated and are summarized in Table VII.

TABLE VII IMMUNOELECTROPHORESIS SAMPLE CONTENT

Sample Number	Protein Content
1	Y-globulin dissolved in .01 M phosphate buffer
2	Precipitate obtained during dialysis procedure
3	Y-globulin, dissolved in .01 M phosphate buffer, and dialyzed
4	Eluent from column separation
5	Eluent from column separation
Mines	Y-globulin, dissolved in .01 M acetate buffer

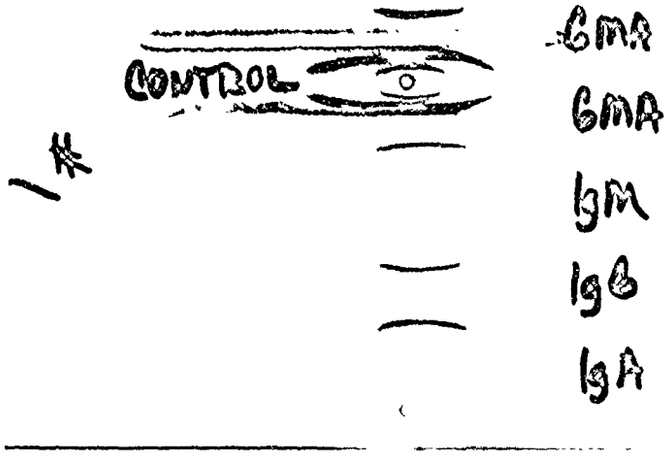

Figure 10a γ-globulin dissolved in .01 M phosphate, pH 6.3

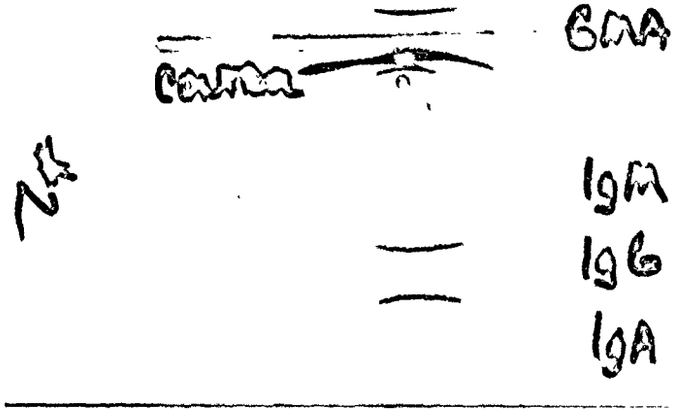

Figure 10b Precipitate from dissolution in Figure 10aabove

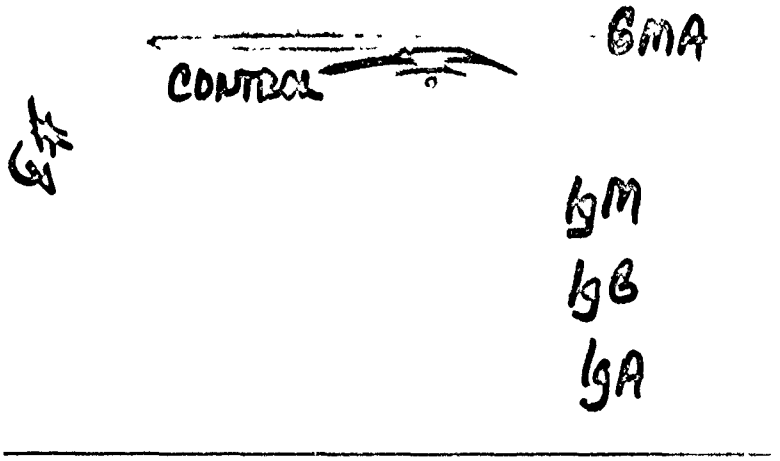

Figure 10c Sample of dialysed γ-globulin

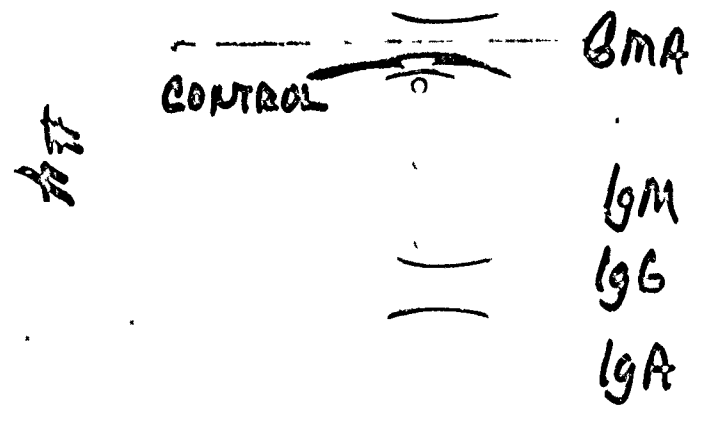

Figure 10d Sample collected after column separation

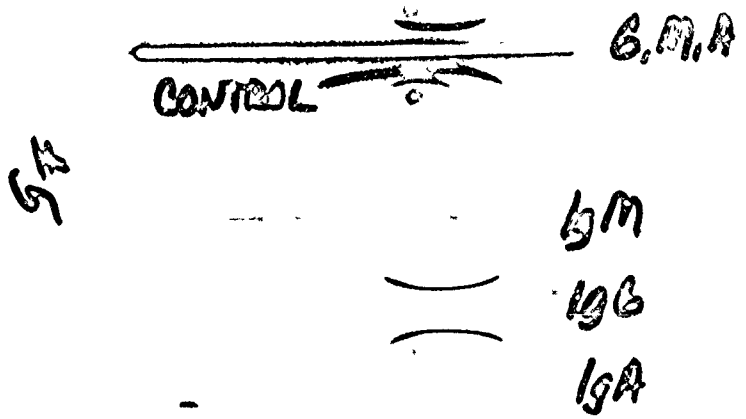

Figure 10e Sample after column separation

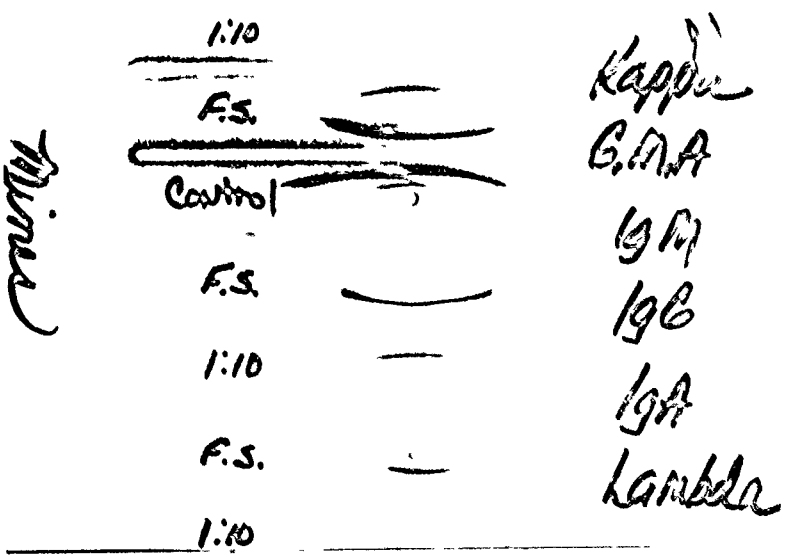

Figure 10f γ-globulin in .01 M acetate buffer, prior to dialysis and column separation

A summary of the immunoelectrophoresis results is compiled in Table VIII.

TABLE VIII SUMMARY OF IMMUNOELECTROPHORESIS RESULTS

	Anti Sera in Trough				
Sample	IgA	IgG	IgM	κ	λ
1	No	Yes	No	(1)	
2	No	Yes	No	(1)	
3	No	Yes	No	(1)	
4	No	Yes	No	(1)	
5	No	Yes	No	(1)	
Mines	Yes(2)	Yes	No	Yes	No

(1) No assay made
(2) Band was visible on original plate, but was too light for photo copy reproduction.

Results of this data indicate that all column isolated proteins and phosphate-buffer γ-globulin samples contained only IgG immunoprotein. γ-globulin samples dissolved in acetate buffer prior to DEAE purification contained mainly IgG, with a slight presence of IgA from the analysis of the IEP plate labelled "Mi·es," Figure 10f.

EPR RESULTS AND DISCUSSION

Experimental EPR Spectra of Metal Ion IgG Systems

The studies conducted at the University of New Hampshire fesulted in the measurement of EPR spectra of selected metal ion-IgG complexes.

