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The Arrangement of the Disulfide Bonds in a γG Immunoglobulin Molecule

William Einar Gall

THE ARRANGEMENT OF THE DISULFIDE BONDS IN A γG IMMUNOGLOBULIN MOLECULE

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by

W. Einar Gall, A.B.

Gerald M. Edelman
Professor

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The Rockefeller University
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PREFACE

The immune system by which the body recognizes foreign materials and destroys or eliminates them presents one of the most intriguing problems in modern biology. One of the key features of the immune response is the production of antibodies or immunoglobulins, i.e. proteins that can bind specifically to a wide range of different antigens. Immunoglobulins also participate in several other reactions of immunity which do not directly depend on the specificity of the antigen-combining site. Analysis of the structure of immunoglobulins can provide important information on the functions and mechanisms of the immune system.

During the last decade, the structure of immunoglobulins has been subjected to closer scrutiny as techniques of protein chemistry have developed, and recently the complete covalent structure of a single immunoglobulin has been determined (Edelman et al., 1969). The studies presented in this thesis formed a part of this structure determination.

This work was performed in the laboratory of Professor Gerald M. Edelman. I am grateful to him for his continued guidance, encouragement, criticism, and support. I am also grateful to the other members of the laboratory, in particular Dr. Bruce A. Cunningham and Dr. Myron J. Waxdal, for their advice and cooperation. Mr. Paul D. Gottlieb and Mr. Urs Rutishauser have also made valuable contributions to the determination of the amino acid sequence of the protein Eu, without which the exact location of the disulfide bonds would not have been possible.

SUMMARY

The human γG1 myeloma protein Eu was isolated in pure form, and the protein was characterized to provide data for more detailed structural analyses. The molecular weight of the native molecule was 154,000 ± 8,000, as measured by sedimentation equilibrium. The component light (molecular weight 22,500) and heavy (molecular weight 51,600) chains were isolated in high yield by gel filtration after reduction and alkylation under mild conditions. Amino terminal analyses and the amino acid compositions of the native molecule and its chains indicated that there were two light and two heavy chains per molecule of Eu. Limited digestion of Eu with trypsin yielded Fab(t) and Fc(t) fragments, which were immunologically identical to Fab and Fc fragments produced by papain digestion.

Amino acid analysis showed 32 residues of half-cystine per mole of Eu, with 5 in each light chain and 11 in each heavy chain. The half-cystinyl residues in the light chain are located at positions 23(I), 88(II), 134(III), 194(IV), and 214(V). The half-cystinyl residues in the heavy chain are located at positions 22(I), 96(II), 144(III), 200(IV), 220(V), 226(VI), 229(VII), 261(VIII), 321(IX), 367(X), and 425(XI). There were no free sulfhydryl groups in the protein, indicating that there are 16 disulfide bonds.

Reduction of Eu under mild conditions indicated that there were four interchain bonds, and peptides containing these bonds were isolated. A bond between half-cystines L-V and H-V links each light chain to its corresponding heavy chain; this bond is in the Fab(t) region of the molecule. There are two bonds linking the heavy chains between corresponding half-cystines H-VI and H-VII; these bonds are in the Fc(t) region of the molecule. All of the half-cystinyl residues forming the interchain bonds are located between residues 220 and 229 of the heavy chain in what may be a "hinge" region of the molecule.

The remaining twelve half-cystines form intrachain bonds. The two intrachain bonds in each light chain are formed by half-cystines L-I and L-II, and by L-III and L-IV. The four intrachain bonds in the heavy chain are made up of half-cystines H-I and H-II, H-III and H-IV, H-VIII and H-IX, and H-X and H-XI. These bonds have a linear and periodic arrangement. Each bond forms a loop about 60 residues long. This structure provides evidence for theories on the evolution of immunoglobulins by gene duplication. This pattern also suggests that the immunoglobulin molecule may be folded in compact domains and that each domain contains one disulfide loop. Each variable region of the molecule contains one disulfide loop which may serve to fix the position of the antigen-binding site in the tertiary structure of the molecule.

