RECHT, BERNARD

THE CHEMICAL CHARACTERIZATION BY AMINO ACID SEQUENCE OF AN UNUSUAL IMMUNOGLOBULIN G MYELOMA PROTEIN

City University of New York PH.D. 1980

PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark ✓.

1. Glossy photographs _____
2. Colored illustrations _____
3. Photographs with dark background _____
4. Illustrations are poor copy ✓
5. Print shows through as there is text on both sides of page _____
6. Indistinct, broken or small print on several pages ✓ throughout _____
7. Tightly bound copy with print lost in spine _____
8. Computer printout pages with indistinct print _____
9. Page(s) _____ lacking when material received, and not available from school or author _____
10. Page(s) _____ seem to be missing in numbering only as text follows _____
11. Poor carbon copy _____
12. Not original copy, several pages with blurred type _____
13. Appendix pages are poor copy _____
14. Original copy with light type _____
15. Curling and wrinkled pages _____
16. Other _____

THE CHEMICAL CHARACTERIZATION BY AMINO ACID SEQUENCE OF AN UNUSUAL IgG MYELOMA PROTEIN

By

Bernard Recht

A dissertation submitted to the Graduate Faculty in Biomedical Science in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1979

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy

12/7/79
date

Jerome L. Schulman
Chairman of
Examining Committee

12/7/79
date

Executive Officer

Supervisory Committee

-Abstract-

The Chemical Characterization by Amino Acid Sequence of an Unusual Myeloma Protein

by
Bernard Recht

Adviser: Blas Frangione.

The partial amino acid sequence of the Fc region of two monoclonal immunoglobulin molecules were determined. One of the myeloma proteins, Goe, was previously shown to possess an IgG3-like hinge region and an IgG1-like carboxy-terminus octadecapeptide. It was therefore suggested that protein Goe provided yet another example of a Lepore-type immunoglobulin similar to the IgG4-IgG2 or the IgG3-IgG1 previously described. The amount of myeloma protein in whole Goe sera, however, was relatively low and therefore methods for purification and characterization had to be carefully designed prior to handling of the myeloma sera. As a result, sera from patient Kup possessing an IgG3-K monoclonal cryoglobulin was utilized. Kup sera was in relatively good supply and the monoclonal component, due to its cryoprecipitability, was easily isolated. Most of the initial experimental conditions were subsequently arrived at through studies on protein Kup.

Protein A, a Staphylococcal aureus cell wall component, was utilized in further purification of the IgG3 myeloma component from Kup because it had been reported that all of the IgG subclasses except IgG3 showed a strong binding capacity to this bacterial protein. After protein Kup was found to be pure by immunological and electrophoretic criteria, it was subjected to various fragmentation procedures including cyanogen bromide and a unique method of pepsin digestion requiring the presence of 8 M urea. Specific fragments were then isolated and sequenced.

It has been shown that particular amino acid residues may be related to specific isotypic and/or allotypic variations. All of the fragments studied from protein Kup agreed with past correlations made in IgG3 molecules with similar genetic make up.

Protein Goe, because it lacked the cryoglobulin ability shown in protein Kup, was initially isolated by starch block electrophoresis or ion exchange chromatography. However when attempting to further separate out contaminating, normal IgG1 by passage through protein A, it was found that the IgG3-like protein Goe also possessed the capacity to bind to protein A. Passage through hydroxylapatite was found to be a simple and fast procedure which substantially purified the myeloma protein. The "subclass specific" fragments of Goe were isolated and sequenced in a similar manner as in Kup. The results from these and other

experiments indicated that protein Goe consisted of a γ3-like hinge region and an Fc region that possessed both γ1 and γ3-related amino acid substitutions. The following particular residues were identified because they had been shown to be variable between subclasses: Residues 274, 276, 296 and 300 are all within the intrachain disulfide loop of the CH2 domain and contain γ3 related amino acids; residue 339, which is located between the intrachain disulfide loops of the CH2 and CH3 domains, has a γ1-related amino acid; residues 397 and 422, which reside within the CH3 domain disulfide loop, are γ3-related; and finally, residues 435 and 436, which lie outside the CH3 disulfide bridge in the carboxy terminal octadecapeptide, have previously been correlated with γ1 heavy chains.

Three possible explanations were discussed which attempted to explain the existence of such an immunoglobulin molecule. Protein Goe may be the result of multiple crossovers at the gene level as was the proposed mechanism for the existence of other hybrid-like molecules. Or, Goe may be the result of a splicing mechanism at the level of the RNA which has been used to explain other sorts of immunoglobulin variants. Finally, Goe simply could be the result of the expression of a normal, but as yet undetected gene. Supportive evidence for the last possibility was given by the isolation and characterization of a component of normal IgG3 from normal sera, that has the capacity to bind to protein A, like that of protein Goe.

Acknowledgements

I wish to acknowledge Dr. Blas Frangione and Dr. Edward C. Franklin for their supervision and guidance during my research and thesis preparation and for generously providing the opportunity and facilities to work on this project. I am also indebted to all those others that have passed through Irvington House Institute for their friendship and advice. Thanks are also due to Ms. Fran Prelli, Mrs. Ellen Rosenwasser and Ms. Joan Zaretzky for their excellent technical assistance throughout and for their enlightening company during coffee times. Thanks also to Mrs. Diane Horner for her secretarial assistance.

Most especially I would like to thank my wife, Ditza, for her constant love and encouragement and for the major role she played in the finalization of this thesis, and my parents who have seen me through this and more.

Dedication

To Miriam

To Mendel

Table of Contents

List of Tables

List of Figures

-Chapter 1-

Introduction

1.1 Immunoglobulin structure

1.2 Variable and constant regions

1.3 Immunoglobulin domains

1.4 Organization of immunoglobulin genes

1.5 Classes of immunoglobulins

1.6 Biology and function of immunoglobulin classes

1.7 Isotypes

1.8 Allotypes of immunoglobulin G

1.9 Detection of allotypes

1.10 Allotype groups

1.11 Antigenic markers & structure

1.12 Chemical, biological and structural differences in IgG subclasses

-Chapter 1-

Introduction

Immunoglobulins make up a heterogeneous class of large molecular weight glycoproteins of characteristically slow electrophoretic mobility. This class of serum proteins, originally named "globulins" because of their noted insolubility in water, are found in relatively high concentration (10 mg/ml) in all normal sera and most likely in all other vertebrate species. In 1937 Tiselius separated serum proteins grossly according to their electrophoretic mobility into albumin, α -, β - and γ-globulins, and later in 1939, Tiselius and Kabat showed by adsorption experiments that it was the γ-globulin fraction that contained the antibody activity. Thus, these molecules are important because they possess the property of being able to bind specifically to and eventually eliminate invading foreign macromolecules or antigens. Heremans, in 1960 coined the term "immunoglobulins" for this biologically essential class of macromolecules that are found in both normal or pathologic sera.

Immunoglobulins from normal sera can express an almost limitless degree of antigenic specificity which is responsible for one type of molecular heterogeneity. Variability in the antigenic specificity of individual antibodies is

the result of variability in amino acid sequence from one immunoglobulin to another. For example, rabbit antibodies possessing different specificities were shown to have different amino acid compositions (Koshland & Englberger, 1963). Amino acid sequence variations of this type are confined entirely to the amino-terminal end of the antibody molecule. The carboxy-terminal portion, however, does not show such a degree of amino acid sequence variation. In fact, variation in this region has been shown to be extremely limited from one antibody molecule to another particularly when studying molecules from the same class. The carboxy-terminal regions are not involved in direct antigen binding but partake in a second group of important biological functions termed effector or secondary functions.

Immunoglobulin molecules therefore can be divided into the amino-terminal or antigen binding region and the carboxy-terminal or effector-function region. Research on immunoglobulin structure and function has therefore usually focused its attention on one of these two regions. This thesis concerns itself with the chemical characterization by amino acid sequence of the carboxy-terminal region of a pathological immunoglobulin (Goe) which appears to share characteristics of both the IgG1 and IgG3 immunoglobulin subclasses. Preliminary studies have shown Goe to possess an IgG3-like hinge region but an IgG1-like carboxy-terminal octadecapeptide. In the Results section of this thesis

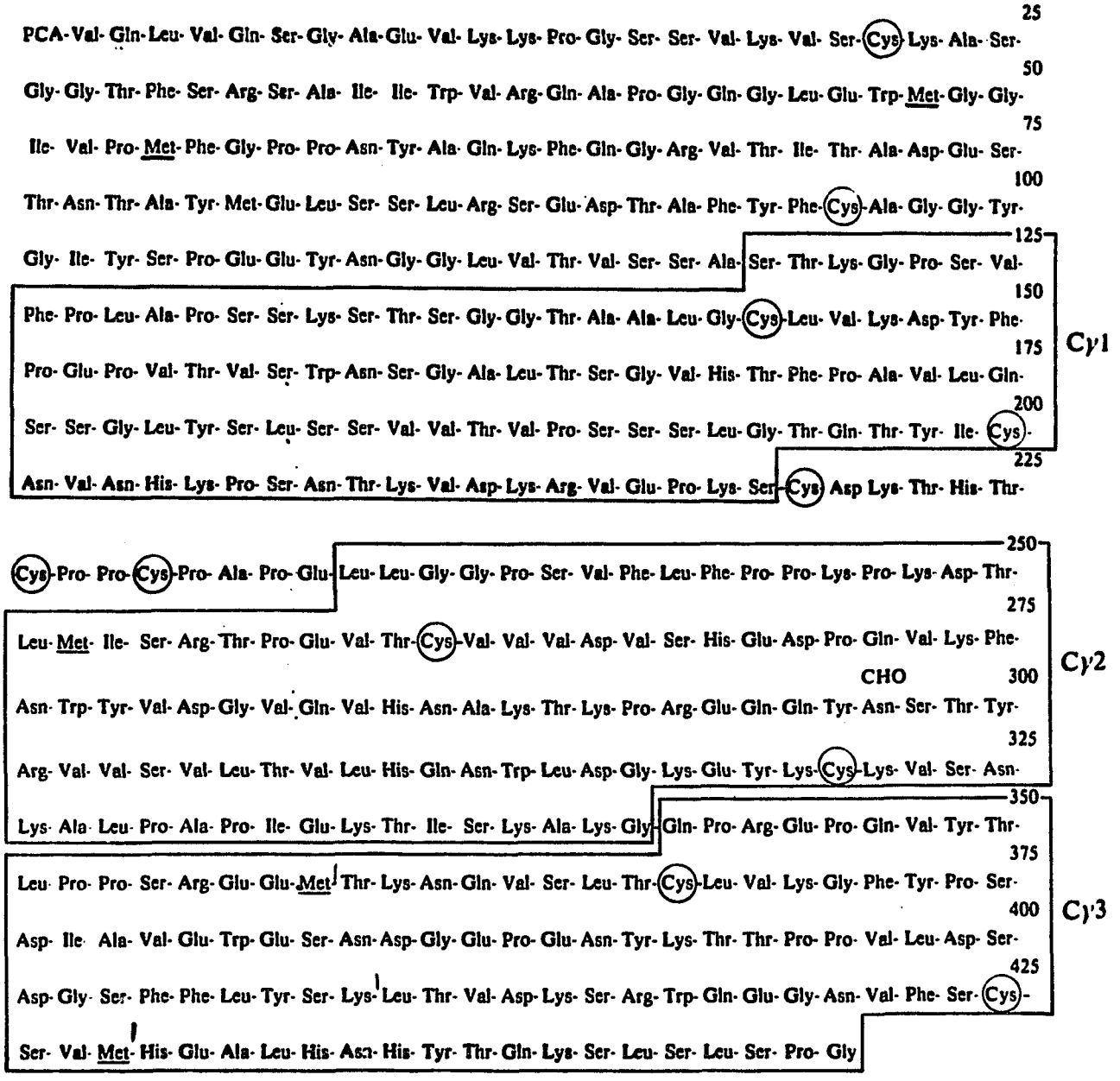
structural studies on protein Goe are outlined and are compared with similar studies done on another myeloma protein of the IgG3 subclass. Presented first is an overview of antibody structure and immunogenetics with a more indepth comparison of the various immunoglobulin classes and subclasses. One is referred to the many recent reviews of these subjects referenced in the following sections for more detailed information.

1.1 Immunoglobulin structure

Normal human IgG1 has a molecular weight of about 150,000 daltons which contains over 1300 amino acid residues (Edelman et al., 1969, Fig. 1-1). Complete sequence studies could only be attempted after such a molecule is broken down into smaller and overlapping fragments. Initial studies on various methods of fragmentation and subunit dissociation were first attempted using rabbit immunoglobulins but the methods can be generally applied to all vetebrate immunoglobulins.

In 1959 Porter successfully cleaved normal rabbit γ-globulin with an enzyme extracted from the plant Carica papaya, called papain. Cleavage of the native immunoglobulin molecule with papain generated two types of fragments whose characterization was extremely important in the delineation of overall antibody structure and function. One of the fragments, weighing 45,000 daltons, was called Fab because it possessed monovalent antigen binding activity but showed no complement fixing activity, and the other, weighing

Fig. 1-1



The complete amino acid sequence of the IgG1 myeloma heavy chain Eu (Edelman et. al., 1969). Each of the constant region domains are enclosed, the cysteines circled and the methionines underlined.

50,000 daltons, was termed Fc because it was readily crystallizable however it did not inhibit the binding of antibody to antigen. Quantitation of the released fragments suggested that there were two similar Fab fragments for each Fc generated and by using gel diffusion it was shown that the two fragments did not share any antigenic determinants (Edelman et al., 1960). In addition other investigators have shown that by using the reducing agent mercaptoethanol, which cleaves disulfide bonds but leaves peptide bonds intact, that immunoglobulins can be dissociated into two subunits termed heavy (H) chains (50,000 daltons) and light (L) chains (23,000 daltons) (Edelman, 1959; Edelman & Poulik, 1961; Edelman et al., 1960; Franek, 1961). Further studies showed that the Fab fragments from normal immunoglobulins could be treated with reducing agents to generate L chains and another fragment, Fd, which was shown to be the amino-terminal half of the heavy chain (Fleischman et al., 1963; Edelman & Bennaceraff, 1962).

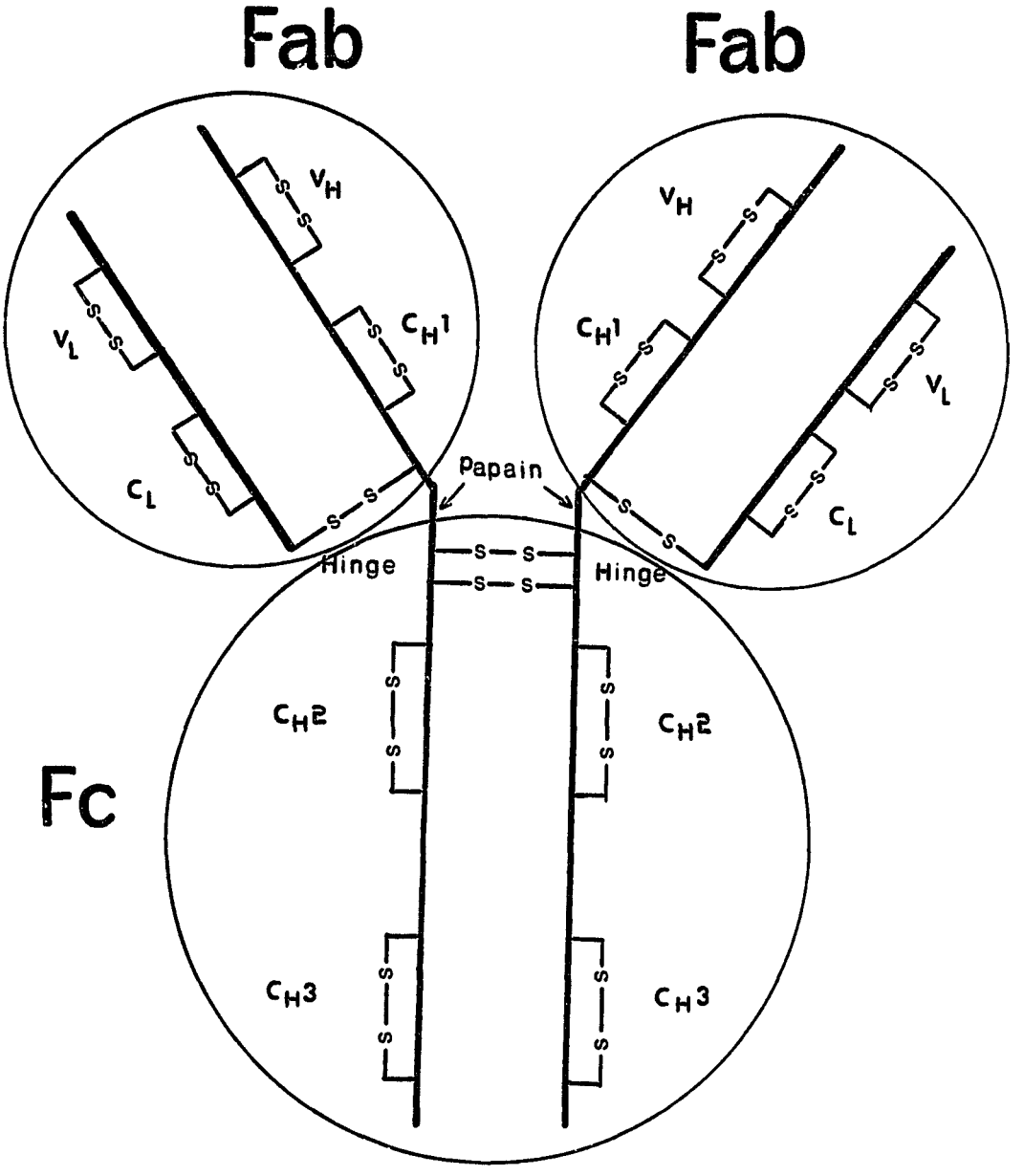
Together with the additional data that each antibody molecule contained two identical combining sites (Karush, 1962) and that for every immunoglobulin molecule there was generated two molecules of heavy chain and two molecules of light chain, a tetrachain immunoglobulin model was proposed (Porter, 1962; Fleischman et al., 1963). It was further generalized that all immunoglobulins have the same fundamental structure (Cohen, 1963b). The basic structure as

diagrammatically presented in Fig. 1-2, shows two identical light chains held together by disulfide bridges. This model also demonstrates how papain cleavage produces the expected Fab and Fc fragments.

Most of the information on immunoglobulin structure has been accumulated through the study of homogeneous protein preparations. These may be obtained by immunizing animals, particularly the rabbit, with certain polysaccharides (Krause, 1970) or more frequently from the sera of humans or other species that become afflicted with multiple myeloma.

Multiple myeloma is a lymphoproliferative disorder of immunoglobulin producing B cells. In this disease it is thought that a single immunoglobulin producing cell, through a mutational event, loses some aspect of its cellular control mechanism and begins to proliferate unceasingly resulting in an enormous overproduction of immunoglobulin. The immunoglobulins secreted from this clone are homogeneous and may account for up to 90% of the total serum immunoglobulin. Frequently multiple myeloma patients excrete into their urine Bence-Jones proteins which are known to be composed of the light chain component of their particular myeloma protein (Edelman & Gally, 1962; Schwartz & Edelman, 1963; Franklin & Stanworth, 1961). Myeloma proteins are thought to be derived from a randomly selected, previously normal immunoglobulin producing cell and consequently its immunoglobulin is believed to be like its normal counterpart

Fig. 1-2



The basic tetrachain structure of an immunoglobulin molecule is shown. Papain cleaves the molecule N-terminal to the hinge region (arrows) and generates the Fc and Fab fragments (circled structures). The various domains (V_H, C_H1, etc.) are noted. Disulfide bridges, both inter- and intrachain, are shown by —S—S—.

(Kunkel, 1968). This notion has been supported by the finding that the frequency of classes and light chain types in multiple myelomas parallel the natural abundance of each class in normal sera (Schur, 1972).

1.2 Variable and constant regions

Primarily through amino acid sequence determinations initially on a number of Bence-Jones proteins (Putnam et al., 1966; Putnam et al., 1967a; Putnam et al., 1967b; Hilschmann & Craig, 1965; Titani & Putnam, 1965; Titani et al., 1965) and later on various myeloma and pooled normal immunoglobulins, it was found that each heavy and light chain could be divided into two very distinct regions. The region that includes approximately the first 110 amino acid residues is termed the variable or V region and the region containing the carboxy-terminal residues (about 110 in L chains and 330-440 in H chains) is called the constant or C region (Dreyer & Bennet, 1965). The amino acid sequence of any particular Bence-Jones or myeloma H or L chain V region is unique. Sequences of different myeloma protein V regions, each of which are thought to be a product of the expression of a different clone of antibody producing cells (Jerne, 1955; Burnet, 1959) have never been shown to be identical except in the extremely rare case of a double myeloma. The amino acid sequence variability within these V regions is shown to be limited only to certain portions

of the chain (Milstein & Pink, 1970). Three such areas, termed hypervariable regions, are found in light chains (Wu & Kabat, 1970; Franêk, 1970; Milstein, 1967; Kabat, 1968) and comprise residues 24-34, 50-56, and 89-97. A similar set of hypervariable regions are also found in heavy chains. The three dimensional folding of the molecule is thought to bring together these hypervariable positions to form the recognition site for antigen (Wu & Kabat, 1970; Kehoe & Capra, 1971; Kabat, 1970). Thus it seems that the heterogeneity inherent in immunoglobulins as mentioned in the Introduction is a result of seemingly innumerable V-region amino acid sequence differences found in antibody molecules in normal sera. It is indeed this variation that is responsible for the capability of an organism to mount an immune response to a multitude of antigenic stimuli. In contrast, the amino acid sequences of C regions are relatively invariant (except for genetic polymorphisms) when chains of the same class, subclass or type are compared. C regions will be discussed further in a later section.

1.3 Immunoglobulin domains

The existence of homology units within the immunoglobulin molecule had been suggested (Hill et al., 1966b; Singer & Doolittle, 1966) and finally became apparent with the elucidation of the complete amino acid sequence of an immunoglobulin by Edelman (1969). Both heavy and light chains are found

to be composed of repetitive units or domains of about 110 amino acids in length which are shown to be related through (1) amino acid sequence homology, (Hill et al., 1966a; Milstein & Pink, 1970); (2) a characteristic intra-chain disulfide loop encompassing 60-70 amino acids, (Milstein, 1964; Hilschmann & Craig, 1965; Frangione et al., 1969b); and (3) a unique three-dimensional folded structure termed the "immunoglobulin fold" (Poljak et al., 1973; Schiffer et al., 1973; Padlan et al., 1973). Edelman, in 1970, postulated that each domain probably possesses its own defined functional properties (see review by Winkelhake, 1978).

The light chains are composed of two domains termed V_L for the variable region of the light chain, and C_L for the constant region of the light chain. Heavy chains possess a variable region domain, the V_H, and in the case of IgG, three constant region domains, C_H1, C_H2, and C_H3. Between the C_H1 and C_H2 domains heavy chains possess an additional region termed the "hinge" region which shows no homology to any of the domains but is important because it contains the interchain disulfide bonds which hold the polypeptide chains together and may play a role in flexibility of the molecule (Wolfenstein-Todel et al., 1976a). Other immunoglobulins contain extra domains (e.g., C_H4 in IgM and IgE) or additional disulfide linked polypeptide chains (e.g., secretory piece in IgA or J chain in polymers of IgM and IgA) which are characteristic of particular classes of immunoglobulins.

Fig. 1-3a, b and Fig. 1-4a, b diagrammatically illustrate the folding patterns of the immunoglobulin domains. Fig. 1-3a shows the basic immunoglobulin fold of a constant region domain. Variable region domains however possess an additional loop as represented by the dotted pattern in Fig. 1-3a. Note also that the folding pattern is stabilized through a single disulfide bridge shown as ⓢⓢ (Fig. 1-3a). Fig. 1-3b demonstrates more clearly the sandwich structure of the domain which is composed of two layers of antiparallel strands (white) while the other sheet has four such strands (shaded) giving an overall cylindrical shape to the domain.

Fig. 1-4a shows the folded structure of the two domains of the light chain. Note that four-stranded sheets in the two domains are rotated with respect to each other. This may be better visualized with the cylindrical diagram, also in Fig. 1-4a, where the four-stranded sheet is represented by the four dark bands on the cylinder. Finally, Fig. 1-4b shows the configuration of each domain in a Fab fragment illustrating that in the constant regions the four-stranded sheets face each other while in the variable region the three-stranded sheets face each other in order to form the antibody combining site.

It appears that immunoglobulins may have developed as a result of random duplication of a primordial gene coding for a 110 amino acid polypeptide chain containing a single

Fig. 1-3
The Immunoglobulin Fold

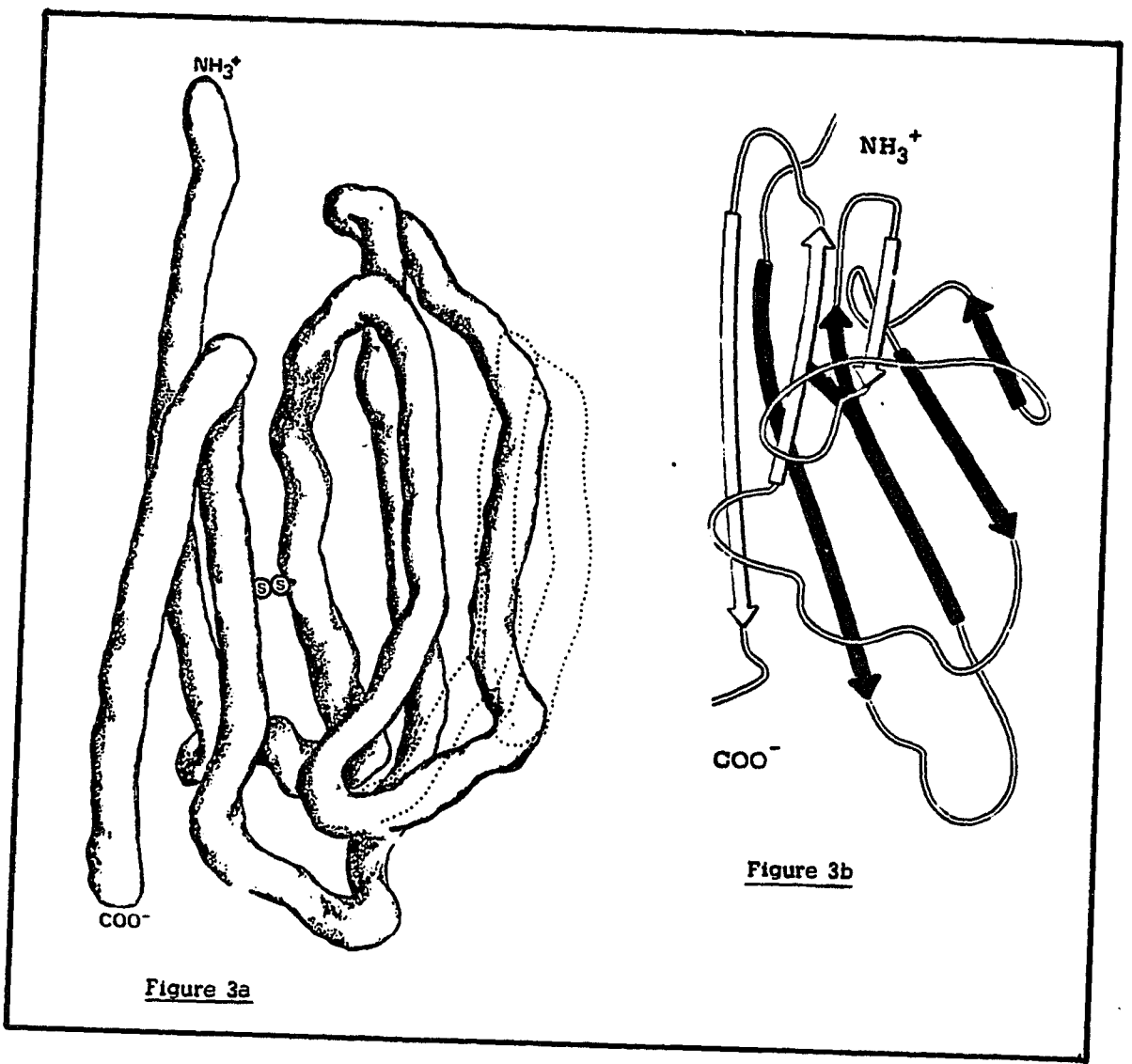


Figure 3a

Figure 3b

Two views of the tertiary structure of a globular domain demonstrating the basic "immunoglobulin fold" (see text) (Poljak et al, 1973; Edmundson et al, 1975).

Fig. 1-4

Folding Patterns of Immunoglobulin Domains

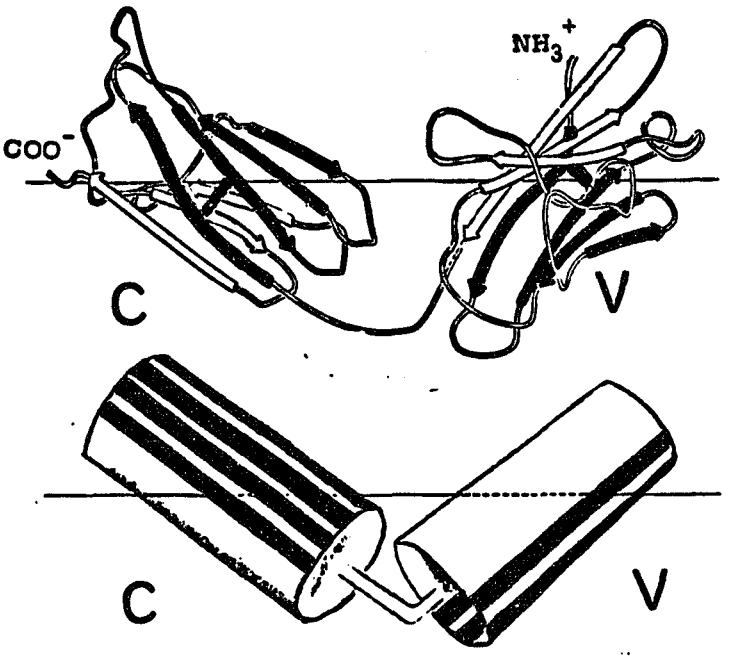


Figure 4a

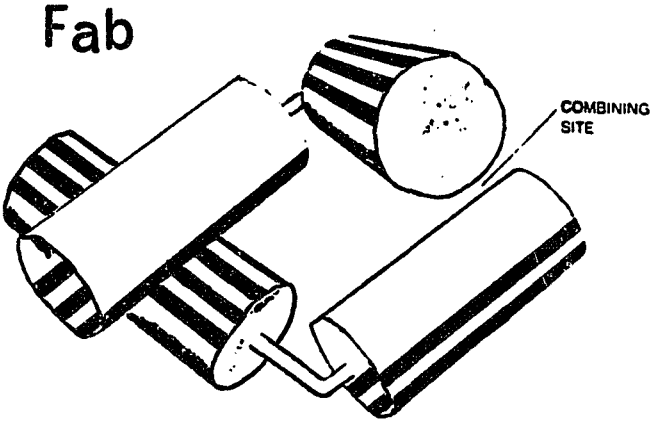


Figure 4b

Figure 4a shows the folding pattern of a light chain (C & V region) depicted by two types of drawings, Figure 4b, shows an Fab fragment consisting of four cylindrical domains, with the two V region domains comprising the antibody combining site (Capra, 1977; Edmundson et al, 1975).

disulfide loop (Doolittle, 1966). Fig. 1-5 shows a possible scheme of the evolution of the various immunoglobulin classes, subclass and types from such an ancestral precursor chain.

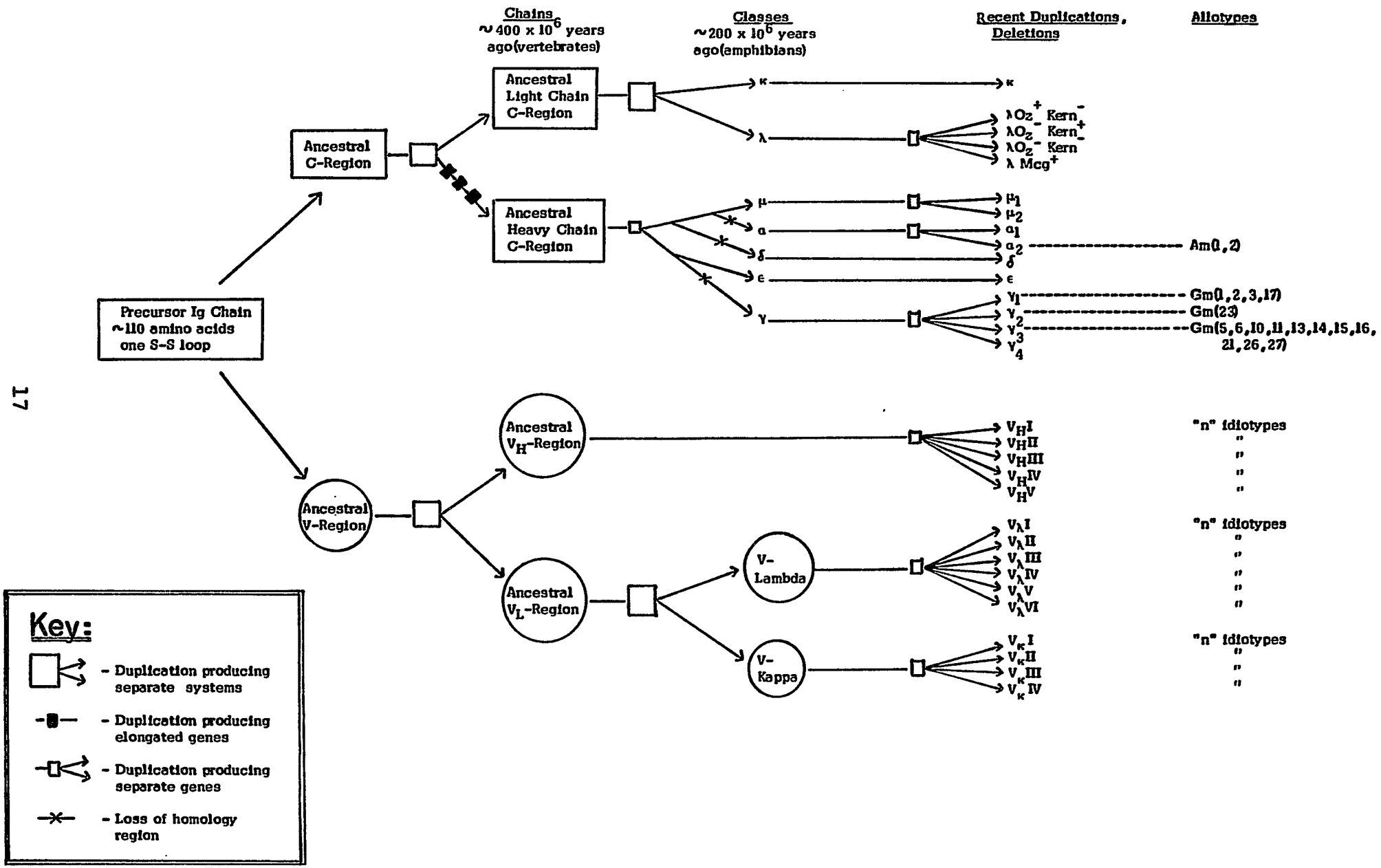
1.4 Organization of immunoglobulin genes

Because V region amino acid sequences vary considerably from one molecule to another while C region sequences from molecules of the same class, subclass or type are for the most part identical, it was originally hypothesized by Dreyer and Bennet (1965) that each heavy or light chain is encoded for by at least two genes - one for the variable region and one for the constant region. Others have also suggested that different V region genes may be associated with the same C region gene (Hood & Ein, 1968; Hood, 1972) for in the mouse it has been estimated that there may be 100 V_K genes per haploid genome but only one C_K gene (Cohn et al., 1974). A translocation event has been postulated that would allow particular V region DNAs, selected from possibly a large number of V region DNAs, to be joined to a specific C region DNA with the concomitant looping out of the genetic material between these two regions (Gally and Edelman, 1972; Sarkar, 1970; Smithies, 1970). In support of the translocation event, Milstein et al., 1974, has shown in mouse that both V and C regions are indeed contained in a single light chain mRNA. To verify this sort

Fig. 1-5

(see following page)

A proposed scheme for the evolution of the various immunoglobulin classes, subclasses and types beginning with a precursor immunoglobulin chain composed of about 110 amino acids and a single disulfide loop. (Adapted from Fudenberg et al., 1978, and Dayhoff, 1978)


Chains
~400 x 10^6 years ago(vertebrates)
Classes
~200 x 10^6 years ago(amphibians)
Recent Duplications, Deletions
Allotypes
Precursor Ig Chain
~110 amino acids
one S-S loop
Ancestral C-Region
Ancestral Light Chain C-Region
Ancestral Heavy Chain C-Region
κ
λ
λO2+ Kern-
λO2- Kern+
λO2- Kern-
λ Mcg+
μ
α
δ
ε
γ
μ1
μ2
α1
α2
δ
ε
γ1
γ2
γ3
γ4
Am(1,2)
Gm(1,2,3,17)
Gm(23)
Gm(5,6,10,11,13,14,15,16, 21,26,27)
Ancestral V-Region
Ancestral VH-Region
Ancestral VL-Region
V-Lambda
V-Kappa
VHI
VHII
VHIII
VHIV
VHV
VλI
VλII
VλIII
VλIV
VλV
VλVI
VκI
VκII
VκIII
VκIV
"n" idiotypes
"n" idiotypes
"n" idiotypes
Key:
- Duplication producing separate systems
- Duplication producing elongated genes
- Duplication producing separate genes
- Loss of homology region

of somatic rearrangement of genetic material, many recent investigations have compared the structure and organization of embryonic or undifferentiated DNA to DNA of the mature antibody producing plasma cell.

At the level of the germ-line gene it has been shown unequivocally by hybridization studies that the C and V region genes are completely separated (Tonegawa et al., 1976; Tonegawa et al., 1977b; Seidman et al., 1978; Hozumi & Tonegawa, 1976). In ontogeny, as the differentiated cell matures to one that produces antibody, a reassortment of DNA takes place that brings the V and C region into close proximity.

Electron microscopic as well as sequence studies of myeloma cell DNA have clearly shown this type of translocation to have taken place (Hozumi & Tonegawa, 1976; Tonegawa et al., 1977a; Rabbitts & Forstor, 1978; Tonegawa et al., 1976; Brack et al., 1978; Lenhard-Schuller et al., 1978). The interesting finding however is that in the case of lambda genes the V and C region DNAs even at the mature antibody-producing stage are not joined and are in fact still separated by about 1250 DNA bases (Brack & Tonegawa, 1977).

Through detailed DNA sequencing studies of both embryonic and mature immunoglobulin DNA it was shown that the classical V region DNA is actually composed of two DNA segments. One codes for about the first 98 amino acids

of the variable region, and the other, now termed the J segment, codes for the remaining 12 or so amino acids (Weigert et al., 1978; Brack et al., 1978). In the germ-line DNA the J segment is separated from the V region DNA by a large yet undetermined DNA region, but is separated from the C region DNA by only 1250 bases (Brack & Tonegawa, 1977). In myeloma DNA the V region and J segment DNA are contiguous but the end of the J segment is still 1250 bases away from the C region DNA (Tonegawa et al., 1978). Precursor segments of light chains are extra polypeptides that precede the amino-termini of mature immunoglobulin chains (Schechter & Burstein, 1976a) but are eventually cleaved before or during the secretion of the shortened chain. These precursor sequences contain a large amount of hydrophobic residues and may play a role in interacting with the cell membrane as a prelude to secretion (Schechter & Burstein, 1976b; Blobel & Dobberstein, 1975a, b). The leader DNA sequence, or that sequence which has been correlated with the amino acid sequence of the precursor segment (Milstein et al., 1972; Swan et al., 1972) is also separated from the V region DNA by 93 bases in both the embryonic and mature DNA (Tonegawa et al., 1978).

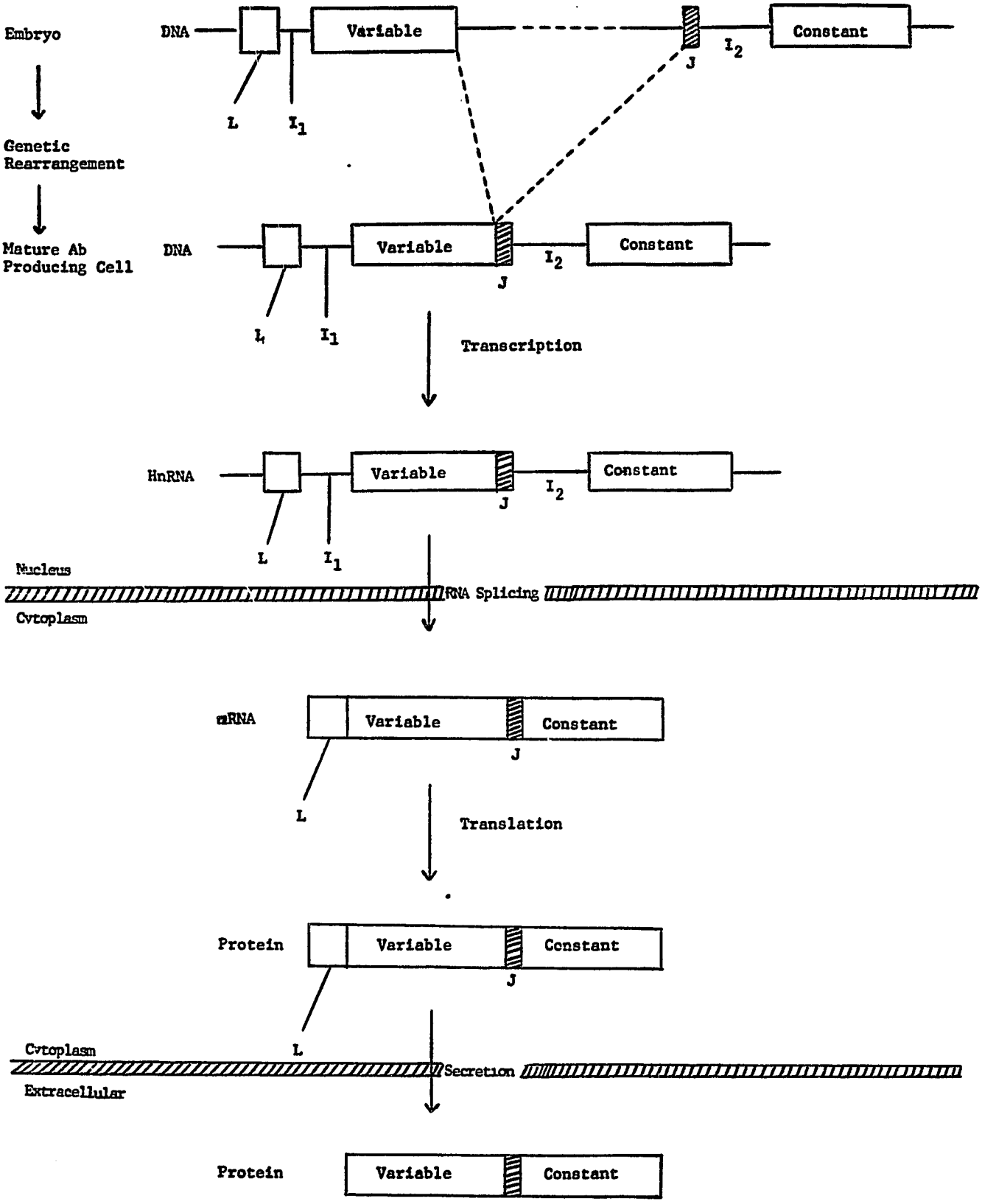
In the mature B cell, transcription of the rearranged DNA would occur in the nucleus to form the large heternuclear RNA (Hn RNA) species. Processing of the Hn RNA by RNA splicing of the intervening RNA sequences to form the

messenger RNA (mRNA) would take place either before or during transport into the cytoplasm. Once in the cytoplasm, translation of the mature mRNA would commence on the rough endoplasmic reticulum (ER). As mentioned previously, before or during secretion the leader sequence, which may be attached to the cell membrane, would be cleaved from the light chain precursor molecule. A similar sequence of events is thought to occur with H chain production and secretion usually occurs only after the covalent linking of the H and L chains to form the tetrameric Ig molecule. The series of genetic events discussed that lead to antibody production is represented schematically in Fig. 1-6.

The DNA sequences that make up the transcription unit of an immunoglobulin polypeptide chain are therefore made up of regions within or between genes ("introns" for intervening sequences) which are eventually excised before translation and of regions which are eventually expressed (exons) (Tonegawa et al., 1978; Gilbert, 1978). In general, introns can range from 10 to 10,000 bases and it is estimated that the DNA encoded for in introns may be ten times the amount in exons (Gilbert, 1978). This type of exon-intron mosaic is being found in many other eucaryotic systems (e.g., ovalbumin genes, β globin genes, and t-RNA genes) as well as in some viral genomes.

Recently it has been reported that a very similar genetic organization exists for heavy chains (Rao et al.,

Fig. 1-6



This figure diagramatically illustrates the series of genetic events that lead to the production of a light chain. A similar series of events is thought to occur with heavy chains.so that at the protein level within the cytoplasm, heavy and light chains combine and are secreted as a tetrameric immunoglobulin.(L-leader sequence; I_1-intervening sequence 1, about 93 base pairs; I_2-intervening sequence 2, about 1250 base pairs in lambda light chain).

1979; Sakano et al., 1979). It was shown that separate exons exist for each of the constant region domains of the heavy chain (i.e., CH1, CH2 and CH3) as well as for the hinge region that lies between the CH1 and CH2 domains. Previous structural studies of a variety heavy chain disease (HCD) proteins (Franklin & Frangione, 1975) in which segments of myeloma immunoglobulin chains are deleted have predicted that individual domains including the hinge region may be under separate genetic control. It has been shown that many HCD proteins contain deletions in the amino-terminal portion of the heavy chains which involve much of the variable and constant regions. Interestingly, the chain usually begins again at a residue homologous to number 216, Eu numbering (Edelman et al., 1969) which may be be considered the beginning of the exon coding for hinge region.

1.5 Classes of immunoglobulins

Immunoglobulins can be divided into five major classes: IgG, IgM, IgA, IgE and IgD. Originally these classes were differentiated serologically and functionally (reviewed by Natvig & Kunkel, 1973; Spiegelberg, 1974) and it was only later that the major classes were shown to vary in their amino acid sequence within their heavy chain constant regions (Natvig & Kunkel, 1973) (γ, μ, α, ϵ, and δ are the heavy chain designation for the IgG, IgM, IgA, IgE and

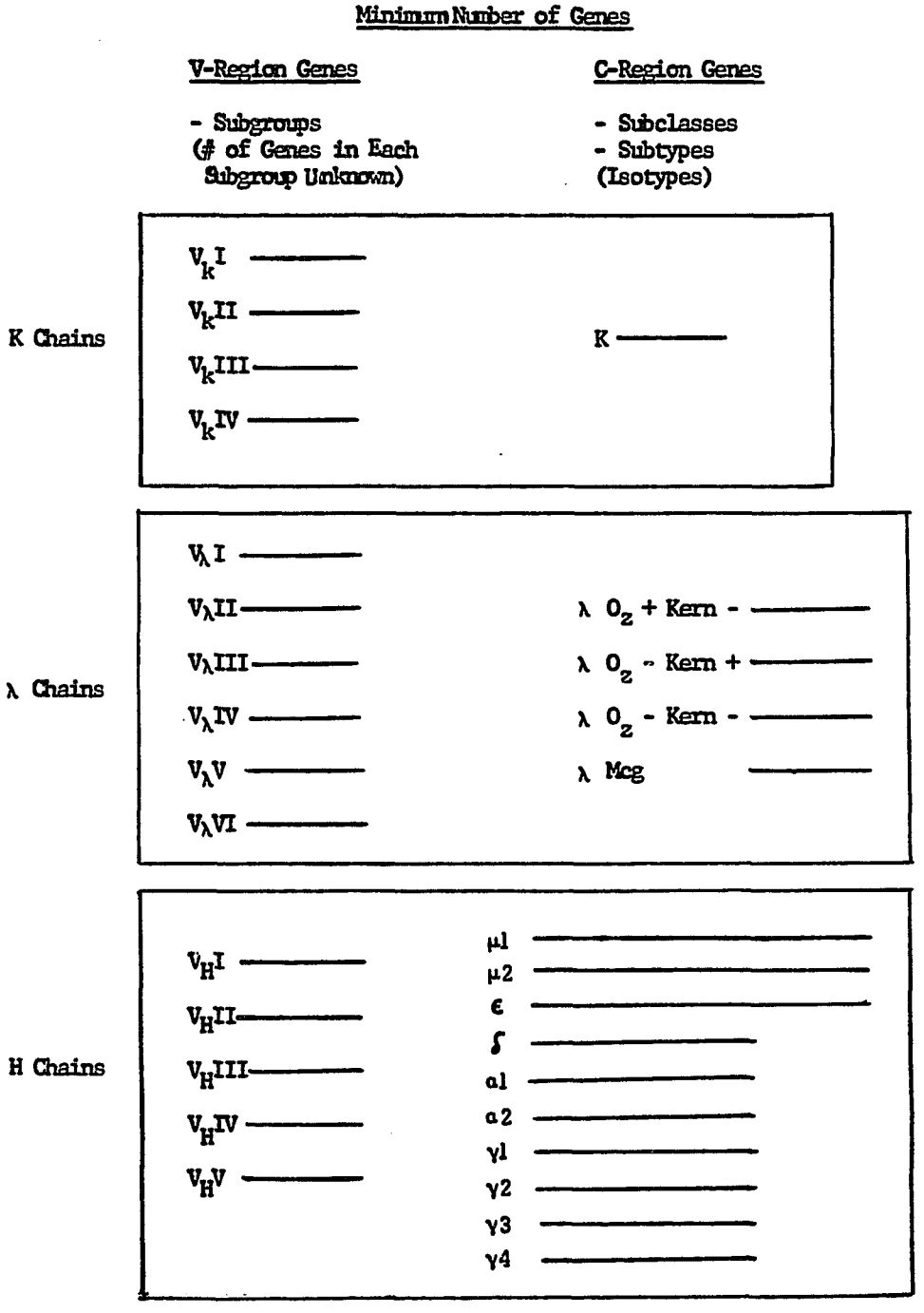
IgD classes respectively). Similarly light chains can be separated into two types, kappa (κ) and lambda (λ) (Mannik & Kunkel, 1963; Fahey, 1963). The genes coding for these individual immunoglobulin polypeptide chains have been divided into three linkage groups: one for κ light chains, one for λ light chains, and one for all heavy chains (Fig. 1-7) (Milstein & Munro, 1970; Gally & Edelman, 1972). The genetic studies by Steinberg and Mutzumoto (1965) have shown that these groups are truly unlinked. All heavy chains are found to be associated with either type of light chain to form complete immunoglobulin molecules. Each class has its own structural properties as a result of differences in (1) amino acid sequence of the constant region, (2) chain length, (3) number and location of inter- and intra-chain disulfide bonds, (4) number, position and composition of carbohydrates, and (5) degree of polymeriation of monomeric units (see Table 1-1). These differences translate into unique serological and biological properties.

1.6 Biology and function of immunoglobulin classes

IgG is the principle immunoglobulin found in normal human serum. IgG, although produced very little in the early portion of a primary immune response, is the major immunoglobulin found in the anamnestic or secondary response and comprises about 80% of the total immunoglobulin.

Fig. 1-7

The Three Linkage Groups for Human Structural Immunoglobulin Genes



The three boxes above represent the three linkage groups (κ, λ, and H chains) that make up the human structural immunoglobulin genes. Each line in a box represents at least one structural gene and the nimber of lines in a box represents the minimum number of genes in that particular linkage group. (Adapted from Fudenberg et. al. (1978).)

Table 1-1

Properties of Human Immunoglobulin Classes

Chemical & Physical Properties	IgG	IgA	IgM	IgD	IgE
Concentration mg/100ml(mg%)	1250	IgA1-300 IgA2-50	125	4	0.03
Subclasses	IgG1,IgG2, IgG3,IgG4	IgA1,IgA2	-?	-	-
L Chain Types	k,λ	k,λ	k,λ	k,λ	k,λ
H Chain Types	γ	α	μ	δ	ε
Size (S_{20})	7S	7S,11S	19S	7S	8S
Molecular Weight	150,000**	(160,000)n	950,000	175,000	190,000
CHO Content(%)	3	7	10	9	13
Extra-Polypeptide Chains	-	J-chain, S piece	J-chain	-	-
Molecular Form	Monomer	Monomer Dimer	Pentamer	Monomer	Monomer
H Chain Domains	4	4	5	4	5
Paraprotein	Myeloma	Myeloma	Macro-globulin	Myeloma	Myeloma
Biological Properties					
C' Fix	+**	0*	++	0	0
Placental Passage	+	0	0	0	0
Skin Sensitization (24hrs)	0	0	0	0	+
Binding to Cells	Macrophages	0	0	?	Mast Cells

* - Not by Classical Pathway
** - See Table on IgG Subclass Properties

At about 10 mg/ml in normal sera IgG is capable of crossing the placenta to provide passive protection to the neonate, it actively fixes complement as a major defense against bacterial infection, and binds well to macrophages. It has a sedimentation coefficient of about 7S and a molecular weight of 150,000 daltons. The general structure of IgG is usually shown as the prototype immunoglobulin molecule, possessing two identical light chains and two identical heavy chains held together by disulfide bonds and non-covalent interactions. Appreciable variability in this general structure among the four IgG subclasses is evident and is discussed later.

IgM antibodies, which comprise about 10% of the total immunoglobulin, are the first class of antibodies to appear in the early stages of an immune response. Possessing a molecular weight of about 900,000 daltons and a Svedberg constant of 19S, this class of immunoglobulin is depicted as a disulfide bonded pentamer of the basic immunoglobulin structure. IgM heavy chains (μ-chains) have a molecular weight of about 70,000 daltons (as compared with 50,000 daltons for γ chains) which is the result of an extra domain ($C_\mu 4$). IgM also contains an extra disulfide linked polypeptide chain of about 15,000 molecular weight termed J chain (for joining chain) which may be involved in initiation of polymerization (Wilde & Koshland, 1978). Phylogenically probably the most primative of the immunoglobulins, the IgM

pentamer is an excellent activator of the complement system (about 15 times more active than IgG1) and is therefore important as a first line of defense against bacterial infection. (Metzger, 1970; Putnam et al., 1971; Marchalonis & Cone, 1973).

IgA is the immunoglobulin class most prevalent in secretions (e.g., milk, saliva, tears, respiratory secretions and perspiration) and constitutes about 20% of the normal serum immunoglobulins. It is usually found in polymerized form as a disulfide-linked dimer. Like IgM, IgA dimers possess a molecule of J chain but in addition also contain another polypeptide termed secretory component (70,000 daltons). The secretory piece is synthesized in a separate cell, unlike J chain, and is added on either as an aid in passing through the epithilial cell or as a protection against extracellular proteolysis. IgA is found in two subclasses, IgA1 and IgA2, with the latter comprising only 10% of the total serum IgA. IgA antibodies are most probably involved in elimination of pathogens from mucosal surfaces (Tomasi & Grey, 1972; Heremans & Vaerman, 1971; Wang & Fudenberg, 1974).

IgE, the heat labile reagenic antibody, is the principle mediator of immediate hypersensitivity or acute allergic reactions. IgE has a high affinity for mast cells and basophils and upon specific combination with antigen will initiate the release of histamine from these cells

(anaphylatic reaction). It is present in sera at very low concentrations (about 0.1 μg/ml) and like μ chains contains an extra domain ($C_\epsilon 4$). Although not active in fixing complement, IgE may be important in the humoral immune response to parasitic diseases for it is often found in high levels in the serum of patients with such infections (Johansson et al., 1972; Ishizaka, 1973).

IgD, found in normal sera at about 30 μg/ml, was not suspected to exist until it was discovered as a rare myeloma protein that failed to react with specific antisera to IgG, IgM, or IgA. IgD, a molecule very prone to proteolysis, is shown to be present on a high proportion of lymphocytes, usually in conjunction with surface IgM, especially in newborns. It has been suggested therefore that IgD may be an ontogenetically primative B cell receptor (Speigelberg, 1972; Leslie & Martin, 1978).

These and other characteristics defining the different immunoglobulin classes are summarized in Table 1-1.

1.7 Isotypes

The antigenic determinants that serologically define the different classes and subclasses of immunoglobulins are called isotypic determinants or isotypes. Subclasses are a further isotypic breakdown of the major classes and have been described in man for IgG, IgA and possibly IgM. IgG-like subclasses have also been found in other mammals

as well (reviewed by Gally, 1973). Because each isotype describes a particular class or subclass which is the product of the expression of a separate structural heavy chain gene, there are at least 10 such heavy chain genes; four genes for the four isotypes of IgG, two for both IgA and IgM, and at least one each for IgE and IgD (see Fig. 1-7). Because all of the members of a species carry and express all of the genes for all the classes and subclasses, the isotypes are accordingly shared by all members of that species.

1.8 Allotypes of immunoglobulin G

The four distinct subclasses or isotypes of the human IgG class have been defined serologically (Ballieux et al., 1964; Grey & Kunkel, 1964; Terry & Fahey, 1964) and are referred to as IgG1, IgG2, IgG3, and IgG4, possessing $\gamma 1$, $\gamma 2$, $\gamma 3$, and $\gamma 4$ type heavy chains respectively. These subclasses can also be distinguished by other chemical and functional criteria (Table 1-2). Limited amino acid sequence variation within each isotype does exist and is probably due to point mutations in the germ line genes. These mutational events, the expression of which can be serologically detected, are unrelated to isotypic determinants and result in the formation of multiple forms of an allele at a single genetic loci. The antigenic differences within an isotype that have been shown to be inherited in a simple

Table 1-2

Properties of Human IgG Subclasses

	IgG Subclasses			
	IgG1	IgG2	IgG3	IgG4
Concentration in serum (mg/ml)	5 - 12	2 - 6	0.5 - 1	0.2 - 1
% of Total IgG	~60	~30	~6	~4
Molecular Weight	150,000	150,000	160,000	150,000
CHO Content (%)	2-3	2-3	2-3	2-3
C'- Fix	++	±	+++	0*
Placental Transfer	+	±	+	+
Skin Sensitization	+	0	+	+
Binds macrophages	+	0	+	0
L-ch Type	k,λ	k,λ	k,λ	k,λ
H-ch Type	γ1	γ2	γ3	γ4
Protein A Binding	+	+	0	+
k/λ	2.0	1.0	1.2	6
PCA Reactivity	+	0	+	+
Ab Activities (dominant)	Anti-Rh	Anti-dextran Anti-Levan	Anti-Rh	Anti-Factor VII

* Not by Classical Pathways

Mendelian fashion as if controlled by allelic genes (Grubb & Laurell, 1956) are termed allotypic differences or allotypes and were first described in the rabbit by Oudin (1956, 1960a, b) and in the human by Grubb (1956). Table 1-3 lists the current notation of the immunoglobulin allotypes in both alphameric and numeric symbols. However, certain genetic markers previously known as "non-markers" appear as isotypes in a few of the subclasses (i.e., antigenically present on all such subclass heavy chains) but behave as allotypes in at least one subclass (i.e., present as alternate alleles in that subclass) (Natvig et al., 1971b; Gaarder & Natvig, 1972; van Loghem and deLange, 1972). These types of markers have now more appropriately been referred to as isoallotypes and are probably due to mutations in a particular subclass of a pre-existing isotypic marker. The recognized isoallotypes are listed in Table 1-4. For reviews and original references on the subject of genetic markers see: van Loghem (1973); Mage et al., (1973); Grubb (1970); Fudenberg et al., (1978).