Experimental Spectra

A summary of the experimental conditions of these investigations is given in Table IX and are grouped on the basis of the metal-ion system. Table lX summarizes the metal-ion and IgG concentrations, the buffer used, and the metal-ion/IgG mole ratio. The metal ion-IgG systems that will be discussed include Cu(II), Cr(III), Co(II), Fe(III), and VO^{+2}. The respective spectra will be presented as each system is discussed. No EPR spectra will be presented in the discussion which indicate vanadyl (IV) -IgG binding.

EPR parameters obtained from the spectra shown in Figures 11-15 include A values, the hyperfine splitting constants, which are measured directly from the spectra, as is H, the magnetic field strength. The Lande' splitting factor, g, is calculated from the equation $h\nu = g\beta H$, with ν equal to the operating microwave frequency. A summary of the experimental conditions for the Spectra of Figures 11-16 is given in Table IX.

TABLE IX METAL ION-IgG EPR STUDIES
Experimental Conditions

Metal Ion	[P]mM.	$[M^{n+}]$mM.	Buffer	$[M^{n+}]/[IgG]$ Ratio
Cu^{2+} (1)	.594	.98	pH = 6.3, no buffer	1.65
Cu^{2+} (1)	.594	.98	pH = 9.3, no buffer	1.65
Cu^{2+}	.297	.49	pH - 7.8, 0.2 M HEPES (2)	1.65
Cr^{3+}	.297	.962	pH = 7.7, 0.2 M HEPES	3.20
Co^{2+}	.297	.764	pH = 7.8, 0.2 M HEPES	2.60
Fe^{2+}	.297	2.16	pH = 7.8, 0.2 M HEPES	7.3

(1) Cu^{2+}-IgG solutions titrated with 0.05 M NaOH in a N_2-purged microtitration cell

(2) HEPES = N-2 Hydroxyethyl piperazine-N^1-2-ethanesulfonic acid

Preliminary Interpretation of Spectra

The EPR spectra of Figures 11-15 are interpreted from crystal field theory and a comparison of the experimental EPR parameters, H, A, and g, to those parameters reported in the literature for isolated metal complexes or metal-protein systems. A brief discussion of crystal field theory and EPR relationships is given in Appendix A and is referred to as necessary. It is imperative to note that this data interpretation is only preliminary and required further experimentation to confirm and refine spectral observations as well

as to develop more rigorous interpretation of the recorded spectra. Each metal ion-IgG system will be discussed separately.

TABLE X METAL ION-IgG EPR STUDIES
Estimated EPR Parameters

Figure	Complex	[M]/[P]	pH	EPR Parameters g values			A values(G)		Temp.
				g_o	$g_{//}$	$g_{\perp}$(1)	$A_{//}$	$A_{\perp}$	
11	$Cu(H_2O)_6^{2+}$	0.00	6.3	2.31	-	-	-	-	RT
11	Cu^{2+}-IgG	1.65	6.3	-	2.26	2.04	185	-	RT
11	Cu^{2+}-IgG	1.65	9.3	-	2.22	2.03	190	-	RT
11	Cu^{2+}-IgG	1.65	9.3	-	2.20	1.9.	?00	-	RT
12 A	Cu^{2+}-HEPES	0.00	7.8	-	2.?6	2.02	175	-	77°K
12 B	Cu^{2+}-IgG(A)	1.65	7.8	-	2.23	2.00	195	-	77°K
12 B	Cu^{2+}-IgG(B)	1.65	7.8	-	2.13	2.00	215	-	77°K
13 B	Cr^{3+}-IgG	3.20	7.8	1.98	-	-	190(^{53}Cr)		77°K
13 B	Cr^{3+}-HEPES	0.00	7.8	1.98	-	-	-	-	77°K
14	Co^{2+}	2.60	7.8	g_1=2.46 g_3=2.01		g_2=2.04			77°K
15	Fe^{3+}-IgG	7.30	7.8	g_1=4.43		g_2=2.05			77°K

(1) $g_{\perp}$ for the Cu^{2+} is estimated from the field position at the minima of the overshoot line at high field

Cu^{2+}-IgG EPR Spectra. Cu^{2+} ion is a d^9 transition metal ion, which is a single unpaired electron (S=1/2). Figure 11 presents a room temperature EPR spectra of a Cu^{2+}-IgG

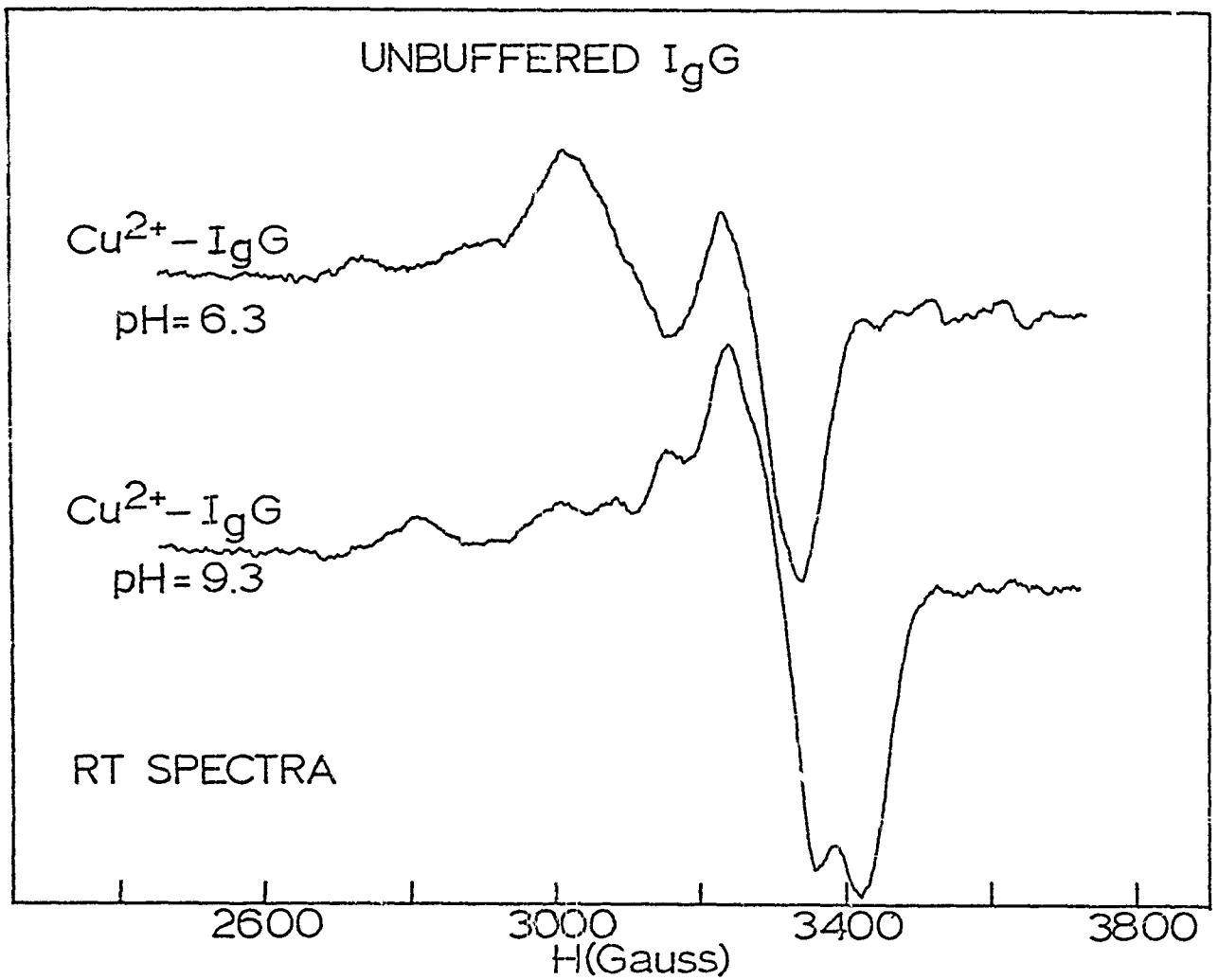

FIGURE 11 Cu^{+2}-IgG EPR Spectra

solution containing a Cu^{2+}: IgG mole ratio of 1.65: 1.00 at pH=6.3 and 9.3. The Cu^{2+}-IgG sample was titrated with 0.05M NaOH from pH=4.5 to pH=9.3. At low pH (~5.0), a typical single, broad EPR first-derivative signal centered at ca. 3050 gauss was observed, corresponding to the free $Cu(H_2O)_6^{2+}$ ion. As the solution is titrated with NaOH to higher pH, the free Cu^{2+} ion spectra is complicated by an overlapping anisotropic Cu^{2+} signal, presumably due to IgG-bound Cu^{2+} ion in an axial ligand environment. At pH 6.3, for example, both the free Cu^{2+} signal and the anisotropic signal are observed in the spectrum shown in Figure 11. At high pH values up to pH 9.3, the isotropic Cu^{2+} ion signal disappears, and a more complex anisotropic spectrum is observed. This spectrum exhibits four $^{63}Cu(I-3/2)$ hyperfine lines in the parallel region from 2750-3400 gauss. Although no computer simulation of this spectrum is available at this time, it appears that this spectrum corresponds to two IgG-bound Cu^{2+} ion EPR signals. Estimated g and A values for the parallel and perpendicular regions of these two anisotropic signals are given in Table X for the higher pH IgG-bound spectra together with the room temperature isotropic parameters of free Cu^{2+} ion. Both the $g_{//}$ and $g_{\perp}$ values, and the $A_{//}$ values are consistent with reported Cu^{2+} Ig-G-bound parameters from the literature.[41,42,43,44]