TABLE OF CONTENTS

Page

Page

I. INTRODUCTION

A. The Functions of Antibodies

The unique aspects of the antibody-forming system are emphasized by the body of facts supporting the theory (Jerne, 1955, 1966; Burnet, 1959) that the immune response is selective, i.e. that the information necessary for synthesizing antibodies to any one of the large number of antigenic determinants is present within the organism before the introduction of antigens. An antigenic determinant serves only to stimulate the increased production of those immunoglobulins which bind to it. This requires that there preexist in each individual a library of information specifying a variety of antigen combining sites. Recent experiments have shown unequivocally that the specificity of an antibody is determined by its amino acid sequence (Haber, 1964; Whitney and Tanford, 1965; Singer and Thorpe, 1968). Moreover, the chemical heterogeneity characteristic of antibodies largely reflects variations in amino acid sequence (Edelman et al., 1961; Koshland and Englberger, 1963) and the diversity of antigen-binding sites required by the selective immune response. (For general reviews, see Frisch (1967), Killander (1967), Cohen and Milstein (1967), Edelman and Gall (1969).)

In addition to binding specifically to a wide range of antigenic determinants (the antigen recognition function), antibodies also participate in other immunological reactions, such as complement fixation and opsonization, which do not depend directly on the specificity of the antigen-binding site. This second type of function has been termed an effector function (Edelman et al, 1969). Recent studies on the structure of immunoglobulins have suggested that the antigen recognition and effector functions are associated with different portions of the antibody molecule. The variable region, which has different amino acid sequences in different molecules, is concerned primarily with the antigen recognition function and reflects the chemical heterogeneity of the immunoglobulins. The constant region, concerned primarily with the effector functions, has a relatively

invariant sequence in a given immunoglobulin class. Despite this simple correlation much remains to be understood of the detailed relationship of the structure of immunoglobulins to the biology of the immune response.

One part of the relation of antibody structure to function is the role of the disulfide bonds in the molecule. In general, disulfide bonds provide important constraints on the three-dimensional structure of proteins and thus may be important in preserving molecular functions and specificity. A disulfide bond forms a covalent bridge either between two polypeptide chains or between different portions of the same chain. The two half-cystines which make up a disulfide bond must be close to each other in the teritary structure of the molecule, regardless of their position in the linear amino acid sequence. Disulfide bonds serve to stabilize the tertiary structure of a protein and may be required for the proper function of the molecule.

Previous studies (Waxdal et al., 1967; Pink and Milstein, 1967b) have suggested that the disulfide bonds of immunoglobulins may be arranged in a linear pattern. The experiments I will describe in this thesis have proven, for the first time, the exact arrangement of the disulfide bonds and the positions of their constituent half-cystines in the amino acid sequence of an immunoglobulin. These results have led to speculations about the tertiary structure of the molecule and its relation to function, and have provided evidence for hypotheses on the evolution of the immunoglobulins. Before discussing the experiments, it is necessary to review briefly the structure of the immunoglobulins and to discuss the methods used for determining the arrangement of the disulfide bonds in proteins.

B. The Structure of Immunoglobulins

Immunoglobulins are multichain proteins, made up of light and heavy polypeptide chains linked by non-covalent interactions and by disulfide bonds (Edelman and Poulik, 1961; Fleischman et al., 1962).

Different classes of immunoglobulins can be separated from serum by physical and chemical techniques (see Williams and Chase, 1967), and five classes of immunoglobulins have been defined (World Health Organization, 1964) according to their general properties (Table I) and the class of their heavy chains. Within a given class of heavy chains, subclasses may be distinguished by their antigenic determinants. For example, there are four subclasses of human γ chains: $\gamma 1$, $\gamma 2$, $\gamma 3$, and $\gamma 4$. These subclasses can also be distinguished by differences in the amino acid sequences of the carboxyl-terminal portions of the γ chains. In contrast to the heavy chain classes, the two major classes of light chains, κ and λ, are found in all immunoglobulins.