1.9 Detection of allotypes

Allotypes can be detected either chemically or serologically. Chemical methods include peptide mapping, amino acid sequence determinations, and differential sensitivity to enzymatic cleavage. However these methods may be useful for only a very few, well characterized allotypes. Some

Table 1-3

IMMUNOGLOBULIN ALLOTYPES CURRENTLY TESTABLE *

Location	Recommended designations: Alphameric	Recommended designations: Numeric	Previous designation	
IgG1	G1m(a)	G1m(1)	Gm(a)	Gm(1)
	(x)	(2)	(x)	(2)
	(f)	(3)	(bw),(b2),(f)	(3)(4)
	(z)	(17)		
IgG2	G2m(n)	G2m(23)	Gm(n)	Gm(23)
IgG3	G3m(b0)	G3m(11)	Gm(bβ),(b0)	Gm(11)
	(b1)	(5)	(b),(b1),(bɤ)	(5)(12)
	(b3)	(13)	(b3),(Bet)	(13)(25)
	(b4)	(14)		
	(b5)	(10)	(bα),(b5)	(10)
	(c3)	(6)	Gm-like,(c),(c3)	(6)
	(c5)	(24)	Gm-like,(c),(c5)	(24)
	(g)	(21)	(g)	(21)
	(u)	(26)	(Pa)	
	(v)	(27)	(Ray)	
	(s)	(15)	(s)	(15)
	(t)	(16)	(t)	(16)
IgA2	A2m(1)	A2m(1)	Am(1)	Am_2
	A2m(2)	A2m(2)		
K chain	Km (1)	Km (1)	Inv, Inv(1)	Inv(1)
	(2)	(2)	(a)	(2)
	(3)	(3)	(b)	(3)

* WHO meeting (Rouen, 1974)

Table 1-4

Suggested nomenclature for the isoallotypic markers of Ig

Designation		
Recommended		
Alphameric	Numeric	Previous
nG1m(a)	nG1m(1)	non-a
nG1m(z)	nG1m(17)	nG1m(z)
nG3m(b0)	nG3m(11)	non-b0
nG3m(b1)	nG3m(5)	non-b1
nG3m(g)	nG3m(21)	non-g
nG4m(a)	nG4m(a)	4a
nG4m(b)	nG4m(b)	4b
nA2m(2)	nA2m(2)	nA2m(2)

Distribution of isoallotypes by Ig subclasses

Alphameric	Numeric	IgG1	IgG2	IgG3	IgG4	IgA1	IgA2
nG1m(a)	nG1m(1)	+f/3 -a/1	+	+	-*	-	-
nG1m(z)	nG1m(17)	+f/3 -z/17	-	+	+	-	-
nG3m(b0)	nG3m(11)	+	+	+g/21 -b0/11	-	-	-
nG3m(b1)	nG3m(5)	+	+	+g/21 -b1/5	-	-	-
nG3m(g)	nG3m(21)	-	+	-g/21 +b1/5	-	-	-
nG4m(a)	nG4m(a)	+	-	+	+a -b	-	-
nG4m(b)	nG4m(b)	-	+	-	-a +b	-	-
nA2m(2)	nA2m(2)	-	-	-	-	+	+A2m(1) -A2m(2)

* Not detectable with all nG1m(a) specific reagents.

allotypes can be serologically detected directly by antigen-antibody precipitation reactions (Natvig, 1966; Kunkel et al., 1966) but precipitating antibodies to most allotypes are extremely difficult to produce. The most common method of allotype detection is by the indirect hemagglutination inhibition system (Steinberg, 1962; Fudenberg, 1963) as outlined in Fig. 1-8 and discussed below.

The hemagglutination system consists of two components: the antigen (in this case IgG) which is immobilized on red blood cells (RBC) and the antibody whose specificity is directed against the immobilized antigen. Mixing the two components together causes the red blood cells to agglutinate which can be observed directly by eye or low powered microscope. Addition of an immunoglobulin test sample which shares the antigenic determinant bound to RBC would block the binding of the antibody to the immobilized antigen and therefore would inhibit hemagglutination. However, if the test sample lacks the determinant, the red blood cells agglutinate (Grubb, 1970).

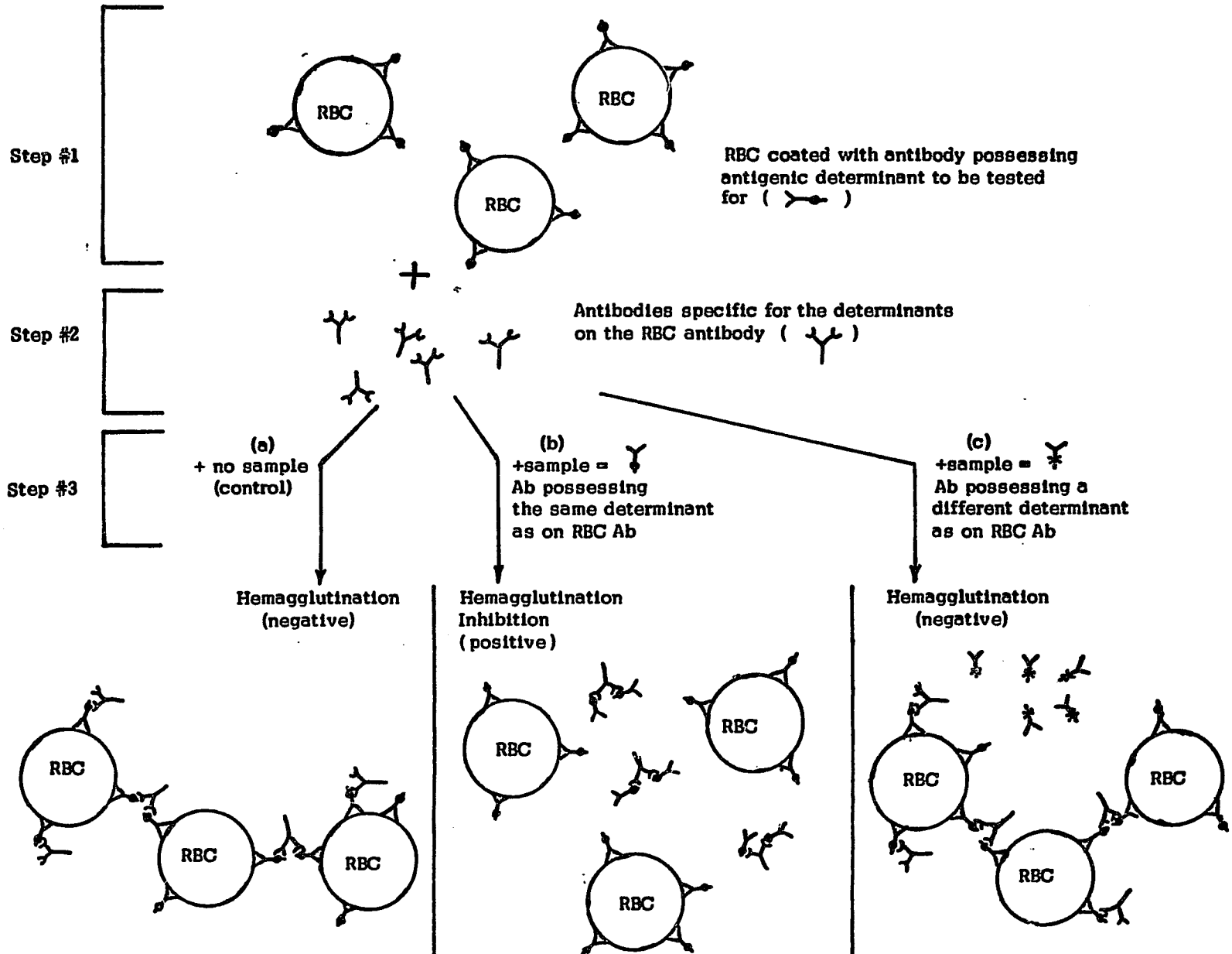
Antisera to allotypic determinants can be obtained from the sera of patients with rheumatoid arthritis (Grubb, 1961) (termed RAGG, for Rheumatoid agglutinator), from normal donors (Ropartz et al., 1960) termed SNAGG, for serum normal agglutinator), from transfused patients, or from sera of subhuman primates or rabbits immunized with normal

Fig. 1-8

(see following page for figure)

The indirect hemagglutination inhibition method for the detection of specific allotypic determinants is diagramatically presented here. In step #1, antibodies possessing the antigenic determinant to be tested for ( )—•) are coated onto red blood cells (RBC). The Y-shaped figures represent antibody molecules while a dark dot (•) and later an "x" represent antigenic determinants found on the antibody molecule. Antibody molecules which are specific for the determinants on the antibody coating the RBC ( )—) are added in step #2 and if no other sample is added (step #3a - control) hemagglutination of the RBC occurs. However, if an immunoglobulin sample is added that possesses the same antigenic determinant as the antibody coating the RBC (step #3b), then hemagglutination is inhibited as diagrammed in the figure. Furthermore, if an immunoglobulin sample is added which contains an antigenic determinant different from that on the antibody coating the RBC (step #3c), then hemagglutination will occur. In this system, therefore, hemagglutination is scored as a negative result indicating the absence of a specific antigenic determinant in an immunoglobulin sample.

Fig. 1-8


Step #1
Step #2
Step #3
RBC
RBC
RBC
RBC coated with antibody possessing antigenic determinant to be tested for ( )
Antibodies specific for the determinants on the RBC antibody ( )
(a)
+ no sample
(control)
(b)
+sample =
Ab possessing
the same determinant
as on RBC Ab
(c)
+sample =
Ab possessing a
different determinant
as on RBC Ab
Hemagglutination
(negative)
Hemagglutination
Inhibition
(positive)
Hemagglutination
(negative)
RBC
RBC
RBC
RBC
RBC
RBC
RBC
RBC

or myeloma proteins. SNAGG reagents are the best because they are usually the only ones that are monospecific.

The immunoglobulins used as antigens can be either incomplete anti-Rh antibodies that by themselves coat the red blood cells but without causing agglutination, or better, they can be monoclonal antibodies that are chemically coupled to the RBC using either tannic acid (Epstein & Fudenberg, 1962), bisdiazobenzidine (Natvig & Kunkel, 1967) or chromic chloride (Gold & Fudenberg, 1967; Vyas et al., 1968).

1.10 Allotype groups

Allotypes, which are defined as multiple alleles at a single genetic loci detected serologically, have probably evolved after the phylogenetic separation of subclasses because all of the allotypes detected so far are confined to particular subclasses (Natvig & Kunkel, 1973). Thus each subclass has evolved, through stable mutational events in the germ line gene, its own set of allotypic markers. Heavy chain subclasses are closely linked, as are their corresponding allotypes (Kunkel et al., 1969b; van Loghem et al., 1970). Therefore, the allotypes on one of the homologous chromosomes corresponding to the four subclasses may be inherited as a group known as a haplotype. As shown in Table 1-5, certain gene complexes that define the haplotype are more prevalent in certain populations than in

Table 1-5

Observed gene frequencies of commonly found Gm complexes haplotypes in different races (Schur, 1972).

IgG1		x								
	a	a			a	a	a	a	a	a
	z	z	f	f	z	z	z	z	z	f
IgG3	g	g	b^0	b^0	b^0	b^0	b^0	b^0	b^0	b^0
			b^1	b^1	b^1	b^1	b^1	s	st	b^1
			b^4	b^4	b^4	-	b^4	-	-	b^4
			b^5	b^5	b^5	c^5	b^5	b^5	b^5	b^5
			b^3	b^3	b^3	c^3	c^3	b^3	b^3	b^3
IgG2			n							n
Caucasian	0.20	0.10	0.52	0.17	0.005	0.001	0.001	0.001	0	0
Negro	0	0	0	0	0.55	0.25	0.08	0.12	0	0
Japanese	0.50	0.15	0	0	0	0	0	0	0.25	0.1
Chinese	0.03	0.02	0	0	0	0	0	0	0.20	0.75

others (Natvig et al., 1967a; Natvig & Kunkel, 1968). The generation and separation of these complexes in different populations most probably occurred as a result of rare recombinational and/or mutational events. A specific haplotype may therefore possibly confer a selective advantage to a particular population in a particular environment.

1.11 Antigenic markers & structure

Through hemagglutination inhibition studies using fragments of immunoglobulin chains as well as amino acid sequence comparisons between chains of differing allotypes, some Gm factors have been localized to specific domains if not to specific amino acid residue interchanges (see Table 1-6). However other Gm factors cannot be defined in such a precise manner probably because the detection of such antigenic determinants may very well be dependent upon the secondary, tertiary or even quaternary structure of the native immunoglobulin molecule. Therefore, amino acid residues from different parts of the polypeptide chain may be brought together to interact to form an antigenic determinant. Modification of the molecule such as chain separation (i.e., reduction of disulfide bridges), peptide bond cleavage, or the presence of denaturing conditions may alter structural characteristics essential for the expression of the Gm factor. For example, the expression

Table 1-6

Allotypes and Isoallotypes correlated to specific amino acid Substitutions

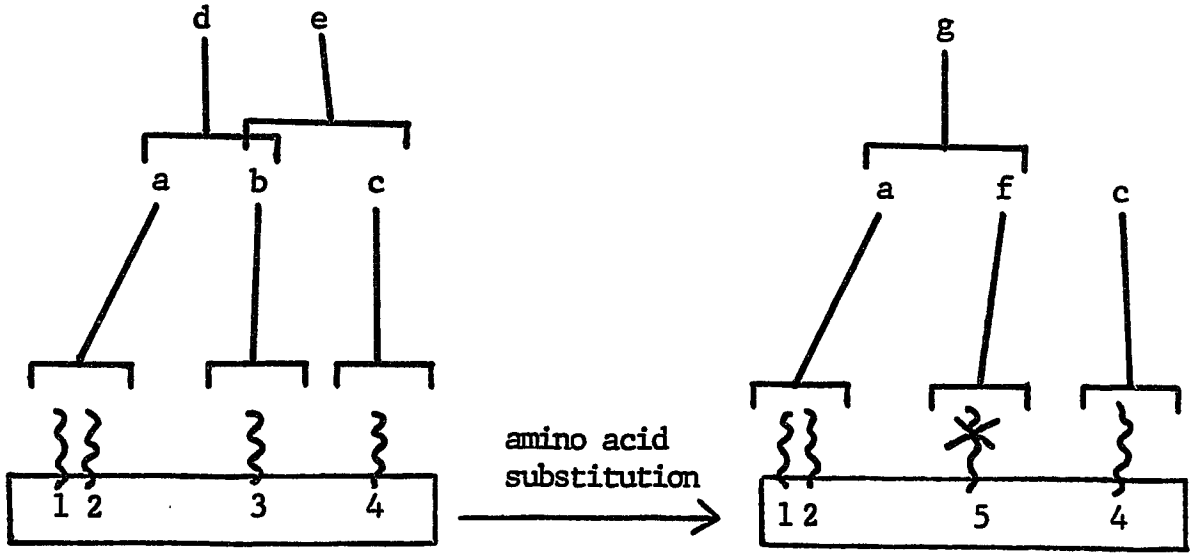
Genetic Marker	Chain	Residue Number (Eu)	Amino Acid	Ref.
G1m(a)	γ1	356/358	Asp/Leu	1
nG1m(a)	γ1,2,3	356/358	Glu/Met	2
G1m(f)	γ1	214	Arg	3
G1m(z)	γ1	214	Lys	2
G3m(g)	γ3	296/436	Tyr/Tyr	4
nG3m(g)	γ2,3	296	Phe	
G3m(b1)	γ3	296/436	Phe/Phe	5
nG3m(b1)	γ1,2,3	436	Tyr	6
nG4m(a)	γ1,3,4	309	Leu	7
nG4m(b)	γ2,4	309	-deleted	7
G3m(b0)	γ3	436	Phe	8
nG3(b0)	γ1,2,3	436	Phe	8

1.Ponstigl & Hilschman, 1972; 2. Edelman, et al., 1969; 3.Press & Hogg, 1969; 4. Grey & Abel, 1970; 5. Prahl, 1967a; 6. Michaelson, et al., 1977; 7. Abel, 1972; 8. Natvig & Kunkel, 1973.

of the Glm(f) and Glm(z) markers of the Fd fragment requires the existence of an intact quaternary structure between heavy and light chains. Conversely, the observation of an amino acid residue interchange that may correlate with the presence of a particular Gm marker does not unequivocally prove that the residue is responsible for the expression of that Gm marker. Therefore although some Gm factors can be correlated with the presence of one or more specific amino acid residues, other Gm markers may detect a particular three dimensional conformation that is the result of interactions of several residues and thus would be difficult to correlate with particular amino acid interchanges.

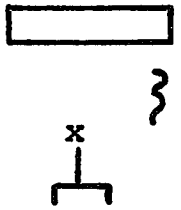
In addition, one cannot rule out the possibility that the reagent that detects the genetic marker is actually picking up more than one determinant (i.e., multispecific) or that different Gm markers may be composed of overlapping antigenic determinants. Such a possibility is schematically envisioned in Fig. 1-9. Therefore one amino acid substitution may result in the loss and/or additional expression of more than one genetic marker. This may be the case in γ3 chains which express many more Gm markers than other subclasses.

Fig. 1-9



Molecule A - Gm (a,b,c,d,e)

Molecule B - Gm (a,c,f,g)



- polypeptide backbone

- amino acid side chain (✱ = amino acid substitution)

- antibody reagent with specificity "x"

Above is shown the possible consequences that a single amino acid substitution may have on the detection of antigenic determinants. For example, determinant "a" is shown to be comprised of amino acids 1 and 2, whereas determinant "d" is comprised of the amino acid combination 1,2, and 3. Therefore determinants "a" and "d" overlap. However a substitution by a mutation of amino acid 3 with amino acid 5 results in the loss of determinants b,d, and e, and the appearance of determinants g and f. This figure also demonstrates that an amino acid substitution may lead to a loss of antigenic determinants (e.g. amino acids 3 + 4 in molecule A make up determinant e, whereas amino acids 5 + 4 in molecule B are <u>not</u> antigenic).

1.12 Chemical, biological and structural differences in IgG subclasses

Apart from differences in isotypic and allotypic markers, the four IgG subclasses also differ in certain biological activities as well as in the primary structure of their heavy chains. Some important biological characteristics of the different classes and subclasses are noted in Tables 1-1 and 1-2. Specific functions or lack of functions can therefore at times be ascribed to specific subclasses.

For example, protein A, a cell wall component of most strains of the pathogenic bacteria Staphylococcus aureus, has been shown to bind specifically to some immunoglobulin classes. Protein A was originally described by Verwey (1940), later studied by Lefkvist and Sjoquist (1963), and named by Grov et al., (1964). The structure of protein A, which has a molecular weight of 42,000, has been studied extensively and recently its amino acid sequence has been worked out (Hjelm et al., 1975; Sjodahl, 1976, 1977a, b). Because of its reactivity with immunoglobulin, protein A has been used in a variety of immunological systems and particularly in radioimmuno assays. Goding (1978) recently reviewed the use of protein A as an aid in immunological assays as well as its specificities of binding to immunoglobulins of species other than humans. Of the four IgG subclasses in humans, only IgG1, IgG2 and IgG4 bind to protein A, while IgG3 molecules do not show an affinity.

It is also of interest to structurally localize various genetic antigens. So far this has been accomplished by testing fragments and subfragments of the various IgG allotypes by hemagglutination inhibition in an attempt to identify the Gm markers in specific domains. Constant region markers that are detected in Fab or Fd fragments are therefore assigned to the CH1 domain while markers detected using pFc' fragments are assigned to the CH3 domains. Markers are assigned to the CH2 domains if they are present in Fc fragments but absent in pFc' fragments. However, the possibility exists that for certain Gm markers, structures on both the CH1 and CH2 domains may be necessary for their expression. Table 1-7 lists the genetic markers and some non-markers in their respective homology regions.

Other examples of variation of biological and chemical characteristics among the subclasses are reviewed by Schur, (1972), Natvig & Kunkel, (1973), Spiegelberg, (1974), and Winkelhake, (1978). Differences among the subclass in enzymatic cleavage by papain with and without cysteine has been reported (Jefferis et al., 1968; Virilla & Parkhouse, 1971; Gergely et al., 1970) as was differences in cleavages with pepsin (Turner et al., 1970). Differences among the subclasses were also reported for catabolic rates (Spiegelberg et al., 1968; Morell et al., 1970). aggregation characteristics (Capra & Kunkel, 1970),

Table 1-7

Distribution of Gm Markers and some Non-Markers among Constant Region Domains

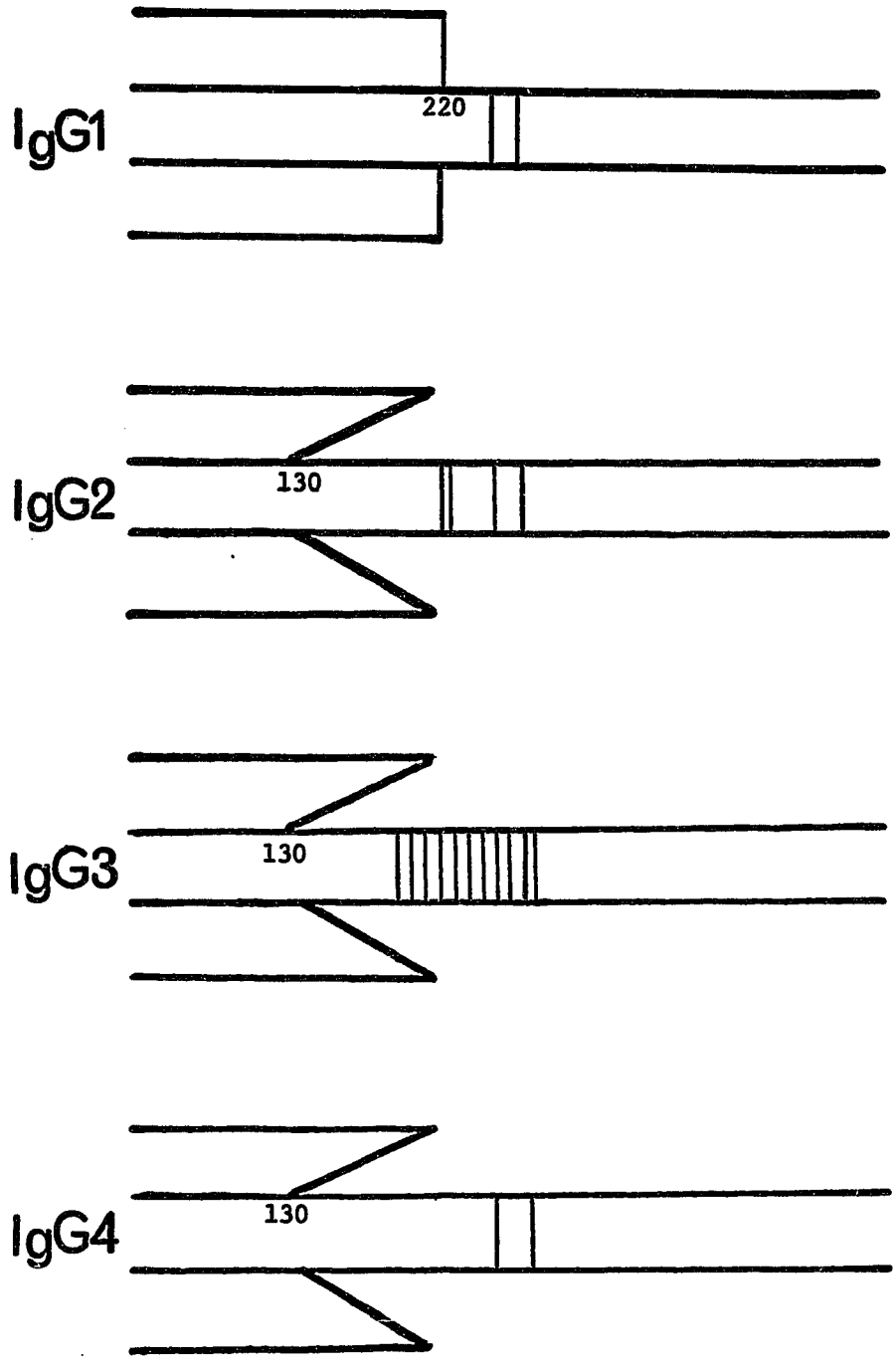
CH1	Fc	CH2	CH3
f	a		a
z	x		x
	n	n	
	g	g	
	s	s	
	t	t	
	u	u	
	v		v
	b0		b0
	b1	b1	
	b3		b3
	b4	b4	
	b5		b5
	c3		c3
	c5		c5
	non-a		non-a
	non-g	non-g	
	non-4a		non-4a

The above figure shows the distribution of the various Gm markers among the constant region domains. The CH2 and CH3 domains, in brackets, constitute the Fc region. Those Fc region genetic markers detected on pFc' fragments were assigned to the CH3 domain, while those which were not found on pFc' fragments were asigned by elimination to the CH2 domain. Therefore some or all of the CH2 markers may define antigens between domains or antigens which need the interaction of both the CH2 and CH3 domains for their expression.
(References: Natvig & Turner, 1971; Natvig & Kunkel, 1968; Grub, 1970; Harboe, 1962; Natvig & Kunkel, et al., 1968; Kronvall, 1965)

complement binding (Ishizaka et al., 1967), and in fixing to receptors on cell membranes (Huber & Fudenberg, 1968; Spiegelberg, 1974). The four subclasses also show unique amino acid sequences possibly relating to specialized functions even when limited to an examination of the constant region domains. Pertinent to this thesis is a detailed discussion comparing the structures of the subclasses.

The most striking structural difference between subclass heavy chains seems to be in the structure of the hinge region which lies between the CH1 and CH2 domains and possesses the interchain disulfide bonds. Structural subclass differences within the hinge have been suspected after examining the susceptibility of cleavage by papain of the different subclasses. IgG1 and IgG3 are readily susceptible to papain cleavage while IgG2 and IgG4 need the addition of cysteine for cleavage. The hinge region is also sensitive to digestion with pepsin and trypsin which cleave in similar but not exactly the same places among the subclasses. Further characterization of fragments from the hinge region by amino acid analysis and sequencing have proven that each subclass has a unique arrangement of interchain disulfide bonds and hence a unique sequence (Frangione, 1969b). Fig. 1-10 diagrams the location and number of disulfide bonds for each of the subclasses.

Fig. 1-10



This diagram demonstrates the differences that exist between the four IgG subclasses with respect to the arrangement and number of interchain disulfide bonds. Note that the location on the γ1 chain of the inter-heavy-to-light chain disulfide bond is positioned at residue 220 while in all the other subclasses this bond is at position 130. Note also the large number of inter-heavy chain disulfide bonds in the IgG3 subclass. In this figure the hinge areas containing the inter-heavy disulfide bonds are somewhat exaggerated in order to compare the subclasses more easily.

γ1 heavy chains are held together by two parallel disulfide bridges located at positions 226 and 229 (Frangione et al., 1969b; Steiner & Porter, 1967). γ2 heavy chains possess four such bonds, two identical to the γ1 positions and the other two at residues 219 and 220. The two interchain disulfide bridges are in the same position as in γ1 heavy chains. The more complex γ3 hinge structure has recently been worked out and is shown to contain eleven cysteine heavy-to-heavy disulfide bridges. Michaelsen et al., 1977a has shown the γ3 hinge region to be made up of a 17 amino acid segment followed by a 15 amino acid segment which is repeated three times over. The γ1 hinge, by comparison, possesses only a 15 amino acid segment that is 60-70% homologous to the repeated 15 amino acid segment. As a result of this extended hinge region (Michaelsen & Natvig, 1972, 1974; Wolfenstein-Todel et al., 1976a; Adlersberg et al., 1975) the molecular weight of γ3 heavy chains is approximately 58,000-60,000 daltons as compared to the γ1, γ2 or γ4 molecular weights of 50,000 daltons (Adlersberg et al., 1975; Saluk & Clem, 1971; Turner et al., 1970).

The arrangement of heavy-to-light interchain disulfide bonds can also serve to distinguish IgG1 from IgG2, IgG3, or IgG4. The γ2, γ3 and γ4 heavy chains are disulfide linked to the C-terminal cysteine of the light chain by a cysteine at position 130 on the heavy chain, but in γ1 this

link is through a cysteine at position 220 (Fig. 1-10). The three-dimensional folding of the chains make either residue position favorable for binding to the light chain (Poljak et al., 1973). However the sequence around these two cysteine positions differ as demonstrated upon isolation of the corresponding cysteine containing peptides.

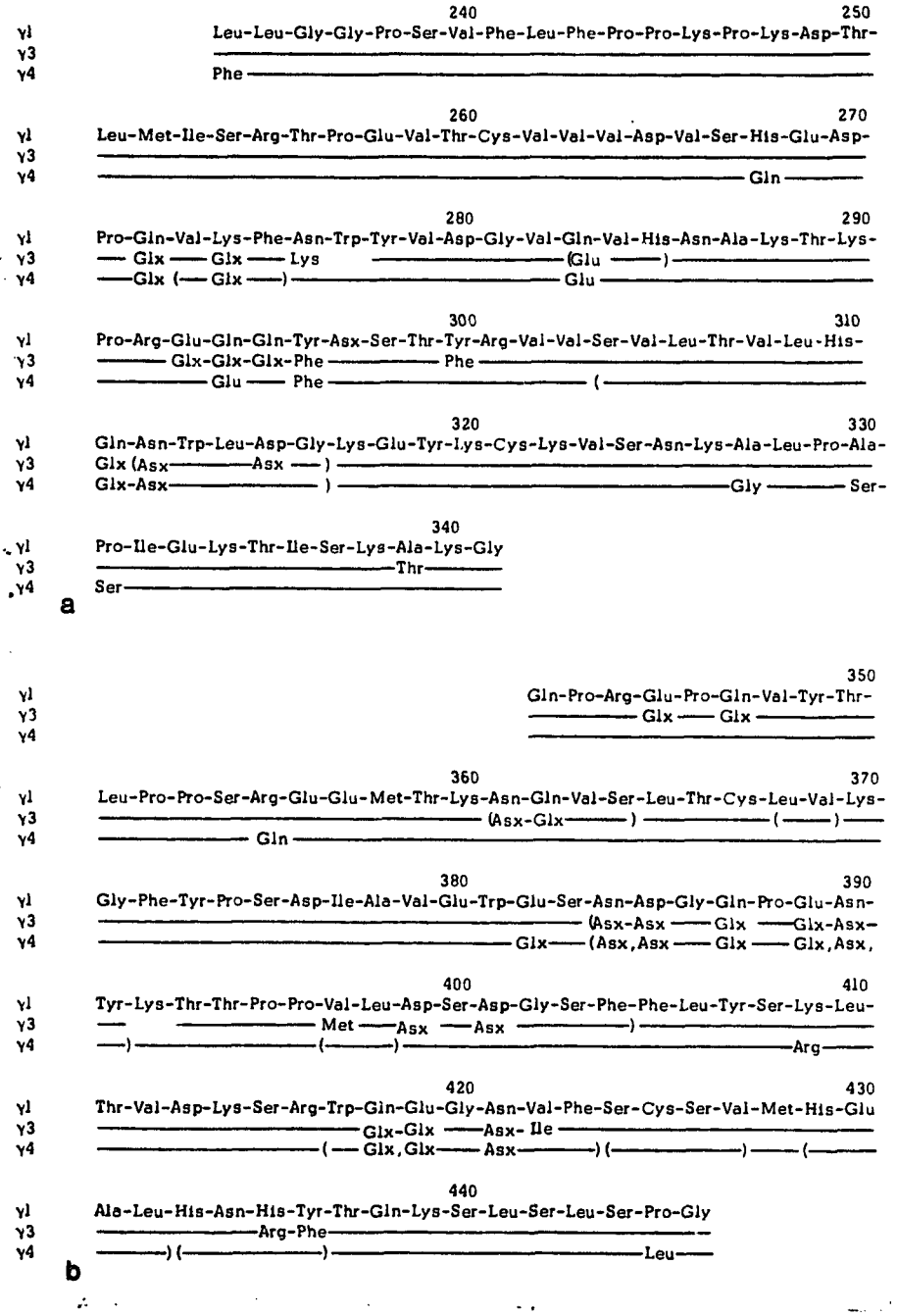
A methodology has been worked out that uses the differences in the amino acid content of these cysteine-containing peptides to distinguish the four subclass heavy chains. This "chemical typing" procedure (see Methods and Materials for details) relies on the fact that peptides of different composition migrate at different rates when applied to an electric field. Therefore if an IgG subclass is partially reduced and radioalkylated to split and radioactively label the interchain disulfide bonds, enzymatically cleaved to generate small peptides, run on high voltage paper electrophoresis, and finally autoradiographed to identify the ^{14}C-labeled cysteine-containing peptides, it can be shown that each subclass presents a unique pattern of radioactive peptide mobilities. Further testing has shown that the two subclasses of IgA, as well as IgM, IgD and IgE show unique distinguishable chemical typing patterns. (Frangione et al., 1969a; Frangione & Franklin, 1972; Mendez et al., 1973).

Somewhat less striking but probably also important in terms of biological function, the subclasses show

differences in a limited number of amino acid residues in their constant (CH1, CH2 and CH3) regions. The complete covalent structure of an IgG1 immunoglobulin heavy chain molecule reported by Edelman et al., (1969) is shown in Fig. 1-1. Although the entire sequence of an IgG3 heavy chain molecule has not yet been reported, a γ3 heavy chain disease protein (Franklin & Frangione, 1973, 1975) which contains an intact Fc fragment has recently been partially sequenced. (Wolfenstein-Todel et al., 1976b). This sequence is shown in Fig. 1-11 and is compared to the γ1 sequence and another partial sequence of a γ4 (Pink et al., 1970).

Table 1-8 lists the specific amino acid residue differences, excluding the unknown amides, between the γ1 and γ3 sequences. Only a limited amount of differences are apparent when either the CH2 or CH3 domains are compared between the subclasses. In the CH2 domain, positions 296 and 300 may be involved with allotypic determinants, whereas residues at position 274 and 276 may be related to isotypic determinants. In the CH3 domain, Met at 397 seems indicative of γ3 heavy chains whereas in γ1 heavy chains, Val is substituted. Also, positions 435 and 436 are His and Tyr respectively in γ1 whereas there is a substitution to Arg and Phe respectively in γ3 which may also correspond to an allotypic determinant. Until complete or nearly complete sequences of all Gm types are

Fig. 1-11



Comparison of (a) the C_H2 region and (b) the C_H3 region of an IgG1, Eu (Edelman et al.,1969), an IgG3, Zuc (Wolfenstein-Todel et al., 1976), and an IgG4 (Pink et al., 1970). Residue numbering according to Protein Eu. The solid lines indicate identical residues. The figure is taken from Wolfenstein-Todel et al., 1976.

Table 1-8

Differences in Amino Acid Residues in C_H2 and C_H3 Domains Between an IgG1 (Eu) and an IgG3 (Zuc)

	Residue in γ3 (Zuc)		Residue Number (Eu)		Residue in γ1 (Eu)
C_H2 (234-341):	Glx	-	274	-	Lys
	Lys	-	276	-	Asn
	Phe	-	296	-	Tyr
	Phe	-	300	-	Tyr
	Thr	-	339	-	Ala
C_H3 (342-446):	Met	-	397	-	Val
	Ile	-	422	-	Val
	Arg	-	435	-	His
	Phe	-	436	-	Tyr

Residue numbering is according to protein Eu (Edelman et al., 1969). Residues 296 and 300 may be related to the G3m(g) allotype while residues 435 and 436 may be related to the G3m(b) allotype.

reported, these substitutions may or may not be relevant in assigning a domain to a particular subclass.

Many partial sequences of regions that contain substitutions have been published. Table 1-9 compares the C-terminal octadecapeptide of the four subclasses and some of their allotypes. Correlations are easily apparent. Table 1-10 lists partial sequences of the carbohydrate containing peptide from different subclasses and allotypes, and once again correlations of particular amino acid substitutions to subclasses or allotypes can be made.

Table 1-9

Sequences of γ-Chain Octadecapeptides Derived from Human Myeloma Proteins

Name	Genetic Markers		Reference
γ1:Eu	Glm(f)	Met-His-Glu-Ala-Leu-His-Asn-His-Tyr-Thr-Gln-Lys-Ser-Leu-Ser-Leu-Ser-Pro-Gly	1
Vill	Glm(f)	—(————)(————)(————)	2
Cor	Glm(f)	—(————————————————)	3
He	Glm(f)	————————————————	4
Daw	Glm(a)	————————————————	5
Löb	Glm(a)	—(————————————————)	6
Par	Glm(a)	—(————————————————)	6
Baz	Glm(a)	————Gly————————————	7
Yok	?	————Gly————————————	8
γ2:Wan	G2m(n)	————————————————	9
Dav	G2m(n)	—(————)(————)(————)	2
Dup	G2m(n)	—(————)(————)(————)	2
Now	-	—(————)(————)(————)	2
Til	-	—(————)(————)(————)	2
Saw	-	—(————)(————)(————)	2
γ3:Zuc	G3m(b1)	————Arg Phe————————	9
Mar	G3m(b1)	————Arg Phe————————	9
Bri	G3m(b1)	—(————Arg Phe————)	6
Vil	G3m(g)	————Arg————————	9
Jon	G3m(g)	—(————Arg————)	6
Sig	G3m(g)	—(————Arg————)	6
γ4:She	-	—(————)(————Leu—)	9
Ger	-	————————————Leu—	9
Row	-	————————————Leu—	2
Mor	-	————————————Leu—	2
Vin	-	————————————Leu—	10

References:

1.Edelman, et al., 1969; 2. Harrington, et al., 1970; 3. Press & Piggot, 1967; 4. Cunningham, et al., 1971; 5. Press, Piggot & Porter, 1966; 6. Werner & Steinberg, 1974; 7. Garver, 1977; 8. Nabeshima, 1976; 9. Prahl, 1967; 10. Pink, et al., 1970.

Brackets, (-), indicate sequences which have been ordered on the basis of amino acid sequence homology.

Table 1-10

Partial amino acid sequence of Fc glycopeptides

Subclass	Gm	Sequence
IgG1	G1m(a) or G1m(f)	Glu-Glu-Glu-Tyr(296)-Asn(CHO)-Ser-Thr-Tyr(300)-Arg
IgG2	G2m(n) or nG2m(n)	(Glx,Glx,Glx,Phe,Asx,Ser,Thr,Phe,Arg)
IgG3	G3m(b1)	(Glx,Glx,Glx,Phe,Asx,Ser,Thr,Phe,Arg)
	G3m(g)	(Glx,Glx,Glx,Tyr,Asx,Ser)-Thr-Phe-Arg
IgG4		(Glx,Glx,Glx,Phe,Asx,Ser)-Thr-Tyr-Arg

This table shows the correlation of amino acid sequences of the carbohydrate-containing peptides with specific IgG subclasses and their genetic markers (Gm). The differences occur at positions 296 and 300. The parentheses inclose sequences that have been ordered on the basis of amino acid composition and homology (from Grey and Abel, 1970). Sequences determined by Edman degradation are indicated with → below the appropriate residue, while ← indicates sequence determination by carboxypeptidase degradation. The carbohydrate at position 297 is indicated by CHO and is present in all of the peptides shown. Sequence numbering is according to Edelman, et al.,1969.

- Chapter 2 -

Methods and Materials

2.1 Automatic sequence analysis

2.2 Identification of PTH derivatives

- 2.2.1 Thin-layer chromatography
- 2.2.2 Gas-liquid chromatography
- 2.2.3 High pressure liquid chromatography
- 2.2.4 Back hydrolysis
- 2.2.5 Liquid scintillation counting

2.3 Manual Edman degradation

2.4 Amino acid analysis

2.5 Polyacrylamide gel electrophoresis

2.6 Carbohydrate stain for polyacrylamide gel electrophoresis

2.7 Affinity chromatography

2.8 Cellulose acetate electrophoresis

2.9 Hydroxylapatite adsorption chromatography

2.10 Reduction and alkylation

2.11 Chemical typing

2.12 Concentration of proteins

2.13 Enzymatic and chemical digestions

- 2.13.1 Urea-pepsin digestion
- 2.13.2 Trypsin digestion
- 2.13.3 Pepsin digestion

2.13.4 Papain digestion

2.13.5 Cyanogen bromide cleavage

2.13.6 Aspartic-proline peptide bond cleavage

2.14 Two dimensional tryptic map

2.15 Immunoelectrophoresis

2.16 Ouchterlony

2.17 Spectrophotometric determination of proteins

2.18 Initial isolation of myeloma proteins

2.19 Antibody production

2.20 Genetic typing analysis

-Chapter 2-

Methods and Materials

2.1 Automatic sequence analysis

Automatic amino acid sequence degradation was performed in a Beckman 890C sequencer which is based on the original manual method for sequential degradation of peptides (Edman, 1957a, b, 1960). The method was further improved by Ilse and Edman (1963) and finally automated by Edman and Begg (1967). The basic chemical reactions as outlined in Fig. 2-1 take place in a spinning glass cylinder reaction cup which allows maximum exposure of the sample to the various reactants involved. Briefly the procedure for sequential residue degradation is as follows:

1. The sample is introduced into the spinning cup dissolved in a volatile solvent, usually 5% formic acid, and subsequently dried by evacuation as a thin film on the inside walls of the cup.

2. The sample is then dissolved in a non-volatile buffer (0.1 M Quadrol) and the coupling process is initiated by introduction of 5% phenylisothiocyanate (PITC) in heptane with 0.1 M Quadrol buffer. The PITC reacts with the free α-amino group to form the phenylthiocarbamoyl derivative of the peptide (Fig. 2-1, step #1).

3. Benzene and then ethyl acetate are added to precipitate the protein and extract excess PITC, Quadrol, and other breakdown products of the reaction.

4. After drying the protein, the cleavage step is initiated by the addition of anhydrous heptafluorobutyric acid (HFBA). This acid treatment cleaves off the N-terminal amino acid as the anilinothiazolinone (ATZ) derivative (Fig. 2-1, step #2).

5. The cleaved ATZ derivative of the amino acid is separated from the rest of the shortened peptide by extraction with butyl chloride and is subsequently flushed out of the cup and deposited into a test tube for further manipulations. The remaining peptide is dried down inside the cup and is ready for the next cycle of coupling and cleavage.

6. Since the ATZ derivative is somewhat unstable, conversion to the more stable isomeric phenylthiohydantoin (PTH) form is desirable and is accomplished by incubating the dried ATZ derivative in aqueous 1N HCl for 10 minutes at 80°C (Fig. 2-1, step #3). Recently, automatic conversion to PTH-amino acids was performed with a Sequemat P-6 Auto Convertor using a modified Tarr procedure (Tarr,1975), making use of anhydrous methanolic hydrogen chloride (a 1N solution of acetylchloride in methanol) instead of the standard aqueous acid. In this automatic procedure glutamic acid and aspartic acid become esterified and the methylester derivatives produced can be identified by HPLC. Two advantages of the modified Tarr procedure is the milder conversion conditions and the greater volatility of the reagent making for shorter drying times after conversion. The PTH amino acids are isolated by extracting twice with ethylacetate. Only PTH-histidine and PTH-arginine remain in the aqueous phase but can also be extracted

The Three Step Edman Degradation

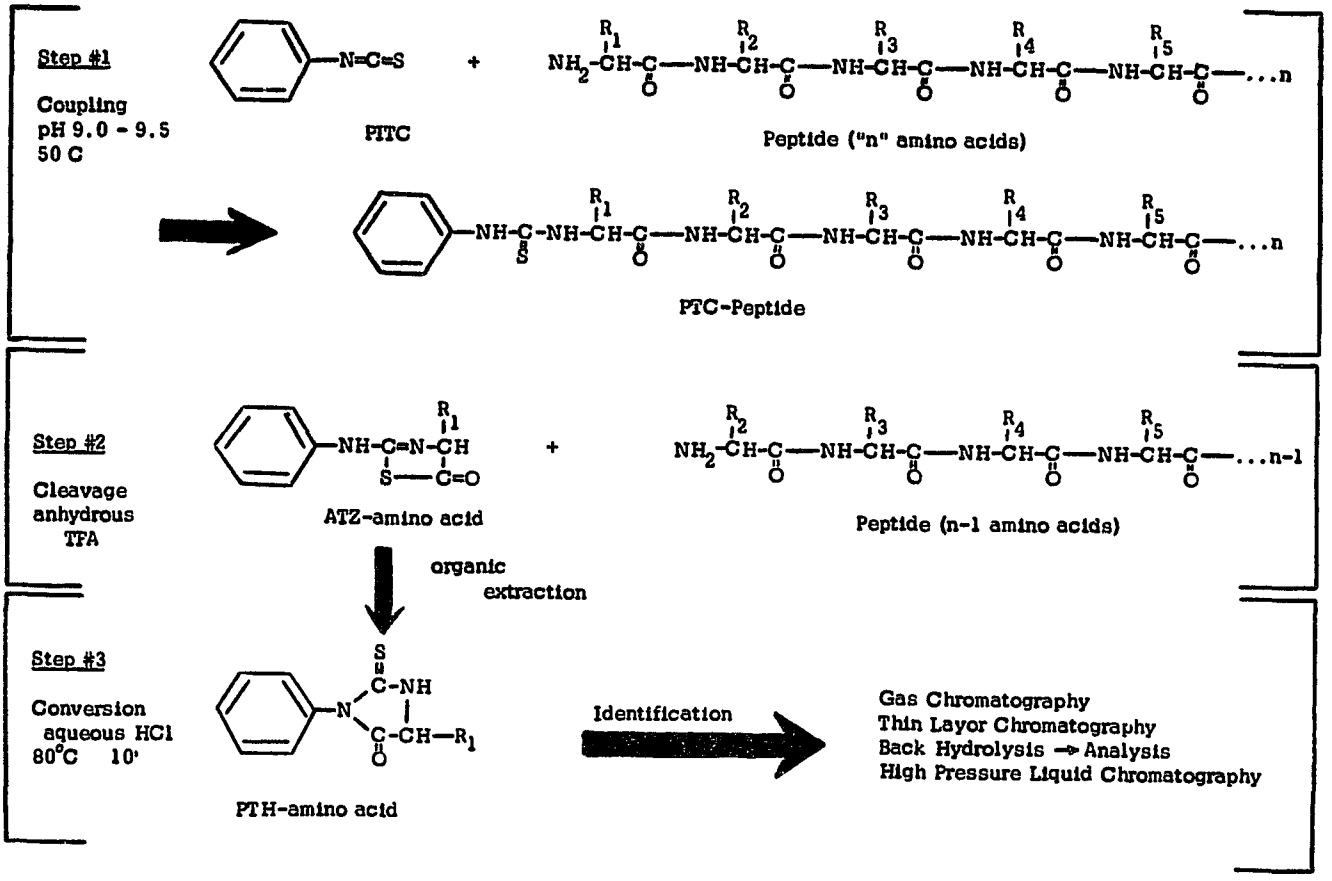


Fig. 2-1

Step 1: The α-amino group of the polypeptide reacts with phenylisothiocyanate (PITC) to produce a phenylthiocarbamoyl (PTC) derivative. The coupling occurs only at alkaline pH 9-9.5 since the α-amino group must be unprotonated.

Step 2: Cleavage takes place upon treatment of the PTC-peptide with anhydrous trifloroacetic acid (TFA) causing a cyclization and simultaneous release of the NH_2- terminal amino acid as an anilinothiazolinone (ATZ) derivative.

Step 3: Since the ATZ derivatives are unstable, they are converted into phenylthiohydantoins (PTH) prior to identification and quantitation.

with ethylacetate after neutralization with IN NH_4OH.

After drying the extracted PTH-amino acids under nitrogen they may be identified and/or quantitated by gas-liquid chromatography, thin layer chromatography, back hydrolysis and amino acid analysis, or high pressure liquid chromatography. In addition, the radioactivity in a 5% aliquot from each cycle was counted to confirm the presence of PTH-S-carboxymethylcysteine.

2.2 Identification of PTH derivatives

The PTH amino acids derived after conversion of the individual steps from the amino acid sequencer were identified using some or all of the following methods:

2.2.1 Thin-layer chromatography (TLC)

TLC of PTH-amino acids were run essentially according to Summers et al.,(1973)(see also Edman & Henschen, 1975; Kulbe, 1974). Polyamide sheets (5 x 5 cm coated with 50 micron polyamide purchased from Chen Chin Trading Co. Ltd., Taiwan) were run in the first dimension using as a solvent tolune, n-pentane and glacial acetic acid in a ratio of 60:30:35 respectively. After drying down, the sheets were run in a perpendicular dimension using as a solvent 35% acetic acid. Individual PTH-amino acid samples were run on one side of the polyamide sheet and were observed as fluorescent spots under short wave ultraviolet light. Each sample was identified by comparison to PTH-amino acid standards which were run on the opposite side of the sheet. Fig. 2-2

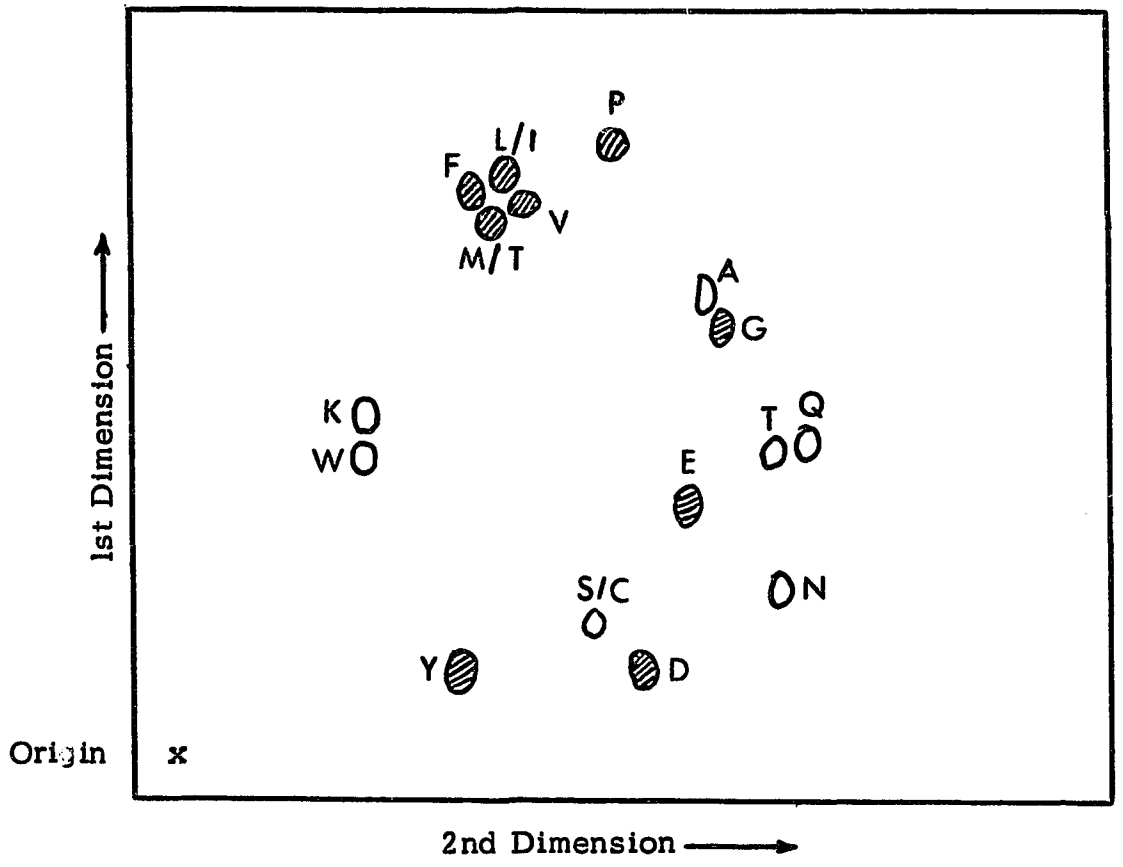


Fig. 2-2

This drawing represents a thin layer chromatograph of phenylthiohydantoin (PTH)-amino acids on a polyamide sheet. The first dimension was run in toluene/n-pentane/glacial acetic acid in a ratio of 60:30:35 respectively and the second dimension in 35% acetic acid. The shaded spots (◍) represent the PTH-amino acids used as standards in this laboratory.

diagrammatically shows where the various PTH-amino acids appear after a standard run. TLC is not a quantitative method and therefore as the sequence progresses, increasing background and overlaps make interpretation more and more difficult and unsure.

2.2.2 Gas-liquid chromatography (GLC)

Gas-liquid chromatography is a physical method of separation in which the different PTH-amino acids are distributed between two phases - one phase being a stationary bed of large surface area, the other being a gas which moves through the stationary phase. Gas liquid chromatography was performed on a 7620-A Hewlett-Packard gas chromatograph which was packed with a Beckman coated support no. 56796 (Pisano & Bronzert, 1969). An isothermal elution procedure as described by Niall,(1973) was utilized. Gas-liquid chromatography is a quantitative method but unfortunately not for all PTH-amino acids. Fig. 2-3 shows a standard 20 minute GLC run of the identifiable PTH-amino acids.

2.2.3 High pressure liquid chromatography (HPLC)

HPLC utilizes a support medium of small, ridged, spherical particles of uniform size to provide a rapid method for separation of many types of small organic molecules (Brown, 1973). PTH-amino acids were separated by HPLC originally on silica columns (Frank & Strubert, 1973; Graffeo et al., 1973; Zimmerman & Pisano, 1973; Frankhauser, 1974; Matthews et al., 1975). Improved separation and identification of most

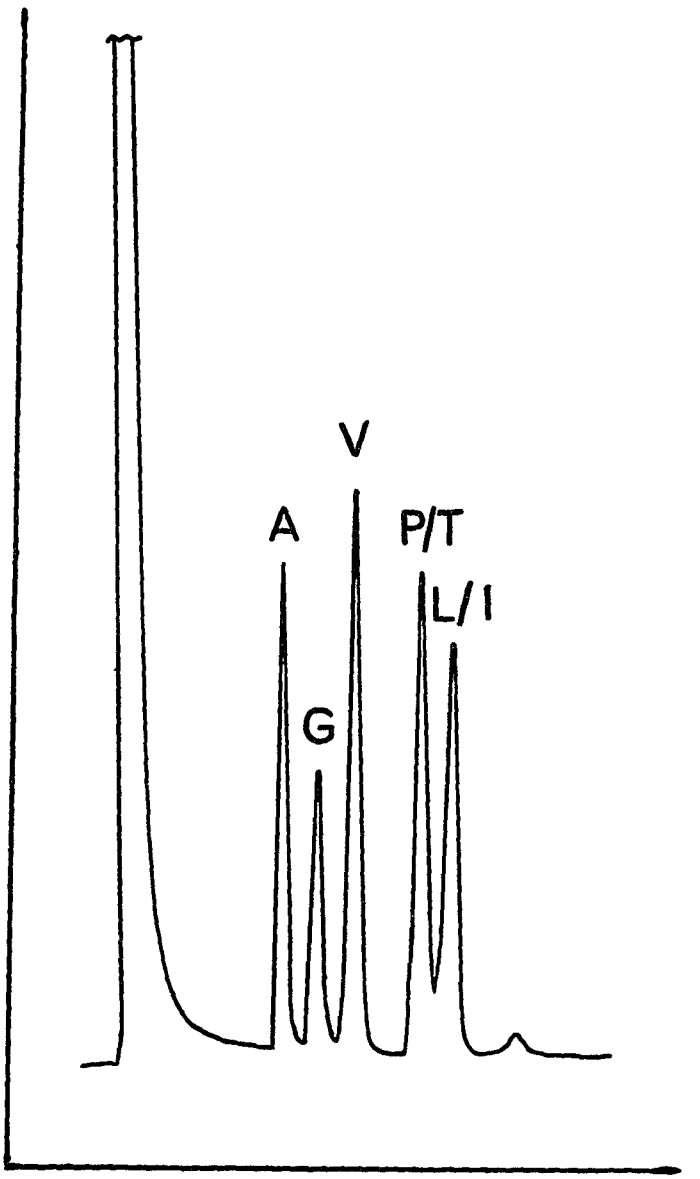


Fig. 2-3

Separation of PTH-amino acids by isothermal elution (20') on gas-liquid chromatography. Proline and threonine as well as leucine and isoleucine run together and must be identified by other means.

PTH-amino acids was accomplished with reversed-phase packings (de Vries et al., 1975; Zimmerman et al., 1976; Downing & Mann, 1976) using as a polar solvent acetonitrile (Margolies & Brauer, 1978) or methanol (Zeeuws & Strøsberg, 1978; Bhown et al., 1978).

Analysis of PTH-amino acids by HPLC in this lab was performed with a Waters HPLC model ALC/GPC - 204 equiped with two pumps (Waters, model 6000 A) a 254 nm adsorbance detector (Waters, model 440), a solvent programmer for gradient elutions (Waters, model 660) a manual sample injector (Waters, model U6K), a programmable automatic sample injector (Waters WISP TM model 710) and a prepacked C_{18}/μ-Bondapak column (30 cm x 3.9 mm I.D.) also by Waters.

The column was developed with a 15 minute linear gradient (gradient curve #6 of the model 660 programmer) of 100% A + 0% B to 55% A + 45% B buffers using a flow rate of 2.5 ml/min. The two buffers used were:

A: 9 liters per batch: 1260 ml of methanol

7740 ml of H_2O

22.5 ml of glacial acetic acid

450 μl of Acetone

B: 4 liters per batch: 400 ml of H_2O

3600 ml of methanol

1 ml of glacial acetic acid

Distilled water was purified further and deionized by passage through a Barnstead water purification system. Methanol was a Waters liquid

chromatography solvent, Acetone and glacial acetic acid were certified ACS reagents from Fisher. All buffers were passed through Millipore, type FH, 0.5 μm filters before use.