A comparison of Cu^{2+} ion EPR signals at 77°K as shown in Figure 12 for Cu^{2+} ion in a 0.2 M HEPES, pH=7.8 buffer (Spectrum A) with the spectrum for Cu^{2+} ion in an identical

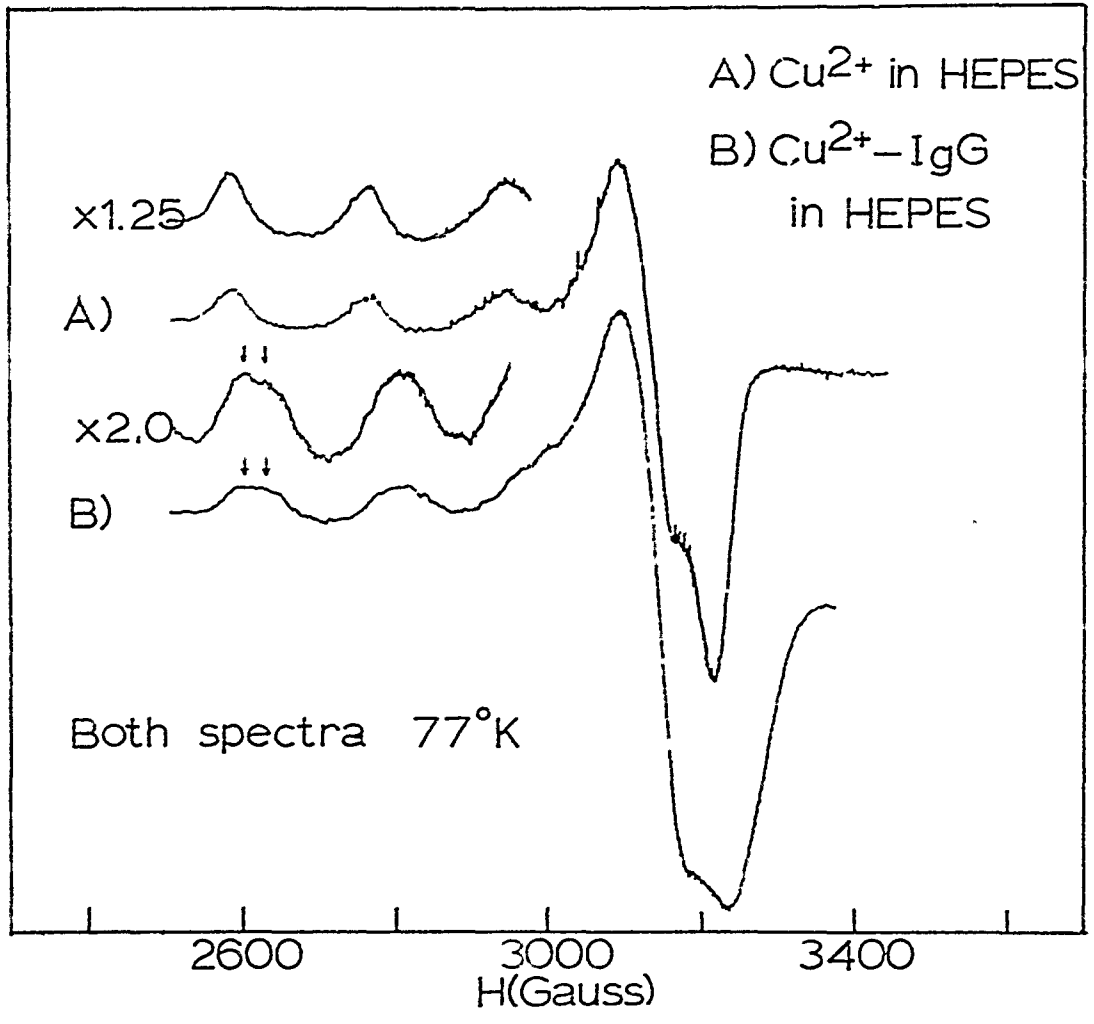

FIGURE 12 Cu^{+2}-IgG EPR Spectra

buffer media containing IgG (Spectrum B) further substantiates the change in Cu^{2+} ligand environment upon addition of IgG. The copper nuclear-hyperfine structure is clearly resolved in the $g_{//}$ region of both anisotropic spectra. A comparison of estimated $g_{//}$ and $A_{//}$ values for Figure 12A and 12B is given in Table X, wherein two sets of parameters are estimated for the Cu^{2+}-IgG signals. The reduction in $g_{//}$ and increase in $A_{//}$ hyperfine values for the Cu^{2+}-IgG signals in comparison with the free Cu^{2+} ion is consistent with an increased ligand field strength [41,42,43,44] for both protein-bound Cu^{2+}-IgG signals of Figure 12B. The pH range (pH=6.3-9.3) of these observed EPR parameter changes likewise suggest coordination of possibly strong nitrogen donors although no $^{14}N(I=2)$ superhyperfine structure have been observed. The two distinct anisotropic Cu^{2+}-IgG signals are moderately resolved in the lowfield, $g_{//}$ hyperfine region as noted by the arrows in Figure 12B, suggesting the existence of two different axial Cu^{2+} ion sites on the IgG protein. Further experimental investigations and computer simulation runs for the Cu^{2+}-IgG system are necessary to completely substantiate this preliminary interpretation. In addition, both pH and metal titrametric experiments are required to define the stoichiometry of the Cu^{2+} binding sites and to estimate approximate pKa values related to the metal coordination phenomenon.

<u>Cr^{3}-IgG Spectra</u>. Chromium (III) ion, a d^3 transition metal, exhibits ligand substitution inertness and contains