Although they share the same overall structure, immunoglobulins within a single class and subclass are a heterogeneous mixture of chemically different molecules. This heterogeneity poses many difficult problems for detailed structural studies. Chemically homogeneous immunoglobulins are, however, produced in large amounts by certain plasma cell tumors, or myelomas, of man and mouse. Although myeloma proteins are not antibodies in the sense that they have been electively induced by stimulation with known antigens, they have the same overall structure as normal immunoglobulins. Each myeloma protein appears to have a unique amino acid sequence and can serve as an example of one of the many different immunoglobulin molecules in the normal heterogeneous population. Thus myeloma proteins provide an ideal source of material for detailed structural studies of immunoglobulins.

The γG immunoglobulins are the most prevalent class of human immunoglobulins, and many features of their overall structure are known from studies on both normal immunoglobulins and γG myeloma proteins. In order to provide an exact picture of the disulfide bond structure of a protein, it is necessary to know its amino acid sequence.

TABLE I

CLASSES OF HUMAN IMMUNOGLOBULINS[a]

CLASS	PER CENT OF TOTAL[b]	HEAVY CHAIN	LIGHT CHAIN	MOLECULAR FORMULA	MOLECULAR WEIGHT
γG	76	γ	κ, λ	$(\kappa_2\gamma_2)$ or $(\lambda_2\gamma_2)$	150,000
γA	17	α	κ, λ	$(\kappa_2\alpha_2)_n$ or $(\lambda_2\alpha_2)_n$	180,000
				$n = 1, 2, 3 \ldots$	$(n = 1)$
γM	7	μ	κ, λ	$(\kappa_2\mu_2)_5$ or $(\lambda_2\mu_2)_5$	950,000
γD	0.2	δ	κ, λ	$(\kappa_2\delta_2)$ or $(\lambda_2\delta_2)$	-
γE	0.1	ε	κ, λ	$(\kappa_2\varepsilon_2)$ or $(\lambda_2\varepsilon_2)$	196,000

[a] For references, see Edelman and Gall (1969). The nomenclature used is that recommended by the World Health Organization (1964).

[b] Based on average concentration ranges in normal human serum. The concentration range for γG immunoglobulin is 800-1680 mg/100 ml.

Because studies on the amino acid sequence of the human γG myeloma protein Eu were being undertaken in the laboratory of Dr. G.M. Edelman, it was reasonable to study the disulfide bond structure of this protein. For these reasons, the following brief review will emphasize the structure of human γG immunoglobulins. Much less is known at present about the structure of other classes of immunoglobulins. The available data do suggest, however, that the other classes of immunoglobulins contain basic structures similar to that of the γG class, and therefore this structure provides a reasonable starting point for examining the structure of all immunoglobulins.

1. The structure of human γG immunoglobulins. Although the four subclasses of human γG immunoglobulins differ in structural details, I will emphasize the γG1 subclass, because it is the most prevalent and most well studied. The overall structure of human γG immunoglobulin is shown in Figure 1.

The molecule consists of two identical light chains, each with a molecular weight of about 22,500, and two identical heavy chains, each with a molecular weight of about 55,000. Each heavy chain and the adjacent light chain interact via non-covalent forces and a single disulfide bond. Two light chain--heavy chain pairs (or half-molecules) are linked together by non-covalent interactions and by disulfide bonds between the heavy chains. If the interchain bonds are broken by treatment with reducing agents under mild conditions, the heavy and light chains may be separated by gel filtration in a dissociating solvent.

Large fragments of the molecule may also be obtained by limited digestion with several proteolytic enzymes to produce Fab and Fc fragments (Fig. 1). The Fab fragment is made up of the whole light chain and the amino-terminal half or Fd portion of the heavy chain. Each of the two Fab fragments from a divalent antibody contains an antigen combining site (Porter, 1958). The Fc fragment is a dimer