This HPLC system separates PTH-amino acids through a reverse phase column packing composed of a silica adsorbant modified by covalently binding it to an organic molecule (C_{18}) to form the following structure:  $-Si-(CH_2)_{17}-CH_3$. This yields the very low polarity bonded packing needed for PTH-amino acid separations.

In the presence of a high polar solvent (H_2O), the PTH-amino acids, which have low polarity, are forced into the packing. In general, upon development of a gradient of decreasing polarity the most polar amino acids are eluted first followed by the less polar ones. However, the actual mechanisms of adsorption and elution also involve properties of solubility, surface tensions, Van der Waals forces and thermodynamics making it very difficult to predict elution patterns (WALCEP resource material, Waters Liquid Chromatography School). Fig. 2-4 shows a typical run of PTH-amino acid standards.

2.2.4 Back hydrolysis

PTH-amino acids were converted back to the parent amino acids by acid hydrolysis (Smithies et al., 1971). An aliquot of each step was transferred to 12 x 75 mm acid washed test tubes and dried down by desiccation over NaOH. 0.2 ml of 6 N HCl and 5 μl of a suspension of 5% stannous chloride in 6 N HCl was added to each

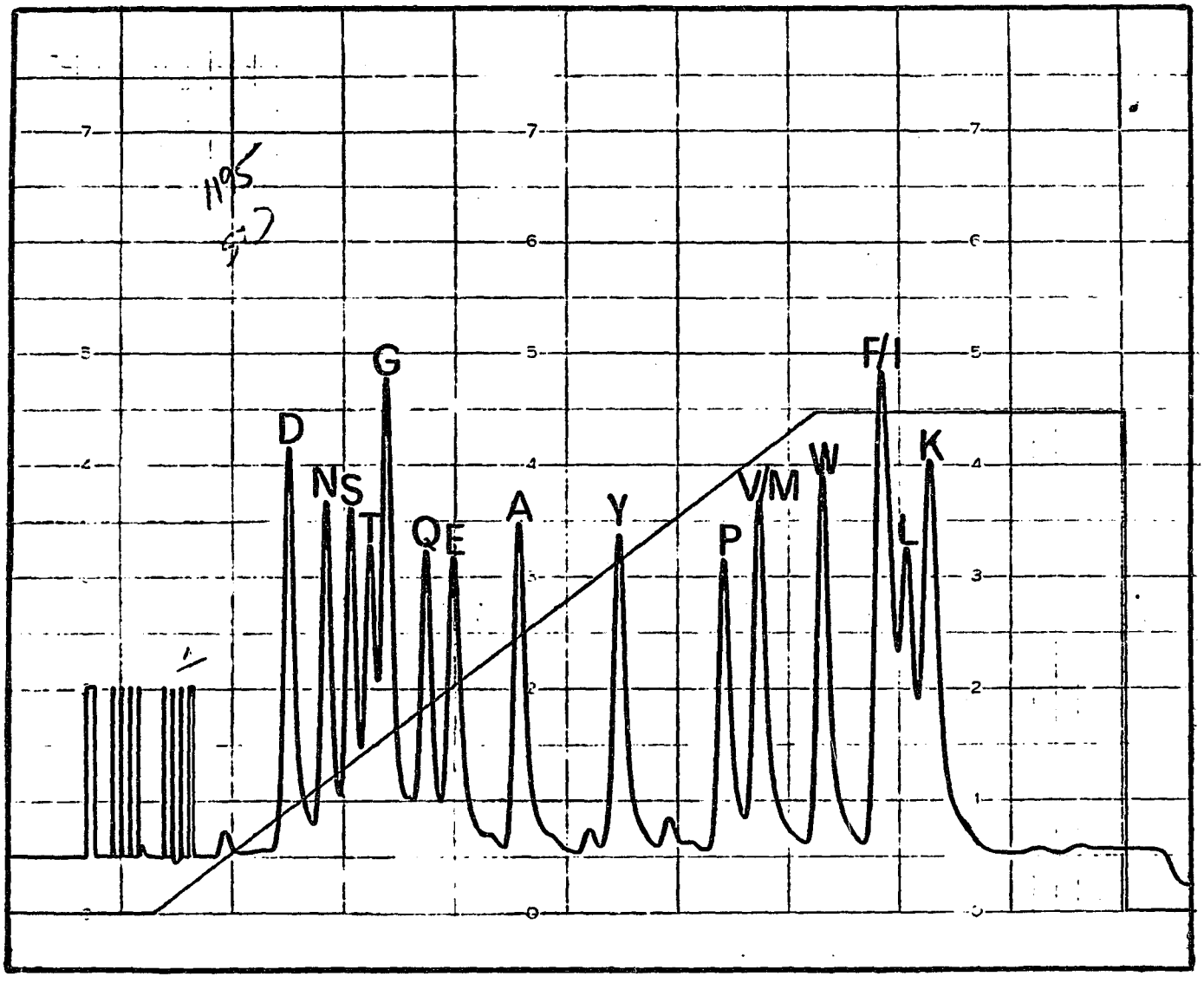


Fig. 2-4

This figure shows the HPLC chromatograph of the various PTH-amino acid standards as they are eluted by a linear methanol gradient, represented by the solid diagonal line. This program does not discriminate between valine and methionine, and phenylalanine and isoleucine, and therefore other methods are needed to identify them. Each peak represents approximately 2.5nmoles of a particular amino acid.

tube prior to being sealed on a vacuum in order to achieve reproducible color values for amino acids monitored with ninhydrin (Moore & Stein, 1954). After 4 hours at 150°C the tubes were broken open and evaporated in a desiccator over NaOH. The dried samples were dissolved in 50 µl of Pierce sodium citrate pH 2.2 buffer and transferred to microfuge tubes which were spun for one minute in a Beckman 152 microfuge. Usually 40 µl of each sample was analyzed on a Durrum model D-500 amino acid analyzer. This method converts PTH-asparagine and PTH-glutamine to their acidic forms - aspartic acid and glutamic acid respectively; PTH-isoleucine to a mixture of isoleucine and allo-isoleucine (which runs between methionine and isoleucine on the amino acid analyzer); PTH-threonine to α-aminobutyric acid (ABA) (which runs like half-cysteine, between alanine and valine). Serine is mostly destroyed, and tryptophan is degraded to glycine and therefore difficult to identify. Hydrolytic yields for each individual residue vary.

2.2.5 Liquid scintillation counting

Counting of ^{14}C-labelled carboxymethylcysteine (CMC) was performed in a Beckman L5-250 liquid scintillation system. Usually 1 to 50 µl of the sample (eg. fractions from the amino acid sequencer or from columns) were mixed with 1 ml of West Chemical Products, Beta Phase, liquid scintillation fluid in 6 ml scintillation vials. Samples were counted for one minute and the total counts recorded.

2.3 Manual Edman degradation

Amino-terminal analysis of proteins and peptides were performed using the manual Edman degradation technique. An aliquot of sample was dried down in an acid washed 12 x 75 mm test tube to which was added 150 µl of 5% phenylisothiocyanate (Pierce) in sequanal grade pyridine. When not used, this solution was flushed thoroughly with nitrogen and stored in a freezer. The sample mixture was then vortexed vigorously, flushed with nitrogen, stoppered, and incubated for one hour in a 45°C water bath. Next, after drying completely over NaOH in a desiccator, 200 µl of trifloroacetic acid (Baker) was added and the mixture incubated for 30 minutes at 45°C. Drying once again was done over NaOH in a desiccator. To extract the cleaved derivatized amino-terminal amino acid, 200 µl of distilled, deionized water was added along with 1.5 ml of sequanal grade n-butyl acetate (Pierce). This solution was stirred vigorously and then centrifuged for at least one minute using a desk-top International clinical centrifuge, (model CL). The top layer (organic phase) which contains the cleaved derivatized amino acid is carefully pipetted to another acid-washed test tube. The extraction was repeated once more and the two organic phases combined, and desiccated to dryness over NaOH. The extracted amino acid was identified by amino acid analysis after back hydrolysis.

If further degradation for the second amino-terminal amino acid

was desired, the remaining aqueous phase was washed by extracting once more and discarding the organic phase. After drying down the remaining aqueous phase, the manual degradative procedure can again be repeated.

2.4 Amino acid analysis

Protein and peptide samples were analyzed for their amino acid content on a Durrum model D500. Samples were hydrolyzed for 24 hours in 12 x 75 mm acid washed tubes by addition of 0.2 ml 6 N HCl. 40 µl of a 10% aqueous solution of phenol was added to prevent degradation of tyrosine (Sanger & Thompson, 1962). The tubes were sealed under vacuum and incubated at 110°C, usually for 24 hours. At the end of the hydrolysis period the tubes were cooled down, broken open and desiccated to dryness over NaOH. The dried residue was dissolved in 100 µl Pierce sodium citrate buffer, pH 2.2, transferred to microfuge tubes and spun for 1 minute in a Beckman 152 Microfuge to settle any insoluble debris or broken glass. 40 µl (40%) of the sample was usually analyzed. The Durrum analyzer is based on classical ion-exchange liquid chromatography for the separation and identification of free amino acids. Elution is a result of the stepwise addition of three buffers. Initially, a pH 3.5, 0.2 M sodium buffer (Durrum) is passed through to allow separation of certain acidic amino acids followed by a pH 4.5, 0.2 M sodium citrate buffer (Pierce) that washes through

a second group of amino acids. The remaining, more basic, amino acids are eluted using a pH 7.9, 1.1 M sodium buffer (Durrum). Each amino acid is identified by its characteristic time of elution under a prescribed set of column conditions (ie. temperature, salt concentration, etc). Fig. 2-5a shows the elution profile of a standard mixture of amino acids.

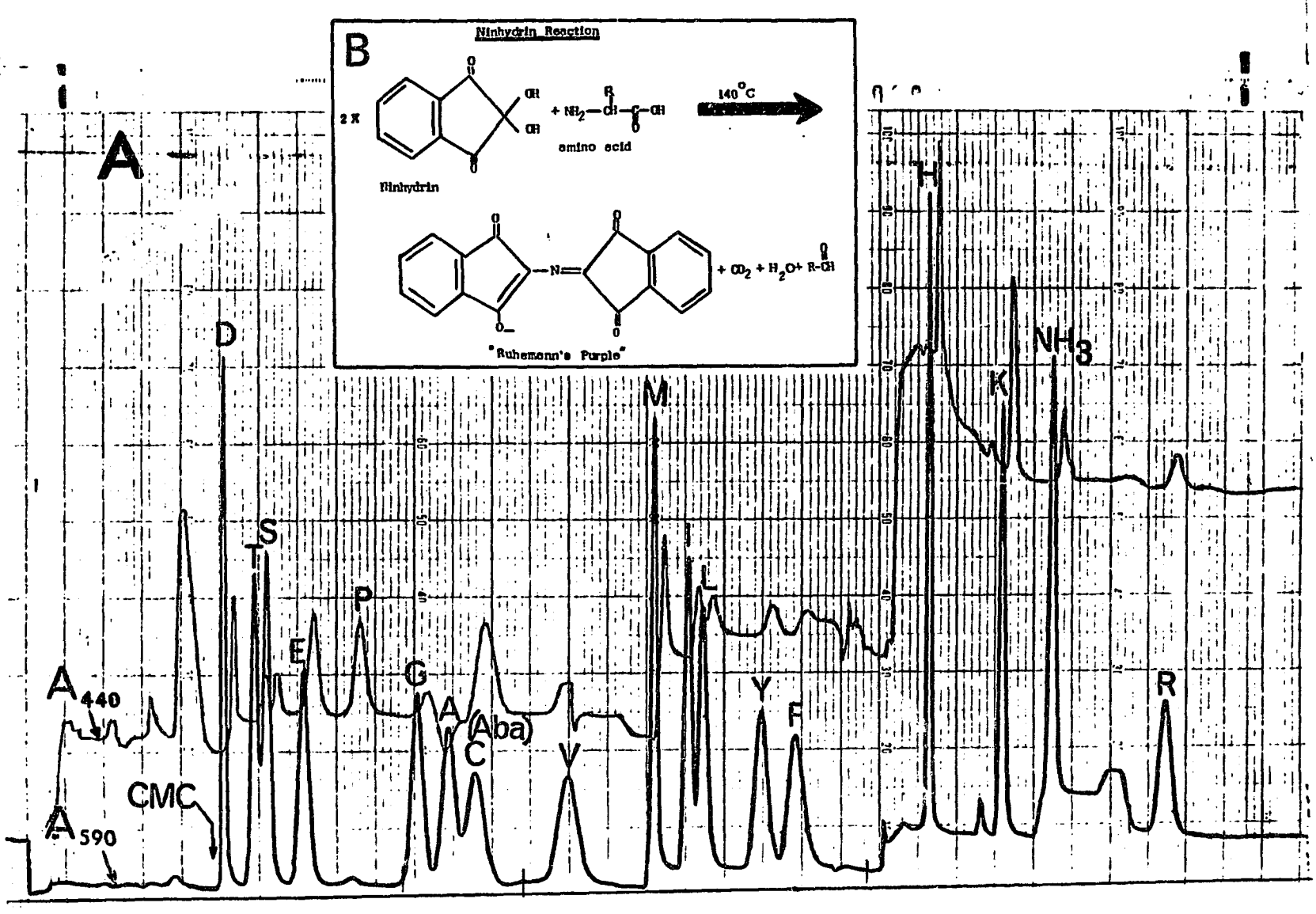
Detection and quantitative determination of the amino acids subsequent to their separation is accomplished through the classic reaction with ninhydrin as first reported by Ruhemann, (1910). Ninhydrin reacts with primary amines to form a colored complex termed "Ruhemann's Purple", the chemistry of which is reviewed by McCaldin, (1960). Fig. 2-5b shows the basic chemical ninhydrin reaction which takes place at 140°C in the Durrum D500 reaction coil and which is detected at a wave length of 590 nm. Ninhydrin also reacts with α-amino acids such as proline and hydroxyproline which yield instead a yellow colored product that adsorbs at 440 nm. The ninhydrin reaction was first used in automated amino acid analyzers by Spackman, Stein and Moore (1958).

The color formed by a given amino acid with ninhydrin is proportional to the amount of the amino acid but differs slightly for each amino acid. Therefore, when correctly calibrated with amino acid standards, the Durrum D500 is able to identify and quantitate a single amino acid or a mixture of amino acids derived from a hydrolysate.

(see following page for figure)

Fig. 2-5

Figure A shows a Durrum D-500 amino acid analyzer chromatograph of a standard mixture of amino acids. Total running time is about 90 minutes. Proline is detected at 440 nm while all other amino acids are detected at 590 nm. This mixture contains approximately 2.5 n moles of each amino acid.
Figure B shows the ninhydrin reaction with primary amines which produces the characteristic purple color which adsorbs at 590 nm.


A
B
Ninhydrin Reaction
2 X
Ninhydrin
amino acid
140°C
"Ruhemann's Purple"
+ CO2 + H2O+ R-CH
D
T
S
E
P
G
A
(Aba)
C
V
M
L
Y
F
H
K
NH3
R
A440
A590
CMC

2.5 Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was run as a slab in the presence of sodium dodecyl sulfate (SDS) (Shapiro et al., 1967; Weber & Osborne, 1969) using a discontinuous (disc) Tris-glycine-HCl buffer system (Ornstein, 1964; Davis, 1964). This system takes advantage of the fact that separation of proteins by SDS-PAGE is dependent on the molecular weights of the corresponding polypeptide chains and that in a discontinuous buffer system the starting zones, even with large sample volumes, become extremely thin. By the use of slab gels, as opposed to separate cylindrical gels, many samples can be run at high resolution and compared accurately.

Table 2-1 lists the stock solutions and the formulas for preparation of 10% or 15% gels. Tris buffers were made from Trizma Base tris(hydroxymethyl)aminomethane obtained from Sigma as reagent grade, with the pH being adjusted by addition of HCl. Glacial acetic acid was reagent ACS grade and methanol, glycerol, and bromphenol blue were certified ACS grades, all from Fisher. Clelands reagent (dithiothreitol - DTT) was purchased from Cal biochem, A-grade. Sodium dodecyl sulfate (SDS), N, N'-Methylene-bis-acrylamide (Bis), Coomassie Brilliant Blue R-250, Glycine, N, N, N' - tetramethylethylene-diamine (TEMED), and ammonium persulfate (APS) were all electrophoresis purity reagents from BioRad.

Table 2-1

Stock Solutions

Acrylamide Stock: 30% Acrylamide
0.8% Bis

Dissolve in distilled H_2O and filter. Store in dark at 4°C.

Tris Buffers: 1 M Tris-HCl, pH 8.8
1 M Tris-HCl, pH 6.8

SDS: 20% SDS in distilled H_2O

Sample Buffer: 2% SDS
10% glycerol
0.1 M Tris-HCl, pH 6.8
0.2% bromphenol blue

Running Buffer: 0.25 M Trizma Base
1.92 M Glycine
1.0% SDS
dilute to 1000 ml with distilled H_2O
dilute 1:10 before use.

Running Gels (30 ml)

	7.5%	10%	15%
Acrylamide Stock	7.5 ml	10 ml	15 ml
1 M Tris-HCl pH 8.8	11.2	11.2	11.2
20% SDS	0.15	0.15	0.15
H_2O, distilled	11.2	8.7	3.7

Stacking Gel (10 ml)

	5%
Acrylamide Stock	1.67 ml
Tris-HCl pH 6.8	1.25
20% SDS	0.05
H_2O, distilled	7.03

	Staining Solution	Destain Solution
Glacial Acetic Acid	5.5%	5.5%
Methanol	45%	45%
Coomassie	0.05%	--

Procedure preparing the gel

Fig. 2-6A diagrams how the two glass plates in which the slab gel is formed and Fig. 2-6B is a drawing of the custom made electrophoresis cell into which the glass plates are positioned. After the plexiglass strips are clamped between the glass plates (Fig. 2-6A), 1% agar is used to form a seal on the three closed sides. When the appropriate running gel is prepared in a beaker, polymerization is initiated by addition of 100 µl APS and 20 µl of TEMED and the solution is poured between the glass plates. To insure an even interface, 2 ml of distilled water is layered on top of the more dense running gel before polymerization. Upon polymerization (about 30 minutes) the water layer is poured off and the stacking gel is prepared. 50 µl APS and 8 µl TEMED is added to initiate polymerization and after the comb has been placed between the plates, the stacking gel is poured in. After polymerization the removal of the comb forms the slots where the sample will be applied. The bottom plexiglass spacer is removed and the glass plates are clamped to the cell in the appropriate fashion. Running buffer is then poured in the top and bottom half of the cell.

Sample preparation

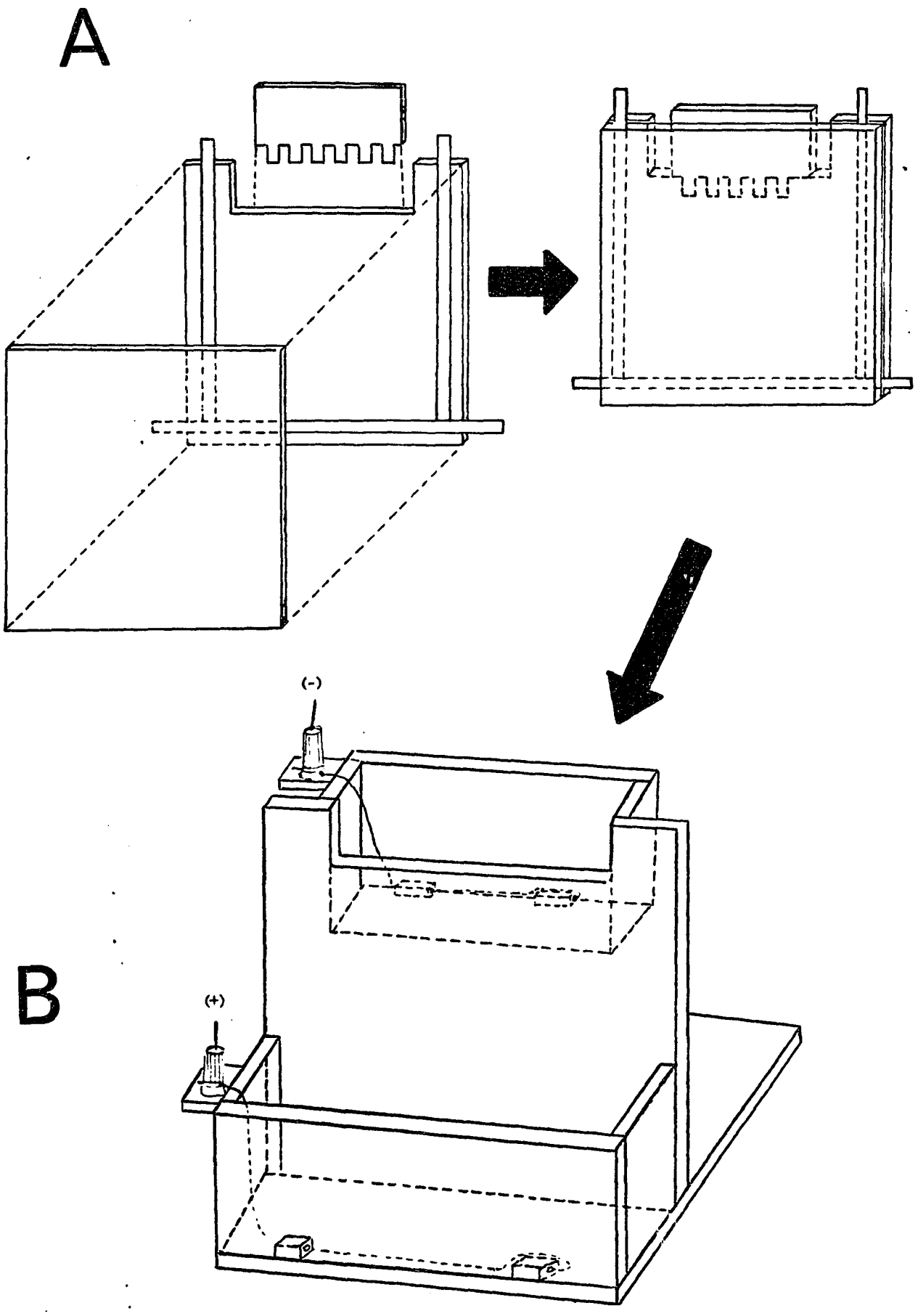
The lyophilized sample containing 20-100 µg of protein is dissolved in 50 µl sample buffer. If reducing conditions are required, the solution is made 0.1 M DTT. The samples are then totally denatured at 100°C in a boiling water bath for two minutes and then applied to

(see following page for figure)

Fig. 2-6

Figure A shows the diagram of how the two glass plates together with the side and bottom plexiglass spacers are assembled to form the mold where the polyacrylamide will be formed. The comb, which forms the gel slots, is placed as indicated before the gel polymerizes and is removed after it polymerizes so that the sample may be applied. Figure B shows the drawing of the electrophoresis cell into which the assembled glass plates are placed. Running buffer is poured into the upper and lower chambers before electrophoresis. The anode and cathode are marked.

Fig. 2-6



individual slots on the slab.

Running the gel

The gel is run at 50 volts for one hour then at 100 volts until the tracking dye is approximately 5 mm from the bottom of the gel. The gel is then removed after separating the glass plates and then placed in staining solution overnight at 37°C with continuous shaking. Destaining is accomplished over a period of hours at 37°C by frequently replacing the used destaining solution with fresh solution.

Photography

The gel can be photographed either wet or dry (drying is done overnight using a Pharmacia Gel Slab Dryer model GSD-4) with a Poloroid Land Camera mounted over an X-ray illumination Box. Pictures were taken using a Polaroid 4 x 5 Land Film type 55 with a red filter and a setting of F 4.5, at 1/2 second.

2.6 Polyacrylamide gel stain CHO

Carbohydrate containing proteins were specifically stained on SDS-polyacrylamide gels using the procedure described by Teramoto *et al.*, (1974). After electrophoresis, the polyacrylamide slab was fixed overnight in 50% methanol and 7.5% acetic acid and then washed five times in water, each time for twelve minutes. The gel was then soaked for one hour with 1% periodic acid in 3% acetic acid. The gel was washed again five times in water and then stained for 30 minutes with

0.4% Basic Fuchsin in water. The stained gel was then washed five times in freshly prepared 0.5% aqueous metabisulfate. Finally the gel was washed thoroughly in water and the carbohydrate staining bands were observed. To stain for protein, the gel was first decolorized with 20% trichloroacetic acid and then emersed in the Coomassic stain as previously described.

2.7 Affinity chromatography

Buffers used:

1 mM hydrochloric acid (HCl)

0.1 M sodium acetate, 0.5 M NaCl (coupling buffer)

1 M ethanolamine (Sigma)

0.1 M acetate buffer, pH 4.0, 1 M NaCl

0.1 M borate buffer, pH 8.0, 1 M NaCl

phosphate buffered saline (PBS), pH 7.0, 0.02% NaN_3

3.5 M NaSCN, pH 6.5 (Mallinckrodt)

1 M acetic acid (HOAc)

Cyanogen bromide (CNBr)-activated Sepharose 4B used in preparing the immunoadsorbant was purchased from Pharmacia Fine Chemicals. The protein to be coupled was dissolved in coupling buffer at 2-3 mg/ml (10-15 mg of protein was bound per gram of CNBr-activated Sepharose 4B). Each gram of Sepharose 4B was swollen for 15-30 minutes in 50 ml of 1 mM HCl and then washed on a fritted disc funnel with 200 ml

of 1 mM HCl and finally dried by vacuum. The protein solution was then added to the Sepharose and mixed gently by rotation overnight at 4°C and then spun at 500-1000 rpm for 5 minutes in an International Centrifuge, model PR-2. The adsorbance of the supernatant was read at 280 nm to check for the completeness of coupling (less than 0.10 adsorbance units at 280 nm was acceptable). To block any remaining active groups, 5 ml of 1 M ethanolamine was added per gram of Sepharose and gently rotated overnight at 4°C. The Sepharose was then filtered to dryness and washed with 50 ml of coupling buffer. To completely wash away any excess blocking agent and unadsorbed protein, the Sepharose was washed alternately with 50 ml acetate buffer and 50 ml borate buffer. This was repeated 4 times. Next, the Sepharose was washed with 3.5 M NaSCN and the adsorbance of the effluent at 280 nm was monitored until it was equal to that of the 3.5 M NaSCN. It was then washed thoroughly and resuspended in PBS and poured into an appropriate sized column. For use of the immunoadsorbant, the sample protein, dissolved in neutral buffer (i.e. PBS, pH 7.0, 0.02% NaN_3), is added directly to the column. Fractions are collected and read at 280 nm until adsorbance reaches background. The column is stripped by eluting with 1 M HOAc and again fractions are collected and read at 280 nm until background readings are reached. The column is washed once again with PBS, pH 7.0, 0.02% NaN_3 and can be used again or stored at 4°C until needed.

2.8 Cellulose acetate electrophoresis

Cellulose acetate electrophoresis of serum samples was performed using a Beckman R-101 Microzone Electrophoresis Cell Set and run in Beckman's B-2 barbital buffer, pH 8.6, $\mu = 0.075$. The power supply (model R-120), sample applicator, blotters, and cellulose acetate electrophoresis membranes were all from Beckman. Membranes were presoaked in barbital buffer and placed in the membrane bridge assembly of the cell prior to sample application. Serum samples were applied once and electrophoresis was run at 250 volts for 20 minutes. After the run, the membrane was stained black in a solution of 0.2% Aniline Black, 3% trichloroacetic acid and 3% sulfosalicylic acid for 5 minutes, destained using three washes of 5% acetic acid, blotted dry, and finally photographed.

2.9 Hydroxylapatite adsorption chromatography

Hydroxylapatite, a form of calcium phosphate was purchased from BioRad as Bio-Gel HPT, a dry free-flowing powder prepared by the method of Tiselius *et al.*, (1956). Its use as a method in protein purification and separation has been reviewed extensively by Hjerten, (1956) and Bernardi, (1971). One part of Bio-Gel HTP was added to six parts of 0.01 M phosphate buffer (PB), pH 6.5, (starting buffer) and gently swirled. The resulting slurry was allowed to settle for ten minutes then decanted and repeated once more. The hydroxylapatite was resuspended again, poured into a column and allowed to settle

by gravity. At least two column volumes of starting buffer were eluted before loading the sample. After application of the sample, which had been previously dialyzed against starting buffer, the column was washed until the adsorbance at 280 nm reached background. At times a stepwise increase in PB concentration was used to elute the various protein fractions. This was accomplished by successively increasing the concentrations of the phosphate buffer each time the adsorbance reached background. At other times a linear gradient of increasing phosphate buffer concentration was used. In this instance, after the initial 0.01 M PB wash, an additional 0.03 M PB, pH 6.5, wash was done. Then the linear gradient was initiated which was made up of 250 ml of 0.03 M PB, pH 6.5,and 250 ml of 0.2 M PB, pH 6.5. To reuse the column, 0.2 M PB, pH 6.5, was run through until no adsorbance at 280 nm was noted and then at least four column volumes of starting buffer were eluted.

2.10 Reduction and alkylation

Partial Reduction: The protein sample was dissolved at 10-20 mg/ml in 0.275 M Tris-HCl, pH 8.2 (partial reduction buffer) and made 5 mM with respect to dithiothreitol (DTT-Cleland's reagent, Calbiochem, A-grade). Reduction took place for one hour at 37^{o}C.

Complete reduction: The "partial reduction" procedure was followed except that the protein was dissolved at 10-20 mg/ml in "complete" reduction buffer: 100 gm guanidinium chloride (Research

Plus Lab-Absolute grade), 1.3 gm tris (Trizma Base, Segma), 0.2 ml of 0.1 M EDTA (ethylenediaminetetraacetate, Fisher, certified ACS), and 100 ml distilled water.

Alkylation - "cold": To alkylate either a partially or completely reduced protein sample, the reduced protein solution was made 11 nM with respect to iodoacetic acid (Fisher) which was recrystallized three times before use. The reaction took place at room temperature for one hour, in the dark immediately after reduction.

Alkylation - "hot": To radioalkylate the cysteines which were reduced, the cold alkylation procedure was followed except that a ^{14}C-labeled iodoacetic acid solution was used and prepared as follows: One vial of Iodo $2-^{14}C$ acetic acid containing 250 μCi (Amersham, 54 mCi/m mole was mixed with 3 ml of 22 mg/ml recrystallized iodoacetic acid made up in 0.275 M tris-HCl, pH 8.2. This made a 120 mM ^{14}C-labeled iodoacetic acid solution and when not in use it was stored frozen

After reduction and alkylation the solution was either dialyzed against water or desalted on Sephadex G25-fine in 5% formic acid and then lyophilized.

2.11 Chemical typing

Chemical typing was performed on 7-8 mg of a partially reduced and radioalkylated (PR/A*) immunoglobulin sample. The PR/A* lyophi-

lized protein was dissolved at 20 mg/ml in 5% formic acid, digested with pepsin (Sigma, from hog stomach mucosa, 2 x crystallized and lyophilized, 3180 units/mg protein) at a 1/50 enzyme: protein (w/w) ration and incubated overnight at 37°C. After complete drying by lyophilization, the pepsin digested protein was dissolved in 0.2 M NH_4HCO_3 at 20 mg/ml and digested with trypsin at a 1/50 enzyme: protein (w/w) ratio. The digest was allowed to proceed at 37°C for 6 hours and terminated by freeze drying. The pepsin-trypsin digest was then dissolved in about 100 µl of 0.01 M NH_4OH and applied as a band about 5 cm long on a sheet of Whatman 3 MM Chromatography paper 15 cm from the positive end. Amino acid standards were spotted 2 cm on either side of the sample. After the samples and standards were dry, the paper was wet down with pH 3.5 pyridine-acetate buffer and then blotted with Whatman #1 Qualitative paper. The blotted chromatography paper was then carefully placed into a Savant paper electrophoresis tank and run in pH 3.5 pyridine-acetate buffer at 3000 volts. When the blue colored marker (Cyanol F-F) migrated 8-10 cm toward the positive electrode away from the point of origin (about one hour), the electrophoresis was stopped, the paper removed from the tank, dried thoroughly, and then exposed overnight to X-ray film (Kodak X-O-mat S film) in order to visualize the radioactive peptide banding pattern. Strips of paper that contained

the electrophoresed amino acid standard were cut away and stained with ninhydrin in order to visualize where they migrated.

To purify the cysteine containing peptides for amino acid analysis, each radioactive band was cut out and sewn on to 1MM Chromatography paper and run at pH 6.5 with high voltage. The radioactive bands were again identified by X-ray, cut out and electrophoresed once more at pH 2.1. The resulting pure radioactive peptides were cut out, eluted with 6 M HCl, and hydrolyzed for 24 hours at 110°C in preparation for analysis.

2.12 Concentration of proteins

Proteins were concentrated, if salt free, by lyophilization and redissolved in a smaller aliquot of buffer or, if not salt free, by negative pressure using 8 mm dialysis tubing (Beckman) which has a 10,000 dalton molecular weight cut off.

2.13 Enzymatic and chemical digestions

2.13.1 Urea-pepsin digestion

Digestion with pepsin in the presence of urea and subsequent fractionation was carried out essentially as described by Parr *et al.*, (1976a, b , 1977). Lyophilized protein samples were dissolved in 0.1 M sodium acetate buffer, pH 4.3, at 20 mg/ml. Enough solid urea (Aldrich, ACS reagent, 99+%, Gold Label) was added to make the concentration slightly above 8 M. The mixture was

adjusted to pH 3.6 with the addition of concentrated HCl. Pepsin (Sigma, from hog stomach mucosa, 2 x crystallized and lyophilized, 3180 units/mg protein) was then added to give an enzyme/substrate ratio of 1:100 (w/w). The reaction was allowed to take place at room temperature (23°C) for one hour with continuous, slow stirring. At the end of this time the reaction was stopped by addition of two drops of 2 M Tris-HCl, pH 9.0, followed by enough 50% NaOH to bring the reaction mixture to pH 9.0. The digest was initially separated by gel filtration on Sephadex G-150 (Pharmacia Fine Chem. Inc.) equilibrated with 0.1 M sodium acetate buffer, pH 5.0, in 6 M urea. Elution of the peaks was monitored by UV adsorption at 280 nm. Further separation and purification procedures are described in the section on Results.

2.13.2 Trypsin digestion

See procedure under "chemical typing"

2.13.3 Pepsin digestion

See procedure under "chemical typing"

2.13.4 Papain digestion

Whole immunoglobulin was digested with papain to generate Fab and Fc fragments (Gergely et al., 1967). The protein was dissolved at 25 mg/ml in pH 7.0 buffered saline and made 0.01 M in cysteine-HCl and 0.002 M in EDTA . It was then digested with papain (Worthington Biochemical Corp.) at an enzyme/substrate ratio of

1:100. Digestion was allowed to proceed at 37°C for 30 minutes. The digested protein was then dialyzed against pH 8.0, 0.01 M sodium phosphate buffer overnight and separated into Fab and Fc fragments on DEAE cellulose equilibrated in the same buffer. The Fab fragment was recovered in the fall-through peak, whereas the Fc fragment that was still bound to the ion-exchange resin was eluted with 0.3 M NaCl. The fractions were pooled, dialyzed against water, and lyophilized for further study.

2.13.5 Cyanogen bromide cleavage

Partially reduced and alkylated heavy chains were dissolved at 40-50 mg/ml in 70% trifloroacetic acid (TFA) to which was added a three fold excess (w/w) of cyanogen bromide (CNBr) (Pierce). The reaction was allowed to proceed at room temperature for 16-20 hours and stopped by the addition of ten volumes of water followed by lyophilization. Separation of the generated CNBr fragments was done on Sephadex G-100 equilibrated in 30% formic (Schroder et al., 1969). However, where the carboxy-terminal octadecapeptide (ODP) was specifically sought, the lyophilized CNBr digest was initially washed twice with 0.01 M NH_4HCO_3. This allowed only the small peptides (i.e. the ODP) to go into solution while the large fragments, as well as small fragments still disulfide linked, would remain as a precipitate. The supernatant was then applied to a column of Sephadex G-50 in 0.01 M NH_4HCO_3, pH 8.2. Protein peaks were detected by adsorbance readings

at both 280 nm and 230 nm, the latter detecting adsorbance of peptide bonds.

2.13.6 Aspartic-proline peptide bond cleavage

Limited acid hydrolysis of aspartic-proline peptide bonds was performed essentially as described by Mole et al., 1977 . The protein sample was dissolved in 50% formic acid (Fisher, Certified ACS) at 10 mg/ml in a 25 x 75 mm acid washed tube. After sealing the tube at atmospheric pressure, the reaction mixture was incubated at 40°C for 96 hours. After the incubation period the tube was broken open and the solution applied directly to a gel filtration column in 10% formic to separate the peptides.

2.14 Two-dimensional tryptic map

The completely reduced and radioalkylated protein sample was dissolved at 1 mg/ml in 0.2 M NH_4HCO_3, pH 8.5. Trypsin was added at an enzyme/protein ratio of 1:100 (w/w) and the reaction mixture was incubated at room temperature for two hours. After lyophilization 2-3 mg of the digested protein was dissolved in about 100 µl of 0.01 M NH_4OH and applied as a spot to the corner of a Whatman 3 MM Chromatography paper. Chromatography was run in the first dimension in a BAWP buffer system (Butanol/Acetic acid/Water/Pyridine in a 15:3:12:10, v/v ratio). After overnight chromatography, the paper was dried at room temperature and then wet with pyridine-acetate buffer, pH 3.5. Electrophoresis was

then run in the second dimension in pyridine-acetate buffer, pH 3.5, at 3500 volts. The paper was then dried thoroughly and stained with ninhydrin to visualize the peptides . The radioactively-labeled cysteine-containing peptides were localized by exposure of the paper overnight to X-ray film. The appropriate peptides after staining with ninhydrin were cut out, eluted with 6 M HCl, and hydrolyzed for 20 hours for amino acid analysis.

2.15 Immunoelectrophoresis

Immunoelectrophoresis was performed in 1% agar made up in 0.05 M barbital buffer, pH 8.5. 3.5 ml of melted agar was poured onto 25 x 75 mm microscope slides and allowed to gel at room temperature at which time the appropriate pattern (i.e. wells and troughs) was cut out. The antigen samples were applied into the wells and electrophoresed for three hours at 4 mamps per slide. After electrophoresis, the troughs were filled with antisera and the slide was placed overnight at 37°C in a closed, moist container.

2.16 Ouchterlony

3.5 ml melted 1% agar in 0.05 M barbital buffer, pH 8.5, was poured onto 25 x 75 mm glass slides. Wells were cut out after the agar solidified. Antigens were placed in the outer wells and antisera in the center well. The precipitate lines were developed overnight at 37°C in a moist chamber (Ouchterlony, 1949)

2.17 Spectrophotometric determination of protein

Determination of protein concentrations as well as screening column effluents were performed spectrophotometrically. To determine concentration of whole immunoglobulin solutions an extinction coefficient of 1.4 was used. Protein peaks from column effluents were determined spectrophotometrically using a Gilson spectrophotometer (model 250) equiped with adjustable wave length, and automatic sampler and data lister, Model 4019. Adsorbance at 280 nm were usually determined on every column fraction, and when appropriate, adsorbances at 230 nm were determined to detect small peptides that do not contain the aromatic amines which adsorb around 280 nm.

2.18 Initial isolation of myeloma proteins

Plasma from myeloma patients was stored at 4°C in sterile 400 ml plastic bags until needed. Clotting was initiated by adding two drops of thrombin (1000 units/ml) and 0.5 ml of 10% $CaCl_2$ to 35 ml aliquots of plasma. The samples were left for one hour at 37°C and then centrifuged for 15 minutes at 12,000 rpm in a Beckman model L ultracentrifuge using a type 30 rotor. The sera was separated from the clot and kept frozen until needed.

The serum from patient Kup contained a monoclonal cryoglobulin protein and therefore was initially isolated by repeated cold precipitation. Kup serum was kept overnight at 4°C and then centrifuged at 3000 rpm

for ten minutes in an International Centrifuge model PR-2. The supernatant was discarded and the precipitate washed at least four more times by mixing it with cold normal saline and centrifuging.

The myeloma protein from patient Goe and the IgG from normal serum was initially isolated by ion exchange chromatography (Peterson & Sober , 1956 . The serum was first dialyzed against distilled water for two hours at room temperature and then overnight against 0.01 M sodium phosphate buffer, pH 7.4. The anion exchanger, DE-52 (diethylaminoethyl cellulose), was purchased pre-swollen from Whatman and was initially washed by suspending it in 0.3 M sodium phosphate buffer, pH 7.4, for at least two hours and then washed thoroughly in a fritted disc funnel with 0.01 M sodium phosphate buffer, pH 7.4, and finally poured into a column. The dialyzed serum sample was then applied to the ion exchange column and washed through with the 0.01 M sodium phosphate buffer. IgG was predominantly eluted from this column in the fall-through wash whereas almost all of the other more negatively charged serum proteins were still bound.

At other times IgG was isolated by zone electrophoresis carried out on blocks of starch (Kunkel, 1954a,b). Potato starch, purchased from Fisher, was washed well in a Buchner funnel with 0.05 M barbital buffer, pH 8.6. The starch was then poured as a smooth suspension into the mold and allowed to dry at room temperature for 3-4 hours.

The serum sample was applied in a 1" slit cut in the starch block 7" from the anode side and then run at 400-450 volts (60-100 mamps) for 21 hours at 4°C. After the run, the block is allowed to dry at room temperature and 1/2" wide strips of starch are cut out and eluted separately by suspension in diluent followed by centrifugation and decantation of the supernatant. Each strip is eluted with about twice the sample volume. An aliquot (0.1 ml) of each eluant is quantitated for protein content by the Folin method. The IgG containing fractions were pooled, dialyzed against water, and then lyophilized.

2.19 Antibody production

Antibodies to purified myeloma proteins were produced by immunization of rabbits. The protein antigen was dissolved at 1 mg/ml in normal saline and an equivalent amount of complete Freund's adjuvant (Difco) was added. The mixture was homogenized on a Virtis homogenizer for at least 10 minutes at high speed until a thick suspension was formed. The homogenized antigen was stored at +4°C when not in use. Initial immunizations took place by injecting a total of 1ml. of the homogenate per rabbit in the foot pad area. The rabbits were then boosted each week for one month with 1 ml. of the homogenate divided into 4-6 places on the back and neck areas. Every other week following the first month, the rabbits were similarly boosted and 50 ml. of blood was collected through an ear vein. The blood was immediately

centrifuged at 2000 rpm for 20 minutes in an International, model L, centrifuge and the serum stored frozen until needed.

2-20 Genetic typing analysis

Typing of immunoglobulin samples for their genetic markers was kindly performed by Dr. E. van Loghem in the Department of Immunogenetics at the Central laboratory of the Netherlands, Red Cross Blood Transfusion Service, Plesmanlaan 125, Amsterdam W., The Netherlands. The hemagglutination inhibition technique was used for the detection of the antigens as described in Chapter 1 (see Fig. 1-8).

-Chapter 3-

Studies on Myeloma Protein Kup

-Chapter 3-

Studies on Myeloma Protein Kup

3.1 Introduction

The purpose in this chapter of characterizing a typical IgG3 myeloma protein, Kup, is two-fold. First, since the strategy of this thesis is to examine the structure of an unusual myeloma protein, Goe, an indepth knowledge of the structure of normal immunoglobulins is essential. Second, the serum from patient Goe was available only in limited amounts. Since preliminary work in finding ideal conditions for fractionation and purification of the various protein components usually involves a large expenditure of starting material, the large quantities of Kup serum available made it more practical to use in the initial structural studies. The results from the studies on protein Kup in this chapter, along with those already published for other IgG3 and IgG1 myeloma proteins, will be compared to the results of similar studies performed on the myeloma protein from patient Goe (Chapter 4).

Protein Kup, an IgG3 monoclonal cryoglobulin, has previously been studied for other purposes. Kup has been shown to possess potent anti-γ globulin activity (Grey et al., 1968). It has also been shown that upon papain digestion of intact protein Kup, an unusual fragment is

generated which consists primarily of the V_H region (Dammacco et al., 1972). In addition, the antigenic determinants of this V_H region fragment have recently been studied and characterized (Michaelson et al., 1977). Earlier studies have characterized Kup as being an IgG3-K type immunoglobulin (Frangione et al., 1969a). Chapter 3 will describe further chemical characteristics of this protein.

3.2 Cellulose Acetate Electrophoresis

Fig. 3-1 shows the electrophoretic pattern on cellulose acetate of a number of different sera. This electrophoretic method separates serum proteins into five distinct fractions termed albumin, alpha 1, alpha 2, beta and gamma-globulins going from the most negatively charged to the most positively charged species respectively. Cellulose acetate electrophoresis of serum samples can detect abnormalities related to the presence of extra components or the increase or absence of normally occuring components. The principle disturbance in the gamma globulin fraction is related to gammopathies in which one or more of the immunoglobulins are elevated, or in the case of genetic deficiencies, absent. Fig. 3-1 therefore compares several myeloma sera with a number of normal sera. The serum samples Goe, Kup and T.Sm. show typical multiple myeloma profiles with a marked increase of protein staining material of

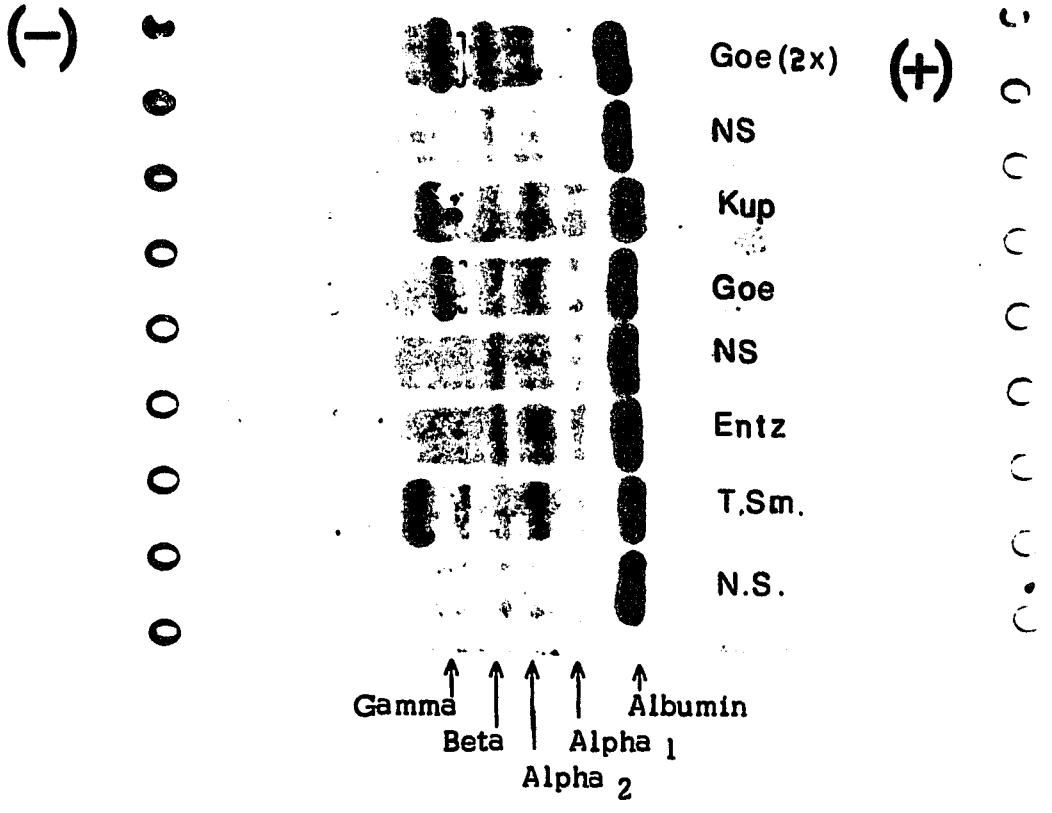


Fig. 3-1

Cellulose acetate electrophoresis profile of various serum samples. Each of the indicated serum samples was applied once except for Goe (2x) which was applied twice in the same spot. The slight mark in the gamma region indicates the sample application point. N.S. indicates normal sera, each of which was from a different individual. Sample Kup, Entz and T. Sm. represent separate individual serum samples. The anode and cathode are marked.

restricted mobility in the gamma-globulin fraction. The normal sera, indicated by N.S., show in contrast the typical diffuse gamma-globulin fraction characteristic of normal immunoglobulin heterogeneity. It is of interest to note that each of the myeloma proteins possess their own characteristic mobility. Protein Kup, is an IgG3 monoclonal cryoglobulin that will be discussed further in this chapter. The other myeloma protein, Goe has some unusual properties that will be discussed in Chapter 4. Protein Entz will also be discussed later. T.Sm. serum possess an IgG1 myeloma protein and is shown for comparison purposes. Each normal serum (N.S.) was taken from separate individuals.

3.3 Polyacrylamide Gel Electrophoresis

Fig. 3.2 presents a 10% polyacrylamide gel electrophoresis (PAGE) profile run under reducing conditions of the same serum samples as in Fig. 3-1. In this system all serum proteins are split into their polypeptide subunits (i.e., they are no longer disulfide bonded) and separate according to their individual molecular weights. Only those proteins that are in relatively high concentration in the serum can be visualized in this gel. The individual normal sera in slots 1, 2, 6 and 8 in Fig. 3-2 show a profile in which the major polypeptide is albumin ($\sim$4.0 mg/ml in serum) which possess a molecular weight of

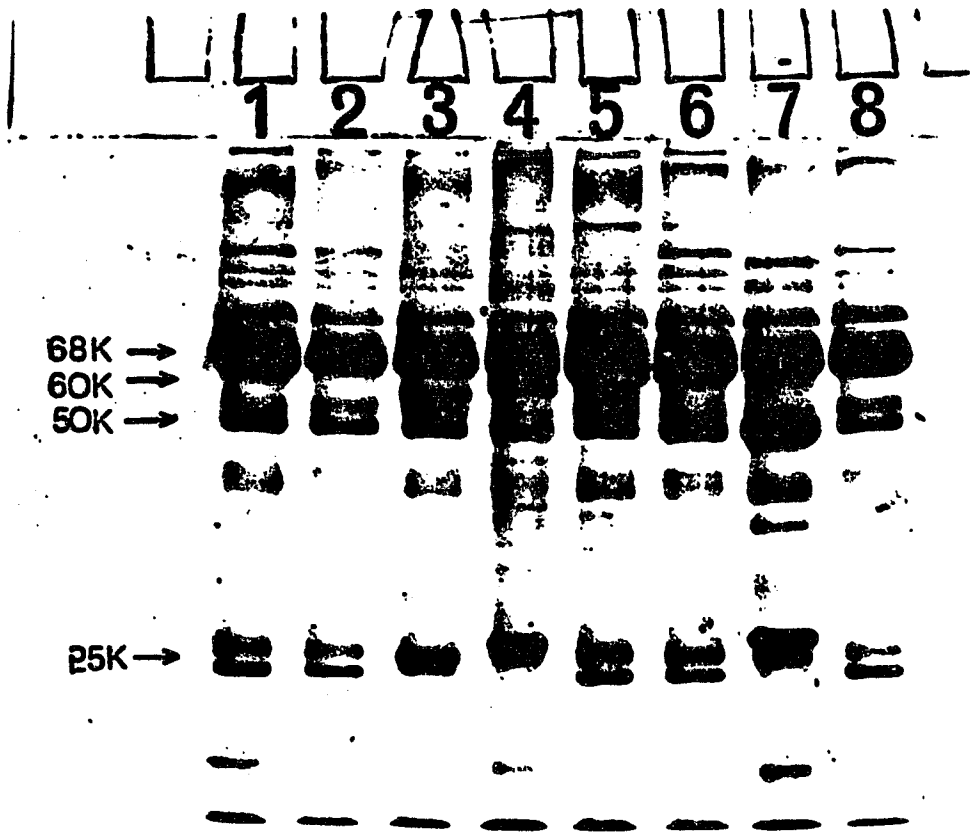


Fig. 3-2

10% PAGE in 0.1% SDS of various serum samples after reduction in 0.1 M dithiothreitol (DTT). 10µl of a serum sample was mixed with 100 µl of sample buffer with DTT and heated to 100°C in a boiling water bath for two minutes. 5 µl of each reduced mixture was loaded onto the gel. The gel slots contained the following samples: 1) Normal Serum 2) Normal Serum 3) Goe Serum; 4) Kup Serum; 5) Entz Serum; 6) Normal Serum; 7) T. Sm. Serum; 8) Normal Serum. Molecular weights are indicated by arrows on the left (eg. 68K = 68,000 daltons).

about 68,000 daltons. Immunoglobulins in this system are separated into their heavy and light chains which band at 50,000 daltons and 25,000 daltons respectively. The monoclonal proteins in the serum of myeloma patients are readily apparent in this gel system because of the relative increase in their concentration and therefore these proteins are detected as darker staining bands in the area where heavy and light chains migrate. In addition, this method can differentiate between heavy chains of the IgG3 subclass versus heavy chains of the IgG1, 2 and 4 subclasses. As previously published, γ3 heavy chains possess a higher molecular weight (58-60,000 daltons) as compared with the other IgG heavy chain subclasses (50,000 daltons). In normal serum, however, the IgG3 subclass is a minor component of the total immunoglobulin and therefore it cannot usually be visualized in this gel system unless the serum sample is overloaded on the gel.

Therefore, by examining the SDS-PAGE banding profiles of the reduced myeloma sera in Fig. 3-2, it can be concluded that the myeloma heavy chain present in Goe and Kup sera (slots 3 and 4 respectively) is γ3-like in molecular weight, while the myeloma heavy chain in T.Sm. serum (Slot 7) is γ1-like in molecular weight.

3.4 Isolation and purification of myeloma protein Kup

Kup, a monoclonal cryoglobulin was initially isolated by repeated cold precipitation (see Methods and Materials). Slot A of the SDS-PAGE in Fig. 3-3 shows the profile of the cold-precipitated Kup. The major heavy chain band has a molecular weight of 58-60,000 daltons which is characteristic of a $\gamma 3$ heavy chain. However there is also present another heavy chain band of about 50,000 daltons which is characteristic of $\gamma 1$, $\gamma 2$, and $\gamma 4$ heavy chains. Even after further repeated cold precipitation Kup still seemed to be contaminated with normal IgG. Further purification was attempted by passage of the partially purified preparation through a column of protein A isolated from Staphylococcus aureus that has been covalently bound to Sepharose-4B beads. The left portion of Fig. 3-3 shows the results of this experiment. The unbound material that was eluted with neutral buffers, peak B, and the material that was bound and subsequently eluted by increasing the hydrogen ion concentration, peak C, were run under reducing conditions on SDS-PAGE (slots B and C respectively) alongside the starting material in slot A (see the center of Fig. 3-3 for gel). Only the IgG3 subclass, which is represented by the higher molecular weight $\gamma 3$ band, was unbound (slot B) whereas almost all of the contaminant 50,000 dalton molecular weight heavy chains were bound to protein A-Sepharose (slot C).

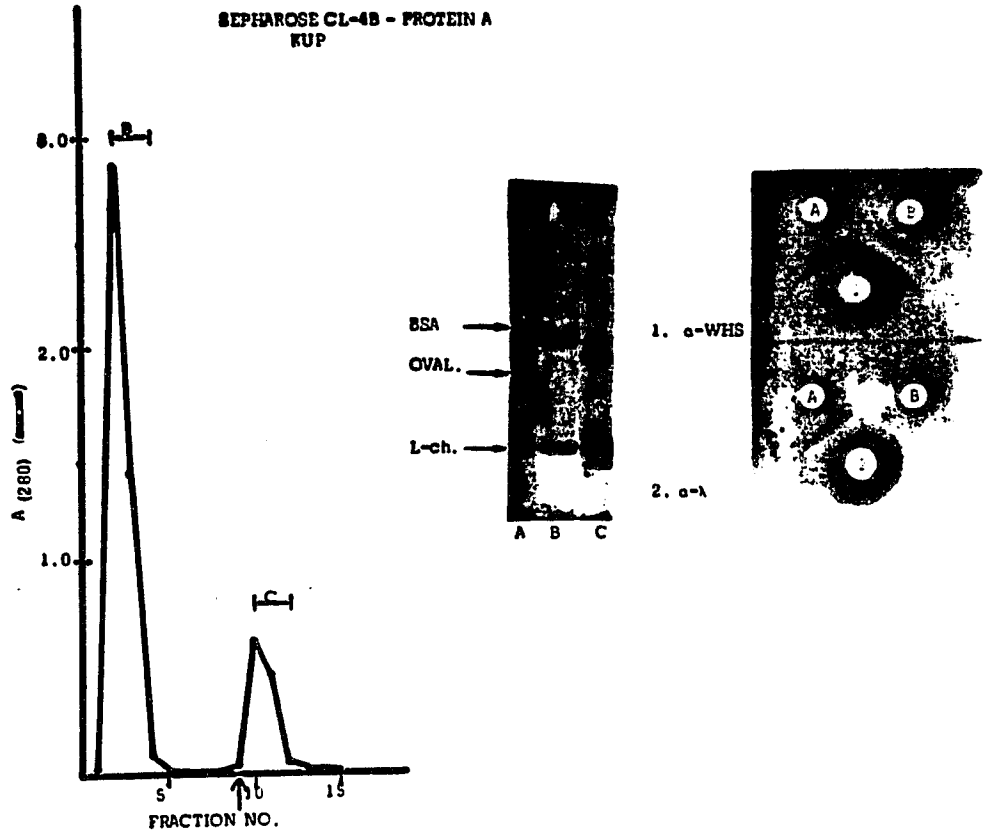


Fig 3-3

The graph on the left shows the profile of cold-precipitated Kup on a column of Staph. protein A covalently linked to Sepharose 4B. Pool B indicates the material that was not bound to protein A and Pool C is the material that was bound but eluted off with 1M acetic acid. The column is run initially in phosphate buffered saline, pH 7.0, with 0.02% NaAzide. The arrow indicates the change to 1M acetic acid. Column dimensions: 1.5 cm x 4 cm. 2 ml fractions were collected at 60 ml/hr.
Pictured in the middle are the protein A fractions analyzed by 10% polyacrylamide gel electrophoresis in SDS. The following samples were run after reduction with DTT: A) Kup isolated by repeated cold precipitation; B) Pool B from protein A column; C) Pool C from protein A column. The following proteins were used as molecular weight markers: Bovine serum albumin (BSA) - 68,000; Ovalbumin (Oval.) - 45,000; light chain (L-ch) - 25,000.
The picture to the right is a double diffusion analysis in agar of A) Kup isolated by repeated cold precipitation, and B) Pool B from protein A column set against the following antisera: 1) anti-whole human sera; 2) anti-lambda light chain.