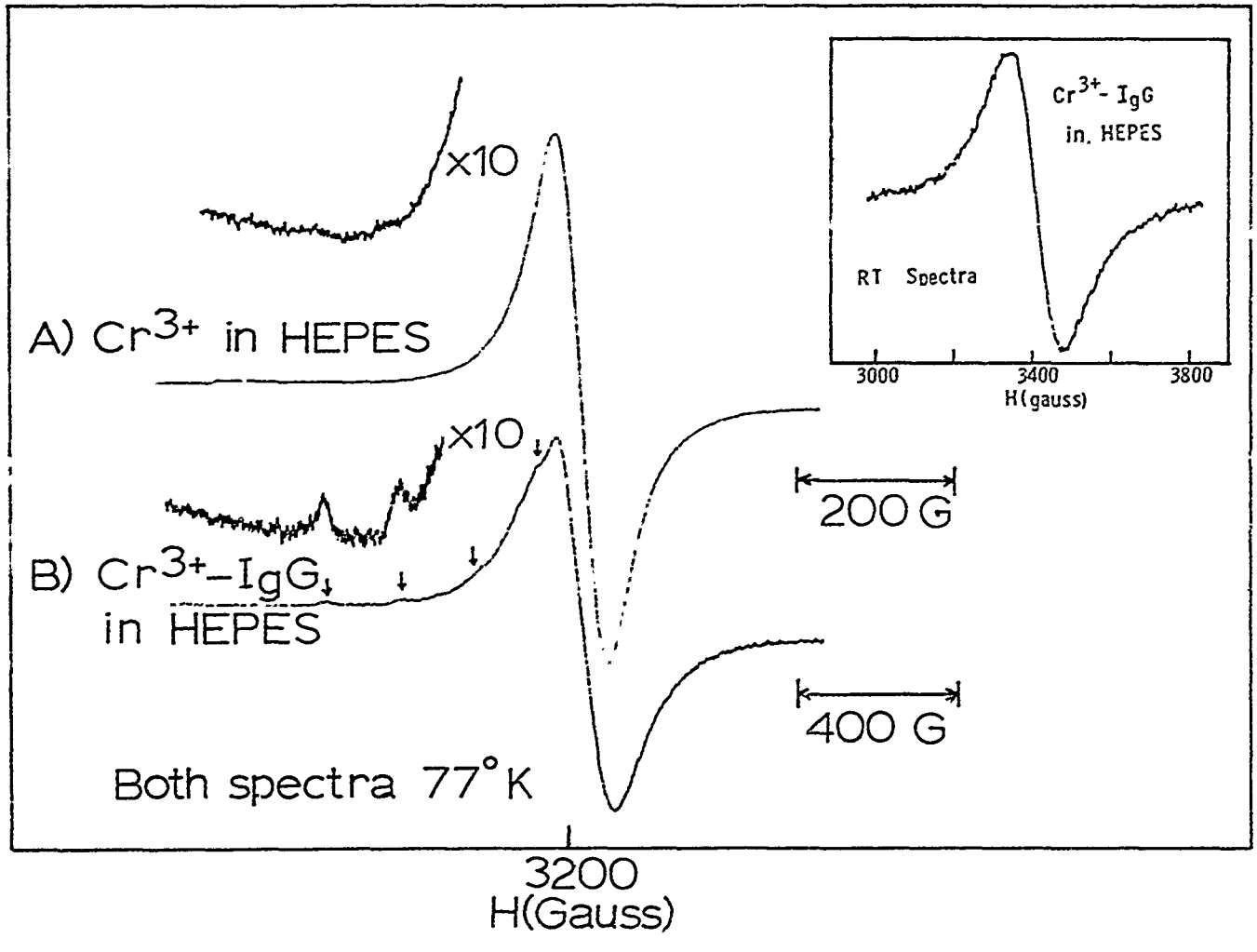

FIGURE 13 Cr^{+3}-IgG EPR Spectra

three unpaired electrons (S=3/2). EPR spectra of various Cr(III) complexes have been extensively studied,[45] whereas few Cr^{3+}-protein bound signals have been observed.[46] The titration of "metal free" IgG solutions in HEPES, pH=7.8 buffer was carried out up to 3.2g-atoms of Cr^{3+}/ IgG Room temperature and 77°K EPR spectra of the Cr^{+3}-IgG solutions (See Table IX for experimental) incubated for 48 hours are presented in Figure 13, together with estimated EPR parameters in Table IX. The room temperature spectra of Cr^{3+}-IgG is rather featureless (See insert, Figure 13) but may be contrasted with an identical 9.62×10^{-4}M Cr^{3+}-HEPES solution without the IgG protein. At room temperature, Cr^{3+} ion exhibits a broad, single line EPR signal centered at g=1.98 (line width, Hpp = 75G), whereas the Cr^{3+}-IgG sample exhibits a much broader signal (g=1.98,Hpp=145 gauss). Liquid nitrogen spectra of these analogous samples are shown in Figure 13A and 13B. Although the resonance g values (g= 1.98) for these spectra are identical, two quite distinct aspects of the Cr^{3+}-IgG spectrum deserve noting. First, the line width of the Cr^{3+}-IgG spectrum is much greater (160 vs. 60G) than the corresponding Cr^{3+}-HEPES spectrum; and second, four hyperfine lines are observed on the low field side of the principle resonance peak with quite large hyperfine splittings of 190 gauss. Similar hyperfine splittings have been observed for a 1:1 Cr^{3+}-IgG sample in a Tris-HCl buffer, pH 8.5 at Denver University. In this case, at least nine hyperfine lines of ca A=73G were observed. Although the

origin of these hyperfine lines is not readily apparent, reported ^{53}Cr(I=3/2, 9.5 % natural abundance) nuclear hyperfine line splittings much smaller (10-60G) than this have been observed in various Cr^{3+}-complexes.[46] The present Cr^{3+} EPR spectra are rather limited at this time, not allowing for zero-field splitting calculations; however, the apparent alterations of the Cr^{3+} EPR signal do suggest molecular interactions of an undefined nature with the IgG protein. Further EPR experiments such as those recommended for the Cu^{2+}-IgG system are necessary.

<u>Co^{2+}-IgG and Fe^{3+}-IgG Spectra</u>. Figures 14 and 15 represent EPR spectra obtained for Co^{2+}-IgG and Fe^{3+}-IgG samples, respectively, obtained at 77°K in a frozen 0.2 M HEPES, pH= 7.8 solution. Although our preliminary investigations are limited, the recorded spectra at 77°K suggest undefined interactions of these aquated metal ions with "metal free" IgG. No room termperature spectra were observed for either sample.

The Co^{2+} ion is of d^7 electron configuration with a cobalt nuclear spin value of I=7/2. Both high-spin (S=3/2) and low spin (S=1/2) complexes are known, although most reported EPR signals have been for low-spin Co^{2+} ion in a planar, square pyramid or octahedral ligand environment.[44,45] The Co^{2+}-IgG EPR signal reported in Figure 14 consists of a broad resonance feature at g_1 =2.46, with a sharper signal at g_2 =2.04, and g_3 =2.01 (See Table IX). These EPR signals are similar to other low-spin Co^{2+}-protein complexes such as various vitamin B_{12} complex spectra,[41] indicating possibly a