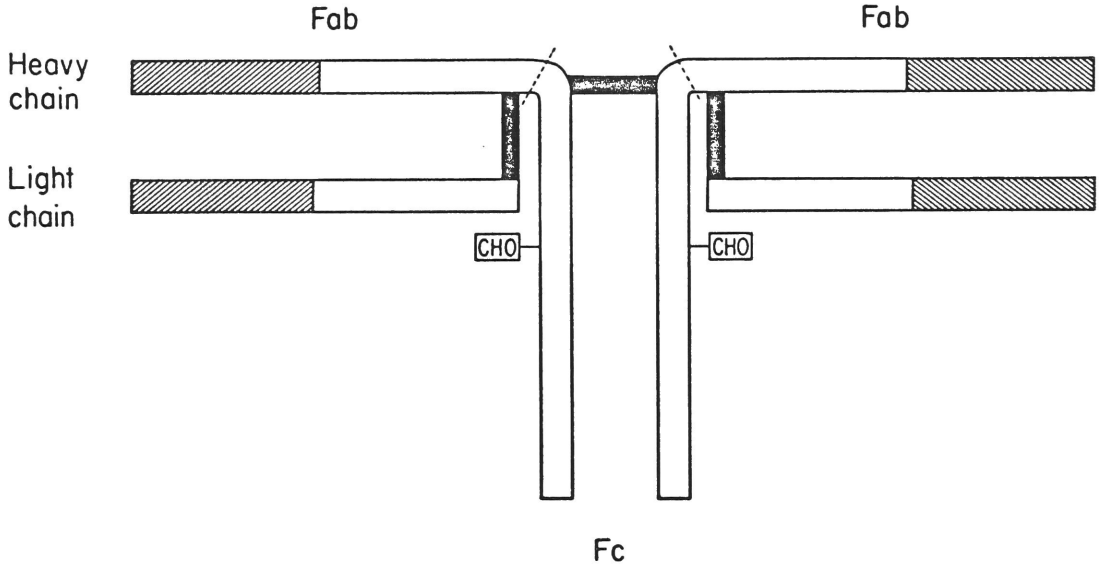


Figure 1. A model of the structure of human γG immunoglobulin. Interchain disulfide bonds are represented by the heavy bars. The site of cleavage to produce Fab and Fc fragments is indicated by the dotted lines. CHO marks the approximate position in the heavy chain of the carbohydrate moiety. The variable regions (at the amino terminal ends of the chain) are indicated by the slanted lines.

of the carboxyl-terminal half of the heavy chains and it can be crystallized (Porter, 1958). The Fc fragment retains the complement fixing and cell binding activities of the molecule (Brambell et al., 1960; Taranta and Franklin, 1961; Morris, 1963; Berken and Benacerraf; 1966).

Immunoglobulins contain carbohydrate, and the amount of carbohydrate varies from one class to another (Müller-Eberhard and Kunkel, 1956). Human γG immunoglobulins contain about 3% carbohydrate, and most of the carbohydrate is attached to the heavy chain at one position in the Fc portion of the molecule (Fig. 1). Recent experiments (Moroz and Uhr, 1967; Melchers and Knopf, 1967; Kern and Swenson, 1967) have shown that the carbohydrate is attached to the γG molecule after the synthesis and assembly of the polypeptide chains but before the molecule is secreted from the cell. Although the attachment of the carbohydrate moiety may be necessary for the secretion of the molecule from the cell, there is no evidence that the carbohydrate is concerned with antibody specificity or the genetic basis for the diversity of structure required for this specificity. For these reasons, the carbohydrate moiety of immunoglobulins will not be considered further.

2. The amino acid sequence of light chains. Light chains have an unusual structure as shown by studies on amino acid sequence carried out in several laboratories (Hilschmann and Craig, 1965; Putnam et al., 1967; Milstein, 1966; Hood et al., 1966; Niall and Edman, 1967; Hilschmann, 1967). Each κ or λ chain may be divided into an amino-terminal variable or V region (residues 1 to about 108) and a carboxyl-terminal constant or C region (residues 109-214) (Hilschmann and Craig, 1965). V regions of different proteins have diverse amino acid sequences; C regions differ in only a few residues, and some of these differences have been shown to reflect genetic polymorphism.

The diversity of the amino acid sequences of V regions is primarily a result of the substitution of one amino acid residue for another at certain positions in the linear sequence, although small changes in the length of the chain have also been observed. Different light chains can be classified into subgroups according to the amino acid sequences of their V regions (Milstein, 1967; Niall and Edman, 1967; Gray et al., 1967; Langer et al., 1968); three subgroups of κ chains have been proposed (Milstein, 1967). On the average, members of different subgroups differ in about 40 of the first 107 residues, while any two members of the same subgroup differ in about 10 of the first 107 residues. The types of substitution and the positions in which variations have been observed in one subgroup of κ chain variable regions are compared with the sequence of the Eu light chain in Figure 2. The amino acid substitutions are not made according to any obvious pattern, and they seem to be distributed throughout the first 108 residues. According to present data, certain positions show no variation at all, possibly because they are important for light chain folding and function. It should be emphasized that the sample may be too small to make valid generalizations about the nature and distribution of the variation.