The right side of Fig. 3-3 shows the results of double diffusion precipitations (Ouchterlony) which tested the immunologic purity of the starting material (well A) and the protein A-purified material (well B) developed against anti-whole human sera (well #1). The starting material shows two strong bands which represent the IgG3 monoclonal immunoglobulin reactivity and the contaminant (i.e., normal) IgG reactivity. The purified material shows very little of the contaminant band. Against anti-lambda light chain antiserum(well #2), the starting material (A) shows reactivity whereas the purified material (B) does not. The myeloma protein from Kup has been shown to possess a κ-type light chain. Therefore the myeloma protein from Kup serum which has been partially purified by repeated cold precipitation, can be isolated free of normal immunoglobulin contaminants by passage through a protein A-Sepharose column. Fig. 3-4 shows that the immuno-electrophoretic pattern of Kup serum (well #1) and protein A-isolated Kup (well #2) developed against anti-whole human serum. The restricted precipitin line against well #2 is characteristic of purified myeloma proteins.

3.5 Isolation and fragmentation of Kup heavy chains

Intact Kup protein was partially reduced and alkylated in order to cleave the interchain disulfide bonds. Heavy and light chains were then separated by passage of the

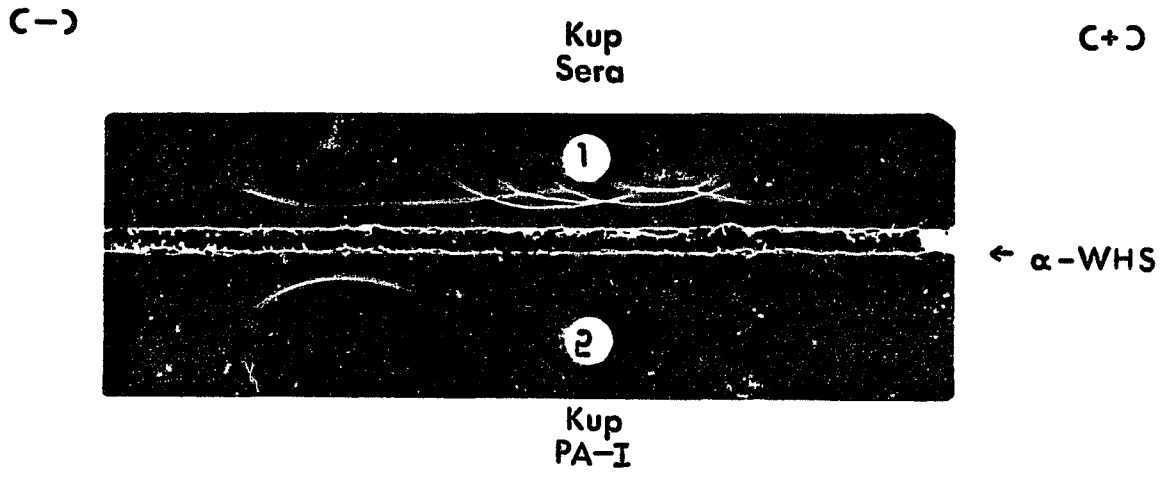


Fig. 3-4

Immunoelectrophoresis of: 1) Kup whole serum and 2) Kup protein after purification through protein A - Sepharose column (Kup, PA-I). After electrophoresis the trough was filled with anti-whole human sera. The anode and cathode are marked.

reduced and alkylated protein through a sephadex column in acid (Fig. 3-5). ^{14}C-labeled iodoacetic acid was used as the alkylating agent in order to follow the interchain cysteine-containing fragments. The amino terminal analysis of the intact protein gave only the single amino acid, valine. Isolated light chains also gave valine after a single manual Edman degradative step, but isolated heavy chains did not show an amino terminal and these chains were therefore considered blocked by the presence of pyrrolidonecarboxylic acid (PCA), a cyclized form of glutamic acid. Because of the presence of a blocked amino terminal and from the amino acid composition of the V_H fragment (Dammacco et al., 1972), Kup heavy chain was assigned to the V_H II subgroup.

Isolated Kup heavy chains were initially fragmented using cyanogen bromide (CNBr) which splits at the carboxyl side of methionine residues. The procedure involves the use of 70% trifloroacetic acid (TFA) as the reaction solvent instead of the more commonly used 70% formic acid (FA) because it has been shown that cleavage at Met-Thr bonds proceed more efficiently in TFA than in FA (Schroeder et al., 1969). Fig. 3-6 shows the column chromatography profile after digesting Kup heavy chains with CNBr, plotting both the radioactivity and adsorption at 280 nm.

Pool I of the CNBr digest most probably contains partially digested and/or aggregated material. Pool II contains a fragment with a very high specific radioactivity

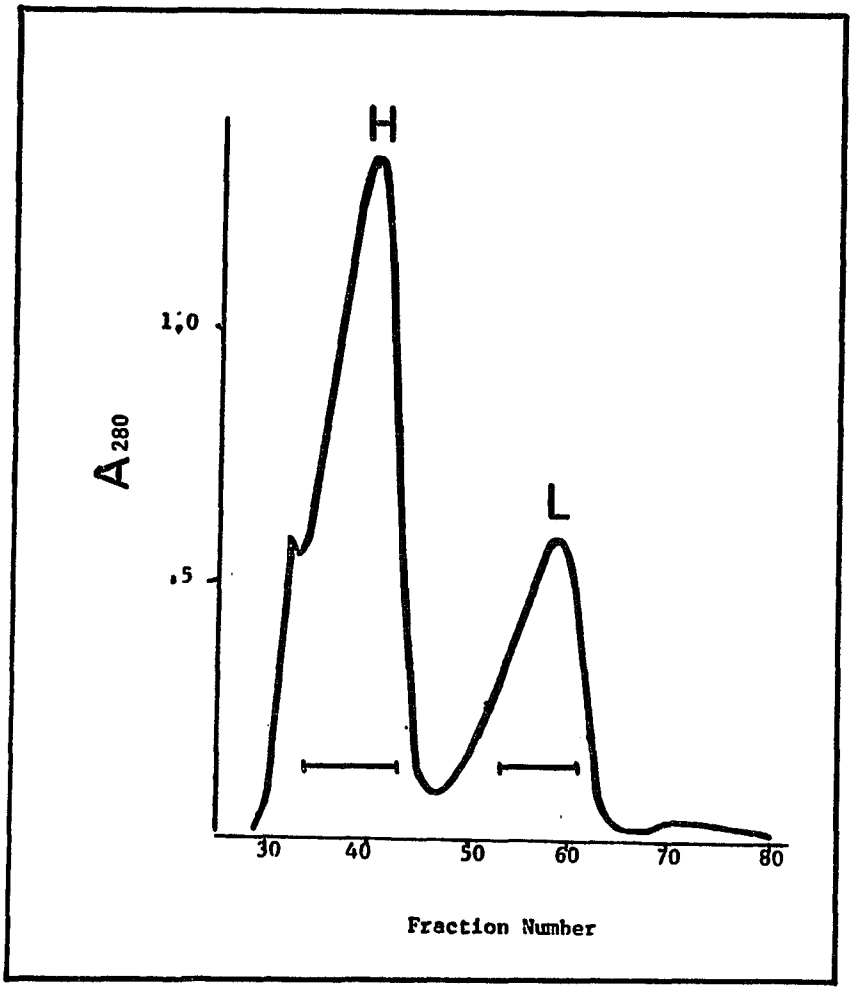
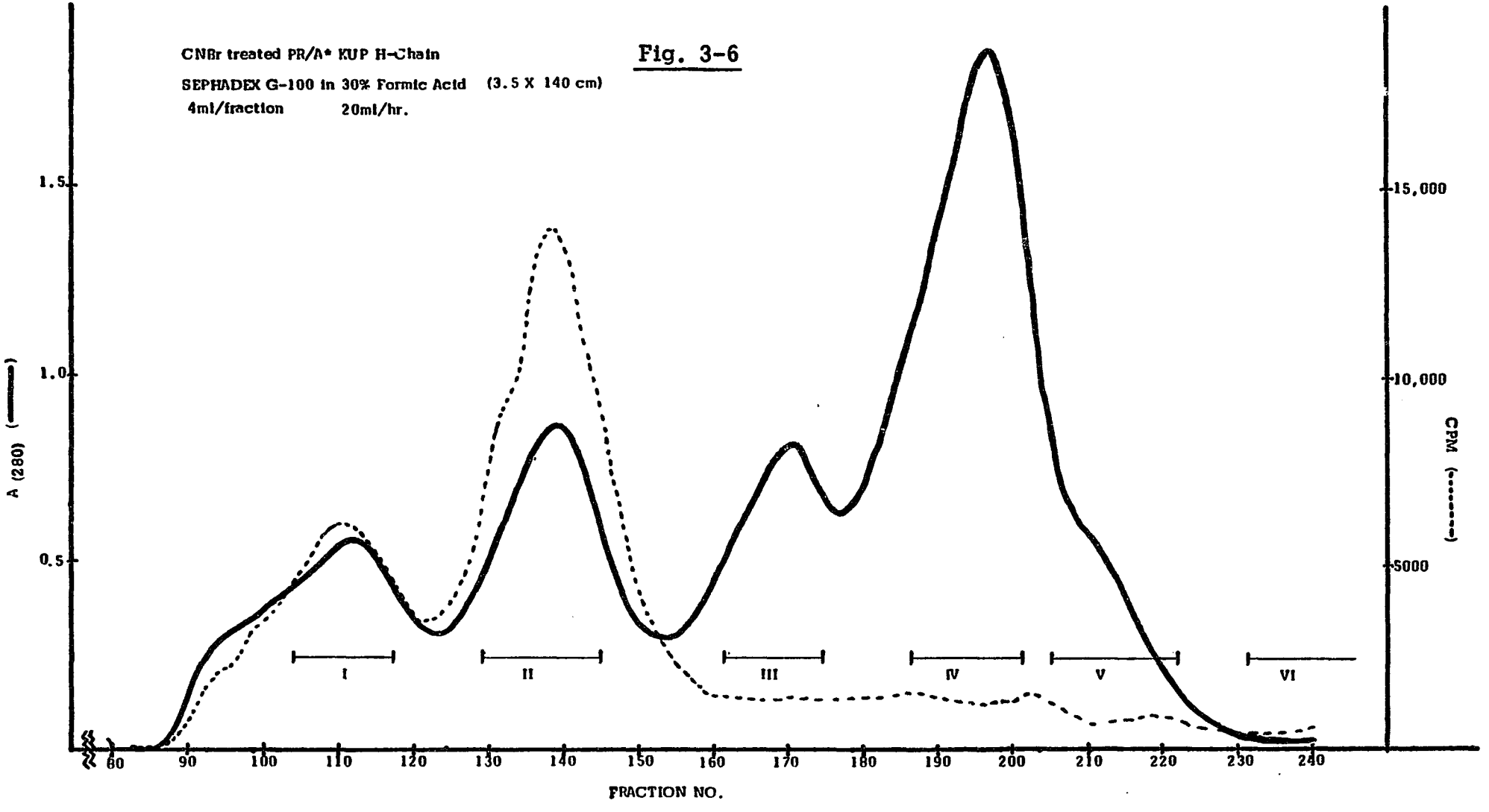


Fig. 3-5

Separation profile of heavy (H) and light (L) chains from partially reduced and radioalkylated Kup on Sephadex G-100 (2.5 cm x 85 cm) equilibrated in 1M acetic acid. 2 ml fractions were collected at 15 ml/hr. The sample contained 3 ml of 20 mg/ml partially reduced and radioalkylated Kup purified by passage through protein A-Sepharose. The heavy and light chains were separately pooled and lyophilized for further study.



Separation of cyanogen bromide digest of partially reduced and radioalkylated Kup heavy chain on Sephadex G-100 equilibrated in 30% formic acid. 25µl of every other fraction was counted for radioactivity and plotted along with the adsorbance at 280 nm. The indicated fractions were pooled and subsequently lyophilized. Pool VI showed no adsorption at 280 nm and included fractions 231-300. Pool III was studied further and is referred to as CNBr-III.

as well as a high molecular weight and therefore most likely contains the CH1 homology region together with the cysteine-containing hinge region. In IgG3 molecules the hinge region contains eleven interchain dissulfide bridges which would account for the high radioactivity in this pool. The amino terminal of the fragment in pool III, after a single manual Edman degradative step, was primarily isoleucine. Therefore this pool was thought to contain the CNBr fragment consisting of most of the CH2 domain from residues 253-358 (Eu numbering, Edelman et al., 1969). Pool IV and V probably contain a mixture of variable region fragments together with CH3 region fragments. Pool VI, upon further study was found to contain the carboxy-terminal octadecapeptide, which will be discussed later. Because of the specific interest in the Fc region, the material in pool III (termed CNBr-III) was further purified and characterized.

Fig. 3-7 shows the SDS-PAGE pattern of the CNBr-III fragment run both unreduced (UR) and reduced (R). Although the whole protein was previously partially reduced and alkylated, the intrachain disulfide bonds remained intact. Reduction in the presence of SDS would cleave all interchain disulfides and therefore the presence of an extra band below Ribo A in the reduced slot (R) indicates that partial peptide bond cleavage has probably occured within the disulfide loop. The lower molecular weight, lighter

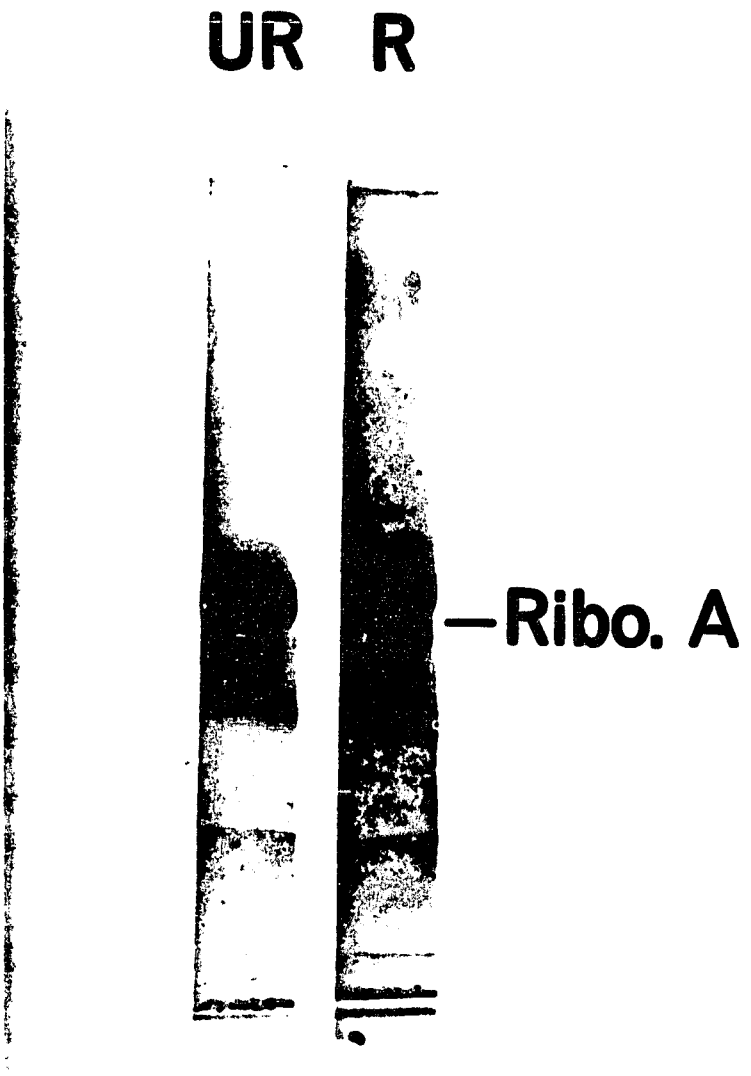


Fig. 3-7

10% PAGE in SDS of CNBr-III (Fig. 3-6, pool III) run without DTT (unreduced - UR) and in the presence of DTT (reduced - R). The marker, ribonuclease A (Ribo. A), has a molecular weight of 13,000 daltons. All three samples (UR, R, Ribo. A) were run on the same slab gel.

staining material seen in both samples is a contamination from pool IV (Fig. 3-6) and is eliminated by rechromatography.

To determine the source of the extra fragment, CNBr-III was completely reduced and radioalkylated (CR/A*) and then chromatographed on G75 (Fig. 3-8). Manual Edman degradation of the CR/A* CNBr-III fragment gave a major amino terminus of isoleucine along with a minor residue of proline. An aliquot of the CR/A* CNBr-III fragment was run on 15% PAGE in SDS under reducing condition and as before at least two major bands were readily apparent (Fig. 3-10, slot A). (The 3rd, lower band in slot A is removed by rechromatography). These two bands stained for carbohydrate indicating that they are both derived from the CH2 domain, which contains the carbohydrate moiety of the molecule at position 297 (Eu numbering). In an attempt to separate these two species, the CR/A* CNBr-III fragment was passed through an ion exchange column initially equilibrated in a Tris-urea buffer and developed with a linear NaCl gradient. This elution procedure produced two peaks, one which is eluted immediately in one column volume in the initial buffer wash and another which is eluted with about 0.1 M NaCl (Fig. 3-9). Fig. 3-10 shows the reduced SDS-PAGE profile of these two pools. Pool I from Fig. 3-9 shows a molecular weight equal to that of the smaller band and had an amino terminus of

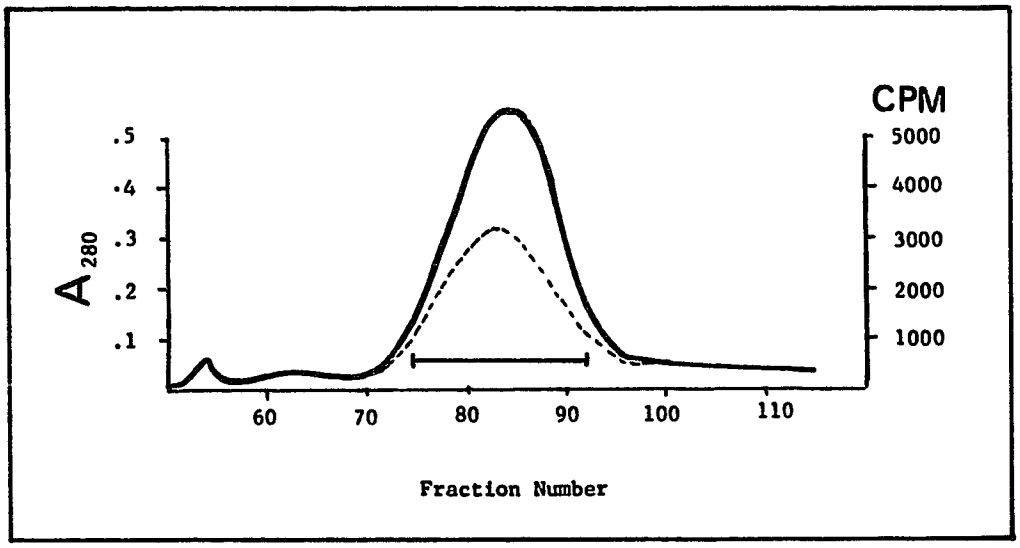


Fig. 3-8

Gel filtration on Sephadex G-75 (2 x 95 cm.) in 10% formic acid of CNBr-III (Fig. 3-6, pool III) after complete reduction and radioalkylation. 2 ml./fraction were collected at about 20 ml./hr. 10% of each fraction was counted for radioactivity. The solid bar indicates the pooled fractions. The solid line indicates adsorption at 280 nm. The dashed line indicates radioactivity in counts per minute (CPM).

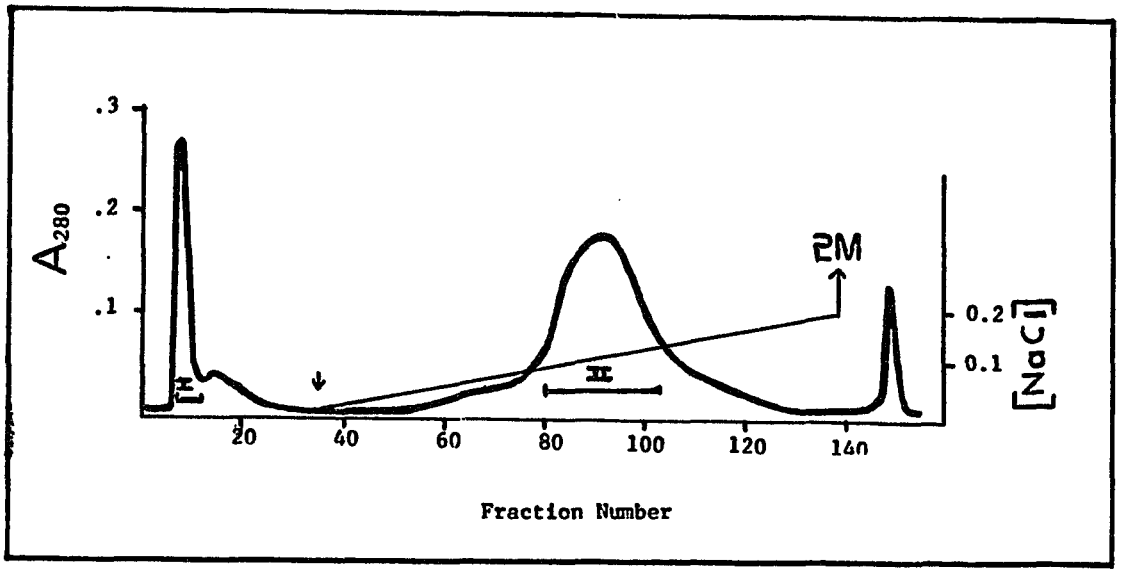


Fig. 3-9

Separation of the CR/A* CNBr-III fragment (Fig. 3-8) on Sephadex DEAE A-25 (1.4 x 30 cm.) equilibrated in 0.02 M Tris-HCl, pH 8.5, with 3 M urea (starting buffer). A linear gradient of 100 ml. of starting buffer to 100 ml. of starting buffer with 0.2 M NaCl was initiated at the arrow. 2.1 ml. fractions were collected at 28 ml./hr. The gradient ended at fraction 136 at wich time 2.0 M NaCl in starting buffer was passed through. The fractions indicated by the solid bars were pooled, desalted on Sephadex G-25 fine in 5% formic acid, and lyophilized.

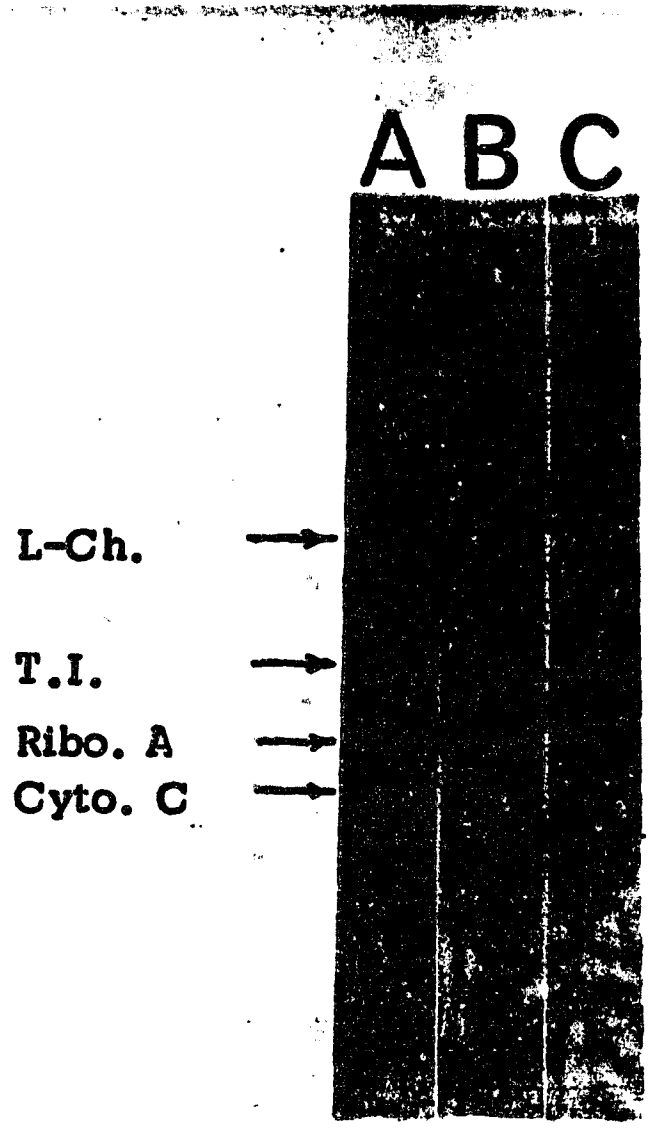


Fig. 3-10

15% PAGE in SDS of the two components separated by ion exchange of the CR/A* CNBr-III fragment (Fig. 3-9). Slot A: CR/A* CNBr-III. Slot B: pool I, Fig. 3-9. Slot C: pool II, Fig. 3-9.

proline, while pool II, the high molecular weight species, gave only isoleucine as an amino terminus.

An examination of the CH2 region sequence in protein Eu, indicated the presence of an aspartic-proline peptide bond eighteen residues from the beginning of the cyanogen bromide fragment at Ile-253. It has been previously reported (Piszkiewicz et al., 1970) that Asp-Pro peptide bonds are selectively susceptible to acid hydrolysis when compared with other peptide bonds. Since the cyanogen bromide reaction is itself performed in 70% trifloroacetic acid, and since gel filtration of the resulting fragments was performed in 30% formic acid, it was assumed that the Asp-Pro bond was partially split during the isolation and purification procedure.

To see if the CNBr-III fragment was indeed partially acid hydrolyzed at the Asp-Pro bond, the CR/A* CNBr-III fragment, which contains the two molecular weight species, was incubated at 10 mg/ml in 50% formic acid at 40°C for 96 hours. Fig. 3-11 shows a picture of the SDS-PAGE of the CR/A* CNBr-III fragment (A) and the same fragment after limited acid hydrolysis (B). The acid treatment converted almost all of the higher molecular weight material into the lower molecular weight material. After gel filtration on Sephadex G-50 (Fig. 3-12) to separate out the small digested fragments, the amino terminus of this acid-derived product was determined to be proline. Amino acid sequence

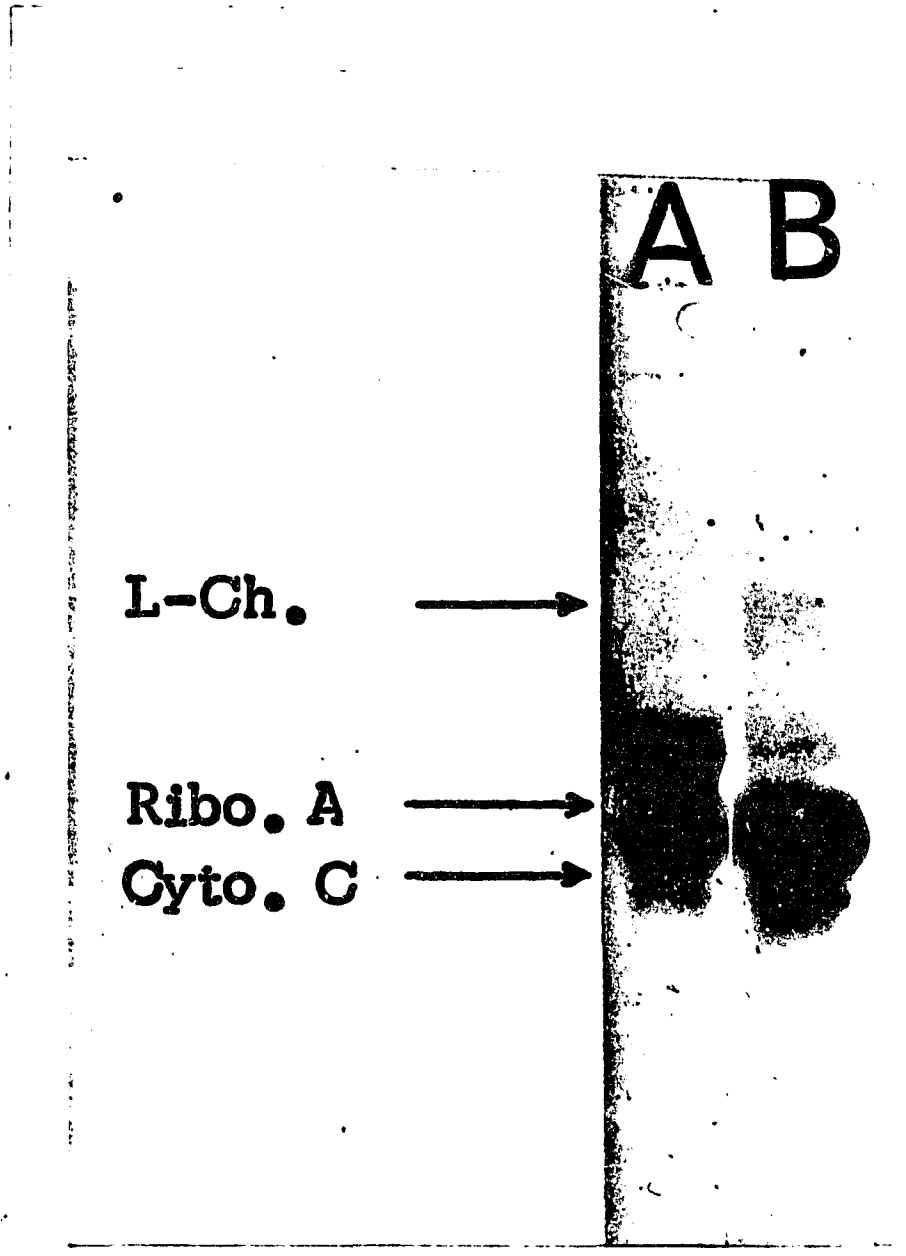


Fig. 3-11

SDS-PAGE (15%) of CR/A* CNBr-III fragment (A) before and (B) after limited acid hydrolysis. Both samples were run under reducing conditions. Proteins run as molecular weight markers were: Light chains (L-Ch.) - 25,000; Ribonuclease A (Ribo. A) - 13,700; Cytochrome C (Cyto. C) - 11,700.

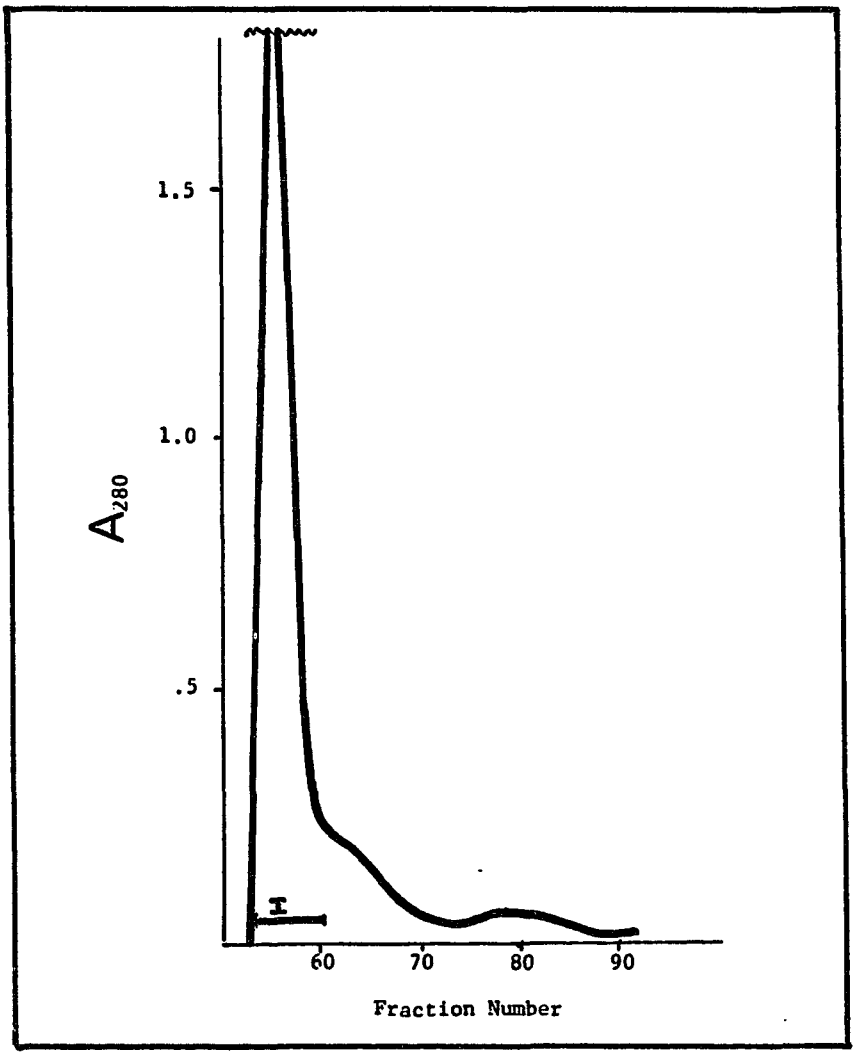


Fig. 3-12

Gel filtration on Sephadex G-50 in 0.01 M NH_4HCO_3, pH 8.5, of the CR/A* CNBr-III fragment after limited acid hydrolysis. The column dimension was 1.8 x 96 cm. . 2ml. fractions were collected at a folw rate of 25 ml/hr. The solid bar indicates the pooled fractions.

studies on the CNBr-III fragments before and after limited acid hydrolysis are summarized in Tables 3-1 and 3-2 respectively.

Table 3-1 summarizes the sequence data obtained from the CR/A* CNBr-III fragment before it was realized that Asp-Pro bond cleavage had taken place during its purification. Two sequences were readily apparent when the major sequence and the contaminating or minor sequence were listed separately as shown in Table 3-1. The major sequence started at Ile-253, while the minor one started at Pro-270. The sequence of the proline fragment derived from acid treatment of CNBr-III is shown in Table 3-2. Fig. 3-13 presents these data in linear sequence form along with other sequences which will be described later.

3.6 Two-dimensional fingerprint of CH2 region

Two dimensional maps of the CR/A* CNBr-III fragment after trypsin digestion were run to see if fragments could be isolated that were not sequenced previously. Fig. 3-14a shows a photograph of the map after staining with ninhydrin. The tracing in Fig. 3-14b indicates the peptides that were recovered in large enough yield to allow their placement by amino acid composition. Table 3-3 lists the composition of these fragments while in Fig. 3-15 they are placed by composition and homology in relation to an IgG3 sequence, Zuc (Wolfenstein-Todel *et al.*, 1976b).

(see following page for Table)

Table 3-1

Footnotes

a) PTH-amino acids are indicated by the one letter code except that C denotes S-carboxymethylcysteine.
b) Gas-liquid chromatography
c) Thin-layer chromatography
d) Back hydrolysis
e) The radioactivity (counts per minute) in an aliquot (5%) from each cycle was determined to confirm the presence of PTH-S-carboxymethylcysteine.
f) A horizontal line indicates that no new PTH-amino acid was seen by this identification method at this cycle.
g) These two residues coeluted using the indicated method and were distinguished by at least one other method.
h) α-amino-butyric acid is derived from threonine following back hydrolysis.
i) PTH-serine is detected as alanine after back hydrolysis
j) Residues in parentheses were very low yield.
k) High pressure liquid chromatography
l) Where more than one residue is noted, the first one is the highest yield.

Table 3-1

Automated Edman Degradation of Kup CNBr-III[a]

	Major Sequence				Minor Sequence		
Cycle No.	GLC[b]	TLC[c]	BH[d]	CPM[e]	GLC	TLC	BH
1	L/I[g]	-[f]	I	133	-	-	P
2	S/C[g]	S/C	A[i]	150	-	E	E
3	-	-	R	131	-	V	V
4	P /T[g]	M/T[g]	Aba[h]	178	-	-	E
5	P/T	P	P	237	-	-	F
6	-	E	E	569	-	-	K
7	V	V	V	297	-	W	-
8	P/T	M/T	Aba	417	-	Y	-
9	A	S/C	G,A	5965	V	-	-
10	V	V	V	1629	-	M/T	D
11	V	V	V	558	-	G	G
12	V	V	V	388	-	-	G
13	-	D	D	199	-	-	-
14	V	V	V	294	-	-	H
15	-	S/C	A	304	-	-	H
16	-	-	H	340	-	-	D
17	-	E	E	320	-	-	-
18	-	D	D	93	-	P	K
19	P/T	P	P	373	-	E	-
20	-	E	E	447	-	-	-
21	V	V	V	559			
22	-	Q	E[j]	317			
23	-	(M/T)	F	399			
24	-	K	K	472			
25	-	(K)	(K)	426			
26	-	Y	Y	54			
27	V	V	V	644			
28	(V)	D	D	410			
29	G	G	G	403			
30	Y	V	V	394			

Continued:

Cycle No.	Major Sequence GLC	TLC	BH	CPM
31	V	E	D	410
32	-	V	G	403
33	-	-	(V)	394
34	-	N	E	330
35	-	A	V	366
36		K	H	342
37		M/T	-	391
38		K	K	382
39		P(V)	P	350
40		K	R	313
41		-	E	640
42		E	E	318
43		Q	E	299
44		-	-	168
45		-	-	321
46		-	-	334
47		M/T	-	407
48		-	(F)	350
49		-	(K)	123
50		-	-	304
51		(V)	(V)	263
52		S/C	-	166
53		-	-	390
54		-	(L)	477

Table 3-2 *

Automated Edman Degradation of Kup CNBr-III Fragment After Acid Hydrolysis [a]

Cycle No.	GLC [b]	TLC [c]	BH [d]
1	P/T [g]	P	P
2	V	E	E
3	V	E	V
4	V	Q	E
5	- [f]	F	F
6	-	K	K
7	-	W	G
8	-	Y	Y
9	V [j]	(S/C)	V
10	(V)	D	D
11	G	G	G
12	V	-	V
13	-	E	E
14	V	(V)	V
15	-	(D)	H
16	A	N	D
17	A	A	A
18	-	-	K
19	P/T	M/T [g]	Aba [h]
20	-	K	K
21	P/T	(P)	P
22	-	E	R
23	-	E	E
24	-	E	E
25	-	E	E
26	(L/I)	F	F
27	-	-	D
28	-	-	S
29	-	M/T	T
30	-	F	F

*) see Table 3-1 for footnotes

Continued

31	-	-	E,R
32	V	V	V
33	V	-	V
34	-	-	-
35	-	(V)	V
36	L/I	-	L
37	-	M/T	(Aba)
38	-	-	(E)
39	(L/I)	M/T	L
40	-	-	(H)
41	-	E	-
42	-	(M^T)	-
43	-	-	D
44	-	-	-
45	-	-	-
46	-	-	-
47	-	-	(K)

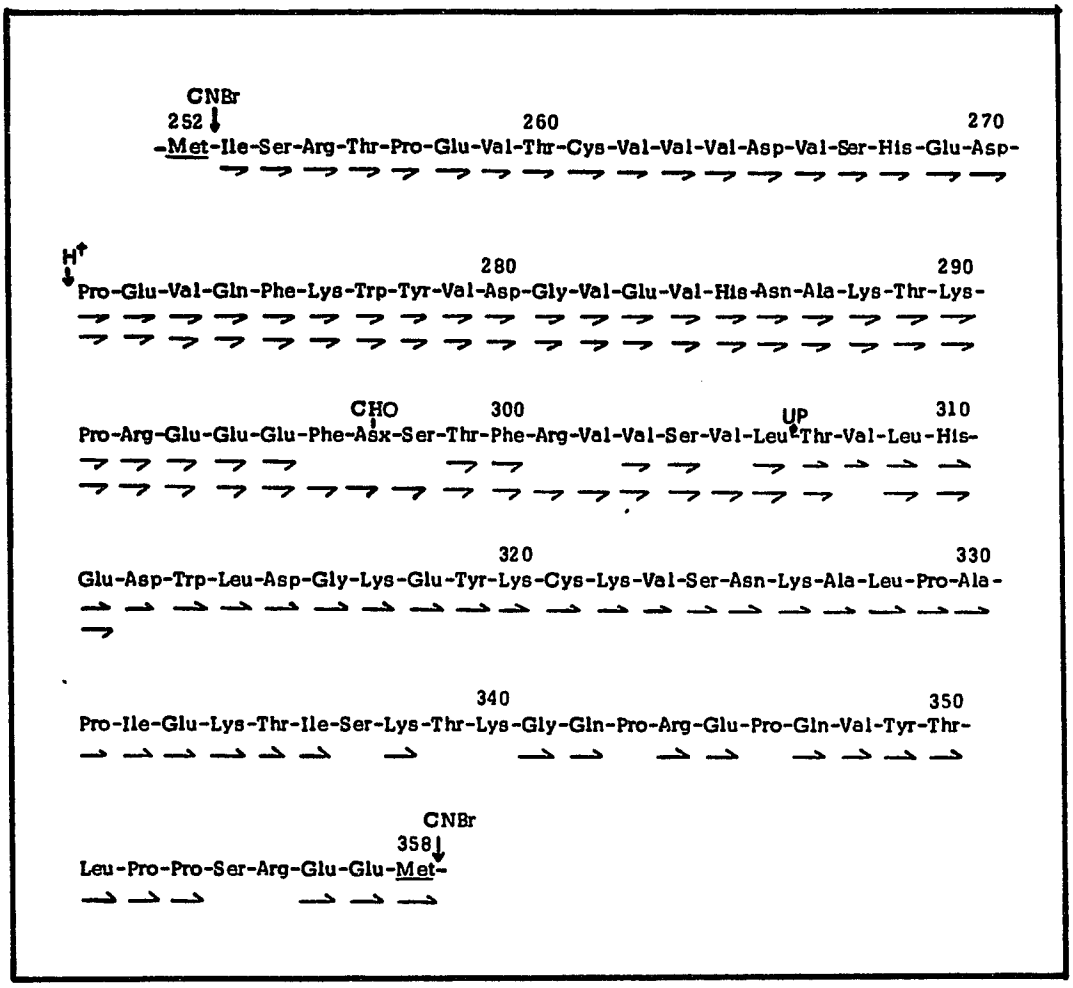


Fig. 3-13

The proposed amino acid sequence of the cyanogen bromide fragment of protein Kup extending from residues 253 to 358. The sequences were derived from automatic degradation of the following fragments: The CR/A* CNBr-III fragment (Table 3-1) which begins at Ile-253 and is indicated by the first row of arrows (→); The CR/A* CNBr-III fragment following limited acid hydrolysis (Table 3-2) which begins at Pro-271 and is indicated by the second row of half arrows (→); The urea-pepsin derived CM-3 fragment (Table 3-6) which begins at The-307 and is indicated by the first row of half arrows (→). Residues with no half arrows under them were not identified and are placed at this time purley by assumption with other published sequences of this region. Cleavage sites are indicated by verticle arrows (↓): cyanogen bromide - CNBr; limited acid hydrolysis - H^+; urea-pepsin digest - UP. CHO indicates the position of the carbohydrate moiety. Numbering is according to protein Eu (Edelman, et al., 1969).

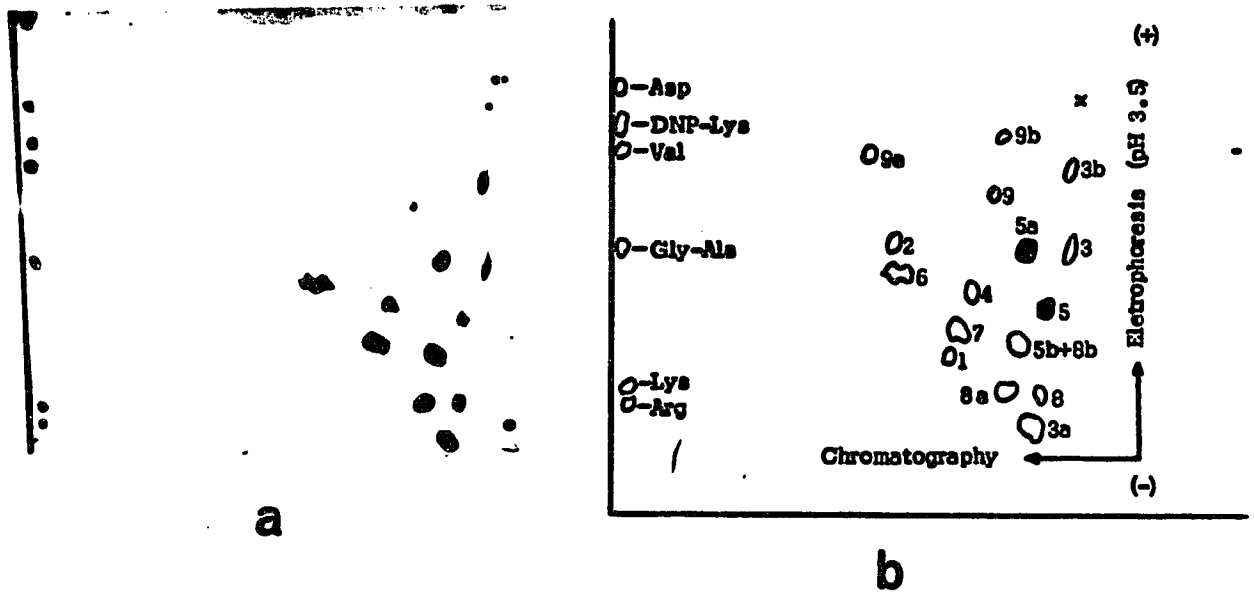


Fig. 3-14

Figure a shows the peptide map of a tryptic digest of the CR/A* CNBr-III fragment. Figure b is a tracing of the map indicating the peptides that were in high enough yield to place according to amino acid composition. Some peptides were too faint to be visualized after photography in figure a although they were indicated by circles in figure b as being in high enough yield to place. The hatched circles, ◍ , indicate the radioactive peptides that were detected by overnight exposure on X-Ray film. The left side of figure b shows the electrophoretic mobility of various markers at pH 3.5. The X indicates the point of origin. The amino acid composition of the indicated peptides are listed in Table 3-3 and are placed by amino acid composition and homology in relation to an IgG3 sequence shown in Fig. 3-15.

(see following page for table)

Table 3-3

The amino acid composition of the tryptic fragments (T1 through T9b) generated from the CR/A* CNBr-III fragment and isolated from a two-dimensional map (Fig. 3-14). Numbers in parentheses ( ) indicate the expected number of that particular residue in the peptide. n.d.=not done. a) Phe was elevated because of the coelution of the carbohydrate moiety on the amino acid analyzer. b) Met was identified as homoserine which runs as a shoulder under Glu on the amino acid analyzer, hence its low value. The peptides are aligned according to amino acid composition and homology in Fig. 3-15.

Table 3-3

Amino Acid	T1	T2	T3	T3a	T3b	T4	T5	T5a	T5b & T8b	T6	T7	T8	T8a	T9	T9a	T9b
CMC							0.7 (1)	0.9 (1)								
Asp		1.9 (2)	0.9 (1)		1.0 (1)		1.2 (1)		1.2 (1)							
Thr			1.7 (2)	0.7 (1)	0.9 (1)						0.8 (1)	0.8 (1)	0.8 (1)	0.8 (1)		
Ser	1.0 (1)	0.1	0.9 (1)		0.9 (1)		1.2 (1)		0.8 (1)		1.0 (1)			0.9 (1)		
Glu		1.1 (1)	2.8 (3)		2.7 (3)	0.8 (1)	0.2		1.0 (1)	1.1 (1)		1.1 (1)		3.5 (3)	1.7 (2)	2.0 (2)
Pro			0.9 (1)	1.0 (1)					0.9 (1)	2.1 (2)		1.1 (1)		2.7 (3)	1.2 (1)	
Gly		1.0 (1)	0.2	0.1	0.1		0.3		0.7 (1)			1.1 (1)			0.2	0.3
Ala		0.9 (1)								1.9 (2)						
Val		2.5 (3)					1.0 (1)		1.2 (1)					0.8 (1)	0.9 (1)	
Met														0.4[b](1)		0.3[b](1)
Ile	1.0 (1)									0.9 (1)	1.1 (1)					
Leu		0.1			0.2		0.1			1.0 (1)				0.9 (1)		
Tyr		0.9 (1)				1.0 (1)	0.3							1.0 (1)	1.1 (1)	
Phe			4.2[a](2)		3.7[a](2)											
His		1.0 (1)	0.1													
Lys		0.7 (1)	1.3 (1)	1.0 (1)	0.2	1.2 (1)	1.8 (2)	1.2 (1)	1.2 (1)	1.1 (1)	1.1 (1)	0.9 (1)	1.2 (1)			0.8
Arg	1.1 (1)		2.0 (2)	1.1 (1)	1.0 (1)				1.0 (1)			1.1 (1)		1.0 (1)		
Trp	n.d.	n.d. (1)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

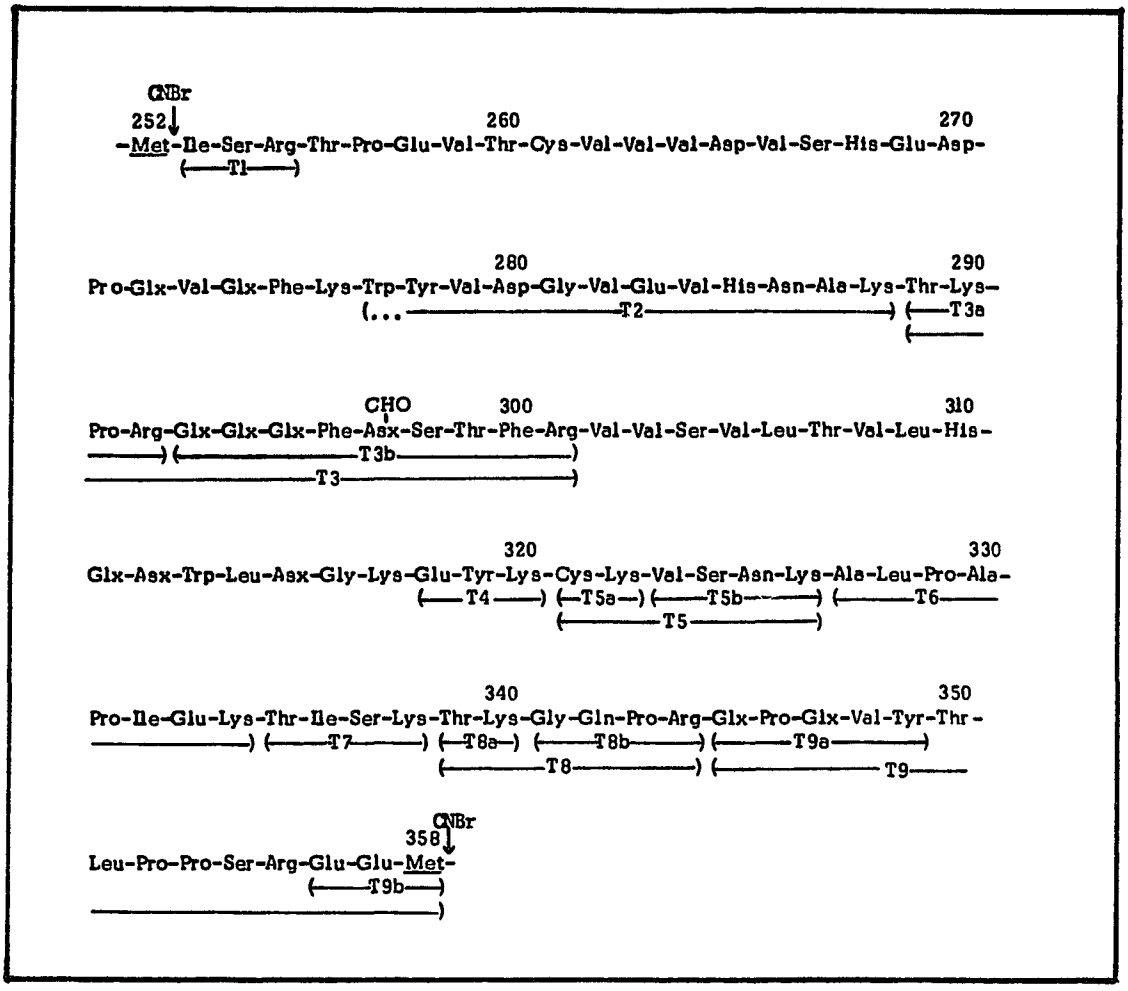


Fig. 3-15

The tryptic fragments of CR/A* CNBr-III isolated from a peptide map (Fig. 3-14) are placed according to amino acid composition with respect to a known IgG3 sequence, ZUC (Wolfenstein-Todel, et al., 1976). The arrows indicate the sites of cleavage by cyanogen bromide (CNBr). Numbering is according to protein Eu.

3.7 Carboxy-terminal octadecapeptide

Pool VI from the cyanogen bromide cleavage of Kup heavy chain (Fig. 3-16) was pooled, lyophilized and passaged through Sephadex G-50 equilibrated in 0.01 M NH_4HCO_3 from which two peaks were eluted (Fig. 3-16). The first peak, pool I, after amino acid analysis could not be placed with any known sequence and may therefore represent a variable region peptide. It was not studied further. The second peak, pool II, upon amino acid analysis was shown to be the carboxy-terminal octadecapeptide (ODP) of the heavy chain. The composition of the ODP is presented in Table 3-4. Also presented in this table is the composition of the homologous fragment generated from human Cohn's fraction-II (CF-II) by a similar isolation method. Both of these fragments are compared to a known γ1 (Eu) ODP fragment as well as a known γ3, G3m(b), (Zuc) ODP fragment. Differences in Tyr, Phe, His and Arg are apparent between the γ1 and γ3 subclasses. Kup shows no apparent differences with the known γ3 G3m(b) fragment, while CF-II, which is primarily IgG1, shows no apparent differences with the known γ1 fragment.

3.8 Urea-Pepsin digests

In an attempt to obtain large fragments for sequencing from other parts of the Fc region, a unique method of digesting intact immunoglobulins with pepsin in the presence

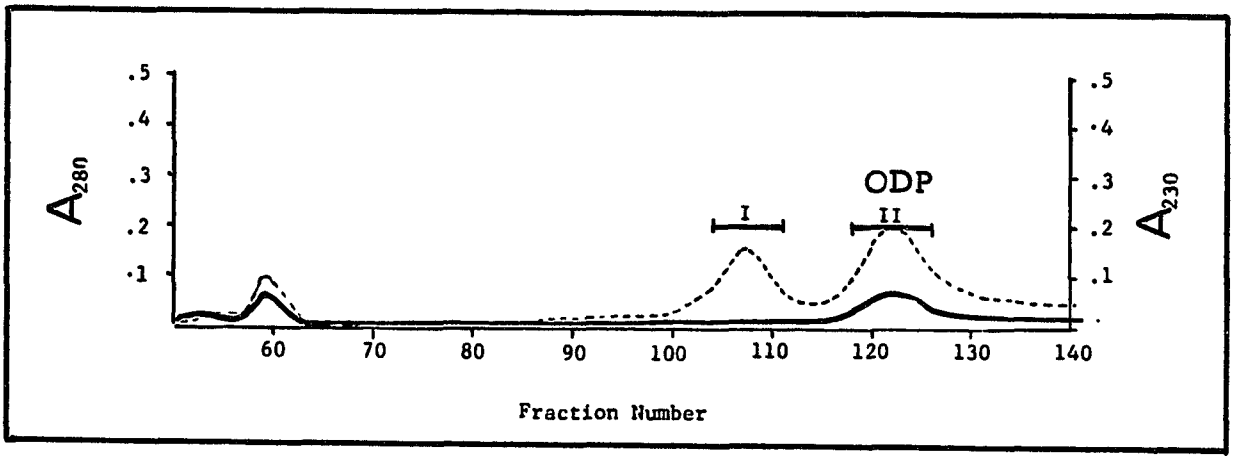


Fig. 3-16

Separation of pool VI (Fig. 3-6) on Sephadex G-50 in 0.01 M NH_4HCO_3, pH 8.5. Fractions of 3.4 ml. were collected at 70ml/hr. The solid bar indicates the fractions which were pooled and lyophilized. Pool II was found to be composed of the C-terminal octadecapeptide (ODP). Column dimensions were 1.8 x 96 cm. The solid line indicates adsorption at 280 nm while the dashed line indicates adsorption at 230 nm.

Table 3-4

Amino Acid Composition of Two C-Terminal Peptides of Protein Kup

Amino Acid	Urea-pepsin generated G50-II fragments (36aa)			CNBr generated ODP fragments (18aa)			
	Kup	γ_3	γ_1	Kup	γ_3	γ_1	CF-II
CMC	0.7	1	1	-	-	-	-
Asp	3.3	3	3	1.2	1	1	1.0
Thr	1.7	2	2	1.1	1	1	1.2
Ser	4.9	6	6	2.6	3	3	2.3
Glu	4.2	4	4	2.2	2	2	2.1
Pro	1.1	1	1	1.1	1	1	1.0
Glu	2.4	2	2	1.4	1	1	1.1
Ala	1.0	1	1	0.9	1	1	1.0
Val	2.1	2	3	0.3	-	-	-
Met	1.0	1	1	-	-	-	-
Ile	0.8	1	-	-	-	-	-
Leu	3.3	3	3	2.9	3	3	3.1
Tyr	0.3	-	1	0.2	-	1	1.0
Phe	1.8	2	1	1.2	1	-	-
His	2.1	2	3	1.6	2	3	3.2
Lys	2.1	2	2	1.1	1	1	1.0
Arg	1.9	2	1	0.9	1	-	-

Table 3-4

Comparison of the amino acid composition of two different carboxy terminal fragments from the heavy chain of protein Kup compared to homologous fragments from a γ1(Eu) a γ3 (Kup) and from Cohn's fraction-II (CF-II) which is primarily γ1. The octadecapeptide contains 18 amino acids (18aa) while the G50-II fragment contains 36 amino acids.

of a high concentration of urea was recently reported. Parr et al., (1976a, b, 1977) has shown that pepsin in the presence of 8 M urea cleaves intact immunoglobulin in unique positions unlike that of the classical aqueous pepsin digest. Fig. 3-17 compares the cleavage sites of the two methods along a heavy chain molecule. Whereas aqueous pepsin digests much of the Fc into small fragments, in the presence of urea, pepsin splits the Fc into a few large unique fragments that are ideal for sequence studies.

Intact Kup, purified on protein A, was digested with pepsin in 8 M urea and initially fractionated on Sephadex G-150 as is shown in Fig. 3-18. Pool I contains the Fb'_2 fragments (Fig. 3-17), whereas pool II contains the fragment termed upFc (urea-pepsin generated Fc fragment). Pool II, after desalting and lyophilizing, was completely reduced and alkylated and passed through Sephadex G-50 as illustrated in Fig. 3-19. Pool II of Fig. 3-19 (termed G50-II) upon amino acid analysis was shown to consist of the thirty-six carboxy-terminal amino acid residues starting at Thr-411. Table 3-4 compares the composition of this fragment with homologous fragments from a γ1 heavy chain (Eu) and a γ3, Gm(b), heavy chain (Zuc). The G50-II fragment from Kup shows an identical composition to the γ3 heavy chain fragments and both fragments possess at least six differences with respect to the γ1 fragment.