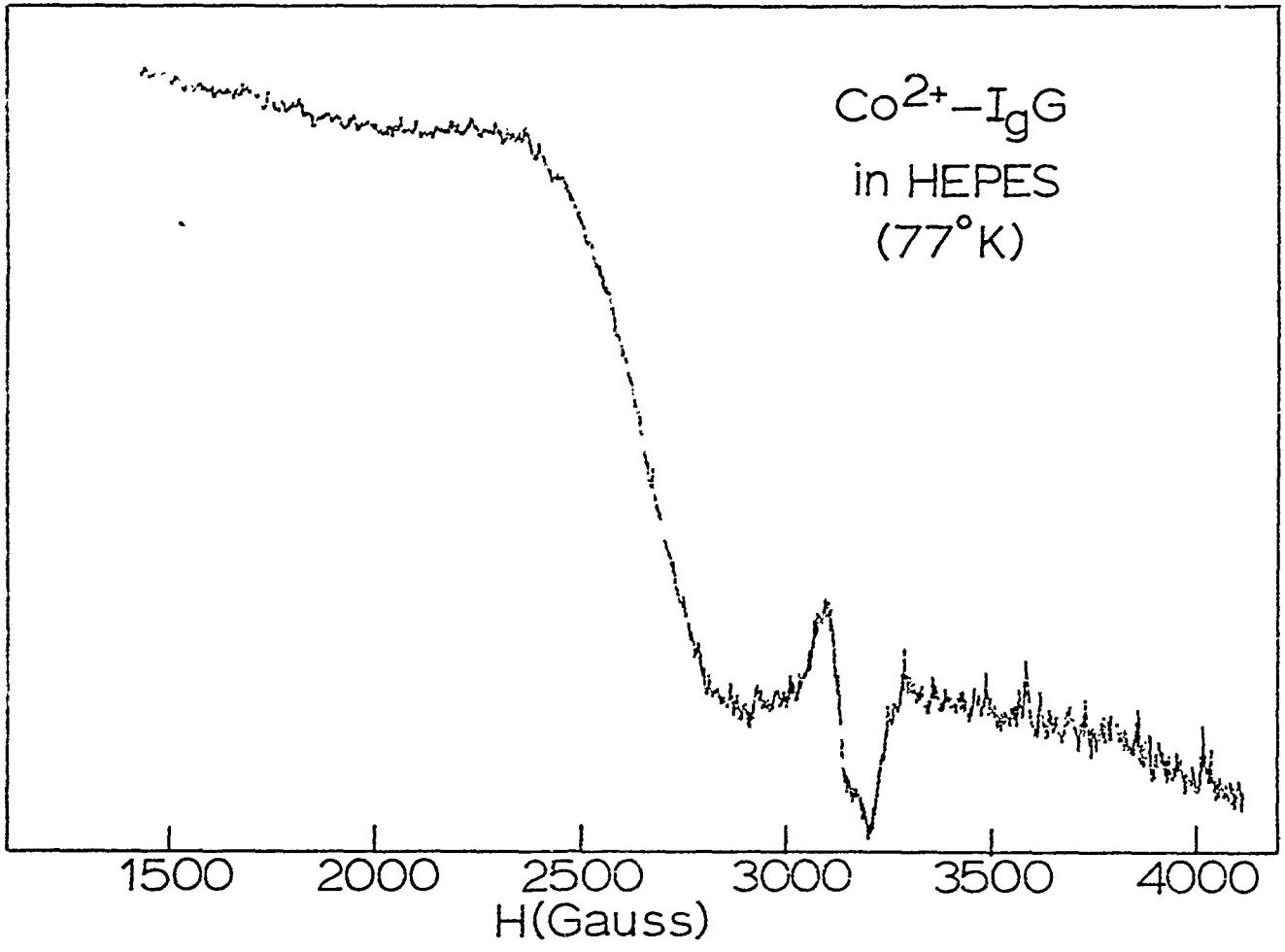

FIGURE 14 Co^{+2}-IgG EPR Spectra

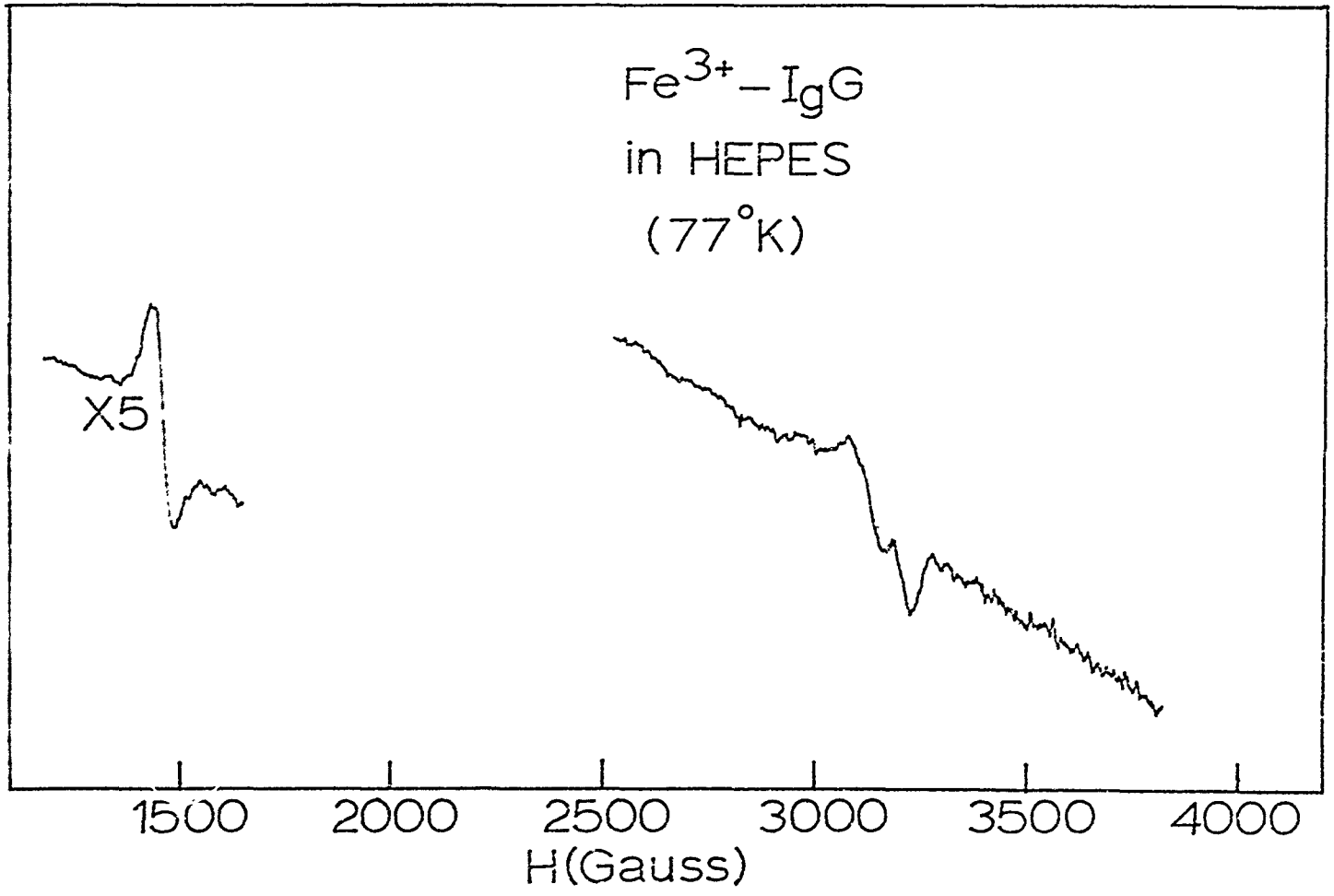

FIGURE 15 Fe^{+3}-IgG EPR Spectra

rhombic ligand field, $g_x \neq g_y \neq g_z$. The nearly identical g_2 and g_3 values of the Co^{2+}-IgG spectra are also consistent with five coordinate tetragonal pyramid complexes such as other B_{12} complexes reported, wherein $g_x \neq g_y = 2.46$, $g_z \approx 2.0$.

The Fe^{3+} ion is of d^5 electron configuration. The Fe^{3+}-IgG spectra is shown in Figure 15, together with estimated EPR parameters in Table X. The Fe^{3+}-IgG spectra were recorded at 77°K in a HEPES, pH=7.8 buffer. The reported EPR spectrum in Figure 15 consists of an intense low-field signal at g=4.43, with poorly defined fine structure as well as a less intense higher field signal possible consisting of two resonance features at g=2.05. The Fe^{3+}-IgG signal corresponds to either a high-spin (S=5/2) Fe(III) ion in at least an axial ligand field [41,43] or two Fe^{3+} signals, one high-spin signal at g=4.43 and a low-spin(S=1/2)signal at g≈2. Although insufficient experimental evidence is available to define whether these signals correspond to one or two Fe^{3+}-IgG bound signals, and do not allow the calculation of zero-field splitting parameters necessary to describe the Fe^{3+} site symmetry, the present spectrum suggests molecular interactions of the Fe(III) ion with IgG in the physiological pH range. Since the metal/IgG ratio or this sample is quite large (≈7.3) in comparison with the other metal-IgG samples investigated, further titrametric data from 1-10 equivalents of Fe^{3+} is necessary to identify whether these two signals appear simultaneously or at different equivalents of metal-ion: IgG ratios.

Additional EPR studies were conducted with VO^{+2}-IgG systems of mole ratios of 6.7 and 1.7 to 1, and solution pH values of 6.3 and 7.8, respectively. The buffer system was double distilled water and HEPES, respectively. No indication of binding by VO^{+2} was apparent at the conditions used in the study.

In summary, the following analysis is offered. Figure 11, the copper(II)-IgG spectra, indicates that at pH 6.3 there is an overlap of signals, from both free copper ion and from bound copper ion, giving a spectra that is a combination of isotropic and anisotropic Cu^{2+} ion signals. At pH 9.3, the free copper ion spectra has disappeared and only bound copper is observed (Figure 11). There is a suggestion that this spectra may be a result of two different copper ions, indicating at least two distinct binding sites.

Figure 12A exhibits a free copper ion EPR spectrum in frozen solution, while Figure 12B shows IgG-bound copper ion in frozen solution. Figure 12B indicates an overlap of spectra, possibly free and IgG-bound copper, rather than two sites as noted in Figure 11B (pH=9.3). Note that this pH is 7.8, midway between the pH values of solution spectra discussed for Figure 11A and 11B. Figure 13A shows an EPR spectra of free chromium(III) ion. Figure 13B presents an EPR spectra that suggests IgG-bound chromium(III) ion with unknown hyperfine structure, although the A values are unlike other reported hyperfine data of ^{53}chromium hyperfine splittings. Reported ^{53}Cr A values of 11 gauss have been reported,

whereas the A_{Cr} values of Table X from spectrum 13B are approximately 200 gauss.

Figure 14 presents the EPR spectrum of IgG-bound cobalt (III) ion and consists of three spectral curves. These three resonance features indicate that the Co(II) ion x, y, and z ligand fields are unequal due to a rhomic ligand configuration about the Co(II) ion. The z axis is severely distorted, while the x and y axes appear nearly identical. Free cobalt (II) ion does not show this distorted EPR spectrum at 77°K.

Figure 15 shows the spectra of iron(III)-IgG. Two possible interpretations of this spectra are plausible:

a) the EPR resonance feature at 1500 gauss could be due to the z axis magnetic field interaction and the signal around 3000 gauss could be the x-y axes interaction, indicating a severely distorted rhomic ligand configuration for a high field Fe^{3+} ion, or

b) the curve at 1500 gauss represents the spectrum of a protein bound Fe(III) ion while the curve around 3000 gauss is the spectra of a free low-spin Fe(III) ion in a cubic ligand field.

The analysis of these spectra definitely provide EPR evidence of coordination by the metal ions, Cu(II), Cr(III), Co(II), and Fe(III) ions to pure non-immune IgG. Further detailed study is required.