Although the similarity of C regions is as striking as the diversity of V regions, C regions of light chains do exhibit limited variations in amino acid sequence. Many, if not all, of these differences may be accounted for by classical genetic analyses. A number of the variations have already been related to genetic markers of immunoglobulins.

The genetic markers, called Inv and Gm groups in humans (Grubb, 1956) and allotypes (Oudin, 1956) in other animals, can be detected by a variety of serological analyses and are inherited in a straightforward Mendelian fashion. For detailed reviews of the genetics of immunoglobulins, see Natvig and Kunkel (1968) and Oudin (1966). Inv markers

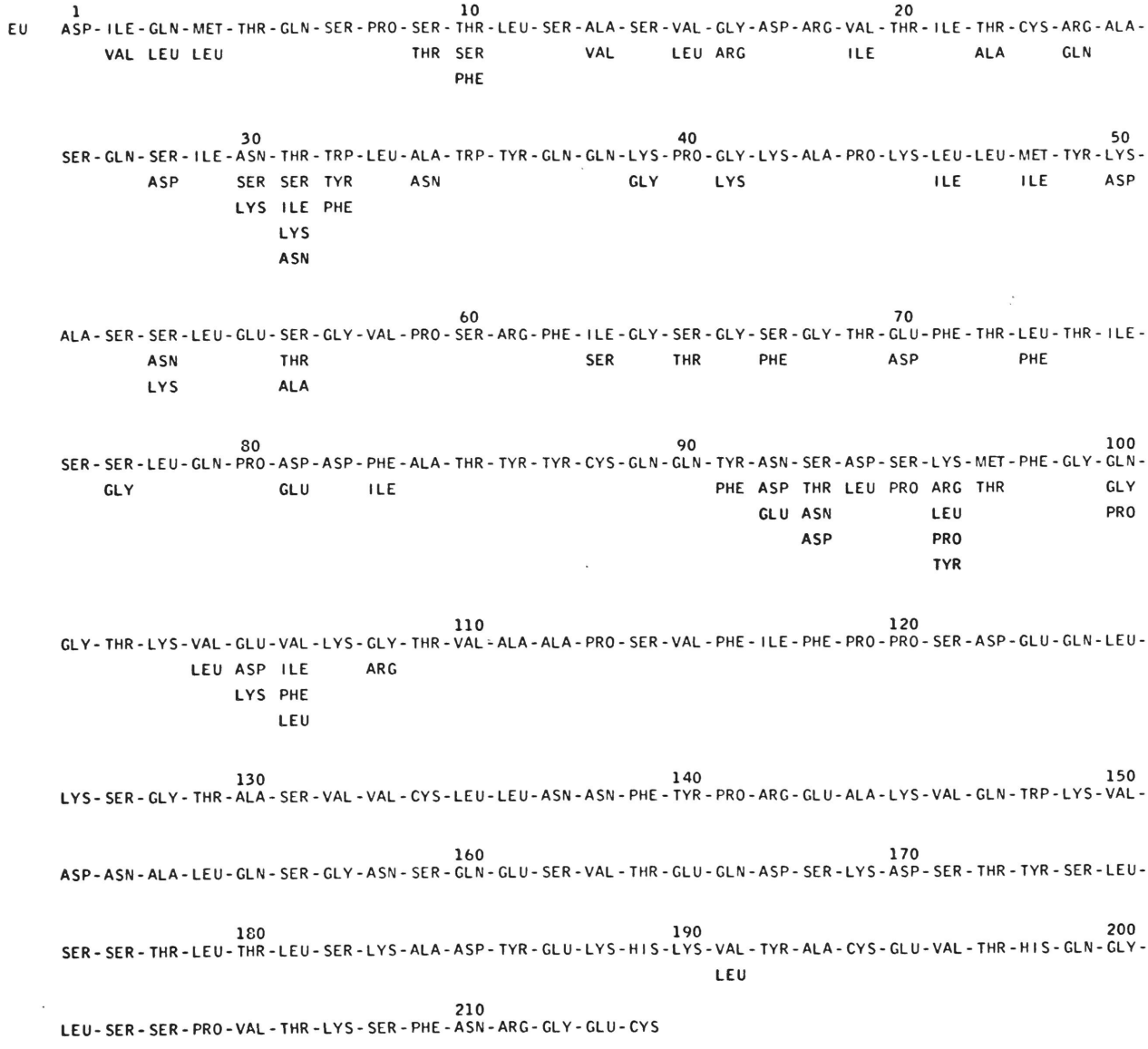


Figure 2. Variations in amino acid sequence within one subgroup of κ chains. Amino acid replacements which have been observed are shown in the appropriate positions under the amino acid sequence of the Eu light chain.

are associated with the κ class of light chains, and the Gm markers are associated only with γ chains. No linkage has been found between the Inv and Gm markers, indicating that the structural genes for light and heavy chains are at distant loci. Variation in the C region of κ chains can be correlated with the Inv marker (Baglioni et al., 1966): residue 191 (cf. Fig. 2) is leucine in Inv(2) proteins and valine in Inv(3) proteins. It has been noted (Natvig and Kunkel, 1968) that although the Inv(1) antigenic marker is linked (Litwin and Kunkel, 1967) to Inv(2), position 191 is the only position which has been observed to vary in the C region. Other variations involving glutamic acid and aspartic acids and their amides have not been excluded, however.

Variations have also been observed in the C regions of λ chains. Although the significance of many of these variations is not understood, one set of variations in C regions of λ chains has been analyzed. Two forms of λ chains have been distinguished by a rabbit antiserum produced against λ Bence-Jones proteins (Ein and Fahey, 1967), and a correlation has been demonstrated (Appella and Ein, 1967) between the different antigenic properties and the presence of a lysine (Oz+) or an arginine (Oz-) at position 191. Genetic data indicate that the Oz groups do not behave as alleles (Ein, 1968), and thus differ from the Inv groups of κ chains. These data suggest that there may be duplicated genes for the constant region of λ chains, in analogy to the duplicated genes for the subclasses of γ chains (Kunkel et al., 1964; Terry et al., 1965).