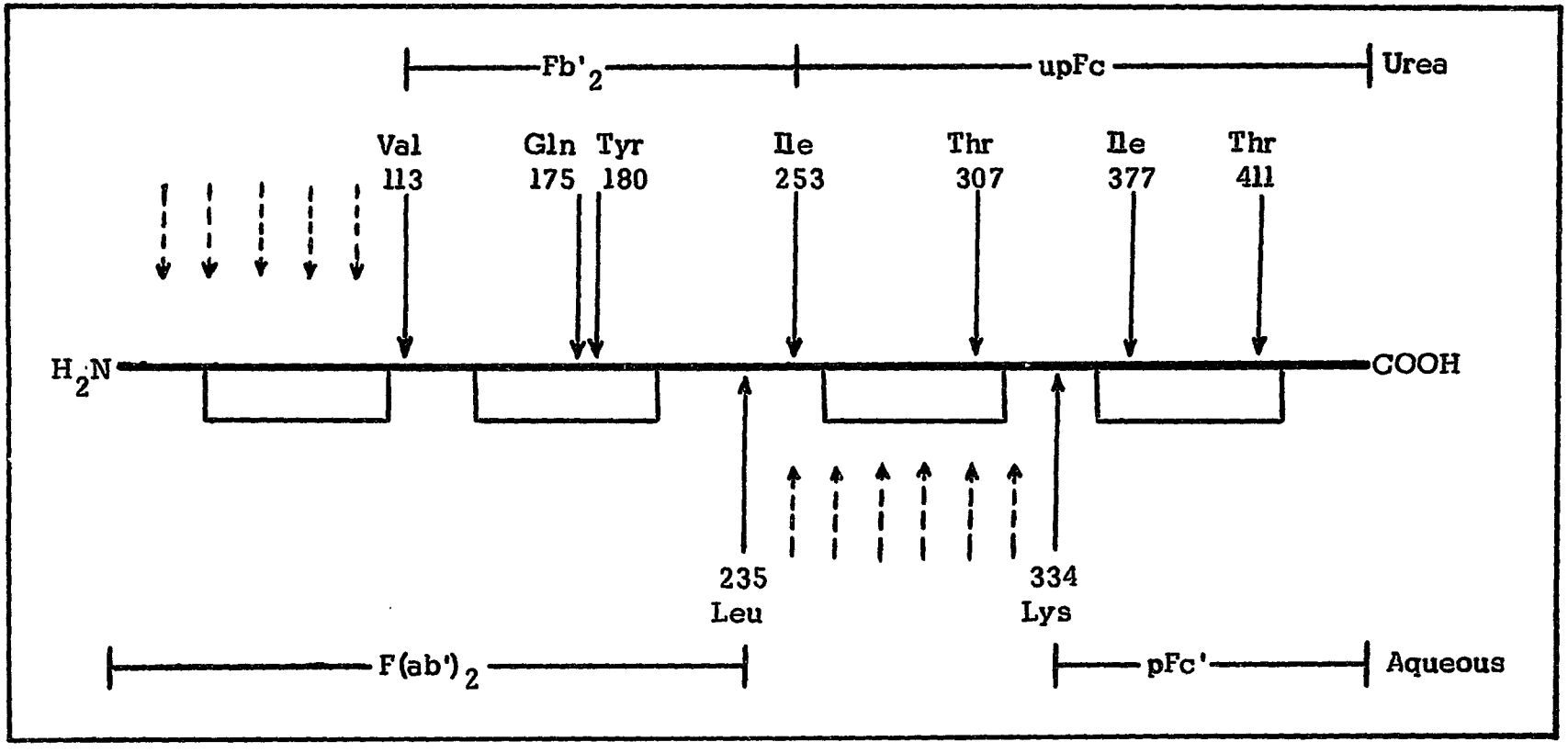


Fig. 3-17

This figure schematically presents the sites of cleavage by pepsin along a γ chain in the presence of urea or aqueous buffer. The peptic fragments generated in the presence of urea are shown on the top, while those in the presence of an aqueous buffer are shown on the bottom. The broken arrows indicate multiple cleavage sites that have not been characterized. The solid arrows indicate the sites of cleavage on the N-terminal sides of those amino acids shown. (Adapted from Parr, et al.,1976)

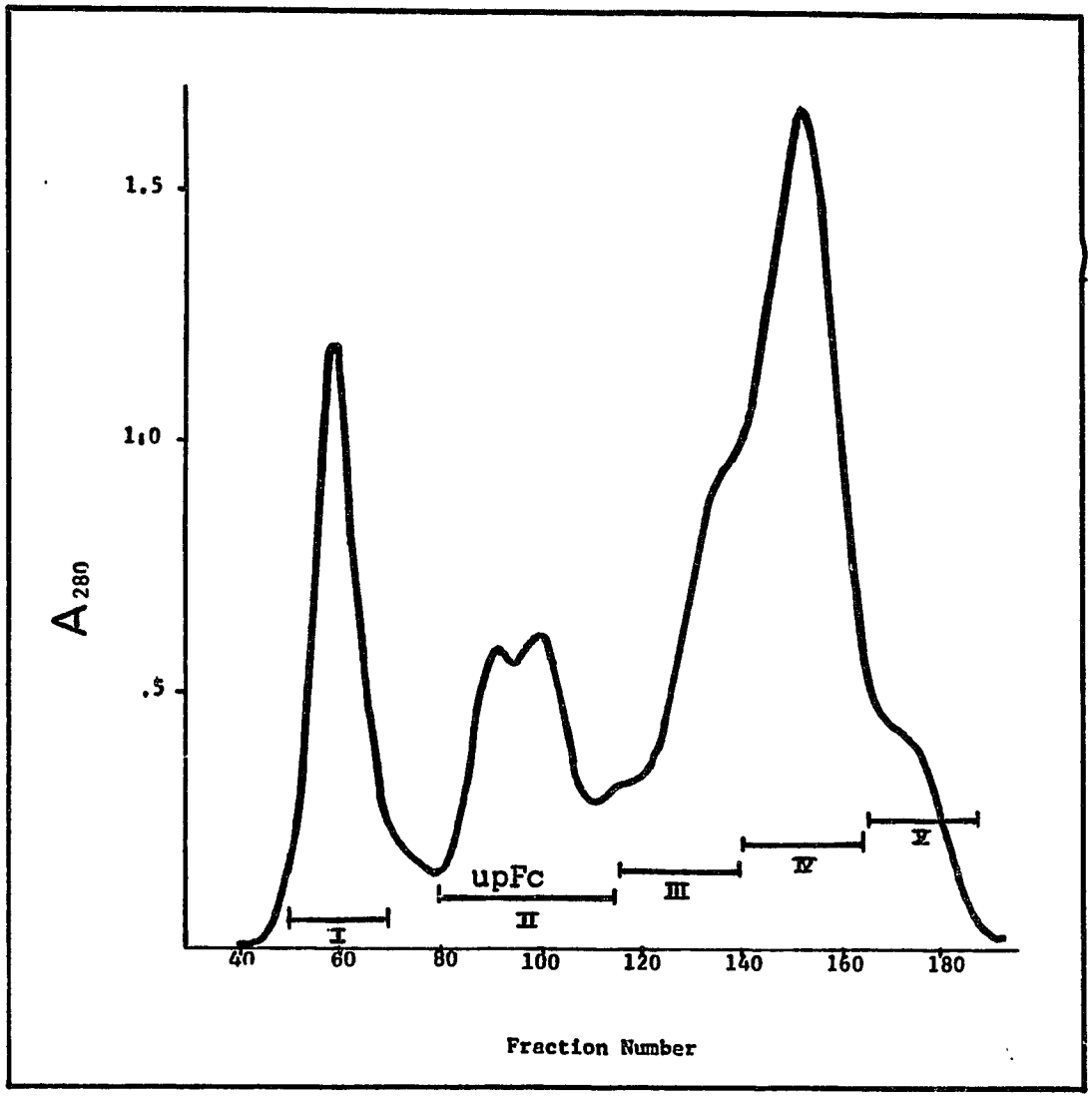


Fig. 3-18

Purified protein Kup (250 mg.) was digested by the urea-pepsin method and was loaded at 25 mg/ml onto a 3.5 x 90cm. Sephadex G-150 column equilibrated with 0.1 M sodium acetate buffer, pH 5.0, in 6 M urea. 3.5 ml. fractions were collected at a flow rate of 8 ml/hr. The fractions indicated by the solid bars were pooled, desalted on Sephadex G-25 fine in 5% formic acid, and finally lyophilized for further study. Pool I contains the Fb'_2 fragment while pool II consists of the urea-pepsin (up) Fc fragment.

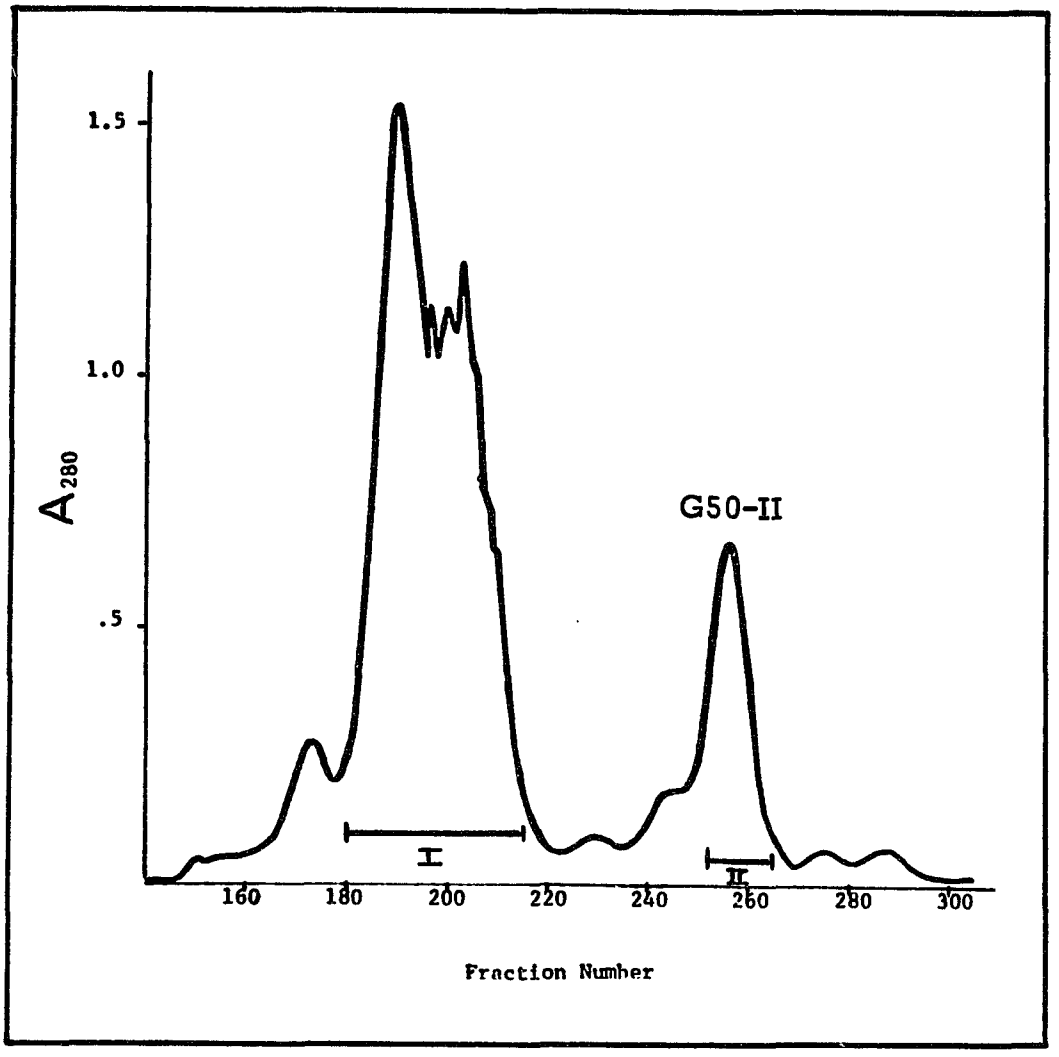


Fig. 3-19

Sephadex G-50 gel filtration of upFc fragment (Fig. 3-17, pool II) after complete reduction and radioalkylation. The column (2.6 x 170 cm.) was equilibrated with the same buffer as in Fig. 3-17. 2 ml. fractions were collected at 20 ml./hr. Pool II was desalted on Sephdex G-25 fine in 5% formic acid and lyophylized, and pool I was passed directly through carboxymethyl-Sephadex for further separation. Pool II was termed Kup G50-II.

Automated Edman degradation was performed on Kup G50-II and the results are presented in Table 3-5 and shown in linear sequence form in Fig. 3-20. The Kup G50-II fragment is identical in sequence to the homologous fragment of Zuc, a G3m(b) heavy chain disease molecule. When compared to Eu, a $\gamma 1$ heavy chain subclass molecule, the six differences in amino acid composition can be explained by three amino acid substitutions: Val → Ile, His → Arg, and Tyr → Phe. Arginine at position 435 has been correlated with γ_3 subclass heavy chains whereas all other subclasses possess His at this position. Phe at position 435 has been correlated with γ_3 molecules possessing G3m(b) markers only. G3m(g) γ_3 molecules as well as all other subclass molecules have Tyr at this position. The Val → Ile substitution at position 422 may also be related to γ_3 isotypes, for all other subclasses possess Val at this position. All the above amino acid interchanges can be accounted for by single base mutational events.

Pool I from the Sephadex G50 column (Fig. 3-19) was shown to contain more than one component. Pool I was therefore further separated by passage through carboxymethyl (CM)-Sephadex, a cation exchanger. Fig. 3-21 shows the elution pattern of this pool on CM-Sephadex developed with a linear salt gradient. CM-I, the first peak, was eluted by washing with starting buffer without salt. The amino terminus of CM-I was shown to be isoleucine by a

Table 3-5*

Automated Edman Degradation of Kup Urea-Pepsin G50-II Fragment[a]

Cycle No.	TLC[c]	HPLC[k]	BH[d]	CPM[e]
1	E,K,(P,M/T)[j]	T,P,V/M[l]	Aba[h],A	62
2	V	V/M	V(G)	80
3	V,D	D,(V/M,L)	D(A)	119
4	K,(P,D,G)	K,(V/M,D)	K,D	177
5	P,A	-[f]	S,(G,A)	80
6	(V)	A,(V/M)	R	132
7	W	W,(L,V/M)	G,A	130
8	E,Q	E,(Q,V/M)	E	145
9	E	E,(Q,V/M)	E	134
10	G	G,(V/M)	G	105
11	N,G	-	D	122
12	I,N	-	I(V)	240
13	F,Y	-	F	442
14	S/C	-	A[i]	746
15	S/C[g]	-	A	12,698
16	S/C(L/I)	-	S	2983
17	(V)	-	V	871
18	M/T	-	M	510
19	E	-	H	436
20	E,A	-	E	361
21	A,L/I	-	A,L	383
22	L/I,A	-	L	288
23	N	-	D,H	276
24	N	-	D	233
25	F,Y	-	R,F(L)	181
26	(Y)	-	F	145
27	M/T[g]	-	Aba,E	296
28	E,Q	-	E	16
29	K	-	K	191
30	-	-	L,A	187
31	L/I[g]	-	L	137
32	-	-	-	146
33	L/I	-	L	225
34	-	-	P(G)	185
35	(P)	-	P	148
36	-	-	G	136
37	-	-	-	142

*)see Table 3-1 for footnotes

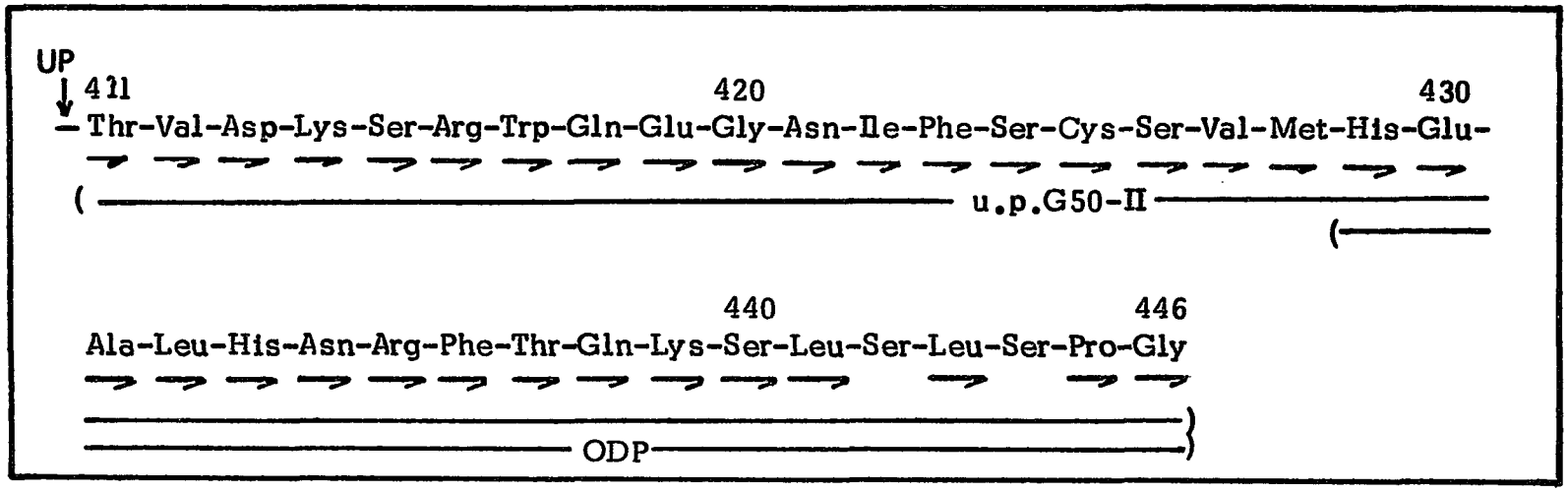


Fig. 3-20

The proposed amino acid sequence of Kup G50-II generated from urea-pepsin (UP) fragmentation protein Kup (Fig. 3-18, pool II). The half arrow (—>) indicates sequence determination by automatic Edman degradation, while the solid line (——) indicates the fragment which was subjected to amino acid analysis.

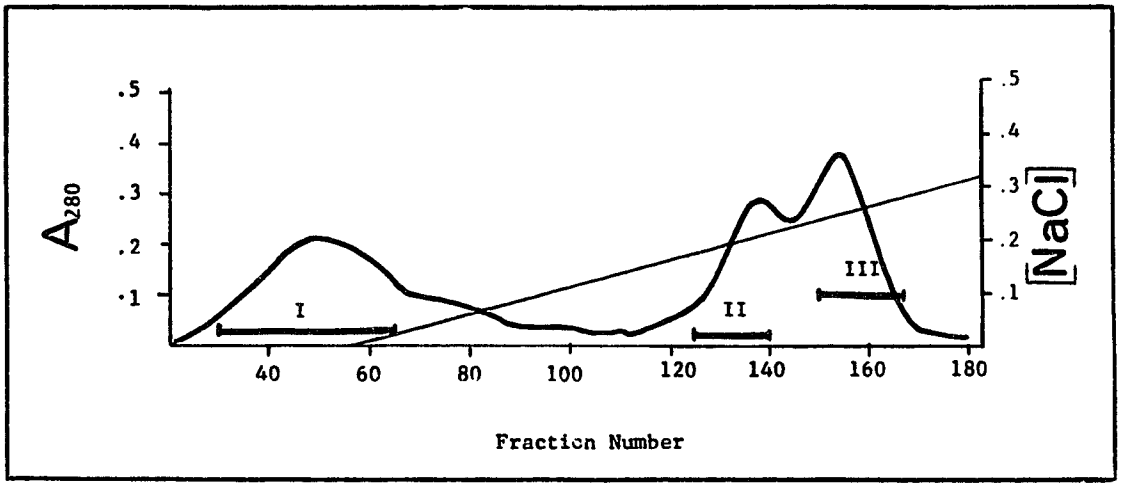


Fig. 3-21

Carboxymethyl (CM)-Sephadex (type C-50) separation of pool I from Fig. 3-18. The column (1.8 x 35 cm.) was equilibrated with 0.1 M sodium acetate buffer, pH 5.0, in 6 M urea (starting buffer). A linear gradient of 250 ml. starting buffer to 250 ml. starting buffer with 0.5 M NaCl was initiated at fraction 56. The solid bars indicate the pooled fractions. Pools I, II, and III are termed Kup CM-I, Kup CM-II, and Kup CM-III respectively. Each pool was desalted on Sephadex G-25 fine in 5% formic acid and lyophilized. Each fraction contained 2.5 ml. and was collected at a rate of 25 ml/hr.

single manual Edman degradation. CM-II was heterogeneous in its amino terminus but CM-III gave only threonine as the amino-terminal residue. Further manual Edman degradations showed CM-I to start at position 253, the identical position of the CNBr-III fragment already sequenced. CM-III was sequenced automatically, the results of which are listed in Table 3-6 and summarized in linear sequence form in Fig. 3-13. CM-III was shown to begin at position 307 with threonine. No unusual Y3 substitutions were observed.

Table 3-6*

Automated Edman Degradation of Kup Urea-Pepsin CM-III Fragment[a]

Cycle No.	TLC[c]	HPLC[k]	BH[d]	CPM[e]
1	P,M/T[g]	-[f]	Aba[h]	56
2	V,P[l]	-	V	41
3	L/I[g]	L[j]	L	90
4	-	(W,F/I)	H	37
5	E,(D)	E	E	43
6	D	D,(W)	D	47
7	D,(L/I)	(W,D)	D(G,A,L)	48
8	L/I	L	L	56
9	D,A	D	D	49
10	G	G	G	58
11	K	K	K	57
12	E,(Y)	E	E	61
13	Y	-	Y	79
14	K,(Y)	-	K	323
15	S/C,(Y,K)	-	A[i]	1799
16	K	-	K	452
17	V	-	V	184
18	(V)	-	A(S)	104
19	N	-	D	79
20	K	-	K	101
21	A	-	A	62
22	L/I	-	L	69
23	P	-	P	60
24	A	-	A	50
25	P	-	P	37
26	I	-	I	58
27	E	-	E	55
28	K	-	K	60
29	M/T	-	(G)	49
30	I	-	I	60

*) see Table 3-1 for footnotes

Continued

31	K(I)	-	-	47
32	K	-	K	68
33	K(Q)	-	(K,L)	60
34	-	-	-	77
35	G,E	-	G	36
36	Q	-	E	47
37	-	-	-	101
38	-	-	(R)	59
39	E	-	(E)	72
40	E	-	E(P)	50
41	Q	-	-	46
42	V	-	-	68
43	Y(M/T)	-	(Y)	54
44	M/T	-	-	47
45	L/I	-	-	49
46	P	-	-	66
47	P(D)(Y)	-	-	51
48	-	-	-	33
49	-	-	-	67
50	E	-	-	41
51	E	-	-	46
52	M/T	-	-	47
53	(M/T)	-	-	43
54	(K)	-	-	47
55	N(A)	-	-	62
56	A	-	-	45
57	(E,P,V)	-	-	48
58	-	-	-	35
59	-	-	-	41
60	-	-	-	60

-Chapter 4-

Studies on Myeloma Protein Goe

-Chapter 4-

Studies on Myeloma Protein Goe

4.1 Introduction

Protein Goe has been typed as an IgG3-κ myeloma which possesses a rare combination of genetic markers seen only in Mongoloid populations. Preliminary structural studies on the isolated protein have shown that Goe possesses a γ3-like hinge region and a γ1-like carboxy-terminus (Prelli et al., 1975). Goe heavy chains may therefore represent either a γ3-γ1 hybrid immunoglobulin molecule or the expression of a rare heavy chain gene indicating the existence of another IgG isotype. If the former is true, the determination of its sequence would be essential in order to ascertain the extent of its hybrid nature and to identify the location of the "switch" where γ3-like sequences hybridize to γ1-like sequences. If the latter is true, its amino acid sequence would be invaluable in distinguishing an unrecognized IgG isotype.

4.2 Characterization of Goe serum

Analysis by both cellulose acetate electrophoresis and polyacrylamide gel electrophoresis (PAGE) demonstrated the presence of a myeloma protein component in Goe serum. The cellulose acetate electrophoresis of Goe serum (Fig. 3-1) shows a sharp, dark staining band in the gamma globulin region characteristic of a myeloma protein and appears to be a little more negatively charged than the myeloma protein seen in

Kup serum (Fig. 3-1). When run under reducing conditions on PAGE (Fig. 3-2, slot 3), the myeloma heavy chain component in Goe serum can easily be identified when compared to the normal serum run along side of it (Fig. 3-2, slot 2). In this gel it can be seen that the myeloma heavy chain of Goe possess a molecular weight approximately equal to that of a γ3 heavy chain (58 - 60,000 daltons), and therefore probably contains the extended hinge region which γ3 molecules are reported to possess.

4.3 Initial Purification of Protein Goe

4.3.1 Starch block electrophoresis

Fig. 4-1a shows the PAGE profile run under reducing conditions of protein Goe isolated in one step by starch block electrophoresis. For comparison, Fig. 4-1b and Fig. 4-1c shows the pattern of purified IgG1 and IgG3 myeloma proteins respectively which were also run under reducing conditions. Three major bands are apparent for protein Goe in Fig. 4-1a: two bands in the 50,000 - 60,000 dalton molecular weight range, which represent heavy chains, and one band at about 25,000 daltons which represents the light chain. Since the monoclonal protein from Goe was shown to possess a γ3 molecular weight heavy chain of about 58,000 daltons (Fig. 3-2, slot 3), the 50,000 dalton band most probably represents the contribution of normal IgG heavy chains. Preliminary experiments on initial serum samples from patient Goe indicated a high level of myeloma protein.

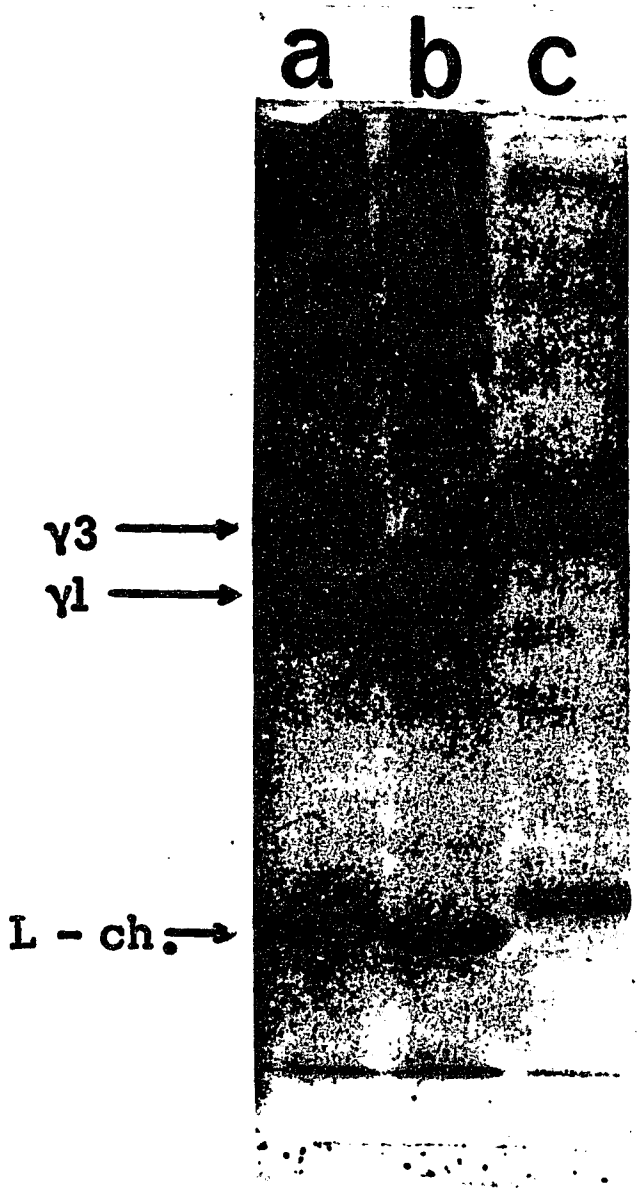


Fig. 4-1

SDS-PAGE (10%) of a) starch block electrophoresis isolated protein Goe, b) a purified IgG1 myeloma protein, c) a purified IgG3 (Kup) myeloma protein. All samples were pre-treated with reducing agent to separate the polypeptide chain subunits. The positions of γ1 and γ3 heavy chains as well as that of light chains (L-ch.) are marked.

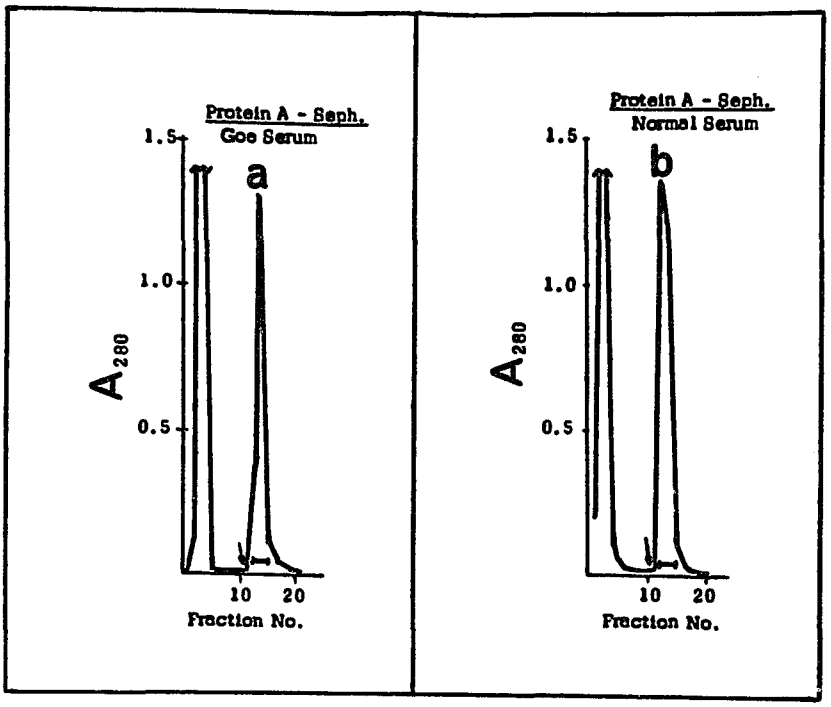
However, in subsequent serum samples, myeloma protein levels were significantly reduced due to successful treatment of the disease. Therefore, most of the serum from patient Goe contained an increased proportion of normal IgG and a relative decrease in the concentration of monoclonal protein (van Loghem, personal communication). It is no surprise, therefore, that the initial gross separation by electrophoretic mobility contained a significant amount of normal IgG from the four subclasses, the most prominent of which is IgG1. In an attempt to purify the IgG3 molecular weight myeloma protein, starch block-purified Goe was passed through a column of protein A-Sepharose. In striking contrast to protein Kup and other IgG3 myeloma proteins which have been shown not to have an affinity for protein A, the myeloma IgG3-like protein in Goe serum was shown to bind strongly to protein A together with normal IgG1, IgG2, and IgG4 subclasses. In a similar experiment, normal human serum was passed through protein A-Sepharose and the resulting bound proteins were examined by SDS - PAGE. Fig. 4-2 shows the protein A-Sepharose elution profile involving Goe serum and normal serum. The SDS - PAGE in Fig. 4-2 shows: slot a) Goe serum proteins which were bound to protein A; slot b) normal serum proteins which were bound to protein A. The serum sample from Kup showed the same PAGE pattern of binding proteins as did the normal serum in slot b. Here again, the IgG3 myeloma component of Goe was shown to bind

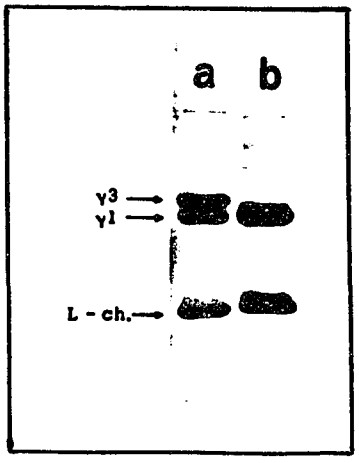
(see following page for figure)

Fig. 4-2

The two upper graphs show the separation of protein A binding and protein A non-binding components from Goe serum (left) and from normal serum (right). Approximately 2 ml. of serum was applied to a protein A-Sepharose column (1.5 x 4 cm.). After sample application the column was washed with 0.15 M phosphate buffered saline, pH 7.0, with 0.02% NaN_3, until the adsorbance at 280 nm. reached backround readings. To elute the bound proteins, the column was washed with 1 M acetic acid (initiated at the arrow shown in the two graphs), and the fractions indicated by the solid bar were pooled. 2 ml. fractions were collected at about 60 ml./hr.
The SDS-PAGE (10%) of pool a (the protein A bound serum proteins of Goe) and pool b (the protein A bound serum proteins of a normal sample) are shown on the bottom of the figure. Both samples were reduced with DTT prior to their application to the gel. The positions of $\gamma 1$ and $\gamma 3$ heavy chains as well as light chanis (L-ch.) are indicated.

Fig. 4-2


Protein A - Seph.
Goe Serum
a
1.5
1.0
0.5
A_{280}
10
20
Fraction No.
Protein A - Seph.
Normal Serum
b
1.5
1.0
0.5
A_{280}
10
20
Fraction No.


a
b
γ3
γ1
L - ch.

to protein A. In light of this observation, protein A could not be used to further purify protein Goe as it was used to purify protein Kup, and therefore other methods of purification were examined.

4.3.2 Hydroxylapatite

Absorption chromatography on hydroxylapatite, a form of calcium phosphate, proved to be an adequate and simple method of purifying the myeloma component in Goe sera. The appropriate fractions from the starch block electrophoresis of Goe serum were dialyzed against distilled water, lyophilized, and then dissolved in 0.01 M phosphate buffer (PB), pH6.5. The protein solution was then applied to a column of hydroxylapatite that was equilibrated with the same buffer and developed by stepwise elution with increasing PB molarites as follows: 0.01 M, 0.05M, 0.07 M, 0.1M, 0.2 M, 0.5 M, and 1.0 M. The results of this elution procedure are shown in Fig. 4-3A. Fig. 4-4 shows the PAGE pattern of each of the protein peaks eluted with the different PB molarity washes.

The material eluted with the initial 0.01 M PB wash contained very little protein as seen by the lack staining bands on PAGE and probably consisted of residual salt that passed through the column upon sample application. The material in the 0.05M PB wash consisted almost entirely of a single band with a molecular weight of about 77,000 daltons, whether reduced or unreduced, and therefore exists as a single polypeptide chain in its native form. The material eluted with 0.07 M PB contained almost entirely the γ3 molecular weight heavy chain band (58-600,000 daltons) as well as the

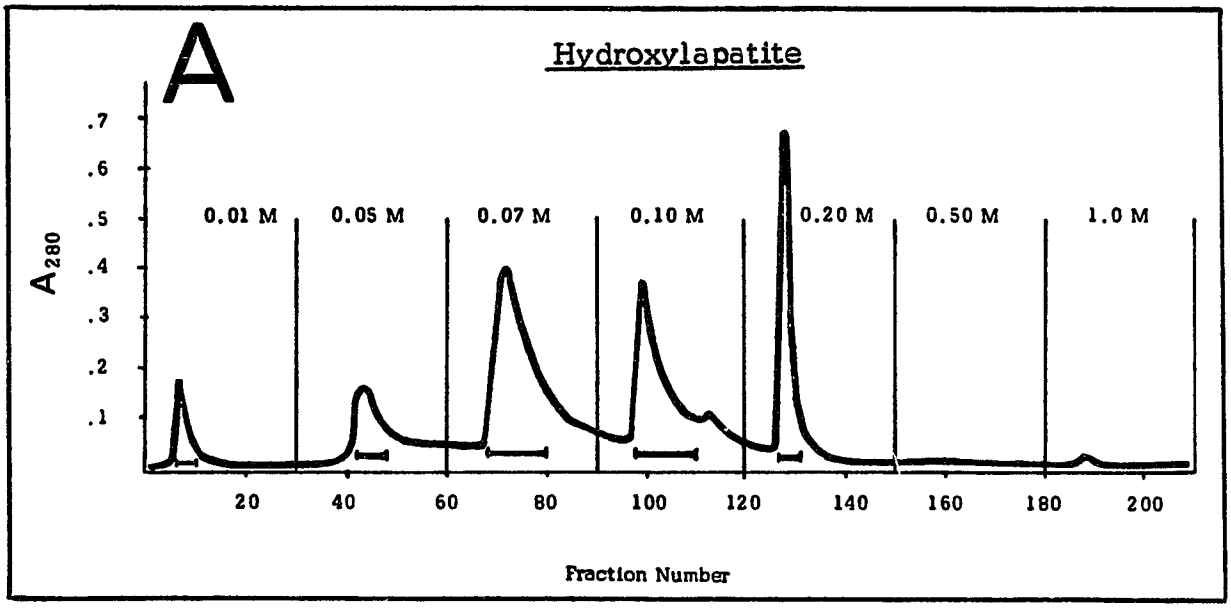
(see following page for figure)

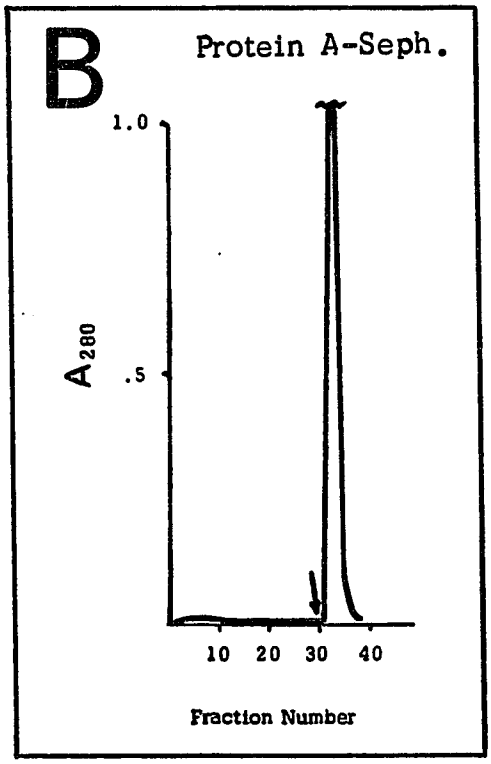
Fig. 4-3

A) Hydroxylapatite elution profile of protein Goe isolated by starch block electroporesis. Elution was carried out by successive washes with increasing concentrations of phosphate buffer, pH 6.5. The numbers (eg. 0.01 M) indicate the molaritiy of the phosphate buffer used to elute a particular peak, and the solid bars indicate the fractions pooled under each peak. The column dimensions were 1.8 x 30 cm. and 3 ml. fractions were collected at 60 ml./hr.

B) Protein A-Sepharose elution of the material eluted from the hydroxylapatite column in Fig. 4-3A using 0.07 M posphate buffer, pH 6.5. The column conditions were the same as in Fig. 4-2. The arrow indicates elution with 1 M acetic acid.

Fig. 4-3


A
Hydroxylapatite
A_{280}
.7
.6
.5
.4
.3
.2
.1
0.01 M
0.05 M
0.07 M
0.10 M
0.20 M
0.50 M
1.0 M
20
40
60
80
100
120
140
160
180
200
Fraction Number


B
Protein A-Seph.
A_{280}
1.0
.5
10
20
30
40
Fraction Number

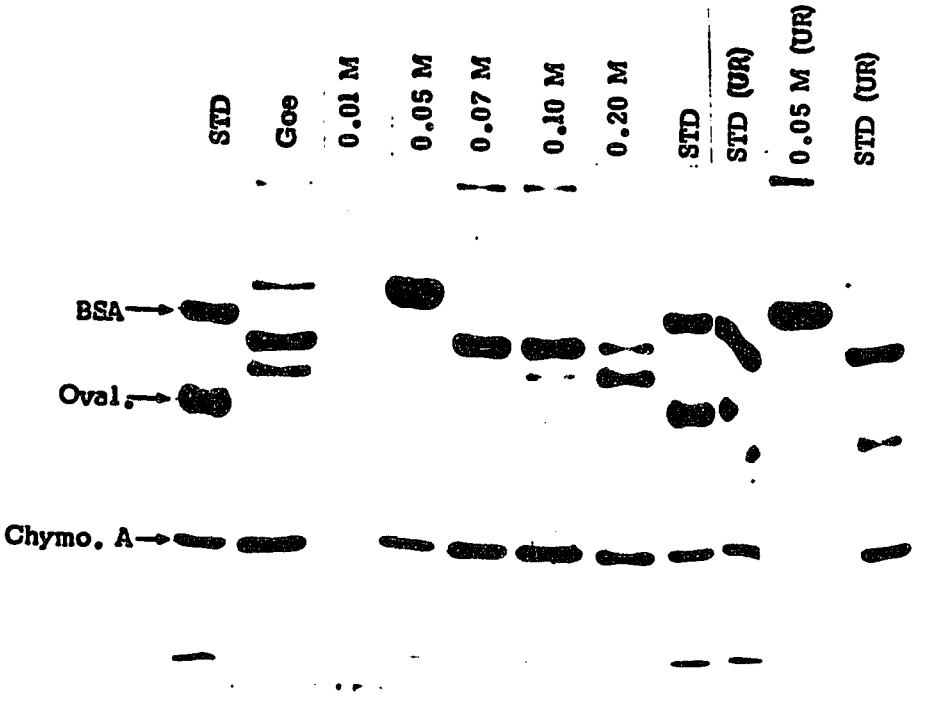


Fig. 4-4

10% PAGE in SDS of the pooled hydroxylapatite fractions shown in Fig. 4-3A. Approximately 40 μg/sample was applied to separate slots in the gel. All samples were reduced with DTT prior to electrophoresis except those marked "UR" (unreduced). The slot labeled Goe designates the material from Goe serum that was isolated by starch block electrophoresis. The molecular weights of the standards (STD) are: Bovine serum albumin (BSA) - 68,000; Ovalbumin (Oval.) - 45,000; Chymotrypsinogen A (Chymo. A) - 25,000.

light chain band (25,000 daltons) indicating that the IgG3 myeloma protein was eluted with this buffer. With increasing PB molarity, the proportion of γ1 molecular weight material (50,000 daltons) increased. Approximately one-fourth of the material eluted with 0.1 M PB possessed a γ1 molecular weight, while as much as three-fourths of the 0.2 M PB wash was of this molecular weight.

Fig. 4-5 shows the immunological analysis of the hydroxylapaptite fractions by immunoelectrophoresis (A) and by double diffusion in agar (B and C). Immunoelectrophoretic analysis demonstrated that the material isolated by starch block electrophoresis had an electrophoretic mobility primarily in the γ-globulin range (well #1) as was also the case with the protein eluted in 0.07 M PB from hydroxylapatite (well #3). Well #2 contained material from the 0.05 M PB wash and in contrast to the other fractions, showed an electrophoretic mobility similar to that of transferrin. The electrophoretic mobility of whole Goe serum (well #4) is also shown for comparison.

Double diffusion analysis in agar of Goe isolated by starch block electrophoresis showed two major precipitin lines when developed against anti-whole human serum (Fig. 4-5B). One of the lines showed identity with the major line of the 0.05 M PB wash, and the other line showed identity with γ1 and the 0.07 M PB wash. In addition the starch block isolated Goe, as well as the 0.05 M PB wash, reacted

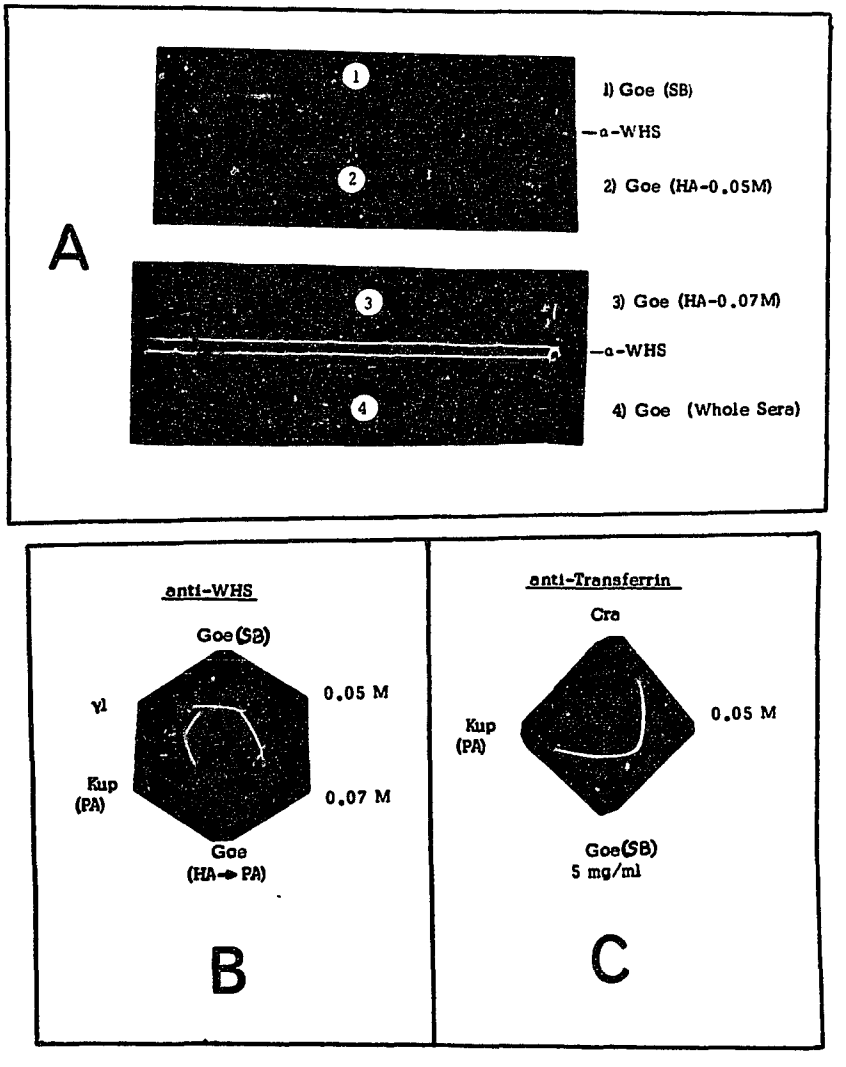
(see following page for figure)

Fig. 4-5

A) Immunoelectrophoresis of: (1) protein isolated from Goe serum by starch block electrophoesis (SB), (2) and (3) the material that eluted from hydroxylapatite with 0.05 M PB and 0.07 M PB respectively (Fig. 4-3A), (4) whole Goe serum. All the samples were reacted against anti-whole human sera (α-WHS) after electrophoresis.

B and C) Double diffusion Ouchterlony of various samples reacted against anti-WHS (B) and anti-transferrin (C). Goe (SB), 0.05 M, and 0.07 M are as described above. Goe (HA→PA) is the starch block isolated material of Goe serum which was eluted on hydroxylapatite with 0.07 M PB and then adsorbed to protein A-Sepharose (Fig. 4-3B). Kup (PA) is protein Kup purified by passage through protein A-Sepharose (Fig. 3-3). γ1 is a purified IgG1 myeloma protein. All samples were approximately 1mg/ml unless otherwise noted in the figure.

Fig. 4-5


A
1) Goe (SB)
α-WHS
2) Goe (HA-0.05M)
3) Goe (HA-0.07M)
α-WHS
4) Goe (Whole Sera)
anti-WHS
Goe(SB)
0.05 M
γ1
Kup
(PA)
0.07 M
Goe
(HA→PA)
B
anti-Transferrin
Cre
Kup
(PA)
0.05 M
Goe(SB)
5 mg/ml
C

strongly with anti-transferrin antiserum (Fig. 4-5c). Since the material from the 0.05 M PB wash was relatively clean by PAGE, automatic Edman degradation was performed and the amino terminal sequence identified was: Val-Pro-Asp-Lys-Thr-Val-Arg-Trp. This sequence was identical to that reported for human transferrin (Sutton & Bren, 1975).

The myeloma protein in Goe serum isolated initially by starch block electrophoresis was therefore heavily contaminated with normal serum IgG and transferrin. Separation and purification was achieved by a single passage through hydroxylapatite in which the Goe myeloma protein eluted after transferrin, but before normal IgG. Fig. 4-3B shows the elution profile of the 0.07 M PB peak when passed through protein A-Sepharose. The profile indicates that this peak indeed contained the unusual IgG3 myeloma that possessed the protein A-binding activity.

When the initial batch of starch block isolated Goe protein was exhausted, a second batch was prepared in a somewhat different but simpler manner. Total IgG was initially isolated from Goe serum by ion exchange chromatography which allows elution of IgG in the fall-through 0.01 M PB wash while all other serum proteins are still bound to the column. The IgG was then directly passaged through hydroxylapatite and eluted with a linear PB gradient starting with 0.03 M PB and ending with 0.1 M PB (Fig. 4-6a). The myeloma protein eluted as a single sharp peak (peak 1, Fig. 4-6a)

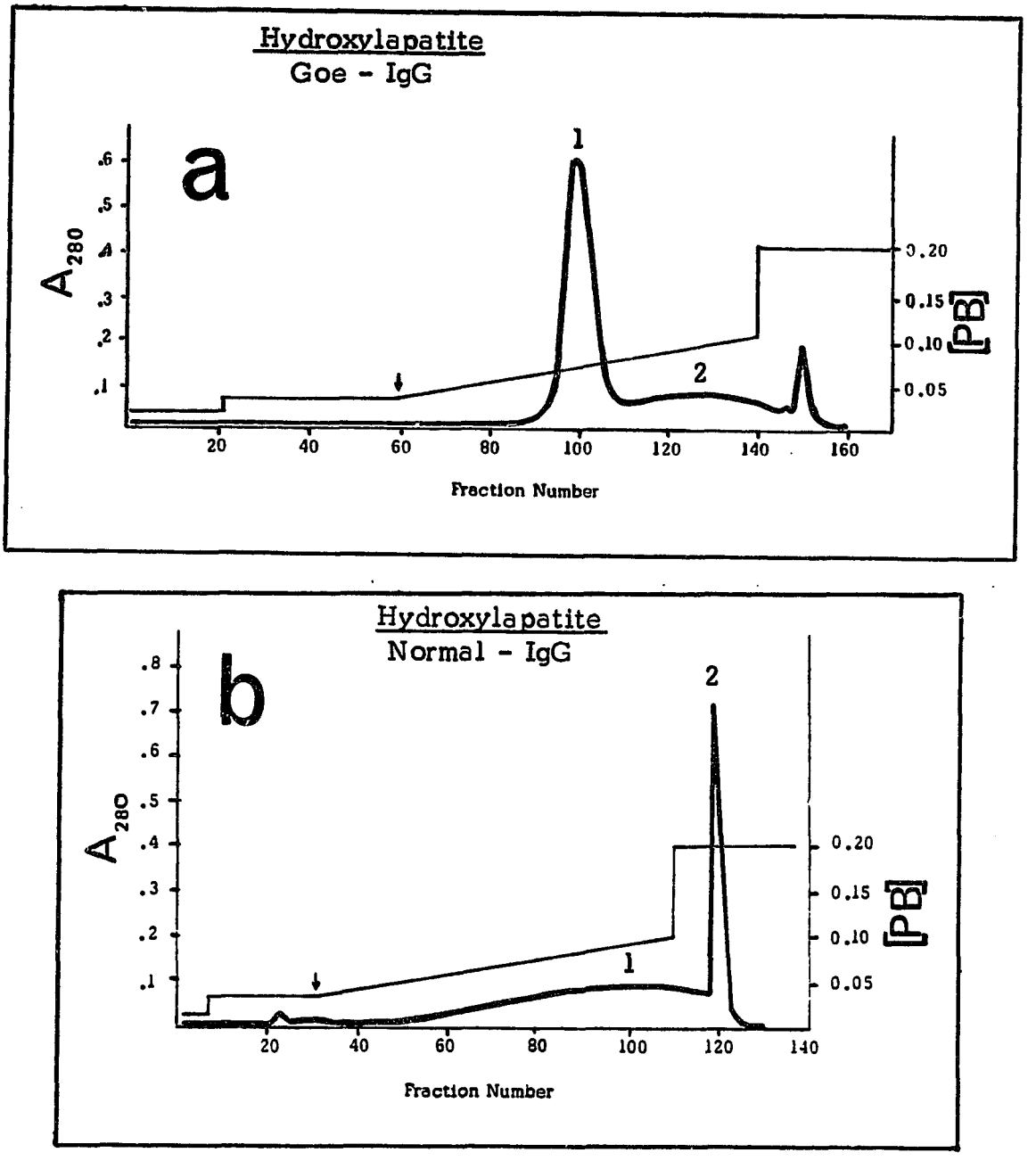


Fig. 4-6

Hydroxylapatite elution of IgG isolated by ion exchange from a) Goe serum and b) normal serum. Elution was carried out by increasing the molarity of pH 6.5 phosphate buffer (PB) as shown by the thin solid lines. The arrows indicate the start of a linear gradient of 0.03 M PB, pH 6.5 to 0.1 M PB, ph 6.5. Otherwise the column conditions are the same as in Fig. 4-3a.

while the normal IgG trailed as a broad peak (peak 2, Fig. 4-6a). For comparison, IgG was also isolated from normal serum by ion exchange in an identical manner and then passaged through hydroxylapatite (Fig. 4-6b). This profile showed only a broad peak during development of the linear gradient (peak 1, Fig. 4-6b) and the absence of a sharp myeloma-related peak was readily apparent. The appropriate IgG hydroxylapatite peaks in Fig. 4-6 from both normal serum (N.S.) and Goe serum were analyzed by SDS - PAGE (Fig. 4-7). The gel demonstrates the successful separation of the two IgG heavy chain molecular weight species originally present in Goe IgG (slot G) after elution through hydroxylapatite (slots 1 and 2). Therefore peak 1 in Fig. 4-6a contains the relatively pure IgG3 myeloma from Goe serum. This material was also shown to possess the protein A-binding activity. Peaks 1 and 2 of the hydroxylapatite separation of normal serum possess primarily $\gamma 1$ molecular weight heavy chains, as expected.

4.4 Carboxy terminal peptide analysis of protein Goe

80 mg of purified protein Goe was digested by the urea-pepsin method as previously described for protein Kup and separated on Sephadex G-100 as shown in Fig. 4-8. The elution profile was similar to that of Kup and other myeloma proteins digested in the same manner (Parr, 1977). Pool II in Fig. 4-8 which contains the up Fc fragment (Fig. 3-14), was desalted, lyophilized, completely reduced and radioalkylated, and passed

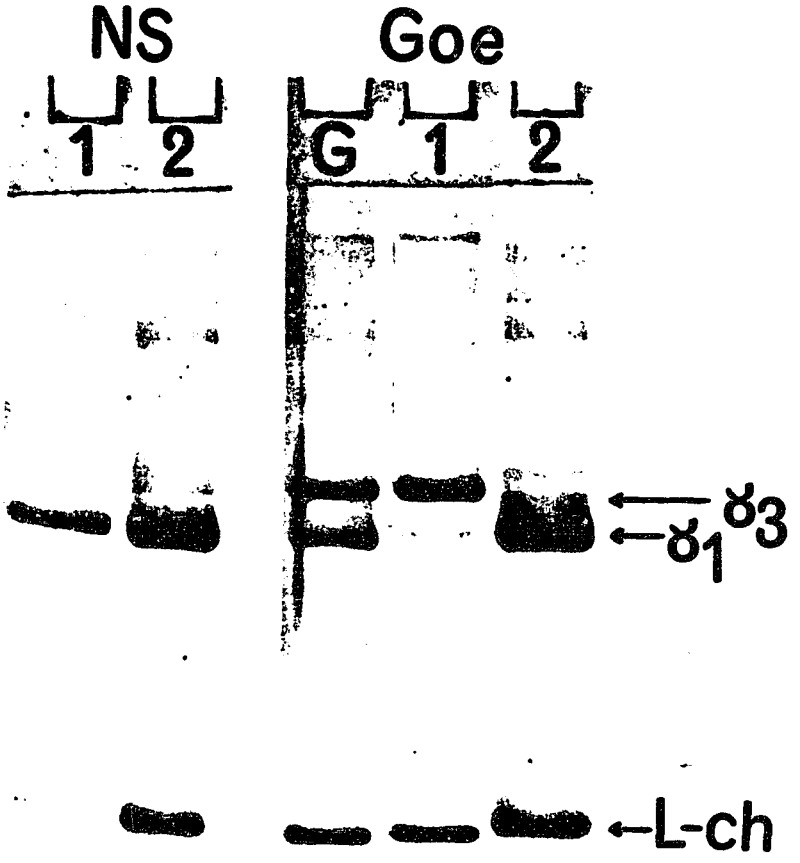


Fig. 4-7

10% PAGE in SDS of the peaks indicated in Fig. 4-6a and b. NS indicates normal sera. G indicates whole IgG isolated from Goe serum by ion exchange chromatography. Slots 1 and 2 in both gels refer to the major peaks separated by hydroxylapatite of normal sera (NS) IgG and Goe IgG in Fig. 4-6b and a respectively. γ3 and γ1 heavy chains as well as light chains (L-ch) migrate as indicated by the arrows.