Ultra Filtration Study of Cu^{2+}-IgG Systems

Limited ultrafiltration studies of Cu^{2+}-IgG were undertaken to confirm the binding of Cu^{2+} ion to non-immune IgG. Prior to introduction of copper-IgG solutions, the eight channels of the MRA cell were flushed twice with .01 M 1,10-phenanthroline to remove any metal contamination. The cell was then flushed with two changes of HEPES buffer to remove the 1,10-phenanthroline. The membranes had been soaked in double distilled, chelexed water overnight prior to installation in the cell. During the cell cleaning process, several experimental complications were noted. Channel five filtered very slowly during both of the HEPES changes, while channel four slowed down considerably during the last HEPES change. A very slight pressure leak around the teflon screw in the sample port was probably the cause of this malfunction. The screws were wrapped with teflon tape to stop any leakage, but the channels still ran slowly. Channel number two had a bad gasket and had been replaced by a non-standard gasket. All gaskets seemed worn and some tended to leave debris in the channel. No debris could pass through the membrane into eluent. The injection ports below the membrane presented a minor problem. If injection ports were not covered by tape during the run, capillary action drew off the eluent. In point of fact, this occurred in channel five and no data were obtained from this channel. The temporary solution was to tape over the injection ports during the remaining runs. This, however, caused the eluent to remain in the channel

and not drop out into the collectors. A procedure adopted to alleviate this problem entailed piercing the tape with a needle after the eluent was through the membrane and in the lower channel. This allowed the eluent to drop into the sample collectors. The MRA instruction book stated that each run should take approximately one hour to run. It was found that each flushing took at least two hours and the metal-IgG run took eighteen hours.

In analyzing the results of the metal-IgG run, an unexplained loss in metal concentration occurred in channel eight, the control designed to measure amount of metal absorbed by the membrane. One additional study was conducted to check the control. As a result, the control performed during the actual metal-IgG study was not considered, and the results from the additional study were used to estimate the amount of metal absorption by the membrane.

The additional study involved mixing copper ion stock solution (2.8 µl) corresponding to similar copper ion concentration as the ultrafiltration run with HEPES buffer (300 µl) and allowing the solution to equilibrate overnight. Three tubes contained copper ion in HEPES buffer; three tubes contained copper ion in HEPES, and one memberane; one tube was copper ion and IgG solution (300 µl) and one tube was HEPES alone.

As a result of an atomic absorption analysis, the difference in copper content of 200 ul of copper-HEPES, diluted to 1 ml, and the copper content of the copper-HEPES in contact

with the membrane was calculated and used to estimate the amount of metal ion absorbed by the membrane. This value was calculated to be 6.3×10^{-9} moles. The copper content in the HEPES was found to be approximately .05 ppm, a value found to be just slightly above background noise and is considered to be insignificant.

The single ultrafiltration study of this work was made with the MRA cell and was accomplished as shown in Table XI. over the initial Cu^{2+}:IgG mole ratios of .55 to 3.9 in a .01 M HEPES buffer (pH = 7.00).

TABLE XI INITIAL Cu^{+2}: IgG RATIOS

Channel	(1) Amount of Cu^{+2} added (µl)	Moles of Cu^{+2}	(2) Mole Ratio Cu^{+2}/IgG
1	.95	9.38×10^{-9}	.55
2	1.9	1.88×10^{-8}	1.1
3	2.85	2.81×10^{-8}	1.65
4	3.8	3.75×10^{-8}	2.2
5	4.75	4.69×10^{-8}	2.76
6	5.7	5.63×10^{-8}	3.3
7	6.65	6.56×10^{-8}	3 9
8	3.8	3.75×10^{-8}	no protein control

(1) Concentration of Cu^{+2} stock solution was 9.87×10^{-3} m/l
(2) Concentration of IgG was 5.67×10^{-5} m/l, or in 200 µl 1.7×10^{-8} moles.

The data summarized in Table XI gives the mole ratios of the Cu(II) ion and IgG system considered for study in the ultrafiltration cell.

Note that the metal ion concentration in the stock solution was high enough to preclude any inordinate dilution effects on the protein, while still achieving the desired mole ratios.

The determination of the amount of copper ion bound to the IgG was made using the equation given in the experimental discussion. Table XII summarizes the added Cu(II), and eluent Cu(II) concentrations and resulting membrane-bound Cu(II) and protein-bound Cu(II).

TABLE XII CALCULATION OF MOLE RATIOS

Channel	Moles of Cu^{+2} added to the IgG	- Moles of Cu^{2} in eluent	= Moles of Cu^{2} held on membrane	Moles of Cu^{2} bound to IgG
1	9.38×10^{-9}	1.57×10^{-9}	6.3×10^{-9}	1.51×10^{-9}
2	1.88×10^{-8}	1.48×10^{-9}	6.3×10^{-9}	1.1×10^{-8}
3	2.81×10^{-9}	1.48×10^{-9}	6.3×10^{-9}	2.0×10^{-8}
4	3.75×10^{-8}	1.42×10^{-9}	6.3×10^{-9}	2.98×10^{-8}
5	4.69×10^{-8}	no eluent retained	- - - - -	- - - - -
6	5.63×10^{-8}	1.57×10^{-9}	6.3×10^{-9}	4.8×10^{-8}
7	6.56×10^{-8}	1.42×10^{-9}	6.3×10^{-9}	5.8×10^{-8}
8	Control	Discarded		

Note that the moles of Cu(II) held on the membrane is constant throughout the series. This is the value estimated from the additional control study. Channel number five eluent was lost because the injection port was not sealed off properly.

The moles of Cu(II) added to the protein increase as the channels increase. The moles of Cu(II) in eluent vary only slightly. The important trend this table shows is that as the moles of Cu(II) added to the protein increase, so the moles of Cu(II) bound to the IgG increase.

Table XIII summarizes the final Cu^{+2}/IgG ratios.

TABLE XIII FINAL MOLE RATIOS, Cu^{+2}/IgG

Channel	Moles of Cu^{+2} bound to IgG	Moles of IgG	Final Mole Ratio Copper/ IgG
1	1.51×10^{-9}	1.7×10^{-8}	.08
2	1.1×10^{-8}	1.7×10^{-8}	.65
3	2.0×10^{-8}	1.7×10^{-8}	1.2
4	2.98×10^{-8}	1.7×10^{-8}	1.75
5	lost		
6	4.8×10^{-8}	1.7×10^{-8}	2.8
7	5.8×10^{-8}	1.7×10^{-8}	3.4
8	Control Discarded		

Table XIII summarizes the final results of the ultrafiltration study. It demonstrates that Cu(II) ion binds to the IgG molecule in increasing mole ratios from .08 to 3.4. The maximum mole ratio of Cu(II) bound to the IgG was not reached in this study. This ultrafiltration data confirms and supports the EPR data.

CONCLUSIONS

Findings

This research effort has demonstrated that two systematic techniques to investigate metal-ion binding to pure, non-immune, IgG are possible. These two techniques are electron paramagnetic resonance spectroscopy (EPR) and ultrafiltration using an MRA cell.

EPR spectroscopy has demonstrated metal ion-IgG binding for Cu(II), Cr(III), Fe(III), and Co(II) ions over the experimental conditions discussed in this report. Metal-ion binding with IgG was not observed for VO^{+2} under analogous conditions used for the above metal systems.

Limited ultrafiltration studies with Cu(II) and IgG confirm the binding of Cu(II) to IgG in mole ratios of .08 to 3.9.

Significance

It appears that the use of procedures to insure metal-free IgG used for study is of prime importance. This allows direct interpretation of metal ion binding to IgG, and eliminates complications arising from metal exchange reactions and mechanisms to determine binding information. Dwek has reported six binding sites for Gd(III), a non-toxic lanthanide metal. Baker and Hultquist reported metal ion-IgG binding for Cu(II), which, in the case reported, is considered a toxic metal.

This research effort shows metal ion-IgG binding for both

toxic[Cr(III), Co(II)], and non toxic[Fe(III), Cu(II)] metal ions.

Non-immune IgG was used in this research effort, as was done in Dwek's work. Baker and Hultquist used immune IgG for their study. The EPR spectrum reported by Baker and Hultquist indicates that the copper of the metal ion-IgG complex is an EPR indeterminable state. It is our opinion, however, that the problem is not the state of the copper ion, but rather the conditions under which the EPR spectrum was produced. Specifically, the concentration of the protein solution was too low to allow an adequate signal from the copper ion-IgG complex.