Light chains from other animals are generally similar to human light chains. Two classes of light chains with variable and constant regions have been found in 15 different species (Hood et al., 1967), although the relative amounts of κ and λ chains in the serum may differ from species to species. Amino acid sequences of two mouse chains have been determined (Dreyer et al., 1967) and the similarity between the V regions of human and mouse κ chains is striking. These data suggest that mouse κ chain variable regions also have subgroups. The homology of the

constant regions is great; about 60 percent of the residues are identical. The differences between the C regions of mouse and human κ chains resemble those found among other homologous proteins from different species, e.g. hemoglobin.

3. The amino acid sequence of heavy chains. Recent studies have shown that heavy chains have V and C regions analogous to those of light chains. The V region of heavy chains is about 115 residues long (Edelman et al., 1969) and begins at the amino terminus; the remainder of the heavy chain is a constant region. Although the data on heavy chain V region sequences are limited, the variations which have been observed are similar in kind and extent to those in light chain V regions. There is some evidence to suggest that there are subgroups of heavy chain V regions (Gottlieb et al., 1968).

Variations in the amino acid sequence of heavy chain C regions may be accounted for in the same way as the variations in light chain C regions, i.e. in terms of known genetic markers. In humans, the genetics of these markers indicate that there are closely linked duplicated genes for the subclasses of γ chains (Kunkel et al., 1964; Terry et al., 1965). The evidence also suggests that the genetic mechanisms which control the synthesis of light chains are the same for heavy chains.

A comparison of the sequences of light chains and portions of heavy chains led to the suggestion that the light and heavy chains originated by duplication of a gene coding for a polypeptide about 110 residues long (Hill et al., 1966; Singer and Doolittle, 1966). The internal homologies in the amino acid sequence of a single myeloma protein provide strong support for this hypothesis (Edelman et al., 1969). As will be discussed in detail below, the arrangement and location of the disulfide bonds also suggests that the molecule may have evolved through gene duplication.