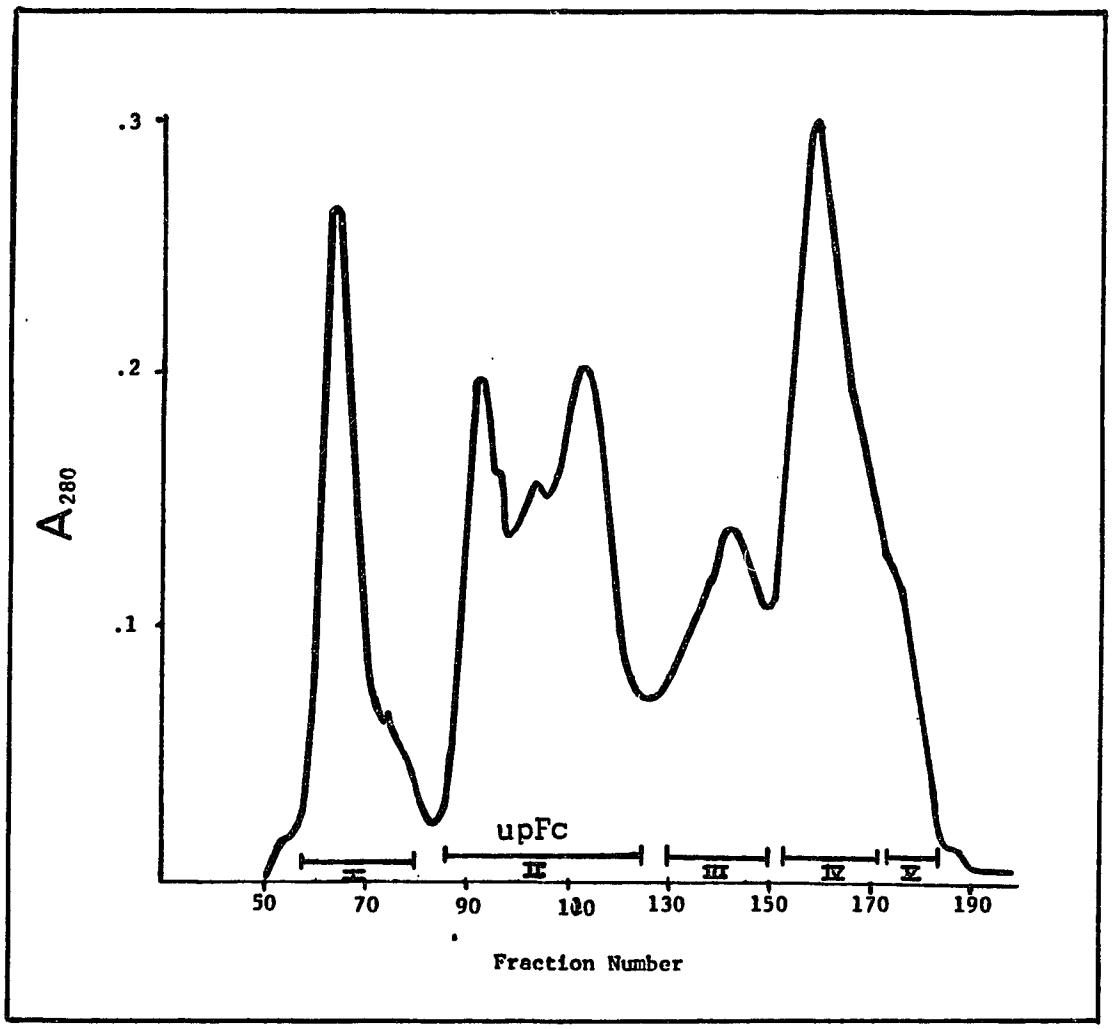


Fig. 4-8

Sephadex G-150 separation of 80 mg of purified protein Goe digested by urea-pepsin method of Parr, et al., 1975. 5 ml sample volume was loaded onto the column (2.5 x 90 cm) equilibrated with 0.1 M sodium acetate buffer, pH 5.0, in 6 M urea and possessing a flow rate of about 10 ml/hr. The indicated fractions, each of which contained 3.5 ml, were pooled, desalted on G-25 fine in 5% formic acid, and lyophilized. Pool II, which was further studied, contained the urea-pepsin (up) generated Fc fragment.

through Sephadex G-50 (Fig. 4-9) from which three peaks were eluted. Upon amino acid analysis, pool III could not be placed and therefore was not studied further. An aliquot of pool II (Fig. 4-9), termed G50-II, was subjected to complete acid hydrolysis and analyzed for its amino acid composition. Table 4-1B presents the analysis data of the G50-II fragment of Goe and compares it with the homologous fragments from proteins Kup (IgG3) and Eu (IgG1). The G50-II fragment of Goe represents the 36 carboxy-terminal amino acid residues of the heavy chain although its amino acid composition does not fit exactly with either the homologous γ1 or γ3 fragments. Six differences occur between the known γ1 and γ3 G50-II fragments. The G50-II fragment of Goe is similar to the γ1 fragment in its content of Tyr, Phe, His, and Arg, although it is more like the γ3 fragment in its content of Val and Ile.

In addition, the cyanogen bromide derived C-terminal octadecapeptide (ODP) was generated as described in Methods and Materials (see Chapter 2.13.4). After isolating partially reduced and alkylated Goe heavy chains (Fig. 4-10, pool I), and digesting them with cyanogen bromide, the fragments which were soluable in ammonium bicarbonate buffer, pH 8.5, were separated on Sephadex G-50 (Fig. 4-11). The material insoluable in this buffer was used later in the isolation of other fragments. Amino acid analysis of the small soluable fragment (Fig. 4-11, pool II) is shown in Table 4-1A and is compared with the C-terminal ODP fragments from a γ1 and a γ3 heavy chain. The amino acid composition of the ODP

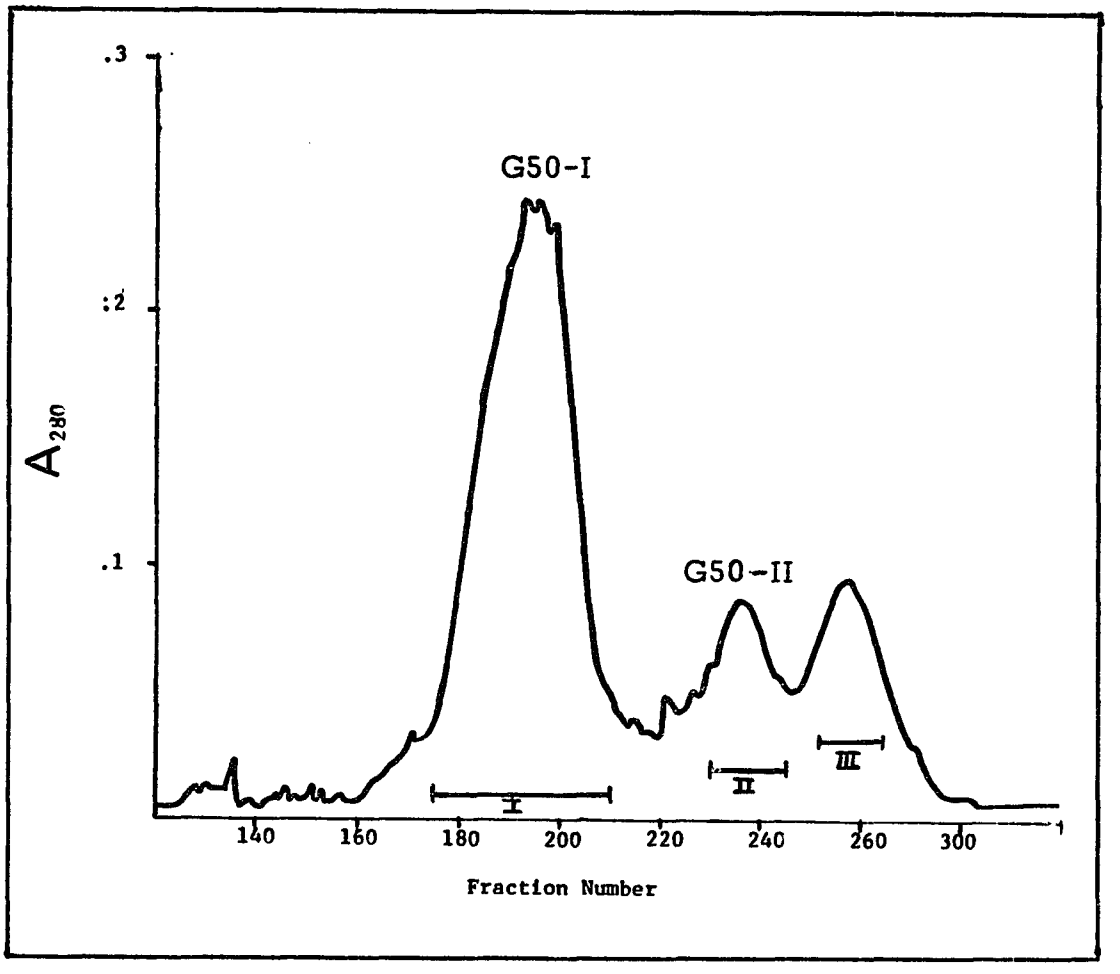


Fig. 4-9

Passage of pool II from figure 4-7 on Sephadex G50 after complete reduction and radioalkylation. The column (2.6 x 160 cm) was equilibrated in 0.1 M NaAcetate, pH 5.0, with 6 M urea. 2 ml fractions were collected at a rate of 30 ml/hr. Pool II and III were desalted on G25 fine in 5% formic acid and lyophilized. Pool I was passaged directly on CM-Sephadex (Fig. 4-15). Pool I and II are referred to as G50-I and G50-II respectively.

Table 4-1

Amino Acid Composition of Two C-Terminal Peptides of Protein GOE

	A CNBr generated ODP fragments (18aa)			B Urea-pepsin generated G50-II fragments (36aa)		
Amino Acid	Eu(γ1)	GOE	Kup(γ3)	Eu(γ1)	GOE	Kup(γ3)
CMC	-	-	-	1	1.0	1
Asp	1	1.1	1	3	3.0	3
Thr	1	1.1	1	2	2.2	2
Ser	3	2.6	3	6	4.3	6
Glu	2	2.0	2	4	4.3	4
Pro	1	1.2	1	1	1.3	1
Gly	1	1.1	1	2	2.4	2
Ala	1	1.0	1	1	1.2	1
Val	-	-	-	3	2.4	2
Met	-	-	-	1	1.0	1
Ile	-	-	-	-	1.0	1
Leu	3	2.7	3	3	3.3	3
Tyr	1	0.9	-	1	1.3	-
Phe	-	-	1	1	1.1	2
His	3	2.7	2	3	2.6	2
Lys	1	1.1	1	2	2.3	2
Arg	-	-	1	1	1.1	2

A. Comparison of the amino acid composition of the cyanogen bromide generated C-terminal octadecapeptide (ODP) from Goe heavy chains (Fig. 4-11) to that of the corresponding sequence in a γ1 (Eu) and γ3 (Kup) heavy chain.

B. Comparison of the amino acid composition of the urea-pepsin generated G50-II fragment of Goe (Fig. 4-9) to the homologous fragments of a γ1 (Eu) and a γ3 (Kup).

The ODP contains 18 amino acids (18aa) while the G50-II fragment contains 36 amino acids (36aa). Both fragments were derived from the C-terminus of the corresponding heavy chains.

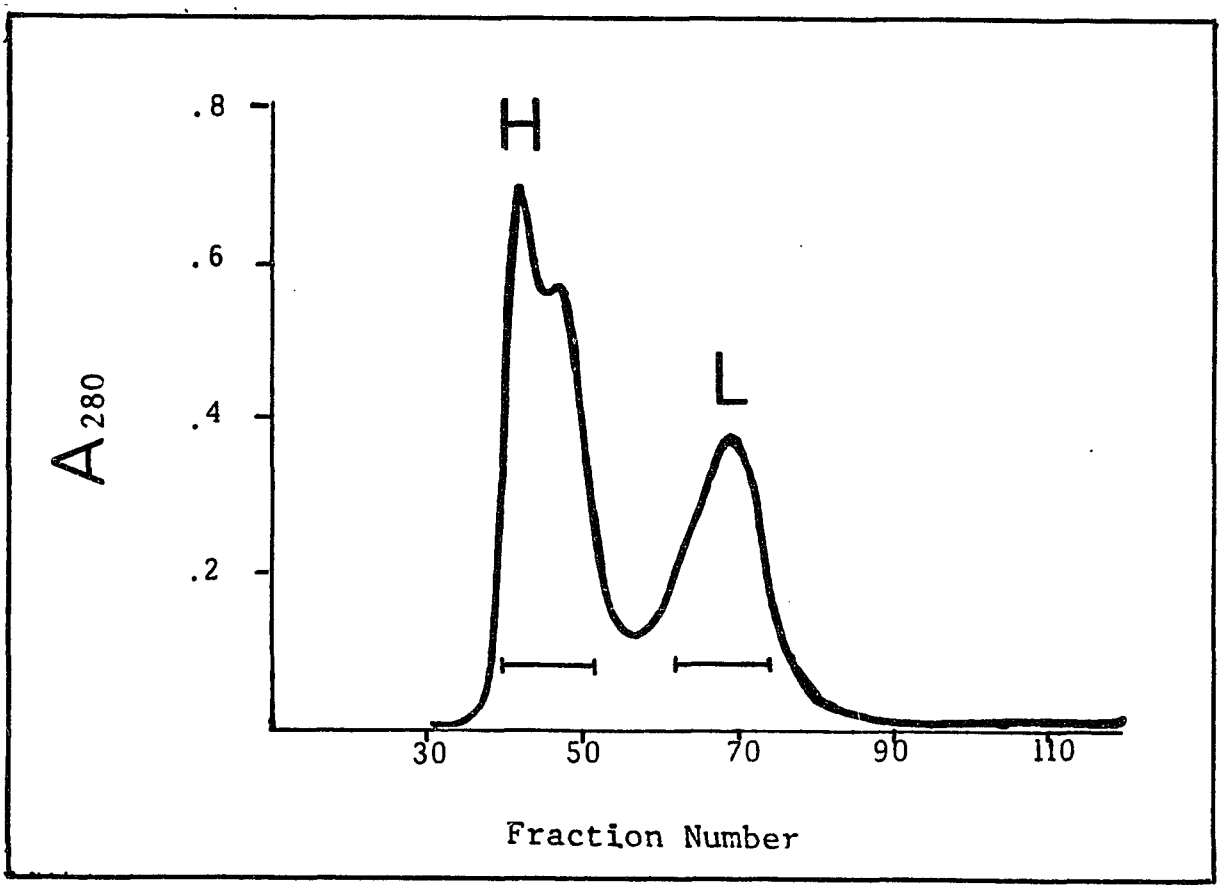


Fig. 4-10

Sephadex 6-100 (2.6 x 90 cm) separation of partially reduced and radioalkylated protein Goe. The column was equilibrated in 1 M acetic acid. 3 ml fractions were collected at 20 ml/hr. The sample contained 50 mg of hydroxylapatite-purified Goe at 15 mg/ml. After partial reduction and radioalkylation protein Goe separated into heavy (H) and light (L) chain peaks which were separately pooled and lyophilized.

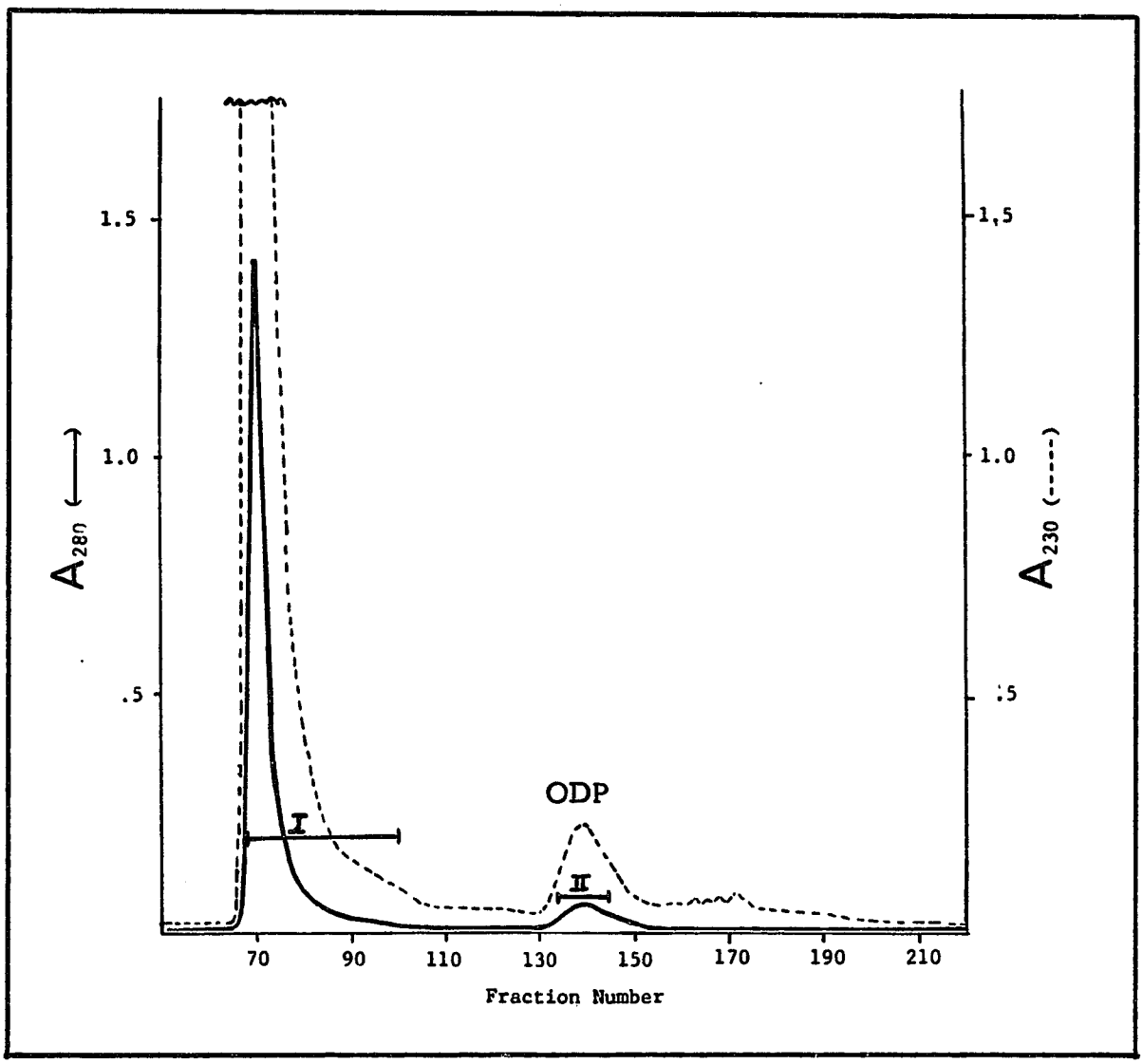


Fig. 4-11

Sephadex G50 (fine) in 0.01 M NH_4HCO_3, pH 8.5, (1.8 x 95 cm) of the fragments which were soluble in the column buffer after digesting partially reduced and alkylated Goe heavy chains with cyanogen bromide. 40 mg of Goe heavy chains were digested 0.8 ml was loaded onto the column and 1.5 ml fractions were collected at a rate of 30 ml/hr. The fractions indicated by the solid bar were pooled and lyophilized. Pool II contained the C-terminal octadecapeptide (ODP).

fragment of Goe agrees well with that of the γ1 ODP fragment in that they both possess 1-Tyr, 0-Phe, 3-His and 0-Arg, whereas the corresponding γ3 fragment consists of 0-Tyr, 1-Phe, 2-His and 1-Arg. The differences between the γ1 and γ3 heavy chains can be accounted for by two amino acid substitutions.

Both the G50-II and ODP C-terminal fragments from Goe heavy chains were sequenced automatically and the data is presented in Table 4-2 and Table 4-3 respectively. Fig. 4-12 presents the proposed amino acid sequence of the 36 C-terminal amino acids of Goe heavy chains by combining the sequence data along with the amino acid compositions of the two fragments and aligning them by homology to known γ1 and γ3 sequences. This sequence is identical to neither γ1 nor γ3 sequences but contains residues that have previously been ascribed to both subclass heavy chains. In particular, Ile at position 422 has previously been seen only in γ3 subclasses while His at position 435 is thought to be specific for the γ1 subclass. Tyr in position 436 has been previously found in all γ1 chains but only in γ3 chains that possess the G3m(g) allotype.

4.5 Asp-Pro Cleavage

The precipitate resulting from the cyanogen bromide digest of Goe heavy chains from which the ODP fragment was extracted was completely reduced and alkylated and passed through Sephadex G-100 (Fig. 4-13). From previous columns run from similarly prepared material and by screening various

Table 4-2*

Automated Edman Degradation of Goe Urea-Pepsin G50-II Fragment[a]

Cycle No.	TLC[c]	HPLC[k]	BH[d]	CPM[e]
1	V	-[f]	Aba[h]	74
2	V	V/M[g]	V	51
3	D	D	D	75
4	K	K	K	60
5	-	-	-	54
6	-	R	R	30
7	W	W	G	44
8	Q	G(E)[j]	E	53
9	(Q)	Q(E)	E	50
10	G	G	G	57
11	N	N	D	35
12	V(L/I)	F/I[g]	I	52
13	F(V)	F/I	F	60
14	-	-	(A)	70
15	A	(E)	-	714
16	-	-	(E)	282
17	V	V/M	-	100
18	-	V/M	M	89
19	-	(Q)	H	94
20	E	E	E	87
21	E,A	A	A	50
22	L/I,F[l]	L	L	56
23	-	-	-	38
24	-	-	-	53
25	-	-	-	70

*) see Table 3-1 for footnotes

Table 4-3

Automated Edman Degradation of Goe ODP Fragment

Cycle No.	HPLC	BH
1	-	H
2	E	-
3	A	A(E)
4	L	L
5	-	H
6	N	D
7	-	-
8	-	Y
9	-	-
10	-	-

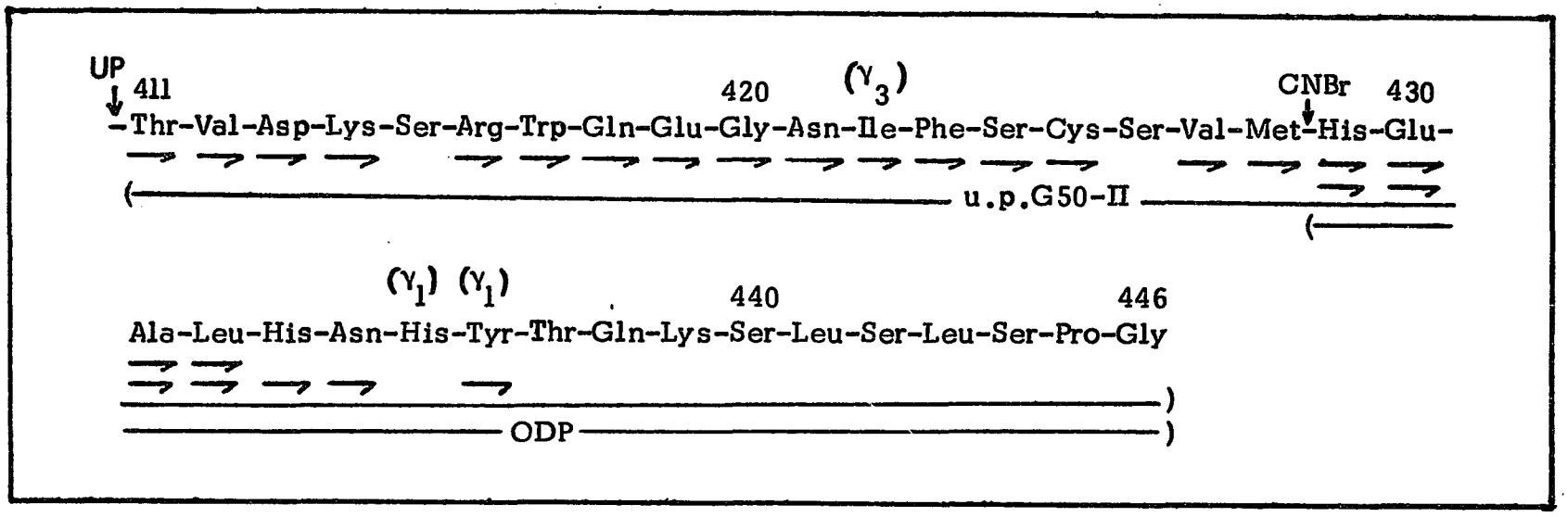


Fig. 4-12

The proposed amino acid sequence for the 36 carboxyterminal amino acid residues of Goe heavy chain. Arrows (→) indicate the residues identified by automatic sequencing. The first row of arrows indicate the sequence of the C-terminal fragment generated by urea-pepsin (UP) digestion of GOE heavy chain which starts at residue 411. The second row of arrows indicate the sequence of the cyanogen bromide (CNBr) derived C-terminus starting at residue 429. The solid lines indicate those residues alligned by homology to known sequences. Ile at position 422 and His at position 435 have previously been found only in γ3 and γ1 heavy chains respectively. Tyr at 436 is found in all γ1 chains but only in γ3 chains that show the G3m(g) allotype. All other residues are common to both γ1 and γ3 heavy chains. Residue numbering is according to protein Eu (Edelman et al., 1969).

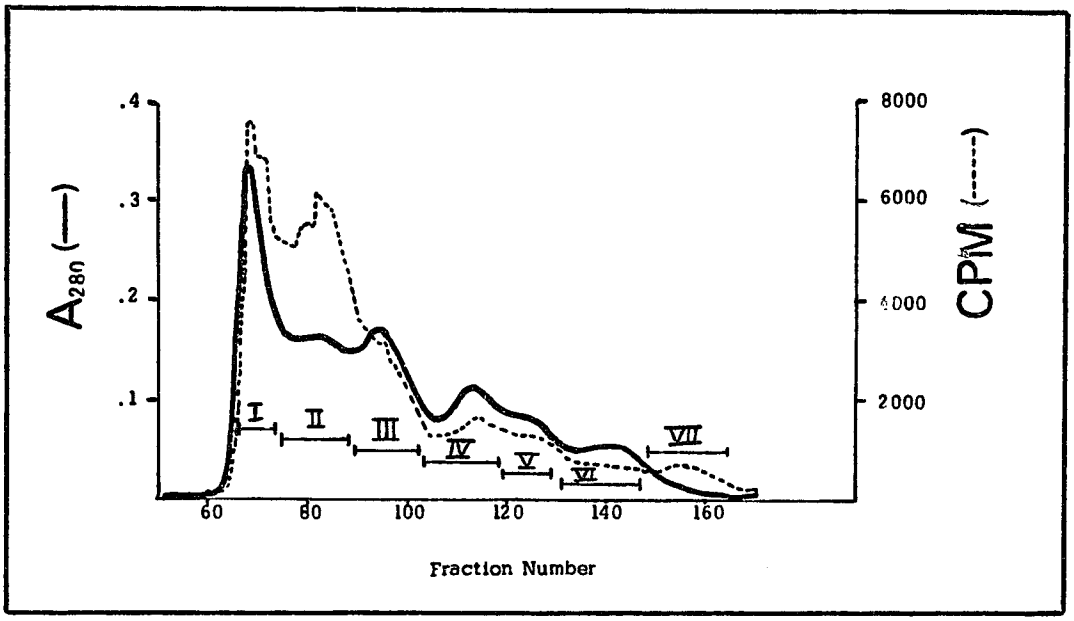


Fig. 4-13

Sephadex G100 in 10% formic acid (2.0 x 95 cm). Partially reduced and alkylated Goe heavy chains were treated with cyanogen bromide and then resuspended in 0.01 M NH_4HCO_3. The supernatant was decanted and fractionated as previously described (Fig. 4-11). The precipitate was then taken and completely reduced and radioalkylated and finally fractionated by gel filtration as shown above. The solid bars indicate the fractions pooled. 50 µl of every other fraction was counted for radioactivity and plotted. 2 ml fractions were collected at about 25 ml/hr.

fractions by PAGE it was determined that pool III most probably contained the CNBr-derived CH2 region beginning at position 253 (Ile). A single manual Edman degradation liberated Ile as the major amino-terminal amino acid. PAGE of this pool showed two bands (Fig. 4-14A) which suggested that cleavage at the Asp-Pro bond also occurred in this molecule. Therefore pool III was lyophilized and subjected to the conditions promoting Asp-Pro bond cleavage. After the incubation period, the mixture was passed through Sephadex G-75 and the major peak lyophilized. Fig. 4-14B shows the PAGE of the acid cleaved fragment. The results in Fig. 4-14 demonstrate the disappearance of the higher molecular weight species found in the starting material (A) and the appearance of the lower molecular weight material (B) after limited acid cleavage. Manual Edman degradation of the fragment shown in Fig. 4-14B gave Pro as the major amino terminus. The sequence of this fragment was determined automatically and is presented in Table 4-4.

The presence of Glu and Lys at residues 274 and 276 respectively are noteworthy because (1) Glu at 274 has previously been found only in γ3 and γ4 chains (in γ1 chains, Lys is the homologous residue) and (2) Lys at 276 has been described only in γ3 chains (Asn is found in the homologous position in γ1 and γ4 chains).

4.6 Further study of other urea-pepsin fragments

Pool I, Fig. 4-9, was passaged directly through an ion-

Table 4-4 *

Automated Edman Degradation of GOE CNBr-III After Limited Acid Hydrolysis [a]

Cycle No.	HPLC [k]	BH [d]
1	- [f]	G [j]
2	E	E(V)
3	-	V(E)
4	V, E	E(V)
5	F/I [g]	F
6	K	K
7	A	G
8	Y	-
9	V	V
10	V(D)	D
11	G	G
12	G	-
13	E	-
14	E	E(V)
15	-	H,K
16	-	-
17	-	-
18	-	-
19	-	Aba [h]
20	(K)	-

*) see Table 3-1 for footnotes

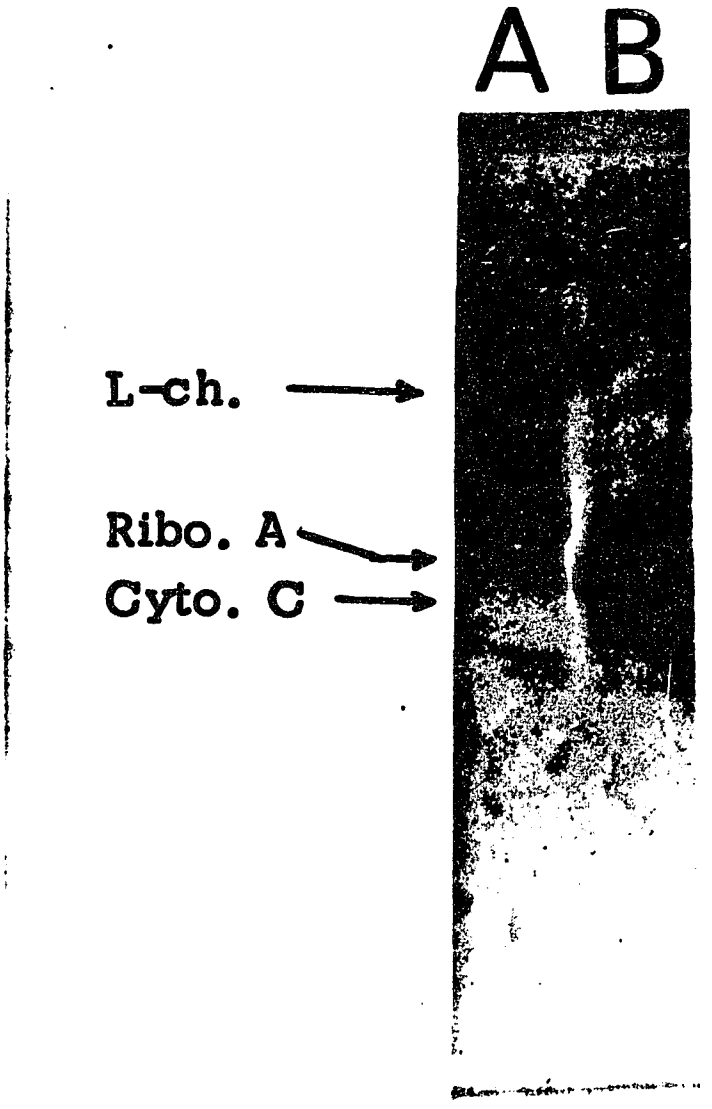


Fig. 4-14

SDS-PAGE (15%) of (A) Pool III, Fig.4-12 and (B) the same material as in (A) but after limited acid hydrolysis and gel filtration. Both samples were run under reducing conditions. The markers, which were run on the same gel slab are: light chain (L-ch.)-25,000; ribonuclease A (Ribo. A)-13,700; cytochrome C (Cyto. C)-11,700.

exhange column and eluted with an increasing linear salt gradient (Fig. 4-15). Pools I and III, termed CM-I and CM-III respectively, were separately desalted an lyophilized and their amino acid sequence determined automatically (Table 4-5 and 4-6). CM-I started at position 253 with isoleucine and CM-III at position 307 with threonine, the same results as with the homologous fragments from protein Kup. CM-I was also sequenced through the subclass related residues at position 274 and 276 which were glutamic and lysine, as determined in the acid cleaved fragment. CM-III was sequenced through position 339 which is also thought to be subclass related. Postion 339 in protein Goe was shown to be alanine, which had previously been found in all the γ1 but none of the γ3 proteins studied. In γ3 position 339 is taken by threonine.

4.7 Tryptic fragments of Goe Fc

Fc fragments from purified protein Goe were generated as described in Methods and Materials. The Goe Fc fragments were then digested with trypsin and the resulting tryptic fragments were initially separated on Sephadex G-50 in IN acetic acid into five peaks. The first peak containing undigested or aggregated material was not investigated further. The other peaks were pooled separately and lyophilized. Each sample was then dissolved in a small volume of 0.01 M NH_4OH and applied to 3 MM chromatography paper and electrophoresed in pyridine-acetate buffer, pH 3.5, at 3,500 volts for one hour. Each of the separated peptides, detected by staining an edge with ninhydrin, was cut out

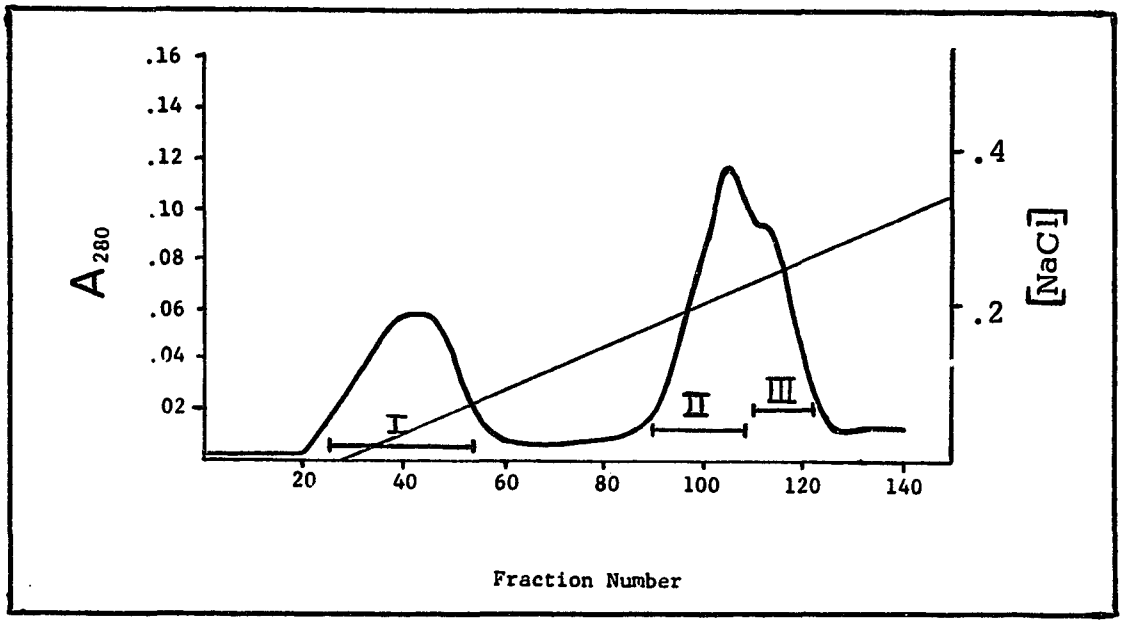


Fig. 4-15

Carboxymethyl (CM)-Sephadex (type-50) ion exchange separation of pool I, Fig. 4-9. The column (2 x 30 cm) was equilibrated with the same buffer as described in Fig. 4-8 and eluted with a gradient of 250 ml of starting buffer to 250 ml of starting buffer with 0.5 M NaCl. The fractions indicated by the solid bars were pooled. Pool I, II and III are referred to as CM-I, CM-II and CM-III respectively. 2.5 ml fractions were collected at 60 ml/hr.

Table 4-5 *

Automated Edman Degradation of Goe Urea-Pepsin Fragment CM-I [a]

Cycle No.	TLC [c]	HPLC [k]	BH [d]	CPM [e]
1	L/I [g]	(W)	I	41
2	(F),	F/I	F(A)	39
3	(V) [j]	- [f]	R	63
4	M/T, (E) [g]	(P)	Aba [h]	43
5	P	P	P	26
6	E	-	E	63
7	V	V/M [g]	V	56
8	M/T, (E)	-	Aba	41
9	S/C	-	A [i]	702
10	V	V/M	-	288
11	V	V/M	V	112
12	V	V/M	V	72
13	D(V)	Q,V/M [l]	D	69
14	V	D	V	61
15	V	V/M	-	50
16	(V,E)	V/M	H	63
17	E	E	E(G)	40
18	D	D	D	109
19	(P,Q)	-	P	85
20	E,Q	E	E	54
21	V	-	V	55
22	Q	E(Q)	E	55
23	F	F/I	F	52
24	F(K)	K	K	41
25	W	W	G	43
26	Y	Y	Y	60
27	V	V/M	V	43
28	V(D)	D	D	52
29	G	-	G	40
30	V	V/M	-	43

*) see Table 3-1 for footnotes

Continued

31	E,Q	E	E,V	50
32	V	V/M	E,V	41
33	V	V/M	-	44
34	V(N)	-	D	45
35	A	A	(A)	36
36	(A)	-	-	45
37	-	-	-	48
38	-	-	-	29
39	-	-	-	35
40	-	-	-	33
41	(E)	-	-	41
42	(E)	-	-	41
43	-	-	-	38

Table 4-6*

Automated Edman Degradation of Goe Urea-Pepsin Fragment CM-III[a]

Cycle No.	TLC[c]	HPLC[k]	BH[d]	CPM[e]
1	M/T[g]	-	Aba[h]	49
2	V	V/M[g]	V	33
3	L	L	L	75
4	L	-	H	64
5	E,Q[l]	E	E	66
6	D		D	50
7	W	-	G	52
8	L	-	L	65
9	N,D	-	D	111
10	G	-	G	69
11	K	-	K	59
12	E	-	E	250
13	Y	-	Y	100
14	K	-	K[j]	96
15	S/C	-	(K)	1140
16	K	-	K	618
17	V	V/M	(V)	418
18	V,K	-	-	210
19	N	-	D	138
20	K	-	K	138
21	A	-	A	90
22	L/I	-	L	72
23	P	-	P	64
24	A,P	-	A	58
25	A(P)	-	P	48
26	L/I	-	I	39
27	E	-	E	59
28	K(P)	-	K	52
29	K,E	-	Aba,(H)	64
30	L/I(M/T)	-	I	43
31	L/I,K	-	-	44
32	K(L/I,M/T)	-	(I)	32
33	A,(K)	-	(K)	54
34	K	-	-	35
35	K,G(Q,A)	-	G,K,(E)	44
36	Q	-	E	46
37	D,N	-	-	50

*) see Table 3-1 for footnotes

Continued

38	-	-	(R)	44
39	E	-	E	39
40	E,Q	-	-	43
41	E,Q	-	E	47
42	-	-	(V)	37
43	-	-	(E,Y)	30
44	-	-	-	55
45	(L/I)	-	-	34

and sewn onto a sheet of 1 MM chromatography paper and electrophoresed a second time at 3,500 volts using pyridine-acetate buffer, pH6.5. The peptides were once again located, cut out, and sewn onto another piece of 1 MM chromatography paper and electrophoresed a final time at pH2.1, pyridine-acetate buffer. This procedure yielded well-separated, ninhydrin-staining spots which were cut out, eluted with 6 M HCl, hydrolyzed for 24 hours at 110 C, and analyzed for thier amino acid composition.

Some ninhydrin-staining spots which were obtained were determined to be single, free amino acids, and others were recovered in too low yield to be properly placed. Table 4-7 presents the amino acid composition data of those peptides which were recovered in high enough yield to be analyzed and placed by homology in either γ1 or γ3 heavy chain sequences.

Two important peptides were identified. T2 and T3 comprise an overlap of the carbohydrate containing peptide from residue 298-300. From Table 1-10, positions 296 and 300 contain residues which have been correlated with specific subclasses or genetic markers. The only sequence that can account for the overlapping peptides is the one correlated with an IgG3 possessing the G3m(g) allotype. Therefore, the proposed sequence for the carbohydrate peptide of Goe has Tyr at postition 296 and Phe at position 300.

4.8 Goe pFc' fragment

Purified Goe protein was dissolved in 0.2 M acetate buffer, pH 4.5 (25 mg/ml) and incubated with pepsin at an enzyme/protein ratio of 1:100 (w/w) for one hour at 37°C After stopping the digestion by addition of crystalline

Table 4-7

Amino Acid Composition of the Tryptic Fragments of the Fc Region of Protein GOE

	Tryptic Peptide and Position								
Amino Acid	T1 249-255	T2 289-298	T3 293-300	T4 318-320	T5 321-322	T6 327-334	T7 356-360	T8 410-414	T9 440-446
CMC					1.0(1)				
Asp	1.2(1)*	1.0(1)	1.1(1)					1.1(1)	
Thr	0.8(1)	1.5(1)	1.7(1)				1.0(1)	1.0(1)	
Ser	1.1(1)	1.1(1)	1.2(1)						2.8(3)
Glu		3.0(3)	2.9(3)	1.1(1)		1.1(1)	2.1(2)		
Pro		0.6(1)	0.4			1.8(2)			0.8(1)
Gly			0.3						1.0(1)
Ala						2.0(2)			
Val								1.0(1)	
Met	0.8(1)						1.0(1)		
Ile	0.9(1)					1.0(1)			
Leu	1.0(1)					1.2(1)		1.2(1)	2.2(2)
Tyr		0.6(1)	0.9(1)	1.0(1)					
Phe			0.8(1)						
His									
Lys		0.6(1)		0.9(1)	1.0(1)	1.0(1)	0.9(1)	1.0(1)	
Arg	0.8(1)	0.6(1)							

* Numbers in parentheses ( ) indicate expected values

Tris-base to achieve a pH of about 7, the sample was applied to Sephadex G-200 equilibrated with 0.3 M NaCl. The elution profile was identical to those digests of other IgG subclasses (Turner et al., 1970) in which the pFc' is eluted well separated from undigested IgG, $F(ab)'_2$ and the small molecular weight fragments. Table 4-8 presents the automatic sequence data of this fragment which starts at position 334 (lysine). This sequence also shows the γ1 related amino acid, alanine, at position 339.

4.9 Summary of sequence and peptide data on Goe

Fig. 4-16 summarizes the sequences and peptide data starting at residue 253 (Ile), and ending at 369 (Val). This sequence, which encompasses most of the CH2 and the beginning of the CH3 region of Goe indicates the specific residues which have previously been correlated to either the IgG1 or IgG^3 subclass but have been identified in Goe regardless. Specifically, the alanine at 339 is correlated to γ1 heavy chains while the glutamic, lysine, and phenylalanine at 274, 276, and 300 respectively, have been correlated with γ3 heavy chains. In addition tyrosine at 296 has been correlated with γ1 chains and G3m(g) allotype γ3 chains, but not with G3m(b1) allotype γ3 chains.

4.10 Hinge region studies

As discussed in Chapter 1, the hinge region of each IgG subclass differs with respect to the number and position

Table 4-8*

Automated Edman Degradation of Goe pFc' Fragment [a]

Cycle No.	GLC [b]	TLC [c]	BH [d]	CPM [e]
1	A,G,L/I [l]	K(G,D,Y)	K,G,E,A	817
2	P/T [g]	(G) [j]	Aba [h]	1124
3	L/I [g]	L/I	I	1016
4	S/C	(D)	A [i]	557
5	(A)	K	K	161
6	A	A	A	326
7	- [f]	K	K	424
8	G	G	G	559
9	(P)	Q	-	479
10	P/T	Y(P)	P	361
11	(V)	-	R	246
12	-	E	E	383
13	P/T	-	P	438
14	-	-	E	485
15	V	-	V	436
16	S/C(V)		Y	462
17	P/T	-	Aba	424
18	L/I	-	L	501
19	P/T	-	P	513
20	P/T	-	P	617
21	S/C	-	(A)	567
22	-	-	R	361
23	-	-	(D,E)	545
24	-	-	E	585
25	(L/I)	-	(L)	605
26	(S/C)	-	Aba	678
27	-	-	K	838
28	-	-	D	963
29	-	-	E	689
30	-	-	V	931

*) see Table 3-1 for footnotes

Continued

31	-	-	-	723
32	L/I	-	L	773
33	(T)	-	Aba	864
34	-	-	A	1862
35	S/C,L/I	-	-	1352
36	V	-	-	340
37	(V)	-	K	-
38	-	-	G	-
39	-	-	F	-
40	-	-	-	-

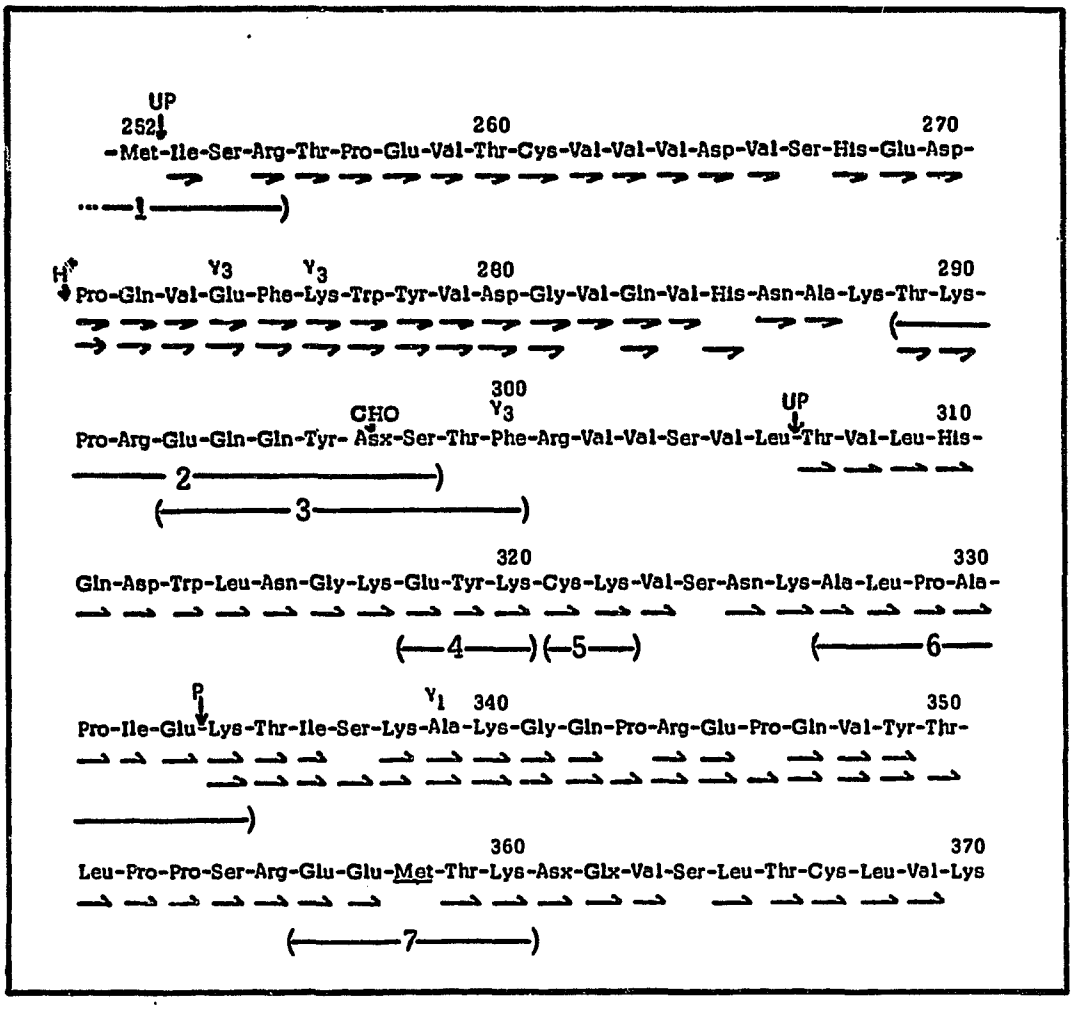


Fig. 4-16

Proposed amino acid sequence of the heavy chain fragment corresponding to most of the CH2 region and the beginning of the CH3 region of protein Goe (numbering taken from protein Eu - Edelman, et al., 1969). Horizontal half arrows indicate amino acid sequence determined by automatic means; horizontal full arrows indicate residues identified by manual means. Solid lines indicate peptides which were placed by homology through amino acid composition (Table 4-7). Residues which have no symbols under them are placed purely by assumption and have been shown to be present in both γ1 and γ3 heavy chains. Residues sequenced in Goe that have previously been correlated with specific subclasses have above them the symbols γ1 or γ3 denoting their correlation with the IgG1 or IgG3 subclass respectively. Tyr at position 296 has been correlated with both γ1 molecules and G3m(g) γ3 molecules. The vertical arrows indicate sequence starting points. UP denotes the cleavage points of digestion with urea-pepsin (see also Fig. 3-17). H^+ denotes the point of cleavage by limited acid hydrolysis. P denotes the cleavage point with aqueous pepsin. The peptides numbered 1-7 indicate the tryptic fragments derived from Goe Fc. CHO indicates the attachment of carbohydrate.

of interchain disulfide bonds. Therefore chemical typing of partially reduced and radioalkylated immunoglobulins (Chapter 2.11) describes the subclass nature of the hinge region. Fig. 4-17 shows the chemical typing autoradiograph of purified monoclonal proteins from each of the four subclasses. Kup was used as the prototype IgG3 subclass myeloma protein. Fig. 4-18 shows a drawing of the autoradiograph demonstrating more clearly the characteristic radioactive banding pattern for each subclass. From this drawing, it is clear that each hinge region generates at least a single major radioactive peptide possessing a unique mobility at pH 3.5 as well as at pH 6.5 Fig. 4-19 shows the amino acid sequence of each of the radioactive peptides isolated from an IgG1 (Bacon) and an IgG3 (Kup) after chemical typing. The two subclass hinge regions are clearly defined by chemical typing especially after the amino acid compositions of the respective radioactive peptides are determined.

To determine the subclass nature of protein Goe, chemical typing was performed as shown in Fig. 4-20. From the autoradiograph, Goe appears to be identical to IgG3 (Kup). The major radioactive peptides from Goe and Kup as well as from the IgG1 myeloma, Bacon, were isolated as described in Methods and Materials. The light chain peptides however were not isolated in this experiment. The amino acid composition of these peptides are shown in Table 4-9.

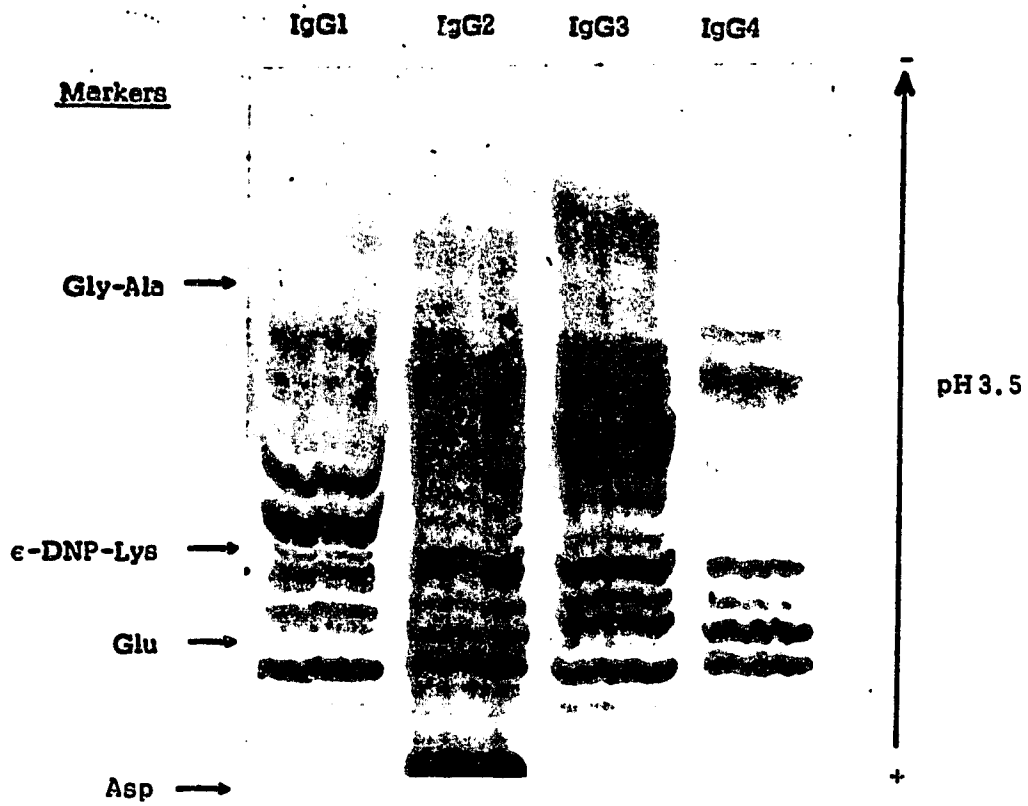


Fig. 4-17

Chemical typing of the four IgG subclasses as described in detail in Methods and Materials. The individual myeloma proteins were partially reduced and radioalkylated and then subjected to pepsin and trypsin digestion followed by high voltage paper electrophoresis at pH 3.5. The photograph above shows a 48 hour autoradiograph exposure of the resulting paper electrophoresis demonstrating the radioactive banding pattern characteristic of each subclass. Markers are indicated on the left and were run together with the samples.

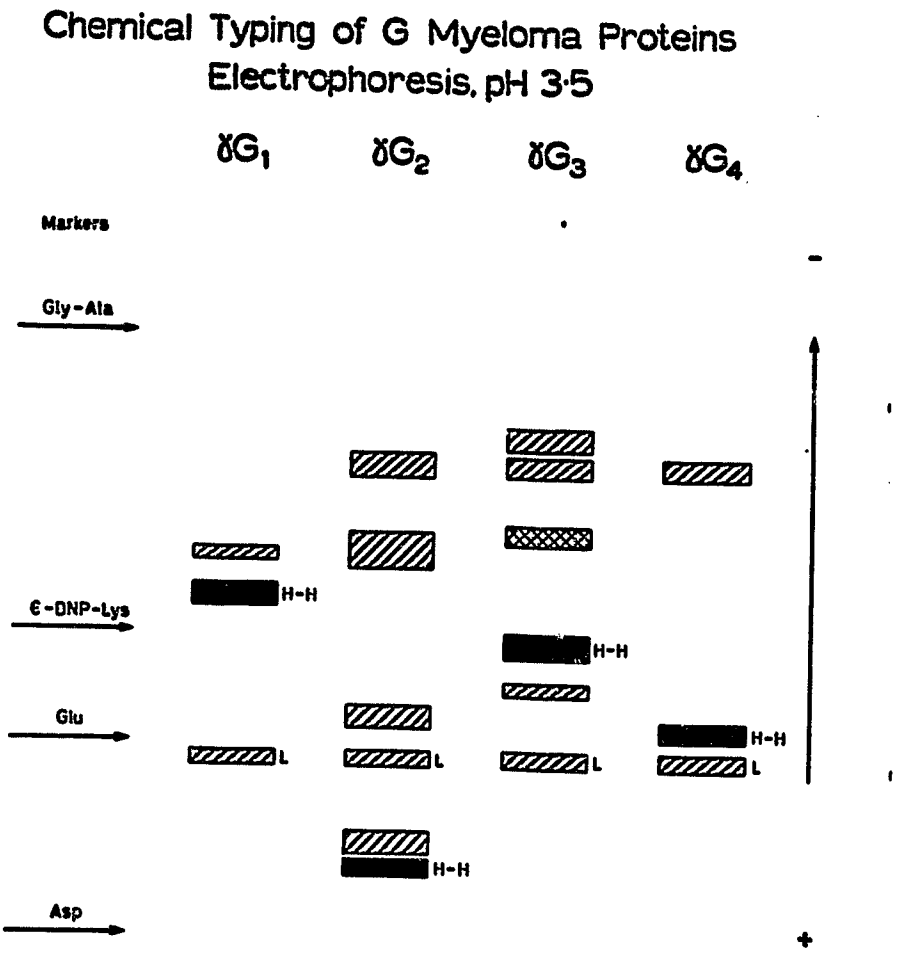


Fig. 4-18

Drawing of the typical chemical typing autoradiograph of the four IgG subclasses. The boxes represent the position of the radioactive peptides. The most radioactive band characteristic of each subclass is represented by the blackend boxes. These major peptides are composed of two ^{14}C-carboxymethylated cysteines which are involved in the interheavy chain (H-H) disulfide bonds. L indicates the radioactive peptide derived from K-type light chains. All other peptides are derived from the corresponding heavy chains.