Directions for Future Study

As a direct result of the variance of EPR data between the Dwek, Baker and Hultquist, and our reports, a correlation between experimental conditions and use of non-immune Cu(II)-IgG versus immune Cu(II)-IgG should be pursued. At the present time too little data is available to contrast these systems.

Both EPR and ultrafiltration studies are recommended to conduct future metal ion-IgG binding research. Using Scatchard plots, binding levels can be determined to accurately estimate the number and stoiciometry of unique metal ion binding sites on the IgG molecule and its fragments. Metal ions for consideration include those of this report as well as others.

The regional location, binding site amino acid sequences, site symmetry, and distribution of the binding sites can be best pursued by EPR and ultrafiltration using the fragments of IgG.

This report noted some difference in the binding process that seems to be directly related to pH. This variable, as well as determination of the K_D for each binding site, requires further study.

REFERENCES

1. Roger A. Goyer et al., Toxicology of Trace Elements, p. xi.

2. Lucy Treagan, "Metals and the Immune Response - A Review," Research Communication in Chemical Pathology and Pharmacology, Vol. 12, No. 2, October 1975.

3. Kenneth David Wilde, PhD Dissertation, "The Effect of Chronic Exposure to Low Levels of Heavy Metals in Immune Mechanism in the Guinea Pig."

4. Alfred Nisonoff et al., The Antibody Molecule, p. 92.

5. Ibid., Chapter 4.

6. Ibid., pp. 2-12.

7. D. H. Campbell et al., Methods in Immunology, 2nd Ed. (1970) and 3rd Ed. (1977).

8. S. R. Dower et al., "The Binding of Lanthanides to Non Immune Rabbit IgG and Its Fragments," Biochem J. 149, (1975), pp. 73-82.

9. R. R. Porter, "The Hydrolysis of Rabbit Gamma Globulin and Antibodies with Crystalline Papain," Biochem J. 73, (1959), pp. 119-126.

10. F. W. Putnam et al., "The Cleavages of Rabbit Gamma Globulin by Papain," J. Biol. Chem. 237, (1962), pp. 717-726.

11. Campbell.

12. Dower.

13. Putnam.

14. Cambell.

15. Putnam.

16. A. Nisonoff et al., "Separation of Univalent Fragments from the Bivalent Rabbit Antibody by Reduction of Disulfide Bonds," Arch. Biochem. Biophys. 89, (1960), pp. 230-244.

17. A. Nisonoff et al., "Evidence for Linkage of Univalent Fragments on Half-Molecules of Rabbit Gamma Globulin by the Same Disulfide Bond," Biochemistry 3, (1964), pp. 1338-1342.

18. Campbell.

19. Nisonoff.

20. R. R. Porter, "Antigen Binding Sites of IgG," Contemporary Topics of Immunochemistry, 1, (1972), pp. 145-164.

21. E. A. Padlec et al., "Immune Systems: Genes Receptors, Signals," ICN-UCLA, Symposium of Molecular Biology, 3rd, (1974), pp. 7-14.

22. J. C. Hsia et al., Biochemistry 10(20),(1971), pp. 3742-3748.

23. R. A. Dwek et al., Philos. Trans. R. Soc. London Ser. B 272(915), (1975), pp. 53-74.

24. J. C. Hsia et al., Arch Biochemistry Biophysics 129(1), (1969), pp. 396-307

25. J. D. Glickson et al., "In Vitro Binding of ^{67}Ga to L1210 Cells," Cancer Research 33, November 1973, pp. 2706-13.

26. R. A. Gans et al., "Serum Inhibition of In Vitro ^{67}Ga Binding by L1210 Leukemic Cells," Cancer Research 35, (1975), p. 1422.

27. P. B. Sigler et al., Journal of Molecular Biology 14(2), (1965), pp. 640-644.

28. Karl D. Hardman, "Crystallography of a Metal Containing Protein, Concavolin A," Advances in Experimental Medicine and Biology, Vol. 40, pp. 103-124.

29. B. L. Baker et al., "A Copper-binding Immunoglobulin from a Myeloma Patient," J. Biolog. Chem., Vol 253(4), (1978), pp. 1195-1200.

30. C. P. Hill, "Toxicology of Copper," edited by R. A. Goyer and M. A. Mehlman, Toxicology of Trace Elements, pp. 123-124.

31. S. K. Dower et al., "The Binding of Lanthanides to Non-immune Rabbit Immunoglobulin G and Its Fragments," Biochem J. 149, (1975), pp. 73-82.

32. D. R. Burton et al., "Difficulties in Determining Accurate Molecular Motion Parameters from Protein Relaxation Embracement Measurements as Illustrated by the Immunoglobulin G·Gd(III) System," Eur. J. Biochem, 71, (1976), pp. 519-528.

33. D. R. Burton et al., "The Determination of Molecular-Motion Parameters from Proton-Relaxation-Embracement Measurements in a Number of Gd(III)·Antibody-Fragment Complexes," Eur. J. Biochem 75, (1977), pp. 445-453.

34. Hsia.

35. Hsia.

36. R. A. Dwek et al., "Interactions of the Lanthanide and Hapten Binding Sites in the F_c Fragment from the Myeloma Protein, MOPC 315," Biochem J., 155, (1976), pp. 37-53.

37. R. A. Dwek et al., "Structural Studies on the Combining Site of the Myeloma Protein MOPC 315," J. Biochem, 53, (1975), pp. 25-39.

38. M. Gavis et al., "Comparison of the Fine Specificity of Anti-Dinitrophenyl-Combining Site Composed of Either V_L Diner or V_L and V_H of Protein 315," Biochemistry Vol. 16, No. 14, pp. 3154-3159.

39. R. A. Dwek et al., "Antibody-Hapten Interactions in Solution," Phil. Trans. R. Soc. Lond. B. 272 (1975), pp. 53-74.

40. R. A. Dwek et al., "Structure of an Antibody Combining Site by Magnetic Resonance," Nature, Vol. 266, 3 March 1977, pp. 31-37.

41. J. S. Garvey et al., Methods in Immunology, 3rd Ed.

42. G. T. Stevenson et al., "The Recombination of Diners of Immunoglobulin Peptide Chains," Biochem J. 118, (1970), pp. 703-712.

43. J. J. Fitzgerald et al., "EPR Studies of the Structure and Metal Ion Exchange of Vanadyl (IV) Bovine Carbonic Anhydrase," Biochem, 13, (1974), pp. 4338-4347.

44. E. Ochiai, Bioinorganic Chemistry, An Introduction and Reference Therein.

45. A. Ehrenburg et al., (editors) Magnetic Resonance in Biological Systems.

46. R. Aasa et al., J. Biol. Chem. 243(9) (1978) pp. 2399-2404.

47. J. S. Taylor et al., Proc. Natl. Acad. Sci. 67(3), (1970), pp. 1410-1416.

48. B. A. Goodman et al., "Electron Spin Resonance of Transition Metal Complexes," Adv. Inorg. Chem. and Radiochem., 13, (1970), pp. 135-362.

49. P. Aisen et al., J. Biol. Chem. 244(17), (1969), pp. 4628-4633.

APPENDIX A

Discussion of Crystal Field Theory and EPR Parameters

Five transition metals are of interest to this research effort. Their electronic characteristics require defining with relationship to their predicted, observed, and reported EPR behavior. All are d orbital metals.

The EPR spectra are analyzed to distinguish the isotropic curves from the anisotropic curves. Isotropic curves indicate free ions, while anisotropic curves indicate bound or hindered movement of the ions.

The electronic configurations of each metal, in high spin state and low spin state, are shown in Figure 16. A high spin state refers to the metal ion influenced by a weak ligand, a weak crystal field. A high spin state approaches a free ion configuration, where the electrons stay spread throughout the entire set of d orbitals. A low spin state refers to the metal ion influenced by a strong ligand, a strong crystal field. In a low spin state the electrons tend to crowd together into the more stable orbitals. The d orbitals have two subsets of orbitals, the e_g and the t_{2g} under an octahedral (O_h) ligand field. The t_{2g} orbitals are filled first in a strong O_h crystal field, while the e_g orbitals are higher in energy and less stable, being filled last, as necessary, in a strong O_h crystal field.

In a weak crystal O_h field, both the t_{2g} and the e_g orbitals contain electrons.