(see following page for figure)

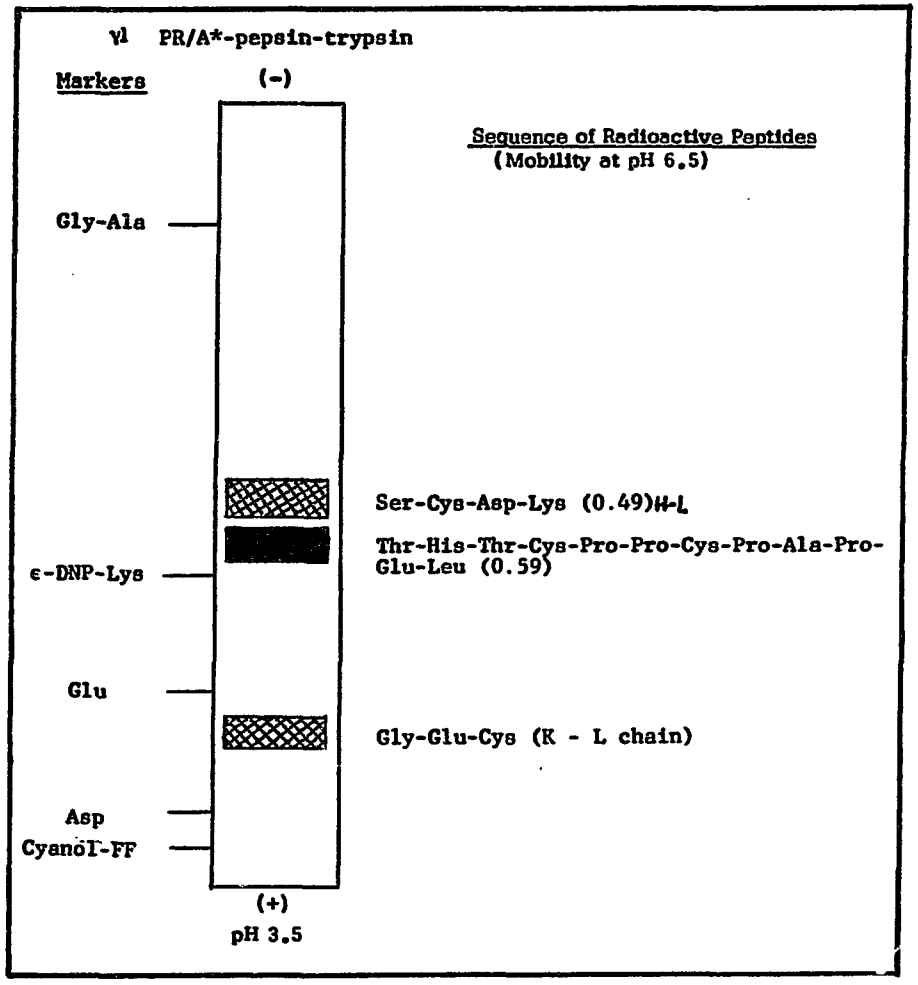
Fig. 4-19

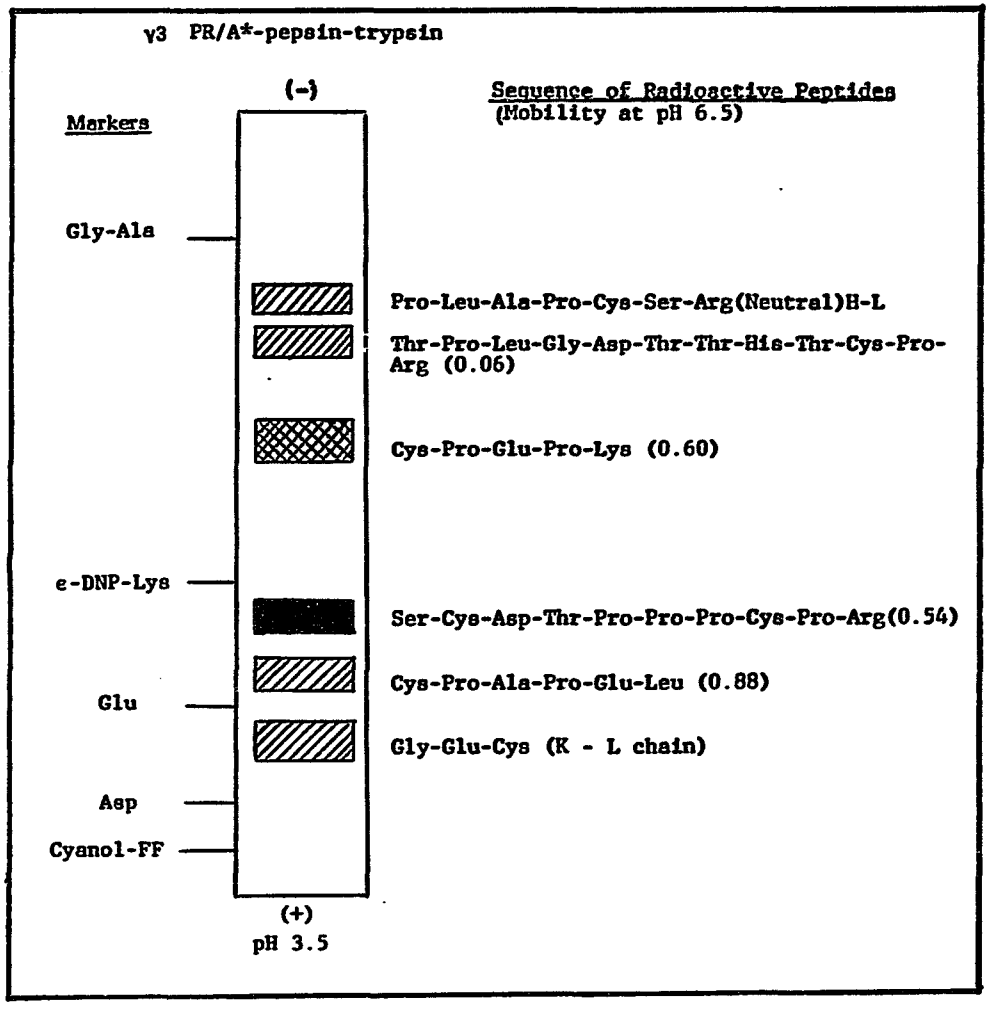
The figures on the following page show the amino acid sequence of the major radioactive paptides found in prototype IgG1 and IgG3 immunoglobulin molecules after pepsin-trypsin digestion of the partially reduced and radioalkyated myeloma proteins followed by high voltage paper eletrophoresis at pH 3.5 (chemical typing). The drawings represent the autoradiograps of the digested proteins after electrophoresis. The radioactive paptides are purified by further paper electrophoresis at pH 6.5 and pH 2.1 before elution and analysis. The radioactive band running below the marker Glu represents the cysteine-containing peptide present in κ-type light chains (K - L chain) which forms the inter-heavy-to-light chain disulfide bridge with a peptide in the heavy chain (H-L). All of the other radioactive peptides are located in their respective heavy chains and form the inter-heavy chain disulfide bridges. The numbers in parentheses after each sequence refer to the approximate mobility of that peptide at pH 6.5 relative to aspartic acid (Asp = 1.0). The anode and cathode are marked in the drawings.

Fig. 4-19

Sequence of Radioactive Peptides Derived After

Chemical Typing of Prototype IgG1 and IgG3 Myeloma Proteins





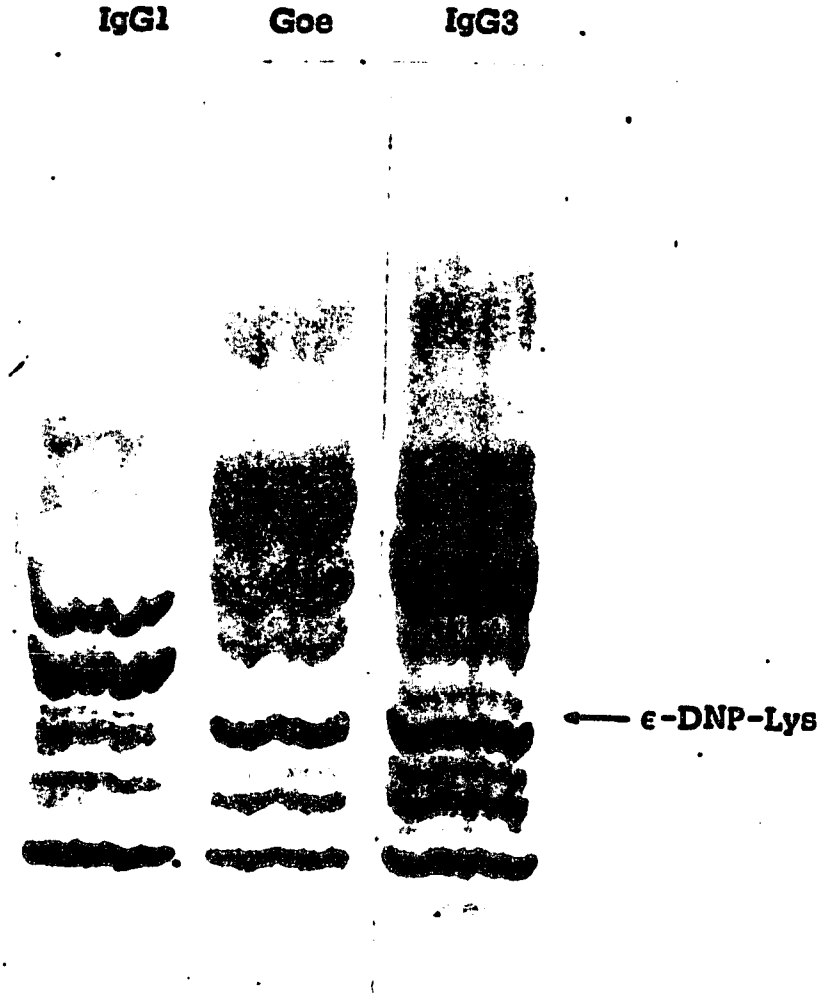


Fig. 4-20

Chemical typing of myeloma protein Goe as compared with an IgG1 (Bacon) and an IgG3 (Kup). All three digests were run on the same paper under identical conditions. The major radioactive peptide in IgG1 has a mobility slightly greater than the marker ε-DNP-Lys, while the IgG3 radioactive peptide has a slightly slower mobility than ε-DNP-Lys. The chemical typing pattern of protein Goe appears to be identical to IgG3 (Kup). The paper was exposed to X-Ray film for 48 hours prior to development.

(see following page for table)

Table 4-9

This table lists the composition of the radioactive peptides isolated from chemical typing of individual myeloma Y1 (Bacon), Y3 (Kup) and Goe proteins. Each radioactive peptide was isolated by repeated paper electrophoresis at three different pH values (pH 3.5, pH 6.5 and pH 2.1). The mobilities at pH 6.5 are noted. Expected composition values for each peptide are indicated in parentheses ( ) next to the appropriate residue. Relative electrophoretic mobility of each peptide is noted by the arrow: + ⟶ -, where (-) shows the fastest mobility at pH 3.5.

Table 4-9

Composition of Radioactive Peptides Isolated from Chemical Typing of Individual Myeloma Proteins (γ1, γ3, Goe)

	Bacon (γ1)		Kup (γ3)					Goe			
	+ → -		+ → -					+ →			
Residue	1	2	1	2	3	4	5	1	2	3	4
CMC	1.5 (2)	0.7 (1)	0.7 (1)	1.3 (2)	0.9 (1)	0.4 (1)	0.5 (1)	0.6 (1)	1.7 (2)	0.9 (1)	0.5 (1)
Asp		1.2 (1)		1.3 (1)		1.3 (1)	0.5		1.7 (1)		1.2 (1)
Thr	1.8 (2)			1.0 (1)		3.3 (4)	0.5	0.7	1.1 (1)		3.3 (4)
Ser				1.0 (1)			1.0 (1)		0.9 (1)		
Glu	1.0 (1)		1.0 (1)		1.0 (1)		0.4	1.1 (1)		1.1 (1)	
Pro	4.6 (4)	0.9 (1)	2.3 (2)	4.0 (4)	2.1 (1)	1.9 (2)	2.1 (2)	2.1 (2)	3.8 (4)	2.0 (2)	2.3 (2)
Gly						1.8 (1)	0.5				1.3 (1)
Ala	1.0 (1)		1.0 (1)				1.2 (1)	0.8 (1)			1.5
Val							0.3				
Met											
Ile											
Leu	1.0 (1)		1.0 (1)			1.5 (1)	1.3 (1)	0.7 (1)	0.4		1.8 (1)
Tyr											
Phe											
His	1.1 (1)					0.9 (1)					0.9 (1)
Lys		1.3 (1)									
Arg				1.0 (1)	1.0 (1)	0.9 (1)	0.8 (1)		0.9 (1)	1.1 (1)	0.9 (1)
Mobility, pH 6.5	0.60	0.37	0.76	0.50	0.53	0.10	0.05	0.76	0.50	0.53	0.10

4.11 Genetic typing

Typing for genetic markers by hemagglutination inhibition was carried out on samples of Kup and Goe whole sera as well as on their respective purified monoclonal proteins. Table 4-10 shows the genetic marker analysis of Kup and Goe whole sera. In addition to the markers shown in this table, the isotypes of all the four subclasses were found to be present in both sera. Table 4-11 shows the results of the genetic marker analysis of the corresponding monoclonal proteins from Kup and Goe sera and lists the source of the antiserum, its specificity (isotypic or allotypic), as well as the suspected region (CH1, hinge, etc.) that the antiserum detects. The IgG1 myeloma protein component from Fishman serum was typed for comparison.

The γ3 allele for the IgG3 myeloma protein from Kup is G3m(b0, b1, b3, b4, b5, u, v). The γ3 allele for the myeloma protein from Goe is G3m(b0, b3, b5, s, t, v). The other allele of Goe is most probably G3m(b0, b1, b3, b4, b5, u, v). The two γ3 alleles from Goe differ only in allotypes of their CH2 domain and not in their CH3 domain. By genetic typing, the isolated myeloma protein from Goe was very pure with only very slight traces of IgG1, IgG2, and IgG4 (personal communication, E. van Loghem). Protein Goe, therefore, does not possess any of the other subclass isotypes or allotypes, although it does possess a rare haplotype found primarily in Mongoloid populations (see Table 1-5).

Genetic Marker Analysis of Myeloma Sera

Sample	Gm Factors																Am fact.		Km fact.	
	γ1				γ2	γ3											α2		κ	
	z	a	x	f	n	g	b^0	b^1	b^3	b^4	b^5	s	t	c^3	c^5	u	v	1	2	1	3
Goe serum	+	+	-	+	+	-	+	+	+	+	+	+	+	-	-	+	+	+	+	-	+
Kup serum	-	-	-	+	+	-	+	+	+	+	+	-	-	-	-	+	+	+	-	-	+

Table 4-10

This table shows the results of the genetic marker analysis of whole Goe serum and whole Kup serum. A (+) indicates the presence of the appropriate marker.

Genetic Marker Analysis of Myeloma Sera

Sample	Gm Factors																		Am fact.		Km fact.	
	γ1				γ2	γ3													α2		κ	
	z	a	x	f	n	g	b^o	b^1	b^3	b^4	b^5	s	t	c^3	c^5	u	v		1	2	1	3
Goe serum	+	+	-	+	+	-	+	+	+	+	+	+	+	-	-	+	+		+	+	-	+
Kup serum	-	-	-	+	+	-	+	+	+	+	+	-	-	-	-	+	+		+	-	-	+

Table 4-10

This table shows the results of the genetic marker analysis of whole Goe serum and whole Kup serum. A (+) indicates the presence of the appropriate marker.

Genetic Marker Analysis of Myeloma Proteins

Antiserum	Specificity	Region	Fishman	Kup	Goe
3272, human	Glm(z)	CH1	-	-	-
3294, human	Glm(a)	CH3	-	-	-
LOE, human	Glm(f)	CH1	+	-	-
SK8, sheep	γ1	CH2	+	-	-
Hei, gibbon	γ1	CH2	+	-	-
KH16-87, rabbit	γ2	CH2	-	-	-
KH16-56, rabbit	G3m(b0)	CH3	-	+	+
MFu, monkey	G3m(b1)	CH2	-	+	-
3057, human	G3m(b3)	CH3	-	+	+
G84, human	G3m(b4)	CH2	-	+	-
2123, human	G3m(b5)	CH3	-	+	+
2624, human	G3m(s)	CH2	-	-	+
2639, human	G3m(t)	CH2	-	-	+
B755, baboon	G3m(u)	CH2	-	+	-
Ray, human	G3m(v)	CH3	-	+	+
Bab 5, baboon	γ3	hinge	-	+	+
SK 3, sheep	γ3	hinge	-	+	+
NR, bovine	γ3	Fc	-	+	+
KH16-102, rabbit	γ4	Fc	-	-	-
K21743, rabbit	nGlm(z)	CH1	+	+	+
B 4, baboon	nGlm(a)	CH3	+	+	+
Ad, chimp	nG3m(b0)	CH3	+	-	-
Z 1, macaca	nG3m(g)	CH2	-	+	+
KH19-06, rabbit	κ	CL	+	+	+
K21744, rabbit	λ	CL	-	-	-
K21778, rabbit	Km3	CL	+	+	+
K21749, rabbit	γ3	CH1	-	+	+

Table 4-11

This table lists the results of the genetic marker analysis of purified protein Goe along with purified Kup protein (γ3) and Fishman protein (γ1). The type of antiserum used, its specificity (isotypic, allotypic) and the proposed region of activity are noted. + indicates the presence of the appropriate marker.

4.12 Anti-Goe antisera

In an attempt to determine if protein Goe possessed any unique or unusual antigens that may possibly be shared by normal immunoglobulins or other myeloma proteins, two rabbits were immunized with purified Goe protein in complete Freunds adjuvant. Absorption of non-specific and cross-reactive anti-IgG activity was accomplished by first passing the antiserum through a column of an IgG-κ myeloma protein bound to Sepharose 4B, and then through a similar column of an IgG1-λ myeloma protein. Fig. 4-21 (top), shows the double diffusion analysis of the absorbed anti-Goe antiserum developed against protein Goe, normal serum, and concentrated Cohn's fraction-II (CF-II). This antiserum gave lines of non-identity between Goe and normal serum and between Goe and CF-II, but normal serum and CF-II gave a line of complete identity. However immunoelectrophoresis (Fig. 4-21, bottom) of normal serum developed against anti-Goe antiserum showed a single arc with a mobility described for transferrin, while against immunoelectrophoresed Goe serum, two arcs were apparent which corresponded to the mobilities of transferrin and γ-globulin. Purified Goe gave a single arc with a mobility in the γ-globulin region. Anti-Goe was then tested by double diffusion analysis against three other IgG3, five IgG1, two IgG2 and four IgG4 myeloma proteins without forming a precipitin line. Anti-Goe antiserum was also tested against a single Mongoloid sera and showed only the anti-transferrin activity. The antibody

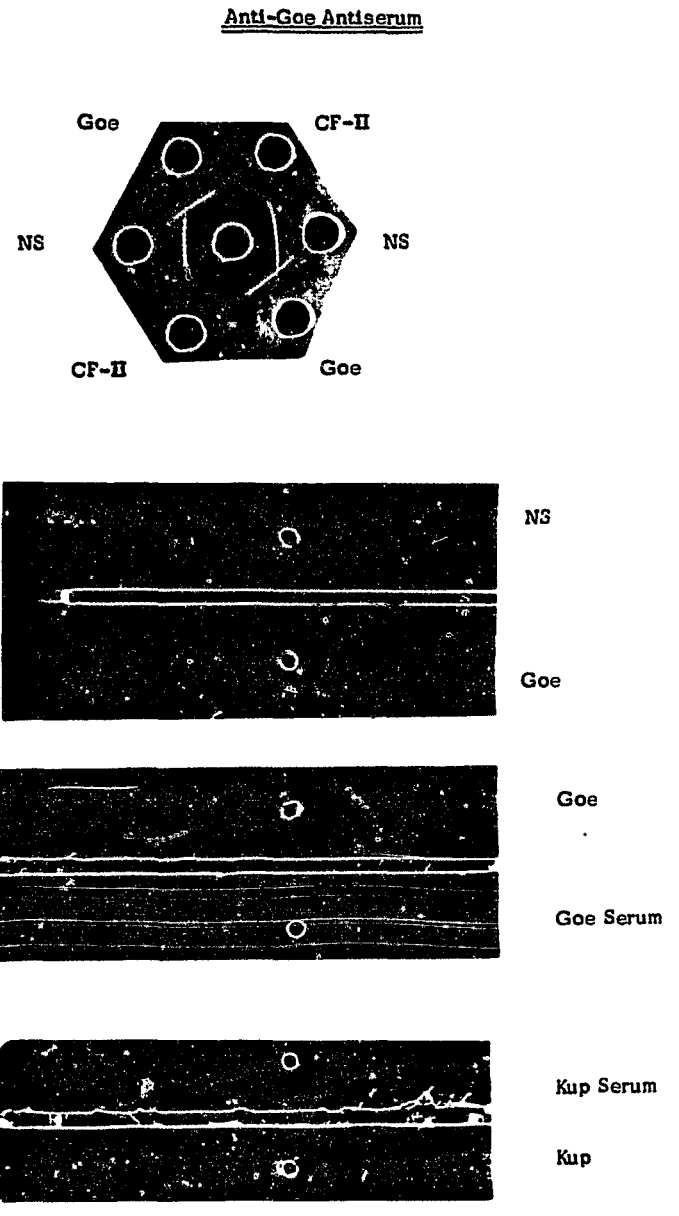


Fig. 4-21

Analysis of anti-Goe antiserum. Top: Double diffusion Ouchterlony against the purified protein Goe (1 mg/mg normal sera (NS), and concentrated Cohn's fraction-II (CF-II) ~ 50 mg/ml. Bottom: Immunoelectrophoresis of normal sera (NS), purified protein Goe, whole Goe serum, whole Kup serum, and purified protein Kup, all against adsorbed anti-Goe antiserum.

activity in anti-Goe antiserum seems specific only for protein Goe and may therefore represent anti-idiotype activity.

4.13 Antigenicity of the F(ab)'$_2$ fragments

To ascertain whether anti-Goe antiserum activity was directed against an Fc determinant, which may describe a new subclass related antigen, or against an Fab determinant, which may describe an anti-idiotypic antigen, precipitation tests were carried out using F(ab)'$_2$ fragments generated from Goe. Protein Goe was digested at 10 mg/ml in 0.1 M sodium acetate buffer, pH 4.2, at an enzyme/substrate ratio of 1:50. The digestion was allowed to proceed for 24 hours at 37°C and finally terminated by bringing the reaction mixture to pH 7.0 with the addition of Tris-base. The neutralized digest was then passed directly through protein A-Sepharose (Fig. 4-22A). Protein samples before and after digestion and after passage through protein A-Sepharose were run on PAGE (Fig. 4-22B). Double diffusion Ouchterlony analysis of the F(ab)'$_2$ fragments generated by the digest was performed using anti-Goe, anti-γ3 hinge, anti-κ light chain and anti Fc antisera. Control IgG1 and IgG3 molecules as well as transferrin and CF-II were also included (Fig. 4-22C).

Unlike intact protein Goe, the pepsin digested material did not bind to protein A-Sepharose (Fig. 4-22A) indicating an alteration (e.g. by digestion) of the Fc-protein A reactive fragments. The SDS - PAGE profile of intact Goe gave the expected γ3 molecular weight heavy chain. Although this

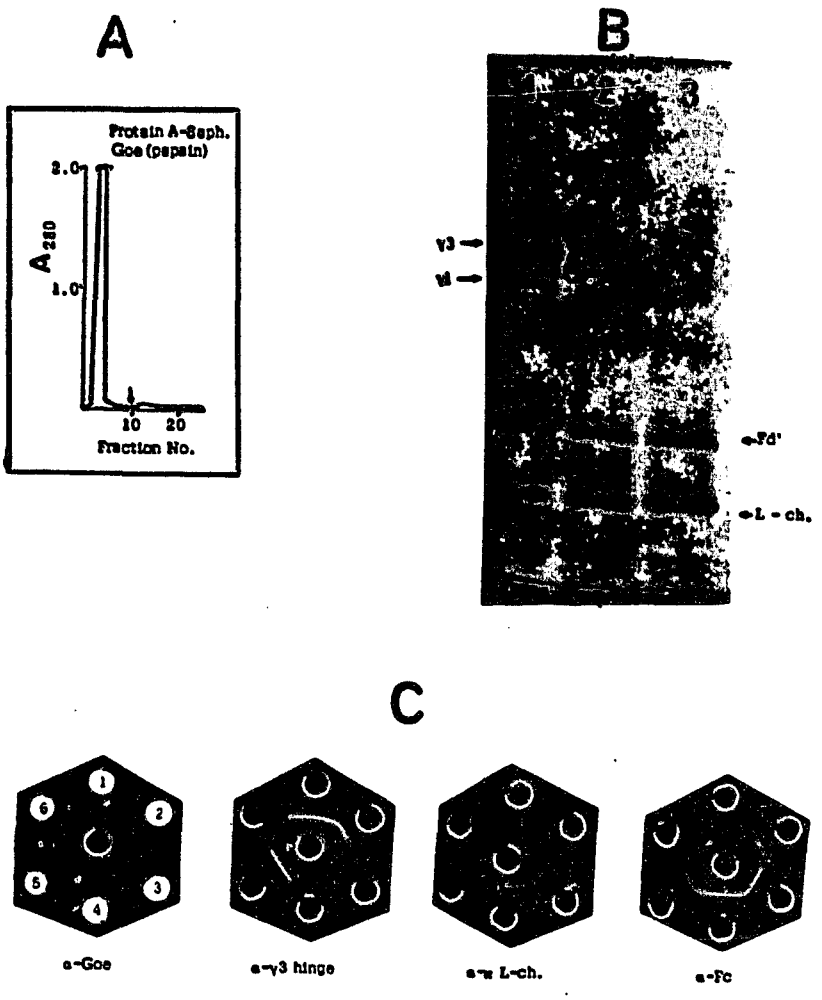


Fig. 4-22

A. Protein A-Sepharose column of Goe digested with pepsin (see text). Column initially eluted with PBSA and then (at arrow) with 1 M acetic acid.
B. 10% PAGE in SDS run under reducing conditions. slot 1: Goe before pepsin digestion; slot 2: Goe after pepsin digestion; slot 3: pepsin digested Goe after passage through protein A-Sepharose (i.e., the first peak in A).
C. Double diffusion Ouchterlony of: well #1 - pepsin-digested Goe; well #2 - purified protein Goe; well #3 - CF-II; well #4 - Fishman (IgG1); well #5 - Kup (IgG3); well #6 - transferrin. These six antigens were run against anti-Goe antisera (α-Goe), anti- γ3 hinge antisera (α- γ3 hinge), anti-κ light chain antisera (α-κ L-ch.), and anti-Fc antisera (α-Fc).

particular sample was somewhat contaminated with γ1 molecular weight heavy chains, their presence does not interfere with the present experiments (Fig. 4-22B, slot 1). The digested material (slot 2, Fig. 4-22B) showed the expected $F(ab)'_2$ subunits which consists of a light chain and an Fd' fragment. The digested material passaged through protein A-Sepharose (slot 3, Fig. 4-22B) was identical to the digested material before passage. In double diffusion analysis, anti-Goe antiserum reacted with whole Goe (well 2) and transferrrin (well 6) but did not react with an IgG3 (well 5), and IgG1 (well 4) or CF-II (well 3). Anti-Goe antiserum did react with pepsin digested protein Goe, which gave a line of non-identity with transferrin and a line of identity with whole Goe. Anti-γ3 hinge antiserum reacted only with IgG3, whole Goe and also pepsin-digested Goe, indicating the presence of γ3 hinge (i.e. Fd' and Fd) in the digested sample. Anti-κ light chain reacted with all samples except for transferrin. Anti-Fc antiserum reacted with all samples except transferrin and pepsin digested protein Goe suggesting the absence of Fc determinants due to digestion by pepsin.

Since the pepsin digested protein Goe contains anti-γ3 hinge and anti-κ light chain activity without any anti-Fc activity, it is reasonable to assume that the anti-Goe activity lies in the $F(ab)'_2$ fragment. The antiserum most probably detects idiotypic determinants that are unique to protein Goe and not a new subclass related antigen which may have been related to the unusual nature of the Fc region of Goe.

-Chapter 5-

Studies on Normal IgG

5.1 Protein A binding of normal IgG

5.2 Isolation of total IgG3

5.3 Protein A binding of normal IgG3

-Chapter 5-

Studies on Normal IgG

5.1 Protein A binding of normal IgG

Protein A affinity chromatography of Cohn's fraction-II (CF-II), which is an enriched Y-globulin fraction prepared by ethanol precipitation (Miles Lab.) yields both a bound and unbound fraction. When run under reducing conditions on SDS-PAGE (10%), CF-II is shown to consist mostly of $\gamma 1$ molecular weight heavy chains and a small amount (Table 1-2) of $\gamma 3$ molecular weight heavy chains (Fig. 5-1A, CF-II). The protein A-unbound fraction, PA-I, yields the $\gamma 3$-like molecular weight heavy chains while the bound material, PA-II, eluted with 1 M acetic acid yields the $\gamma 1$-like molecular weight heavy chains. However, upon overloading the gel with PA-II material, a small amount of a $\gamma 3$-like molecular weight band becomes apparent (Fig. 5-1A, CF-II PA-II). Previously, other investigators have also detected a small portion of IgG3 on the protein A-bound material using specific anti-IgG3 antiserum (Skvaril, 1976; van Kamp, 1979). These observations have raised the possibility that there exists in normal IgG3 a fraction that reacts with protein A. The description in this thesis of an IgG3-like myeloma protein, Goe, that binds to protein A through its Fc region supports this theory. The

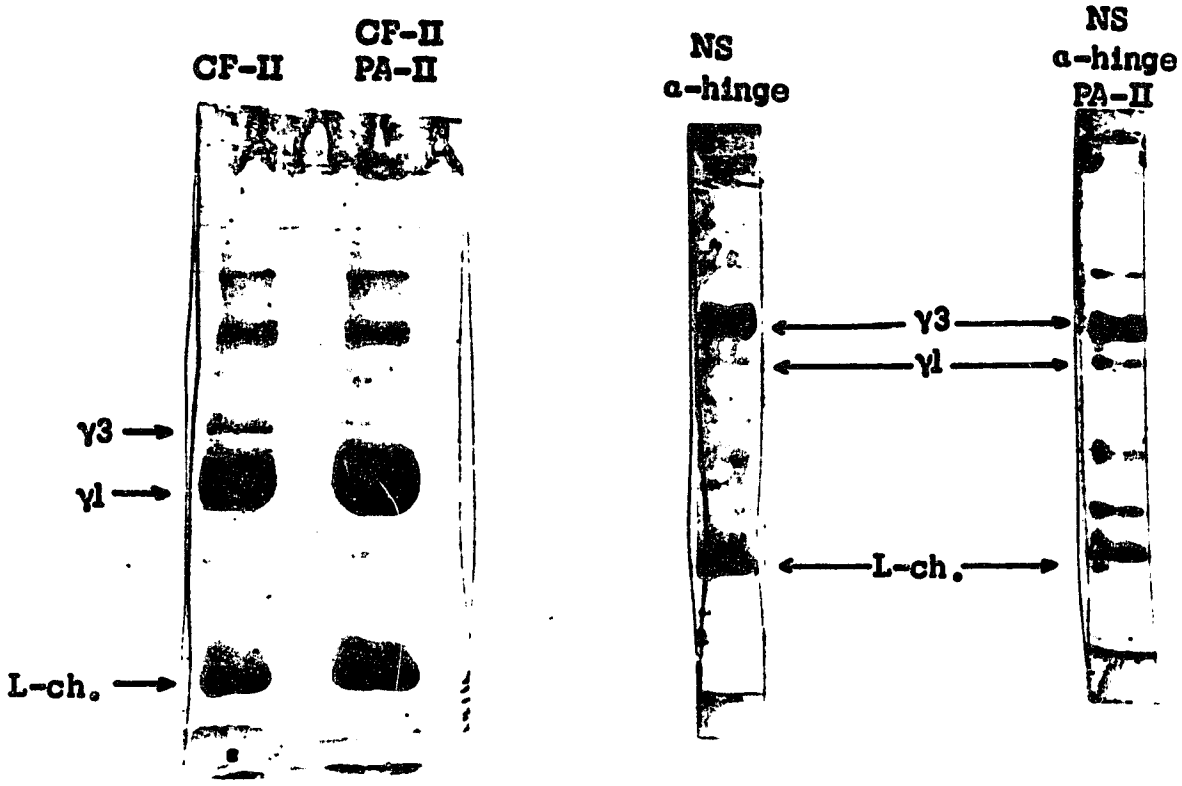


Fig. 5-1

A. 10% PAGE in SDS of Cohn's fraction-II (CF-II) and of Cohn's fraction-II material that binds to protein A-Sepharose (CF-II, PA-II).

B. 10% PAGE in SDS of the fraction of normal sera (NS) that is bound to an anti- γ3 hinge affinity column (α -hinge), and then bound to protein A-Sepharose (PA-II). Reducing conditions were used in all gels.

following sections describe the isolation and characterization of the protein A-reactive IgG3 fraction.

5.2 Isolation of Total IgG3

Previous studies have used protein A-Sepharose to isolate IgG3 from normal human sera (Hjelm, 1975), but as discussed in Sec. 5.1, the IgG3 isolated in this manner would be devoid of the fraction of IgG3 that was bound to protein A. As an initial step in isolating protein A-reactive IgG3, total IgG3 would have to be isolated. Since both the protein A-bound and protein A-unbound IgG3-like molecules contain the extended hinge region (seen as an increase in apparent molecular weight on SDS gels) isolation of total IgG3 was attempted using specific anti- $\gamma 3$ hinge antiserum.

Anti-$\gamma 3$ hinge antiserum (α-hinge) was prepared in rabbits immunized against $\gamma 3$ heavy chain disease protein Omm-s, a protein which lacks the V_H and CH1 domains but contains an intact $\gamma 3$ hinge region (Franklin et al., 1975). Contaminating, non-$\gamma 3$ hinge antibodies were absorbed by passage of the antiserum first through a column of an IgG1-K myeloma protein bound to Sepharose and then through another column with an IgG1-λ myeloma protein. The absorbed antiserum was then passaged through a column of IgG3 (Kup) bound to Sepharose and α-hinge antibodies were eluted with 1 M acetic acid. The α-hinge antibodies were

tested for their specificity by double diffusion against a number of purified myelomas of all four subclasses (Fig. 5-2). The α-hinge antibodies reacted only with the IgG3 subclass including proteins Kup and Goe. These antibodies also reacted against CF-II at 20 mg/ml but not at 1 mg/ml which was expected if IgG3 comprised only about 6% of the total IgG. The α-hinge antibodies also reacted with normal serum in which the IgG3 concentration was about 1 mg/ml and gave a line of identity with a known IgG3 myeloma (Fig. 5-2). However no reaction was seen with transferrin and therefore the reaction with normal serum cannot be a contaminant anti-transferrin antibody. Fig. 5-2 also shows the expected reaction with CF-II (PA-I) - the material from CF-II that does not bind to protein A (i.e., IgG3). Therefore it seems that the α-hinge antibodies were specific for all IgG3 molecules.

To isolate the total IgG3 population, normal serum was passed through an immunoabsorbant made with the purified α-hinge antibodies. Fig. 5-1B shows the SDS-PAGE under reducing conditions of the material from normal serum which was bound and eluted from the α-hinge immunoabsorbant (NS, α-hinge). Almost all of the heavy chain material possessed a γ3-like molecular weight with very little γ1 molecular weight material present. The one-step immunoa sorbant represents at least a thousand-fold purification of normal IgG3 from normal serum.

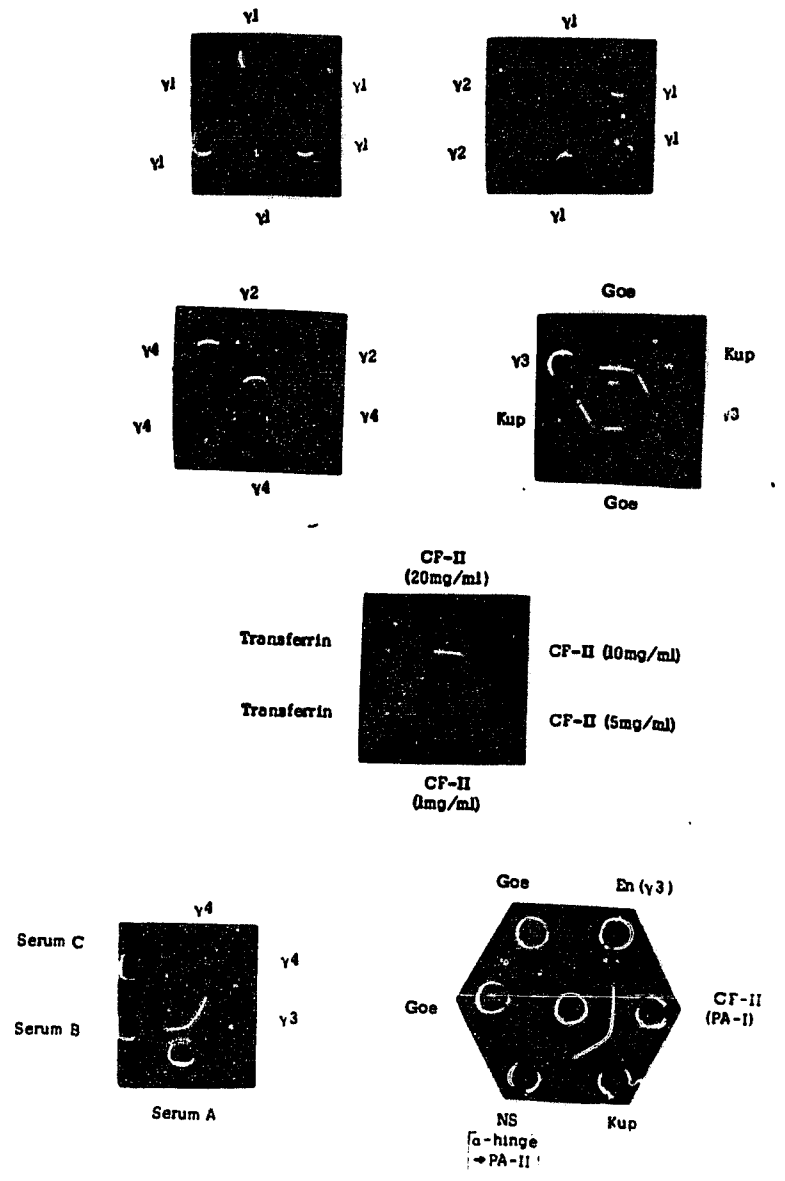


Fig. 5-2

Ouchterlony (double diffusion) analysis of anti-hinge antiserum tested against a number of antigens. Ten different IgG1 molecules (γ1), four different IgG2 molecules (γ2), four different IgG4 molecules (γ4), and three different IgG3 molecules (γ3 and Kup) were tested. Also included was Cohn's fraction II (CF-II) tested at 20, 10, 5 and 1 mg/ml, transferrin and three different serum samples (A, B and C). See text for explanation of other samples.

5.3 Protein A binding of normal IgG3

The purified IgG3 eluted by acid from the α-hinge immunoadsorbant was dialyzed against 0.15 M phosphate buffer, pH 7.0, and passaged through protein A-Sepharose. Most of the material passed through the column, however a minor fraction was retarded and eluted by the addition of 1 M acetic acid. SDS-PAGE (10%) of the protein A-bound IgG3 run under reducing conditions is shown in Fig. 5-1B (NS, α-hinge, PA-II) and consists mainly of γ3 molecular weight heavy chains, although a slightly higher percentage of γ1 molecular weight heavy chains seems to be present than in the starting material (Fig. 5-1B, NS, α-hinge). A limited amount of degradation is also apparent in the IgG3 material eluted from protein A, but previous studies have shown IgG3 molecules to be relatively sensitive to proteolytic cleavage (Natvig et al., 1967b; Virella and Parkhouse, 1971).

Fig. 5-2 shows, by Ouchterlony analysis, that the IgG3 that was bound to protein A was still reactive with α-hinge (NS, α-hinge → PA-II).

Chemical typing was performed on the IgG3 protein A-reactive material (Fig. 5-3). Although a very limited amount of material was available for this study, the chemical typing clearly showed the presence of the major γ3 radioactive peptides, in particular the band representing the major H-H γ3 peptide which migrates just below the ε-DNP-Lys marker.

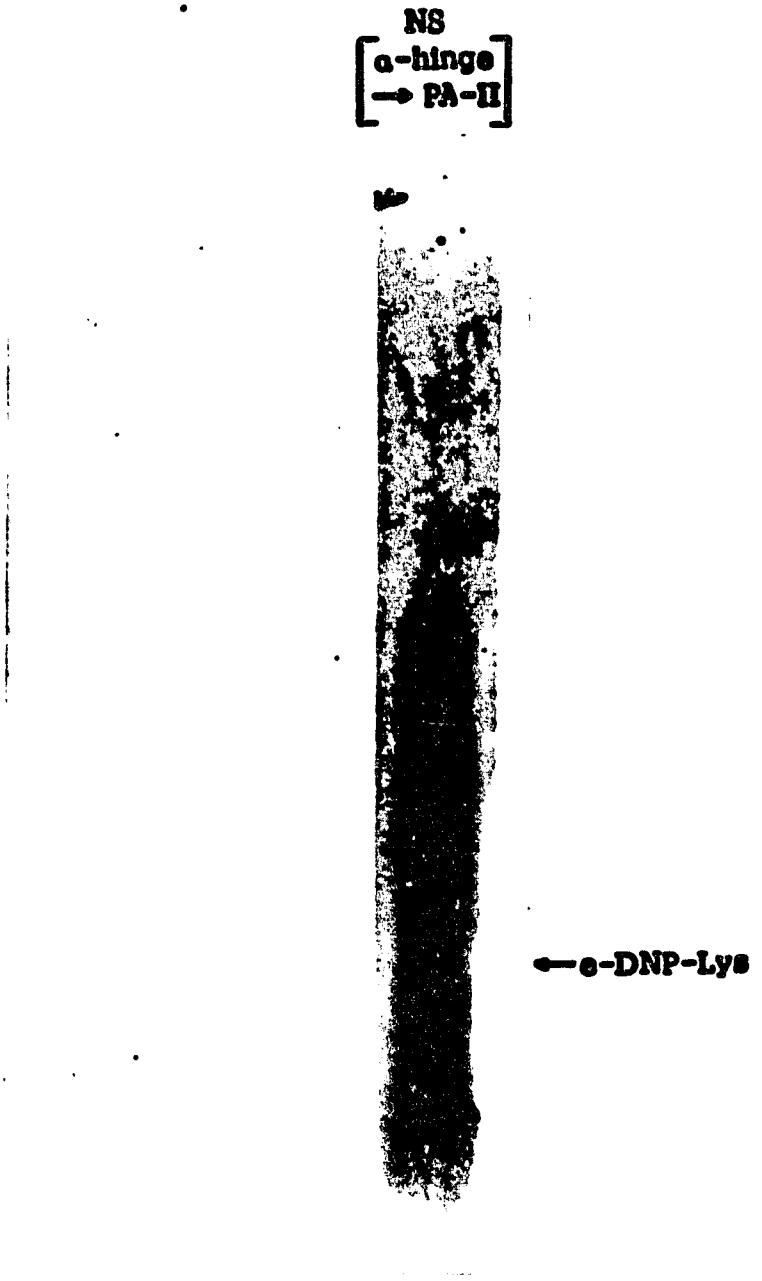


Fig. 5-3

Chemical typing — autoradiograph of the component of normal sera (NS) that binds to both anti-γ3 hinge antibodies and to protein A-Sepharose (α-hinge→PA-II). Note the IgG3-like banding pattern especially the characteristic dark γ3 H-H band immediately below the ε-DNP-lys marker. IgG1 molecules do not show this band.

Genetic typing of the protein A-bound IgG3 disclosed that all γ3 determinants of the CH1, CH2 and CH3 regions were present as were the G3m(b) allotypes as well as G3m(g) allotypes, although less of the latter one. At high concentrations of protein, a trace of the other subclasses was found but could be disregarded in the context of this experiment.

These studies, therefore, demonstrate the existence of IgG3 molecules present in normal sera that possess reactivity to protein A.

-Chapter 6-

Discussion

-Chapter 6-

Discussion

6.1 Introduction

The previous chapters have described in detail particular structural characteristics of two myeloma proteins. In addition, evidence has been presented for the existence of a new type of immunoglobulin present in normal sera, a prototype of which may be represented by protein Goe, the unusual myeloma protein studied in this thesis. Protein Kup, which was studied first as a prototype IgG3-κ immunoglobulin molecule, is a monoclonal cryoglobulin possessing the G3m(b) genetic markers, and has been shown to correspond in amino acid sequence to other G3m(b)-type myeloma proteins. Protein Goe was also found to possess the antigenic characteristics of an IgG3. Further studies showed that the hinge region in protein Goe was similar to that of other γ3 hinge regions, but its Fc region was composed of both γ1-like and γ3-like primary sequences, or sequences which have been previously correlated with those particular subclasses. In addition, protein Goe, unlike other IgG3 subclass immunoglobulin molecules, possesses the capacity to bind strongly to Staphylococcus aureus protein A-Sepharose.

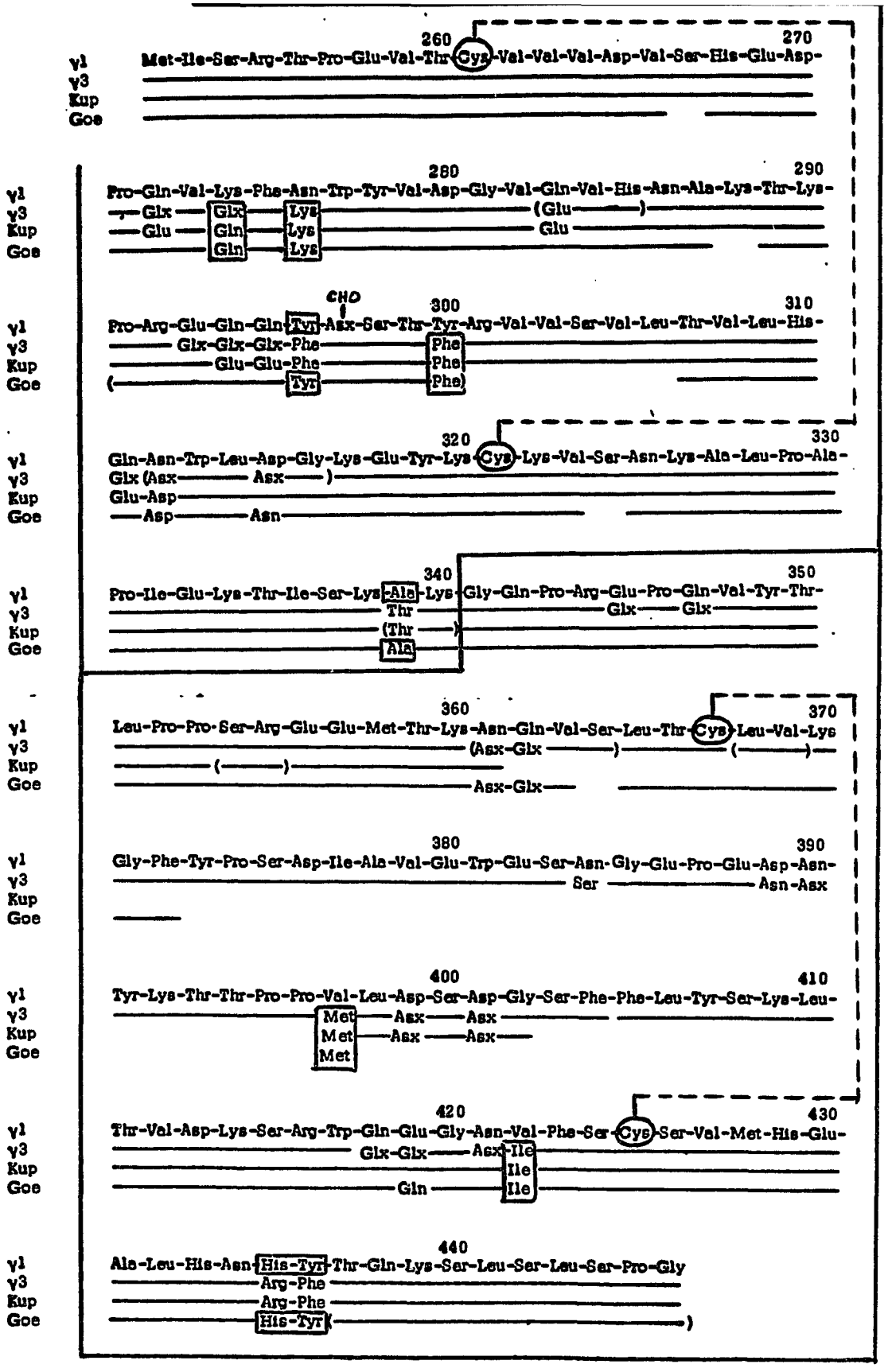
6.2 Sequence studies of the Fc region of Goe

Fig. 6-1 presents the amino acid sequence data of the various Fc region fragments derived from protein Kup and Goe and compares these sequences to both a completed γ1(Nie) Fc sequence (Ponstingl and Hilschmann, 1976) as well as a γ3 (Wis) Fc sequence (Frangion et al., in press). Kup Fc as

(see following page for figure)

Fig. 6-1

Proposed amino acid sequence of Fc fragments of proteins Kup and Goe as compared tc known γ1 and known γ2 sequences. Solid lines indicate residues identical to the γ1 sequence. Absence of a line indicates unidentified residue. Brackets ( ) indicates those residues placed by amino acid composition homology. Similarities in sequence between Goe and the other subclasses where subclass variation occur are inside boxes. Half-cystinyl residues are circled and intrachain disulfide bridges are noted. Each of the constant domains are enclosed by large boxes: CH2-residues (234-341), CH3-(residues 342-446). Attachment of carbohydrate is indicated by CHO above the appropriate amino acid. γ1 sequence (Nie) from Ponstingl and Hilschmann, (1976); $\gamma 3$ sequence (Wis) from Frangione et al., in press.


CH2
CH3
CHO
γ1
γ3
Kup
Goe
260 270
Met-Ile-Ser-Arg-Thr-Pro-Glu-Val-Thr-Cys-Val-Val-Val-Asp-Val-Ser-His-Glu-Asp-
280 290
Pro-Gln-Val-Lys-Phe-Asn-Trp-Tyr-Val-Asp-Gly-Val-Gln-Val-His-Asn-Ala-Lys-Thr-Lys-
300 310
Pro-Arg-Glu-Gln-Gln-Tyr-Asx-Ser-Thr-Tyr-Arg-Val-Val-Ser-Val-Leu-Thr-Val-Leu-His-
320 330
Gln-Asn-Trp-Leu-Asp-Gly-Lys-Glu-Tyr-Lys-Cys-Lys-Val-Ser-Asn-Lys-Ala-Leu-Pro-Ala-
340 350
Pro-Ile-Glu-Lys-Thr-Ile-Ser-Lys-Ala-Lys-Gly-Gln-Pro-Arg-Glu-Pro-Gln-Val-Tyr-Thr-
360 370
Leu-Pro-Pro-Ser-Arg-Glu-Glu-Met-Thr-Lys-Asn-Gln-Val-Ser-Leu-Thr-Cys-Leu-Val-Lys
380 390
Gly-Phe-Tyr-Pro-Ser-Asp-Ile-Ala-Val-Glu-Trp-Glu-Ser-Asn-Gly-Glu-Pro-Glu-Asp-Asn-
400 410
Tyr-Lys-Thr-Thr-Pro-Pro-Val-Leu-Asp-Ser-Asp-Gly-Ser-Phe-Phe-Leu-Tyr-Ser-Lys-Leu-
420 430
Thr-Val-Asp-Lys-Ser-Arg-Trp-Gln-Glu-Gly-Asn-Val-Phe-Ser-Cys-Ser-Val-Met-His-Glu-
440
Ala-Leu-His-Asn-His-Tyr-Thr-Gln-Lys-Ser-Leu-Ser-Leu-Ser-Pro-Gly

far as was determined, was identical in its primary sequence (excluding unknown amides) to the known γ3 sequence of protein Wis, which, like Kup, possessed the G3m(b) allotype. However, protein Goe was identical to neither the previously published γ1 or γ3 sequences.

The hinge region of protein Goe was studied by identification, isolation and purification of radioactive hinge-region peptic-tryptic peptides through a procedure known as chemical typing (see Chapter 4.10). Only γ3-like hinge region peptides were identified when compared with control γ1 and γ3 myeloma proteins. Because the heavy chain of protein Goe possessed a γ3-like heavy chain molecular weight on SDS - PAGE, it was assumed that Goe possessed the typical γ3 hinge primary sequence composed of a 17 amino acid segment followed by a triplicated 15 amino acid segment.

In the CH2 domain, the carbohydrate containing peptide (residues corresponding to positions 296-300) of Goe was similar to the homologous peptide in G3m(g) molecules. Both possess Tyr and Phe at positions 296 and 300, respectively, but differ from G3m(b) molecules which have Phe in both corresponding positions or γ1 molecules which have Tyr in both positions (see Table 1-10). Since the genotype of patient Goe was f; n; b0b1b3b4b5/za; n-; b0stb3b5, there could be no chance of contaminating G3m(g)-like molecules derived from either chromosome. It is possible, therefore, since the paraprotein was typed as having the genetic markers: za;

n-; b0stb3b5, that Tyr at position 296 may in fact represent the non-b1 determinant, an isoallotype which would also be present in all G3m(g) molecules as well as IgG1 molecules (see Table 1-4).

Another position which shows variation among the IgG subclass heavy chains is the residue at position 339 involving the region between the disulfide bridges of the CH2 and CH3 domains (i.e. an inter-domain region). At this position Goe possessed a residue of Ala which is usually found in γ1 heavy chains, whereas all other previously studied γ3 sequences had Thr in this position. Its significance in contributing to an antigenic determinant, whether isotypic or allotypic has not been established, but since protein Goe showed no γ1 subclass determinants, position 339 in itself probably does not contribute to such an isotypic marker. The possibility has not been excluded, however, that position 339 may be involved in the expression of allotypic markers.

Methionine at position 397 has been previously found in all γ3 heavy chains but is substituted for Val in all γ1 heavy chains studied to date. Although not sequenced directly, Met has been assumed to occupy position 397 in both Goe and Kup heavy chains. In Kup, a CNBr-derived fragment from pool IV, Fig. 3-6, possessing a manually-determined amino-terminal sequence of Leu-Asx-Ser-Asx was isolated which corresponds to residues 398-401. Non-specific cleavage of Val-Leu bonds

(i.e., positions 397-398 in γ1 chains) by treatment with CNBr has never been reported. Therefore, Met was tentatively assigned to position 397 in protein Kup. In Goe, as a result of the low amount of starting material and low yield of this peptide as determined when isolating it from protein Kup, the CNBr fragment beginning at residue 398 was not recovered. However, a CNBr fragment was isolated from pool VII, Fig. 4-13, which gave a manual amino-terminal sequence of Aba(Thr)-Lys and an amino acid composition (data not shown) similar to the CNBr fragment comprising residues 359-397. Therefore, Met was tentatively assigned to position 397 in protein Goe.

Position 422 was also found to be variable when γ1 and γ3 chains were compared. γ1 chains possessed Val whereas γ3 chains possessed Ile. Both Kup and Goe possessed the γ3 related substitution of Ile. These latter two substitutions, Met at 397 and Ile at 422 are both contained within the disulfide loop of the CH3 domain.

The two last subclass-related residue substitutions include the residues at positions 435 and 436 which are excluded from the region of the CH3 disulfide loop and therefore lie at the carboxy-terminal end of the domain. As seen in Table 1-9, Arg at position 435 has been found only in γ3 chains whereas His is present in all the other subclasses at this position. Position 436 is occupied by Tyr in all subclasses including γ3 except in γ3 molecules possessing the G3m(b) allotype in which this position is occupied by

Phe. Kup was shown to possess the typical G3m(b) substitutions of Arg-Phe at positions 435-436 respectively whereas Goe was shown to possess the typical γ1 substitutions of His-Tyr at the respective positions.

Fig. 6-2 summarizes the areas on Goe heavy chains where subclass related amino acid substitutions have been localized.

6.3 The binding to Staphylococcal aureus protein A

In humans it has been reported that all IgG subclasses except IgG3 bind to protein A isolated from the cell wall of a pathogenic strain of staphylococcus aureus (Kronwall and Williams, 1969). This binding has been localized to the Fc regions of immunoglobulin heavy chains (Forsgren and Sjoquist, 1966) and therefore is not a typical antibody-antigen reaction involving the antibody-combining (Fab) sites. Testing the binding of various Fc fragments (Endresen et al., 1974; Endresen and Grov, 1976) together with particular physiochemical measurements (Lancet et al., 1978) suggested that protein A binding occurs at the interface of the CH2 and CH3 domains. However, recent studies using crystals of the complex formed by the Fc of IgG and a binding fragment of protein A (FB) have identified residues of mutual contact (Deisenhofer, 1978). Contact residues of the Fc fragment were present in both the CH2 and CH3 domains and include, in the CH2 domain: Met-252, Ile-253, Ser-254, Val-305, Leu-309, His-310, Gln-311, and Asn-312; and in the CH3 domain: His-433, Asn-434, His-435 and Tyr-436. His-435 is present in all γ1,

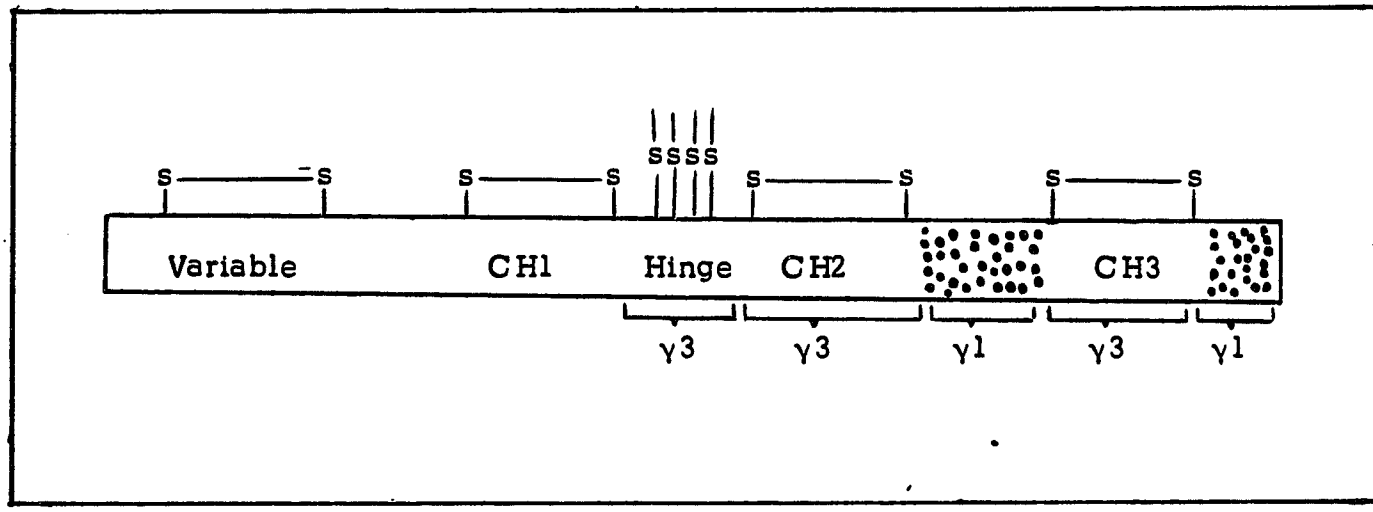


Fig. 6-2

This figure summarizes the areas on Goe heavy chains where supposed subclass related amino acid substitutions have been localized. Strippled areas ▨ are those regions in which γ1-like amino acid substitutions were located. The hinge region as well as the CH2 and CH3 domains encircled by their respective intrachain disulfide loops contains only γ3 related amino acid residues (i.e., residues where subclass related variation has been previously shown to occur.

γ2, and γ4 heavy chains but is substituted by Arg in all previously reported γ3 heavy chains (see Table 1-9). The more positively charged residue substitution of Arg, seen in either G3m(b) or G3m(g) IgG3 allotypes, is most probably responsible for the inability of protein A to bind to IgG3 molecules, since all the other contact residues appear to be constant throughout the subclasses. The non-binding of protein A can therefore be considered a marker for at least the His-to-Arg substitution at position 435. Since protein Goe does not possess this substitution (it has His at 435) it therefore should and in fact does show protein A binding, even though it possesses the antigenic determinants of the IgG3 subclass.

Of particular interest however, are the few recent reports of a minor portion of normal polyclonal IgG3 that seems to bind to protein A along with the IgG1, 2 and 4 subclasses (Skavril, 1976; van Kamp, 1979). These reports were reconfirmed and elaborated upon in this thesis (see Chapter 5) and indicate the existence of a sub-subclass of IgG3 which is distinguished by its ability to bind to protein A.

6.4 Unusual Genes

The alterations that could account for the presence of such an unusual immunoglobulin molecule as protein Goe most likely occurred at the gene level as a result of mutations and/or genetic recombinations. Alterations of genetic

material occurs continuously and examples of specific types of genetic rearrangements and mutations have appeared in the literature (reviewed by Natvig and Kunkel, 1973). Although the actual occurence of such genetic events has not been documented, the existence of rare gene types can often be explained in terms of simple genetic mechanisms. By studying and identifying common haplotypes and by detailed study of the chemical nature of allotypes, rare genes or gene complexes can be sorted out for further in-depth study (van Loghem and Natvig, 1970; Natvig et al., 1971a).

Three basic theories can be proposed to explain the occurence of a gene coding for an unusual IgG molecule that possesses properties of two different subclasses. The first two possible theories include mechanisms of genetic rearrangement and can be characterized as (1) genetic recombination and (2) gene splicing. The third possible theory is that the unusual protein is simply the rare, over expression (i.e., myeloma state) of a previously undetected gene present in most populations. These three theories will be discussed and their relation to the available data analyzed.

6.4.1 Genetic recombination

Two types of hybrid immunoglobulins thought to be due to intragenic recombination have previously been studied in detail.

One type of intracistronic recombination studied in immunoglobulins in the IgG4-IgG2 hybrid described by Natvig

and Kunkel (1972, 1974). This study was mainly concerned with the identification of subclass antigens and the localization of genetic markers in the CH1, CH2 and CH3 domains. Three antigenic markers normally found in the IgG4 subclass were found in the CH1 and CH2 domains of the hybrid protein, called La, which was isolated from the serum of a Negro male. In addition three antigenic markers normally found in the IgG2 subclass were found in the CH3 domain of the isolated La protein. The corresponding IgG4 markers in the CH3 domain and the corresponding IgG2 markers in the CH1 and CH2 domains were not detected as measured by hemagglutination inhibition techniques. Immunoglobulin fragments (Fab, Fc, pFc') were also utilized in inhibition techniques to help assign antigens to particular domains (e. g. markers that are inhibited by Fc but not pFc' fragments were assigned to the CH2 domain by elimination).

It was postulated that a hybridization due to unequal homologous crossing over took place which could have occurred through a misalignment of immunoglobulin genes during meiosis. In the case of La, the point of hybridization seems to be near the junction separating the CH2 and CH3 domains. A genetic event that could have produced an IgG4-IgG2 hybrid is shown diagrammatically in Fig. 6-3 where the first event would be a mispairing of homologous genes. The gene coding for the IgG2 heavy chain would be aligned with the gene coding for the IgG4 heavy chain and a subsequent recombination could have occurred in the region between the CH2 and CH3 domains. As

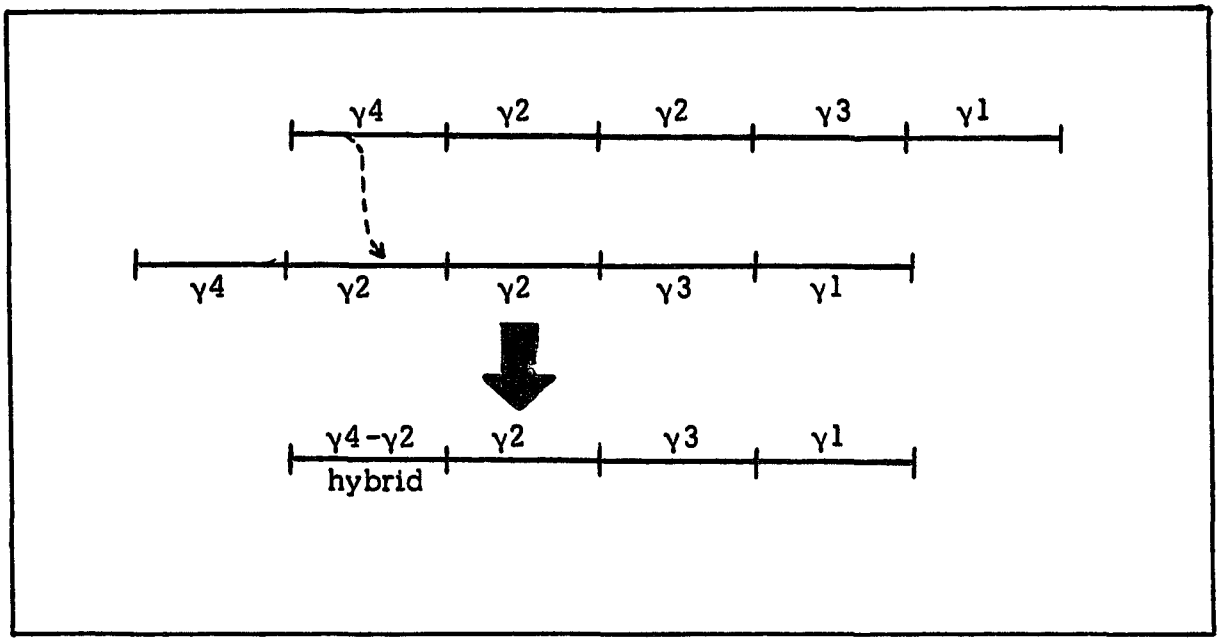


Fig. 6-3

The above diagram shows the possible events that could have taken place to explain the formation of an IgG4-IgG2 hybrid immunoglobulin gene. A mispairing at meiosis could have taken place which would lead to an unequal homologous crossover to produce the IgG4-IgG2 hybrid as shown. The two IgG2 genes are shown because of the finding of normal IgG2 molecules in certain individual sera presumed to be homozygous for the hybrid gene (adapted from Natvig and Kunkel, 1974).

shown in Fig. 6-3 this sort of recombination would result in the absence from the sera of any normal IgG4 molecules. However, normal IgG2 molecules were shown to be present because both IgG2κ-CH1 and IgG2-CH2 isotypic antigens were detected in the whole sera although not detected in isolated La protein. A duplication of the IgG2 gene prior to the formation of the hybrid gene could readily explain this finding. Similar duplications of the IgG1 gene have been reported in other studies (Natvig et al., 1971a). All the findings that produce such an IgG4-IgG2 hybrid molecule can therefore be explained as a result of a single genetic event. Moreover, the normal conterpart of protein La was detected in four out of 150 Negro sera screened but was not found in other populations.

The second type of intracistronic recombination studied was the IgG3-IgG1 type hybrid first described by Kunkel et al., (1969a) which was called 2904. Conclusions concerning the nature and existence of the hybrid molecule were drawn through hemagglutination inhibition and precipitation studies. Hemagglutination inhibition showed the presence or absence of certain antigenic determinants while precipitation studies were used to demonstrate that IgG1 determinants could be precipitated with antisera to IgG3 determinants. There was a curious absence of the major genetic markers in 2904 serum.

The results of the hemagglutination inhibition and precipitation experiments on 2904 showed the presence of an

IgG3-IgG1 hybrid molecule with the concurrent absence of any normal IgG1 or IgG3 molecules. It was proposed that an unequal homologous crossover took place similar to that of Lepore hemoglobins or to that of the IgG4-IgG2 hybrid, with subsequent elimination of multiple genetic antigens. A model for this proposal is shown in Fig. 6-4 in which two major Caucasian gene complexes are drawn. Once again, mispairing of the IgG3 and IgG1 genes at meiosis is followed by an intracistronic crossover to produce the hybrid gene. High nucleic acid sequence homology between IgG3 and IgG1 genes can promote such a mispairing. As shown in this diagram, the regions of the hybrid molecule that are retained just happen to be those that do not possess genetic markers, which would account for their absence in the serum.

The immunological data tell us whether or not certain determinants, which represent only a limited amount of amino acids in the whole sequence, are present. Further structural and chemical studies are necessary to characterize just how much of the amino terminus of the 2906 hybrid molecule is γ3-like and just how much of the hybrid is γ1-like. Werner and Steinberg (1974) have attempted to characterize the γ chain of the 2904 hybrid molecule by performing two dimensional thin layer fingerprints of Fc tryptic peptides and through amino acid analysis of the carboxy-terminal octadecapeptide. The carboxy-terminal octadecapeptide was shown to have a similar amino acid composition to that of the

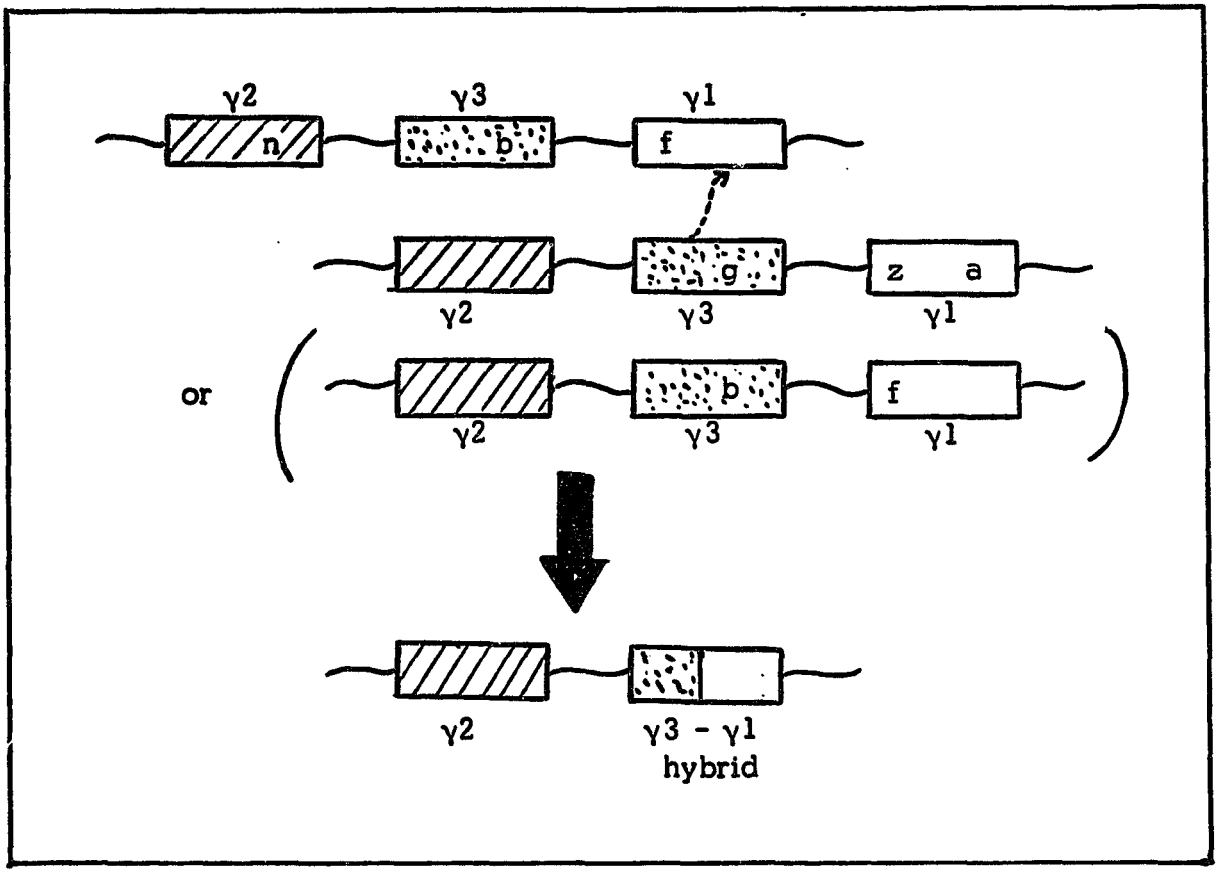


Fig. 6-4

The above figure shows the results of the mispairing and cross-over between an IgG1 gene with either one of the two IgG3 genes shown together with their respective gene complexes. These complexes represent major Caucasian haplotypes with the approximate position (i.e., amino or carboxy-terminal half) of some of the known genetic markers. Note the absence of genetic markers in the hybrid gene haplotype (adapted from Kunkel et al., 1969a.)

octadecapeptide derived from an IgG1 myeloma protein or from normal IgG (which is predominantly IgG1). Peptide maps of the Fc fragment have shown it to be most like a γ1 Fc fragment, although there was one γ3-specific peptide present when the fingerprint was compared to a fingerprint of a known γ3 myeloma Fc fragment. It was suggested that this peptide was a γ3 hinge region fragment although no supportive evidence (e.g., amino acid analysis or sequence studies) were presented.

Studies included in this thesis on the 2904 IgG3-IgG1 hybrid molecule (here called Entz) have shown that it reacts with specific anti-γ3-hinge antiserum (Fig. 5-2, sample En). On cellulose acetate electrophoresis (Fig. 3-1) Entz serum showed no monoclonal protein bond, but rather looked like a normal serum with a diffuse γ-globulin region. On SDS - PAGE (Fig. 3-2, slot 5) Entz serum showed no apparent myeloma band, although there seems to be a greater density of staining material in the γ3 heavy chain region when compared to normal serum (slots 1, 2 and 6). By chemical typing, protein Entz was shown to possess a γ3-like hinge region (data not shown). Moreover when passaged on protein A-Sepharose, both γ3 and γ1 molecular weight components from Entz serum was seen to bind strongly, identical to the situation with Goe serum (Fig. 4-2). Therefore, the γ3-like protein from the non-pathological serum of Entz showed similar protein A binding characteristics as did the myeloma protein from Goe serum.

Although protein Entz and protein Goe possess certain

properties in common in that they both have γ3-like hinge regions and both bind to protein A, Entz is probably a true hybrid since it was shown to contain antigenic determinants from both the IgG1 and IgG3 subclass, a situation that can be explained by a single crossover event at the genetic level (see Fig. 6-4). Goe, on the other hand showed only a normal array of IgG3 antigenic determinants and therefore is not the same type of molecule as Entz. Also by examining the proposed amino acid sequence of Goe (Fig. 6-1 and Fig. 6-2), it can be easily seen that no single crossover event between two known IgG sequences could have produced the desired sequence. Although one can envision multiple crossovers that would produce a γ3-γ1-γ3-γ1 type hybrid molecule as seen in protein Goe, such a series of events would be highly improbable. Genetic recombination alone, in terms of crossingover events, does not therefore satisfactorily explain the existence of the unusual immunoglobulin Goe molecule.

6.4.2 Gene splicing

As described in Chapter 1 (1.4), individual immunoglobulin chains have been shown to be products of multiple transcriptional units or exons which are separated at the DNA level by non-translated-intervening portions or introns (see Fig. 1-6). In general it has been proposed that the intervening sequence and its splicing mechanism may play an important role in the evolution of eukaryotic genes by linking duplicated genes that are not adjacent to each other

(Gilbert, 1978; Darnell, 1978). The recent reports that various mouse immunoglobulin genes are interrupted by intervening sequences at the junction of domains including the hinge region (Sakano et al., 1979; Early et al., 1979; Kataoka et al., 1979) along with the hypothesis that heavy chains have probably evolved by successive duplication of a domain (see Fig. 1-5) makes splicing mechanisms useful in explaining the construction of new immunoglobulin genes.

It has been postulated that gene splicing probably occurs in the joining of V and C exons. The possibility exists, as an alternative to classical genetic recombination, that individual C-region exons can undergo a rearrangement and a subsequent splicing mechanism to form unusual genes. One type of unusual gene would be the direct result of the loss of an exon. As described previously, the existence of molecules with apparent deletions of whole exons (or domains), as seen in heavy chain disease proteins, has been reported numerous times and is probably the most common of the myeloma variants described to date. A mouse $\gamma 1$ chain mutant (IF 2) was also reported to lack the whole CH1 domain (Milstein et al., 1974a). In addition, the hybrid-like variants described in the previous section (6.4.1) could also have been explained as a result of recombination taking place between different constant region genes at the junction of their respective domains. A single reassortment and subsequent splicing of separate constant domain exons from two

different subclasses could easily explain the existence of such hybrid molecules.

The direct splicing of two different immunoglobulin subclass constant region exons cannot fully explain the formation of a molecule structured like protein Goe. But the splicing of a specific number of regions of one subclass to another can result in the γ3-γ1-γ3-γ1 array of subclass related amino acid substitutions. As with genetic crossing over, this random type of splicing seems to be a highly unlikely event. However, upon further examination of where the subclass substitutions lie, one notices that all of the γ1-related amino acid substitutions are found outside of the intrachain disulfide loops which demark the basic CH2 and CH3 domains (Fig. 6-1 and Fig. 6-2). The definitive boundaries between the CH2 and CH3 domains have not yet been established, and furthermore, J segments associated with the CH2 and CH3 coding exons have not yet been identified. Therefore it is possible to postulate that the regions outside the disulfide loops may in fact contain the J segment involved in splice recognition and the joining together of domain region in RNAs. These J segments may be somewhat variable in that different subclass domains may possess different J segments. Protein Goe may therefore possess the intrachain disulfide loop γ3 regions (exons) but as a result of some sort of genetic rearrangement may have acquired the J segments corresponding to γ1 heavy chains which are expressed as γ1-

related amino acid substitutions. Until more DNA sequence studies have been made, especially with human Ig genes, these theories remain speculative.

6.4.3 Rare, unusual gene

It is apparent that no single, simple genetic event, whether by a crossover mechanism or by a gene splicing mechanism, is sufficient to explain the results of the amino acid sequence of protein Goe. Chapter 5 has given convincing proof of the existence of IgG3-like molecules which react with anti-γ3 hinge-specific antiserum and give a chemical typing pattern similar to other IgG3 molecules, but, like myeloma protein Goe, bind strongly to protein A. Therefore it is postulated that there may exist in normal serum a sub-population of IgG3 molecules which possess a γ1-like carboxy terminus which make them protein A reactive. The myeloma protein from the serum of patient Goe therefore represents the chance over-expression of the gene coding for the unusual protein which is normally found in low concentration. The demonstration of this sub-subclass of IgG3 is a direct result of studies undertaken on its myeloma counterpart found in Goe serum.

APPENDIX

The Three - and One - Letter Code for Amino Acids

Alanine	Ala	A
Cysteine (half-cystine)	Cys	C
Aspartic acid	Asp	D
Glutamic Acid	Glu	E
Phenylalanine	Phe	F
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Lysine	Lys	K
Leucine	Leu	L
Methionine	Met	M
Asparagine	Asp	N
Proline	Pro	P
Glutamine	Gln	Q
Arginine	Arg	R
Serine	Ser	S
Threonine	Thr	T
Valine	Val	V
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Aspartic acid or Asparagine	Asx	B
Glutamic acid or Glutamine	Glx	Z
α-Aminobutyric acid	Aba	

Nomenclature of IgG Subclasses

Subclass		
Current	Old	Heavy Chains
IgG1	γG1, We, γ2b	γ1
IgG2	γG2, Ne, γ2a	γ2
IgG3	γG3, Vi, γ2c	γ3
IgG4	γG4, Gc, γ2d	γ4

REFERENCES

Adlersberg, J. P., Franklin, E. C. & Frangione, B.: Repetitive hinge region sequence in human IgG3: Isolation of an 11,000 dalton fragment. Proc. Nat. Acad. Sci., U.S.A., 72: 723-727 (1975).

Ballieux, R. E., Bernier, G. M., Tominaga, K. & Putnam, F. W.: Gamma globulin antigenic types defined by heavy chain determinants. Science, 145: 168-170 (1964).

Bernardi, G.: Chromatography of proteins on hydroxyapatite. In: Methods in Enzymology. (Ed. by Jacoby), pp. 325-339 (1971).

Bhown, A. S., Mole, J. E., Weissinger, A. & Bennett, J. C.: Methanol solvent system for rapid analysis of phenylthiohydantoin amino acids by high pressure liquid chromatography. J. Chromat., 148: 532-535 (1978).

Blobel, G., Dobberstein, B.: Transfer of proteins across membranes. I. Presence of immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. J. Cell. Biol., 67: 835-851 (1975a).

Blobel, G., Dobberstein. B.: Transfer of proteins across membranes. II. Reconstitution of functional rough microsomes from heterologous components. J. Cell. Biol., 67: 852-861 (1975b).

Brack, C., Hirawa, M., Lenhard-Schuller, R., & Tonegawa, S.: A complete immunoglobulin gene is created by somatic recombination. Cell, 15: 1-14 (1978).

Brack, C. & Tonegawa, S.: Variable and constant parts of the immunoglobulin light chain gene of a mouse myeloma cell are 1250 nontranslated bases apart. Proc. Nat. Acad. Sci., U.S.A., 74: 5652-5656 (1977).

Brown, P. R. : In: High Pressure Liquid Chromatography: Biochemical and Biomedical Applications. (Academic Press, New York), p. 128 (1973).

Burnet, F. M.: In: The Clonal Selection Theory of Acquired Immunity. Vanderbilt Univ. Press, Nashville Tennessee (1959).

Capra, J. D. & Kunkel, H. G.: Aggregation of γG3 proteins: Relevance to hyperviscosity syndrome. J. Clin. Invest., 49: 610-621 (1970).

Cohen, S.: Properties of the peptide chains of normal and pathological human γ-globulins. Biochem. J., 89: 334-349 (1963b).

Cohn, M., Bloomberg, B., Geckeler, W., Raschke, W., Riblet, R., & Weigirt, M.: In: The Immune System, Genes, Receptors, Signals. (Ed. by Sercarz, E. E., Williamson, A. R., & Fox, C. F.) Academic Press, New York/London, p. 89 (1974).

Dammacco, J., Franklin, E. C. & Frangione, B.: An unusual papain fragment containing the V_H region of an IgG3 myeloma protein. J. Immunol., 109: 565-569 (1972).

Darnell, J. E., Jr.: Implications of RNA-RNA splicing in evolution of eukaryotic cells. Science, 202: 1257-1260 (1978).

Davis, B. J.: Disc electrophoresis: II. Method and application to human serum proteins. Ann.N.Y. Acad. Sci., 121: 404-436 (1964).

Deisenhofer, J., Jones, T. A. & Haber, A.: Crystallization, crystal structure analysis, and atomic model of the complex formed by a human Fc fragment and fragment B of protein A from Staphylococcus aureus. Hoppe-Seyler's Z. Physiol. Chem., 359: 975-985 (1978).

de Vries, J. X., Frank, R. & Birr, C.: The separation and quantitative identification of PTH-amino acids by high pressure liquid chromatography. FEBS lett., 55: 65-67 (1975).

Doolittle, A.: The amino-terminal amino acid sequence of rabbit immunoglobulin light chains. Proc. Nat. Acad. Sci., U.S.A., 55: 1195-1201 (1966).

Downing, M. R. & Mann, K. G.: High-pressure liquid chromatography analysis of amino acid phenylthiohydantoins: Comparison with other techniques. Anal. Biochem., 74: 298-319 (1976).

Dreyer, J. W. & Bennett, J. C.: The molecular basis of antibody formation: A paradox. Proc. Nat. Acad. Sci., U.S.A., 54: 864-869 (1965).

Early, P. W., Davis, M. M., Kaback, D. B., Davidson, N. & Hood, L.: Immunoglobulin heavy chain gene organization in mice: Analysis of a myeloma genomic clone containing variable and constant regions. Proc. Nat. Acad. Sci., U.S.A., 76: 857-861 (1979).

Edelman, G. M.: Dissociation of γ-globulin. J. Am. Chem. Soc., 81: 3155-3156 (1959).

Edelman, G. M.: The covalent structure of human γG Ig. XI. Functional Implications. Biochem., 9: 3197-3205 (1970).

Edelman, G. M. & Benacerraf, B.: On the structural and functional relations between antibodies and proteins of the gamma-system. Proc. Nat. Acad. Sci., U.S.A., 48: 1035-1042 (1962).

Edelman, G. M., Cunningham, B. A., Gall, P. D., Rutishauser, U. & Waxdal, M. J.: The covalent structure of an entire γG immunoglobulin molecule. Proc. Nat. Acad. Sci., U.S.A., 63: 78-85 (1969).

Edelman, G. M. & Gally, J. A.: The nature of Bence-Jones proteins. Chemical similarities to polypeptide chains of myeloma globulins and normal γ-globulins. J. Exp. Med., 116: 207-227 (1962).

Edelman, G. M., Heremans, J. F., Hermans, M. J. & Kunkel, H. G.: Immunological studies of Human γ-globulin: Relation tc the precipitin lines of whole γ-globulin to those of the fragments produced by papain. J. Exp. Med., 112: 203-223 (1960).

Edelman, G. M. & Poulik, M. D.: Studies on structural units of the γ-globulins. J. Exp. Med., 113: 861-884 (1961).

Edman, P.: Phenylisothiocyanate mechanism. Proc. Roy. Australian Chem. Inst., 24: 434 (1937a).

Edman, P.: On the mechanism of phenylisothiocyanate degradation of peptides. Acta. Chem. Scand., 10: 761-768 (1957b).

Edman, P.: Phenylthiohydantoins in protein analysis. Ann. N.Y. Acad. Sci., 88: 602-610 (1960).

Edman, P. & Begg. G.: A protein sequenator. Eur. J. Biochem., 1: 80-91 (1967).

Edman, P. & Henschen, A.: In: Protein Sequence Determination. (Ed. by Needleman), Springer, New York, p. 232 (1975).

Endresen, C. & Grov, A.: Further characterization of protein A reactive and non-reactive subfragments of Fc from human IgG. Acta. Path. Microbiol. Scand., Sec. C, 84: 397-402 (1976).

Endreson, C., Heggeness, M. & Grov. A.: Tryptic fragments of Fc from normal human IgG and their interaction with Staphylococcal protein A. Scand. J. Immunol., 3: 261-267 (1974).

Epstein, W. V. & Fudenberg, H. H.: Demonstration of Gm1(a) with anti-Gm1(a) specificities by tanned cells coated with individual γ-globulins. J. Immunol., 89: 293-299 (1962).

Fahey, J. L.: Two types of 6.6S γ-globulins and 18S γ-macroglobulins in normal serum and γ-microglobulins in normal urine. J. Immunol., 91: 438-447 (1963).

Fleischman, J. B., Porter, R. R. & Press, E. M.: The arrangement of the peptide chains in γ-globulin. Biochem. J., 88: 220-228 (1963).

Forsgren, A. & Sjoquist, J.: Protein A from Staphylococcus aureus. Pseudo-immune reaction with human globulin. J. Immunol., 97: 822-827 (1966).

Franêk, F.: Dissociation of animal 7S γ-globulins by cleavage of disulfide bonds. Biochem. Biophys. Res. Comm., 4: 28-32 (1961).

Franêk, F.: In: Symposium on Developmental Aspects of Antibody Formation and Structure. (Ed. by Sterzl, J. & Riha, I.), Prague, p. 311 (1970).

Frangione, B. & Franklin, E. C.: Chemical typing of immunoglobulins IgM, IgA1 and IgA2. FEBS lett., 20: 321 (1972).

Frangione, B. & Milstein, C.: Variation in the S-S bridges of immunoglobulins G: Interchain disulfide bridges of γG3 myeloma proteins. J. Mol. Biol., 33: 893-906 (1968).

Frangione, B., Milstein, C. & Franklin, E. C.: Chemical typing of immunoglobulins. Nature, 221: 149-151 (1969a).

Frangione, B., Milstein, C. & Pink, R.: Structural studies of immunoglobulin G. Nature, 221: 145-148 (1969b).

Frangione, B., et al., In Press (1979).

Frank, G. & Strubert, W. Chromatographia, 6: 522 (1973).

Frankhauser, P., Fries, P., Stahala, P. & Brenner, M.: Edman-Abau als analytische methode in der "solid phase" - peptidsynthese. Helv. Chem. Acta., 57: 271-277 (1974).

Franklin, E. C. & Frangione, B.: Structural variants of human and murine immunoglobulins. Cont. Topics in Mol. Immunol., 4: 89-126 (1975).

Franklin, E. C., Frangione, B. & Adlesberg, J. B.: An antigenic determinant unique to the hinge of γ3 heavy chains. J. Immunol., 115: 314-315 (1975).

Franklin, E. C. & Stanworth, D. R.: Antigenic relationships between immune globulins and certain related paraproteins in man. J. Exp. Med., 114: 521-533 (1961).

Fudenberg, H. H.: The hereditary human gamma globulin (Gm) groups: Interpretations and extensions. Prog. Allergy, 7: 1-31 (1963).

Fudenberg, H. H., Pink, J. R. L., Wang, A. C. & Douglas, S. D.: In: Basic Immunogenetics. Oxford University Press, Inc. (1978).

Gaarder, P. I. & Natvig, J. B.: Two new antigens of human IgG "Non b " and "Non b^1" related to the Gm system. J. Immunol., 108: 617-621 (1972).

Gally, J. A.: Structure of immunoglobulins. In: The Antigens. (Ed. by M. Sela), pp. 161-298 (1973).

Gally, J. A. & Edelman, G. M.: The genetic control of immunoglobulin synthesis. Ann. Rev. Genet., 6: 1 (1972).

Gergely, J., Fudenberg, H.H. & van Loghem, E.: The papain susceptibility of IgG myeloma proteins of different heavy chain subclasses. Immunochem., 7: 1-6 (1970).

Gergely, J., Medgyesi, G. A. & Stanworth, D. R.: Structural studies of immunoglobulins - II. The varying susceptibility to papain-digestion of a group of human myeloma γG-globulins. Immunochem., 4: 369-374 (1967).

Gilbert, W.: Why genes in pieces? Nature, 271: 501 (1978).

Goding, J. W.: Use of Staphylococcal protein A as an immunological reagent. J. Immunol. Methods, 20: 241-253 (1978).

Gold, E. R. & Fudenberg, H. H.: Chromic chloride: A coupling reagent for passive hemagglutination reactions. J. Immunol., 99: 859-866 (1967).

Graffeo, A. P., Hagg, A. & Karger, B. L.: High pressure liquid chromatography. Anal. Lett., 6: 505 (1973).

Grey, W. R. & Kunkel, H. G.: H chains groups of myeloma proteins and normal 7S γ-globulin. J. Exp. Med., 120: 253-266 (1964).

Grov, A., Myklestad, B. & Oeding, P.: Immunochemical studies on antigen preparations from Staphylococcus aureus. I. Isolation and chemical characterization of antigen A. Acta. Path. Microbiol. Scand., 61: 588-596 (1964).

Grubb, R.: Agglutination of erythrocytes coated with "incomplete" anti-Rh by certain rheumatoid arthritic sera and some other sera. The existence of human serum groups. Acta. Path. Microbiol. Scand., 39: 195-197 (1956).

Grubb, R.: The Gm groups and their relation to rheumatoid arthritis serology. Arthritis. Rheum., 4: 195-202 (1961).

Grubb, R.: The genetic markers of human Ig. In: Molecular Biol., Biochem. and Biophys. Springer, Berling, p. 152 (1970).

Grubb, R. & Laruell, A. B.: Hereditary serological human subgroups. Acta. Path. Microbiol. Scand., 39: 390 (1956).

Heremans, J. F.: Les Globulines Seriques du Systeme Gamma, leur Nature et leur Pathologie. Editions Arscia, Bruxelles (1960).

Heremans, J. F. & Vaerman, J. P.: Bilogical significance of IgA antibodies in serum and secretions. In: Progress in Immunol. (Ed by Amos), p. 875 (1971).

Hill, R. L., Delaney, R., Fellows, R. E., Jr. & Lebovitz, H.: The evolutionary origins of the immunoglobulins. Proc. Nat. Acad. Sci., U.S.A., 56: 1762-1769 (1966a).

Hill, R. L., Delaney, R., Lebovitz, H. E. & Fellows, R. E., Jr.: Studies on the amino acid sequence of heavy chains from rabbit immunoglobulin G. Proc. Roy. Soc. Lond., 169: 159-175 (1966b).

Hilschmann, N. & Craig, L. C.: Amino acid sequence studies with Bence-Jones proteins. Proc. Nat. Acad. Sci., U.S.A., 53: 1403-1409 (1965).

Hjelm, H.: Isolation of IgG3 from normal human sera and from a patient with multiple myeloma by using protein A-Sepharose 4B. Scand. J. Immunol., 4: 633-640 (1975).

Hjelm, H., Sjodahl, J. & Sjoquist, J.: Immunologically active and structurally similar fragments of protein A from Staphylococcus aureus. Eur. J. Biochem., 57: 395-403 (1975).

Hjerten, S.: Calcium phosphate chromatography of normal human serum and of electrophoretically isolated serum proteins. Biochem. Biophys. Acta., 31: 216-235 (1956).

Hood, L.: Two genes: One polypeptide - fact or fiction? Fed. Proc., 31: 177-187 (1972).

Hood, L. & Ein, D.: Immunoglobulin lambda chain structure: Two genes, one polypeptide chain. Nature, (Lond.), 220: 764-767 (1968).

Hozumi, N. & Tonegawa, S.: Evidence for somatic rearrangement of immunoglobulin genes coding for variable and constant regions. Proc. Nat. Acad. Sci., U.S.A., 73: 3628-3632 (1976).

Huber, H. & Fudenberg, H. H.: Receptor sites of human monocytes for IgG. Int. Arch. Allergy, 34: 18-31 (1968).

Ishizaka, K.: Chemistry and biology of immunoglobulin E. In: The Antigens. (Ed. by M. Sela), pp. 479-528 (1973).

Ilse, D. & Edman, P.: Reactions with phenylisothiocyanate. Aust. J. Chem., 16: 411 (1963).

Ishizaka, T., Ishizuka, K., Salmon, S. & Fudenberg, H.: Biological activities of aggregated gamma globulin VIII. Aggregated immunoglobulins of different classes. J. Immunol., 99: 82-91 (1967).

Jefferis, R., Weston, P. D., Stanworth, D. R. & Clamp, J. R.: Relationship between papain sensitivity of human γG immunoglobulins and their heavy chain subclass. Nature, 219: 646-649 (1968).

Jerne, N. K.: The natural-selection theory of antibody formation. Proc. Nat. Acad. Sci., U.S.A., 41: 849-857 (1955).

Johansson, S. G. O., Bennick, H. H. & Berg. T. The clinical significance of IgE. In: Progress in Clinical Immunology. (Ed. by Schwartz), Vol. 1, p. 157 (1972).

Kobat, E. A.: Unique features of the variable regions of Bence-Jones proteins and their possible relation to antibody complementarity. Proc. Nat. Acad. Sci., U.S.A., 59: 613-619 (1968).

Kabat, E. A.: Heterogeneity and structure of antibody-combining sites. Ann. N.Y. Acad. Sci., 169: 43-54 (1970).

Karush, F.: Immunologic specificity and molecular structure. Adv. Immunol., 2: 1-40 (1962).

Kataoka, T., Yamawak-Kataoka, Y., Yamagishi, H. & Honjo, T.: Cloning immunoglobulin γ2b chain gene of mouse: Characterization and partial sequence determination. Proc. Nat. Acad. Sci., U.S.A., 76: 4240-4244 (1979).

Kehoe, J. M. & Capra, J. D.: Localization of two additional hypervariable regions in immunoglobulin heavy chains. Proc. Nat. Acad. Sci., U.S.A., 68: 2019-2021 (1971).

Koshland, M. E. & Englberger, F. M.: Differences in the amino acid composition of two purified antibodies from the same rabbit. Proc. Nat. Acad. Sci., U.S.A., 50: 61-68 (1963).

Krause, R. M.: The search for antibodies with molecular uniformity. Adv. Immunol., 12: 1-56 (1970).

Kronwall, G. & Williams, R. C.: Differences in anti-protein A activity among IgG subgroups. J. Immunol., 103: 828-833 (1969).

Kulbe, K. D.: Micropolyamide thin-layer chromatography of phenylthio-hydantoin amino acids (PTH) at sub nanomole level. Anal. Biochem., 59: 564-573 (1974).

Kunkel, H. G.: Starch zone electrophoresis. Meth. Biochem. Anal., 1: 140 (1954a).

Kunkel, H. G.: In: Methods of Biochemical Analysis. New York, Interscience, p. 411 (1954b).

Kunkel, H. G.: The "abnormality" of myeloma proteins. Cancer Res., 28: 1351-1353 (1968).

Kunkel, H. G., Natvig, J. B. & Joslin, F. G.: The "Lepore" type of hybrid γ-globulin. Proc. Nat. Acad. Sci., U.S.A., 62: 144-149 (1969a).

Kunkel, H. B., Smith, W. K., Joslin, F. G., Natvig, J. B. & Litwin, S. D.: Genetic markers of the γA_2 subgroup of A immunoglobulin. Nature, 223: 1247-1248 (1969b).

Kunkel, H. G., Yount, W. J. & Litwin, S. D.: A genetically determined antigen of the Ne subgroup of γ-globulin: Detection by precipitation analysis. Science, 154: 1041-1043 (1966).

Lancet, D., Isenman, D., Sjodahl, J., Sjoquist, J. & Pecht, I.: Interactions between staphylococcal protein A and immunoglobulin domains. Biochem. Biophys. Res. Comm., 85: 608-614 (1978).

Lefkvist, T. & Sjoquist, J.: Purification of staphylococcal antigens. Int. Arch. Allergy, 23: 289-305 (1963).

Lenhard-Schuller, P., Hohn, R., Brack, C., Hirama, M. & Tonegawa, S.: DNA clones containing mouse immunoglobulin κ chain genes isolated by in vitro packaging into phage coats. Proc. Nat. Acad. Sci., U.S.A., 75: 4709-4713 (1978).

Leslie, G. A. & Martin, L. N.: Structure and function of serum and membrane D(IgD). Cont. Top. in Mol. Immunol., 7: 1-49 (1978).

Mage, R., Lieberman, R., Potter, M. & Terry, W. D.: In: The Antigens. (Ed. by M. Sela), pp. 299-376 (1973).

Mannik, M. & Kunkel, H. G.: Two major types of normal 7S globulin. J. Exp. Med., 117: 213-230 (1963).

Marchalonis, J. J. & Cone, R. G.: Biochemical and biological characteristics of lymphocyte surface immunoglobulin. Transplant. Rev., 14: 3-49 (1973).

Margolies, M. N. & Brauer, A.: Protein microsequencing using high-pressure liquid chromatography of phenylthiohydantoin amino acids. J. Chromat., 148: 429-439 (1978).

Matthews, E. W., Byfield, P. G. H. & MacIntyre, I.: Separation of phenylthiohydantoin-amino acids by high pressure liquid chromatography. J. Chromat., 110: 369-373 (1975).

McCaldin, D. J. J. Chem. Rev., 60: 39 (1960).

Mendez, E., Frangione, B. & Kokwa, S.: Chemical typing of human immunoglobulin E and D. FEBS lett., 33: 4-6 (1973).

Metzger, H.: Structure and function of γM macroglobulins. Adv. Immunol., 12: 57-116 (1970).

Michaelsen, J. E., Frangione, B. & Franklin, E. C.: Primary structure of the "hinge" region of human IgG3: Probable quadruplication of a 15 amino acid residue-basic unit. J. Biol. Chem., 252: 883-889 (1977a).

Michaelsen, T. E. & Natvig, J. M.: The hinge region of IgG3, an extended part of the molecule. FEBS lett., 28: 121-124 (1972).

Michaelsen, J. E. & Natvig, J. B.: Unusual molecular properties due to an extended hinge region. J. Biol. Chem., 249: 2778-2785 (1974).

Milstein, C.: Disulfide bridges and dimers of Bence-Jones proteins. J. Molec. Biol., 9: 836-838 (1964).

Milstein, C.: Linked groups of residues in immunoglobulin κ chains. Nature, 216: 330-332 (1967).

Milstein, C., Brownlee, G. G., Harrison, T. M. & Mathews, M. B.: A possible precursor of immunoglobulin light chains. Nature New Biol., 239: 117-120 (1972).

Milstein, C., Brownlee, G. G., Cartwright, E. M., Javis, J. M. & Proudfoot, N. J.: Immunoglobulin precursors. Nature, 252: 354-359 (1974).

Milstein, C., Adetugbo, K., Cowan, N. J. & Secher, D. S.: In: Progress in Immunology. (Ed. by Brent, L. & Holborow, J.), North-Holland Amsterdam, Vol. 1, pp. 157-168 (1974a).

Milstein, G. & Munro, A. J.: The genetic basis of antibody specificity. Ann. Rev. Microbiol., 24: 335-358 (1970).

Milstein, C. & Pink, J. R. L.: Structure and evolution of immunoglobulins. Prog. Biophys. Mol. Biol., 21: 209 (1970).

Mole, J. E., Bhown, A. S. & Bennett, J. C.: Structural analysis of the peptides derived from specific acid-catalyzed hydrolysis at aspartylprolyl peptide bonds in human J chain. J. Immunol., 118: 67-70 (1977).

Moore, S., & Stein, W. H.: A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. J. Biol. Chem., 211: 907-913 (1954).

Morell, A., Terry, W. D. & Waldman, T. A.: Metabolic properties of IgG subclasses in man. J. Clin. Invest., 49: 673 (1970).

Natvig, J. B.: Heterogeneity of anti- γ-globulin factors detected by pepsin digested human γG-globulin. Acta. Path. Micro. Scand., 66: 369-382 (1966).

Natvig, J. B. & Kunkel, H. G.: Detection of genetic antigens utilizing gamma globulins coupled to red blood cells. Nature, 215: 68-69 (1967).

Natvig, J. B. & Kunkel, H. G.: Genetic markers of human immunoglobulin: The Gm & Inv Systems. Series Hematol., 1: 66-96 (1968).

Natvig, J. B. & Kunkel, H. G.: A hybrid IgG4-IgG2 immunoglobulin. Scand. J. Immunol., 1: 418 (1972).

Natvig, J. B. & Kunkel, H. G.: Human immunoglobulins: Classes, subclasses, genetic variants and idiotypes. Advan. Immunol., 16: 1-59 (1973).

Natvig, J. B. & Kunkel, H. G.: A hybrid IgG4-IgG2 immunoglobulin. J. Immunol., 112: 1277-1284 (1974).

Natvig, J. B., Kunkel, G. H. & Gedde-Dahl, T.: Genetic studies on the heavy chain subgroups of gamma G-globulin. In: Gamma Globulins, Nobel Symp. No. 3, (Ed. by J. Killander), pp. 313-328 (1967a).

Natvig, J. B., Kunkel, H. G. & Litwin, S. P.: Genetic markers of heavy chain subgroups of human G Globulin. Cold Spring Harbor Symp. Quant. Biol., 32: 173-180 (1967b).

Natvig, J. B., Michaelsen, J. E. & Kunkel, H. G.: Evidence for recent duplication among certain gamma globulin heavy chain genes. J. Exp. Med., 133: 1004-1014 (1971a).

Natvig, J. B., Turner, M. W. & Michaelsen, T. E.: Genetic control of the constant homology regions of immunoglobulin G heavy chain subclasses. Ann. N.Y. Acad. Sci., 190: 150-160 (1971b).

Niall, H. D.: Automated Edman degradation: The protein sequenator. In: Methods in Enzymology. Vol. 27, p. 942 (1973).

Ouchterlony, O.: Antigen-antibody reactions in gels. Acta. Path. Microbiol. Scand., 26: 507-515 (1949).

Ornstein, L.: Disc electrophoresis. I. Backround and theory. Ann. N.Y. Acad. Sci., 121: 321-349 (1964).

Oudin, J.: L'allotpyie de certain antigines proteidiques du serum. C. R. Acad. Sci., 242: 2489 and 2606 (1956).

Oudin, J.: Allotypy of rabbit serum proteins. I. Immunochemical analysis leading to the individualization of seven main allotypes. J. Exp. Med., 112: 107-124 (1960a).

Oudin, J.: Allotypy of rabbit serum proteins. II. Relationships between various allotypes; their common antigenic specificities, their distribution in sample population genetic implications. J. Exp. Med., 112: 125-142 (1960b).

Padlan, E. A., Segal, D. M., Spande, T. F., Davies, D. R., Rudikoff, S. & Potter, M.: Structure at 4.5 Å resolution of a phosphorylcholine-binding Fab. Nature, N. B., 245: 165-167 (1973).

Parr, D.: Fragments produced by digestion of human immunoglobulin G subclasses with pepsin in urea. Biochem. J., 165: 303-308 (1977).

Parr, D. M., Connell, G. E., Kells, D. I. C. & Hofmann, T.: Fb'_2, a new peptic fragment of human immunoglobulin G. Biochem. J., 155: 31-36 (1976a).

Parr, D. M., Hofman, T. & Connell, G. E.: Characterization of upFc, a fragment of human immunoglobulin G1 produced by pepsin in urea. Biochem. J., 157: 535-540 (1976b).

Peterson, E. A. & Sober, H. A.: Chromatography of proteins. I. Cellulose ion-exchange adsorbants. J. Am. Chem. Soc., 78: 751-755 (1956).

Pink, J. R. L., Butterly, S. A., DeVries, G. M. & Milstein, C.: Human immunoglobulin subclasses. Partial amino acid sequences of the constant region of a γ4 chain. Biochem. J., 117: 33-47 (1970).

Pisano, J. J. & Bronzert, T. J.: Analysis of amino acid phenylthiohydantoins by gas chromatography. J. Bio. Sci., 244: 5597-5607 (1969).

Piszkiewicz, D., Landon, M. & Smith, E. L.: Anomalous cleavage of aspartyl-proline peptide bonds during amino acid sequence determinations. Biochem. Biophys. Res. Comm., 40: 1173-1178 (1970).

Poljak, R. J., Amzel, L. M., Avey, H., Chen, B. L., Phizackerley, R. P. & Saul, F.: Three-dimensional structure of the Fab' fragment of a human immunoglobulin at 2.8-A resolution. Proc. Nat. Acad. Sci., U.S.A., 70: 3305-3310 (1973).

Ponstingl, H. & Hilschmann, N.: Zur strukturregel der antikorper. Die primarstruktur eines monoklonalen IgG1-immunoglobulins (Myelomprotein Nei) III. Hoppe-Seyler's Z. Physiol. Chem., 357: 1571-1604 (1976).

Porter, R. R.: The hydrolysis of rabbit γ-globulin and antibodies with crystalline papain. Biochem. J., 73: 119-126 (1959).

Porter, R. R.: The structure of gamma globulins and antibodies. In: Basic Problems in Neoplastic Disease. (Ed. by A. Gellhorn & E. Hirschberg), Columbia Univ. Press, N.Y, pp. 177-194 (1962).

Prelli, F., Frangione, B., Franklin, E. C., Abel, C. & van Loghem, E.: An unusual γ3 human immunoglobulin heavy chain. Fed. Proc. (Abstracts), 34: 970 (1975).

Putnam, F. W., Shimizii, A., Paul, C. & Shinoda, T.: Tentative structure of human IgM immunoglobulin. Prog. in Immunol., Vol. I: 292 (1971).

Putnam, F. W., Shinoda, T., Titani, K. & Wikler, M.: Immunoglobulin structure; variation in amino acid sequence and length of lambda light chain. Science, 157: 1050-1053 (1967a).

Putnam, F. W., Titani, M., Wikler, M. & Shinoda, T.: Structure and evolution of kappa and lambda light chains. Cold Spring Harbor Symp. Quant. Biol., 32: 9-30 (1967b).

Putnam, F. W., Titani, K. & Whitley, E., Jr.: Chemical structure of light chains: amino acid sequence of type κ Bence-Jones proteins. Proc. Roy. Soc. Lond., 166: 124-137 (1966).

Rabbitts, T. H. & Forstor, A.: Evidence for noncontiguous variable and constant region genes in both germline and myeloma DNA. Cell, 13: 319-327 (1978).

Rao, D. N., Rudikoff, S., Krutzsch, H. & Potter, M.: Structural evidence for independent joining region gene in immunoglobulin heavy chains from anti-galactan myeloma proteins and its potential role in generating diversity in complementarity-determining regions. Proc. Nat. Acad. Sci., U.S.A., 76: 2890-2894 (1979).

Ropartz, C., Lenior, J., Hemet, Y. & Rivat, L.: Possible origins of the anti-Gm sera. Nature, 188: 1120-1121 (1960).

Sakano, H., Rogers, J. H., Huppi, K., Brack, C., Traunecker, A., Maki, R., Wall, R. & Tonegawa, S.: Domains and the hinge region of an immunoglobulin heavy chain are encoded in separate DNA segments. Nature, 277: 627-633 (1979).

Saluk, P. H. & Clem, L. W.: The unique molecular weight of the heavy chain from human IgG3. J. Immunol., 107: 298-301 (1971).

Sanger, F. & Thompson, E. O. P.: Halogenation of tyrosine during acid hydrolysis. Biocim. Biophys. Acta., 71: 468 (1962).

Sarkar, S.: Mechanism for joining V and C genes. Nature, 228: 1089 (1970).

Schechter, I. & Burstein, Y.: Identification of N-terminal methionine in the precursor of immunoglobulin light chain. Biochem. J., 153: 543-550 (1976a).

Schechter, I. & Burstein, Y.: Marked hydrophobicity of the NH_2-terminal extra piece of immunoglobulin light chain precursors: Possible physiological functions of the extra pieces. Proc. Nat. Acad. Sci., U.S.A., 73: 3273-3277 (1976b).

Schiffer, M., Girling, R. L., Ely, K. R. & Edmundson, A. B.: Structure of a λ-type Bence-Jones protein at 3.5 Å resolution. Biochem., 12: 4620-4631 (1973).

Schroeder, W. A., Shelton, J. B. & Shelton, J. R.: An examination of conditions for the cleavage of polypeptide chains with cyanogen bromide: Application to catalase. Archives of Biochemistry and Biophysics, 130: 551-556 (1969).

Schur, P. H.: Human gamma G subclasses. Prog. Clin. Immunol., 1: 71-104 (1972).

Schwartz, J. & Edelman, G. M.: Comparisons of Bence-Jones proteins and L polypeptide chains of myeloma globulins after hydrolysis with trypsin. J. Exp. Med., 118: 41-53 (1963).

Seidman, J. G., et al.: Multiple related immunoglobulin variable-region genes identified by cloning and sequence analysis. Proc. Nat. Acad. Sci., U.S.A., 75: 3881-3885 (1978).

Shapiro, A. L., Vinuela & Maizel, J. V.: Molecular weight estimation of polypeptide chains by electrophoresis in SDS - polyacrylamide gels. Biochem. Biophys. Res. Comm., 28: 815-820 (1967).

Singer, S. J. & Doolittle, R. F.: Antibody active sites and immunoglobulin molecules. Science, 153: 13-25 (1966).

Sjodahl, J.: Repetitive sequences in protein A from Staphylococcus aureus: Three highly homologous Fc binding regions. FEBS lett., 67: 62-67 (1976).

Sjodahl, J.: Repetitive sequences in protein A from Staphylococcus aureus. Arrangement of five regions within the protein, four being highly homologous and Fc binding. Eurp. J. Biochem., 73: 343-351 (1977a).

Sjodahl, J.: Structural studies on the four repetitive Fc binding regions in protein A from Staphylococcus aureus. Eur. J. Biochem., 78: 471-490 (1977b).

Skvaril, F.: The question of specificity in binding human IgG subclasses to protein A-Sepharose. Immunochem., 13: 871-872 (1976).

Smithies, O.: Pathways through networks of branched DNA. Science, 169: 882-883 (1970).

Smithies, O., Gibson, D. M., Fanning, E. M., Goodfliesh, R. M., Gillman, J. G. & Ballantyne, D. L.: Quantitative procedure for use with Edman-Begg sequenator. Partial sequences of two unusual immunoglobulin light chains, Rzf and Sac. Biochem., 10: 4912-4921 (1971).

Spackman, D. H., Stein, W. H. & Moore, S.: Automatic recording apparatus for use in the chromatography of amino acids. Anal. Chem., 30: 1190-1206 (1958).

Spiegelberg, H. L.: γD immunoglobulin. In: Cont. Topics in Immunochem. (Ed. by F. P. Inman), Vol. I, p. 65 (1972).

Spiegelberg, H. L.: Biological activities of immunoglobulins of different classes and subclasses. Adv. Immunol., 19: 259-294 (1974).

Spiegelberg, H. L., Fishkin, B. G. & Grey, H. M.: Catabolism of human γ-G immunoglobulins of different heavy chain subclasses. I. Catabolism of γG-myeloma proteins in man. J. Clin. Invest., 47: 2323-2330 (1968).

Steinberg, A. G.: Progress in the study of genetically determined human gamma globulin types. (The Gm and Inv groups) Prog. Med. Genet., 2: 1-33 (1962).

Steiner, L. A. & Porter, R. R.: The interchain disulfide bonds of a human pathological immunoglobulin. Biochem., 6: 3957-3970 (1967).

Summers, M. R., Smythers, G. W. & Oroszlan, S.: Thin-layer chromatography of sub-normal amounts of phenylthiohydantoin (PTH) amino acids on polyamide sheets. Anal. Biochem., 53: 624-628 (1973).

Swan, D., Aniv. H. & Leder, P.: Purification and properties of biologically active messenger RNA for a myeloma light chain. Proc. Nat. Acad. Sci., U.S.A., 69: 1967-1972 (1972).

Tarr, G. E.: A general procedure for the manual sequencing of small quantities of peptides. Anal. Biochem., 63: 361-370 (1975).

Teramoto, Y. A., Puentes, M. J., Young, L. J. T. & Cardiff, R. D.: Structure of the mouse mammary tumor virus: polypeptides and glycoproteins. J. Virol., 13: 411-418 (1974).

Terry, W. D. & Fahey, J. L.: Subclasses of human γ-2 globulin based on differences in heavy polypeptide chains. Science, 146: 400-401 (1964).

Tiselius, A.: Electrophoresis of serum globulin II electrophoretic analysis of normal and immune sera. Biochem. J., 31: 1464-1477 (1937).

Tiselius, A., Hjerton, S. & Levin, O.: Protein chromatography on calcium phosphate columns. Arch. Biochem. Biophys., 65: 132-155 (1956).

Tiselius, A. & Kabat, E. A.: An electrophoretic study of immune sera and purified antibody preparations. J. Exp. Med., 69: 119-131 (1939).

Titani, K. & Putnam, F. W.: Immunoglobulin structure: amino- and carboxy-terminal peptides of type 1 Bence-Jones proteins. Science, 147: 1304-1305 (1965).

Titani, K., Whitley, E., Jr., Avogardo, L. & Putnam, F. W.: Immunoglobulin structure: Partial amino acid sequence of a Bence-Jones protein. Science, 149: 1090-1092 (1965).

Tomasi, T. B. & Grey, H. M.: Structure and function of immunoglobulin A. Prog. Allergy, 16: 81-213 (1972).

Tonegawa, S., Brack, C., Hozumi, N. & Pirrotta, V.: Organization of immunoglobulin genes. Cold Spring Harbor Symp. Quant. Biol., 42: 921-931 (1977a).

Tonegawa, S., Brack, C., Hozumi, N. & Schuller, R.: Cloning of an immunoglobulin variable region gene from mouse embryo. Proc. Nat. Acad. Sci., U.S.A., 74: 3518-3522 (1977b).

Tonegawa, S., Hozumi, N., Matthyssens, G. & Schuller, R.: Somatic changes in content and context of immunoglobulin genes. Cold Spring Harbor Symp. Quant. Biol., 41: 877-889 (1976).

Tonegawa, S., Maxam, A. M., Tizard, R., Bernard, O. & Gilbert, W.: Sequence of a mouse germ-line gene for a variable region of an immunoglobulin light chain. Proc. Nat. Acad. Sci., U.S.A., 75: 1485-1489 (1978).

Turner, M. W., Bennick, H. H. & Natvig, J. B.: Pepsin digestion of human G myeloma proteins of different subclasses. I. The characteristic features of pepsin cleavage as a function of time. Clin. Exp. Immunol., 7: 603 (1970).

van Kamp, G. J.: IgA contamination of IgG prepared on a protein A column. J. Immunol. Methods, 27: 301-305 (1979).

van Loghem, E.: Genetic studies on human immunoglobulins. In: Handbook of Experimental Immunology and Immunochemistry. (Ed. by D. M. Weir), Vol. I, chapter 12 (1973).

van Loghem, E. & de Lange, G.: "Non-b", antigenic determinant of IgG. Vox. Sang., 22: 193-199 (1972).

van Loghem, E. & Natvig, J. B.: Uncommon Gm Gene complexes. Vox. Sang., 18: 421-434 (1970).

van Loghem, E., Natvig, J. B. & Matsumoto, H.: Genetic markers of immunoglobulins in Japanese families. Ann. Hum. Genet., 33: 351-359 (1970).

Verwey, E. F. J. Exp. Med., 71: 635 (1940).

Virella, G. & Parkhouse, R. M. E.: Papain sensitivity of heavy chain subclasses in normal human IgG and localization of antigenic determinants for the subclasses. Immunochem., 8: 243 (1971).

Vyas, G. N., Fudenberg, H. H., Pretty, H. M. & Gold, E. R.: A new rapid method for genetic typing of human immunoglobulins. J. Immunol., 100: 274-279 (1968).

Wang, A. C. & Fudenberg, H. H.: IgA and evolution of immunoglobulins. J. Immunogenet., 1: 3-31 (1974).

Weber, K. & Osborn, M.: The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Sci., 244: 4406-4412 (1969).

Weigert, M., Gatmaitan, L., Loh, E., Schilling, J. & Hood, L.: Rearrangement of genetic information may produce immunoglobulin diversity. Nature (Lond.), 276: 785-790 (1978).

Werner, B. C. & Steinberg, A. G.: Structural studies of a human hybrid immunoglobulin. Immunogenet., 3: 254-271 (1974).

Wilde, C. E., III & Koshland, M. E.: Polymerization of human immunoglobulin M. Biochemistry, 17: 3209-3214 (1978).

Winkelhake, J. L.: Review Article: Immunoglobulin structure and effector function. Immunochem., 15: 695-714 (1978).

Wolfenstein-Todel, C., Franklin, E. C. & Frangione, B.: Structural and evolutionary relationship of the hinge region of the major immunoglobulin classes and subclasses. La Ricerca in Clinica e in Labcratorio, Vol. VI (1976a).

Wolfenstein-Todel, C., Frangione, B., Prelli, F. & Franklin, E. C.: The amino acid sequence of "heavy chain disease" protein ZUC. Structure of the Fc fragment of immunoglobulin G3. Biochem. Biophys. Res. Comm., 71: 907-914 (1976b).

Wu, T. T. & Kabat, E. A.: Analysis of sequences of the variable regions of Bence-Jones proteins and myeloma light chains and their implications for anti-body complementarity. J. Exp. Med., 132: 211-250 (1970).

Zeeuws, R. & Strosberg, A. D.: The use of methanol in high-performance liquid chromatography of phenylthiohydantoin-amino acids. FEBS lett., 85: 68-72 (1978).

Zimmerman, C. L., Appella, E. & Pisano, J. J.: Advances in analysis of amino acid phenylthiohydantoins by high performance liquid chromatography. Anal. Biochem., 75: 77-85 (1976).

Zimmerman, C. L. & Pisano, J. J.: Analysis of amino acid phenylthiohydantoins by high speed liquid chromatography. Biochem. Biophys. Res. Comm., 55: 1220-1224 (1973).