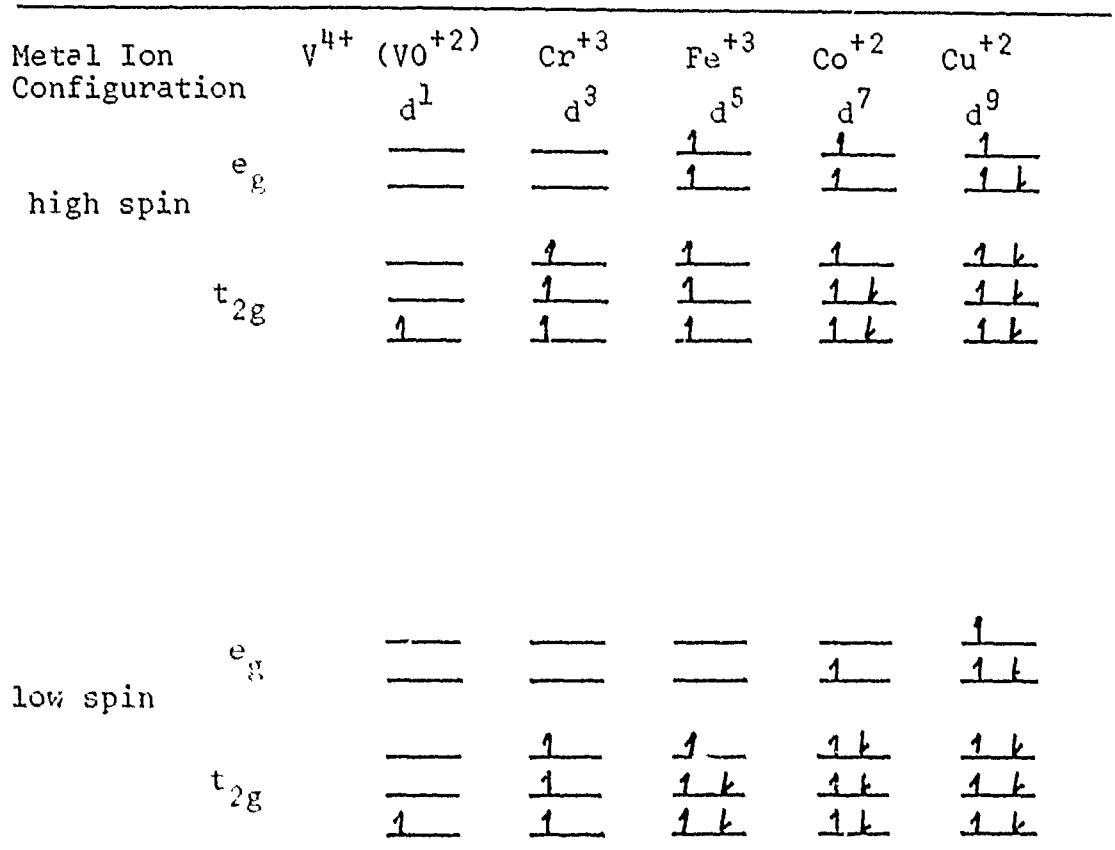

Figure 16 Metal ion configuration, high and low spin states.

As shown in Figure 16 , there is no electron spin difference in spin states for a d^1, a d^3, or a d^9 metal ion. In a d^5 and a d^7 metal ion, however, there is a difference between a high and a low spin state.

Generally, a low spin state denotes a strong ligand field while a high spin state denotes a weak ligand field.

Electron paramagnetic resonance takes advantage of the electron spin state in the orbitals t_{2g} and e_g. To briefly explain, some terms and their relationships are presented.

The following equation describes the magnetic effects on spin and orbital angular momentum of an electron.

$$\Delta E = h\nu = g\beta H m_e + A m_h m_n$$

ΔE is the energy difference between the electron spins in a magnetic field

h is Plank's constant

ν is the frequency of the microwave signal providing energy to the system

g is the Lande' splitting constant

β is the Bohr magneton constant

H is the magnetic field strength

m_e is the total electron spin number

The term $g\beta H m_e$ is called the Zeeman effect, while the term $A m_h m_n$ is called the Fermi contact effect.

The Fermi contact effect describes the interaction of the electron and a neighboring nucleus. This appears on the EPR spectra as hyperfine and superhyperfine splitting. Superhyperfine splitting will not be considered in this discussion.

The Zeeman effect describes the energy difference between the spin states of the electron. In practice, ν is set to a known value and H is measured from the spectra. This allows g to be calculated and is the reported value. For a free electron, g is equal to 2.0023.

The calculation of g values and comparison with other reported g values allows the investigator to make certain

assessments of the ion's magnetic properties, since g is directly related to the magnetic moment, μ. Using g values, the investigator can also make interpretations of the symetry and strength of the ligand field in terms of magnetic spin levels and crystal field theory.

One additional term must be introduced. A, the hyperfine splitting constant, is measured from the spectra and is the distance, in gauss, between the hyperfine and superhyperfine peaks. Evaluation of A allows interpretations to be made of the electron spin-nuclear spin interactions. These interpretations are reported values and can be compared with other values and help to shed light upon the electron's particular environment.

In summary, EPR spectra are analyzed to determine type of curve, either isotropic or anisotropic, and the magnitude of the g values and A values. These three determinations allow one to describe the basic ligand environment of the metal ion and interactions of the electrons on the metal ion with its immediate chemical environment.

ACKNOWLEDGEMENTS

The author wishes to express his sincere appreciation to the United States Army, Chemical Corps, for the opportunity to pursue this degree.

The author especially thanks Dr. J. J. Fitzgerald for guidance, assistance and friendship during the course of this research and to Dr. J. H. Conte and the staff of the Chemistry department for the needed support and encouragement.

Of special note is the prayerful support and caring by the author's wife and friends.

VITA

Nyle Hedin was born 27 August, 1947, in Pierre, South Dakota. He graduated from Rapid City High School in 1965. In 1969 he graduated from the South Dakota School of Mines and Technology, earning a Bachelor of Science in Chemistry. He attended the last two years of college using an ROTC scholarship. He graduated as a Distinguished Military Graduate and with a Regular Army Commission. Graduation was followed by active duty with the United States Army. He remains on active duty to the present, now having the rank of Captain, Chemical Corps. His army career includes tours of duty in the United States, Vietnam, and Germany. This Masters Degree was earned under sponsorship of the U. S. Army.

SECURITY CLASSIFICATION OF THIS PAGE *(When Data Entered)*

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER	2. GOVT ACCESSION NO. / 3. RECIPIENT'S CATALOG NUMBER
4. TITLE *(and Subtitle)* Metal Ion Interactions with Immunoglobulin G (IgG) I. Preliminary Studies with Electron Paramagnetic Resonance (EPR) Spectroscopy and Ultrafiltration	5. TYPE OF REPORT & PERIOD COVERED: Final Report 12 Dec 78 / 6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s): CPT Nyle Hedin	8. CONTRACT OR GRANT NUMBER(s)
9. PERFORMING ORGANIZATION NAME AND ADDRESS: Student, HQDA, MILPERCEN (DAPC-OPP-E) 200 Stovall Street, Alexandria, VA 22332	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
11. CONTROLLING OFFICE NAME AND ADDRESS: HQDA, MILPERCEN, ATTN: DAPC-OPP-E, 200 Stovall Street, Alexandria, VA 22332	12. REPORT DATE: 12 Dec 78 / 13. NUMBER OF PAGES: 130
14. MONITORING AGENCY NAME & ADDRESS*(if different from Controlling Office)*	15. SECURITY CLASS. *(of this report)*: Unclass / 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT *(of this Report)*

Approved for public release; distribution unlimited

17. DISTRIBUTION STATEMENT *(of the abstract entered in Block 20, if different from Report)*

18. SUPPLEMENTARY NOTES

Thesis for Master's Degree in Chemistry, South Dakota School of Mines and Technology, Rapid City, SD 57701

19. KEY WORDS *(Continue on reverse side if necessary and identify by block number)*

IgG, Metal Ion, EPR, Ultrafiltration, Antibody, Metal Ion Binding, Transition Metal Ion Binding, Gamma Globulin.

20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

This report presents a systematic methodology to study the transition metal ions of Cu(II), Cr(III), Co(II), VO^{+2}, and Fe(III), and their binding to immunoglobulin G (IgG). Procedures include dialysis, DEAE-cellulose column chromatography, gel electrophoresis, immunoelectrophoresis, ultrafilitration, UV spectroscopy, atomic absorption spectroscopy, and electron paramagnetic resonance (EPR). IgG used is non specific, metal free, human γ-globulin. Conclusions show that Cu(II), Cr(III), (See reverse)

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

SECURITY CLASSIFICATION OF THIS PAGE *(When Data Entered)*

Block 20 Continued:
Co(II), and Fe(III) do bind to IgG, while VO^{+2} does not under conditions used in this study. Ultrafiltration studies detail Cu(II) binding to IgG in mole ratios of .08 to 3.9. No end point was reached in this study.