4. The tertiary structure of γG immunoglobulins. Knowledge of the arrangement of the disulfide bonds in an immunoglobulin has prompted speculations about the tertiary structure of the molecule and its relation to molecular function. For these reasons, it is pertinent to review other data on the three-dimensional structure of immunoglobulins and the nature of the antigen-combining site.

Early studies on the shape of the γG immunoglobulin molecule indicated that it is elongated, rather than spherical. Low angle X-ray scattering data (Kratky et al, 1955; Kratky and Paletta, 1955) were interpreted to indicate that the molecule was about 240 Å long and 20 Å to 60 Å in cross-sectional diameter. More recent hydrodynamic studies (Noelken et al., 1965) have shown that the molecule has a high intrinsic viscosity and a frictional coefficient ratio of 1.47, substantially higher than the value of 1.1-1.3 expected for a typical globular protein. Fab and Fc fragments had lower frictional coefficient ratios (Noelken et al., 1965) than the whole molecule suggesting that the isolated fragments had a compact structure.

A model with three compact portions (the Fab and Fc regions) linked by highly extended, flexible portions was proposed for γG immunoglobulin by Noelken et al. (1965). This model suggested that free or unhindered rotation of the three compact portions of the molecule was allowed, and it was pointed out that flexibility of this type would be advantageous for the precipitation of various antigens. This flexible model has to be reconciled, however, with an overall average rotational relaxation time of about 220 nsec measured by several methods (Weltman and Edelman, 1967; Wahl and Weber, 1967; Krause and O'Konski, 1967; Ingram and Jerrard, 1962; Edsall and Foster, 1948; Oncley, 1943). This relaxation time is much greater than that of either the Fab or the Fc fragments. Thus, in the whole molecule, the three compact regions do not appear to be completely unhindered in their rotation, although these findings do not exclude the presence of some flexibility.

A more compact but still flexible structure of the molecule has been suggested by electron micrographs of antibodies and antigen-antibody complexes (Feinstein and Rowe, 1965; Valentine and Green, 1967). Micrographs of γG antibody molecules bound to ferritin (Feinstein and Rowe, 1965) show a bend in the middle; the angle of the bend may vary over a wide range, but the maximum length of the antibody molecule was about 200 Å. Micrographs of purified γG antibodies complexed with a divalent hapten show nearly regular closed figures which were interpreted to be made up of 2, 3, 4, or 5 Y-shaped molecules (Valentine and Green, 1967). The divalent hapten links the molecules together to form the figures and each corner in a figure contained a conspicuous projection.

The model proposed by Valentine and Green (1967) to explain these figures places the antigen binding site at the ends of two arms of the Y. The angle between the two arms is variable as if the arms were closely joined by a hinge. The stem of the Y projects from the ring figures and may represent the Fc portion of the molecule, as projections were not observed in figures formed by preparations which have been digested with pepsin. The dimensions of the Fab portions were 60 Å x 35 Å and those of the Fc fragment were 45 Å x 40 Å. Molecular weights calculated from these dimensions on the assumptions that the Fab and Fc fragments are cylindrical were consistent with their observed molecular weights. According to this model, the maximum extended dimension of the molecule in any configuration is about 120 Å.

Low angle X-ray scattering measurements of a γG myeloma protein in solution, however, gave a radius of gyration which is higher than would be expected if the maximal extended length were only 120 Å (Kratky, 1969). The observed radii of gyration of Fab and Fc fragments confirmed that they are compact. A model with three compact regions connected by portions of the polypeptide chain which behave like "stiff" springs to allow limited flexibility in solution could explain the observed high radius of gyration and the high intrinsic viscosity

(Noelken et al., 1965) of the whole molecule. At the same time, collapse of the structure into a more compact form on a surface would account for the electron microscopic data.

Obviously the most detailed information on the tertiary structure of immunoglobulins will come from crystallographic analysis of an entire molecule. Although this is still far off, preliminary data on a crystalline human γG1 myeloma protein have been reported (Terry et al., 1968). One half-molecule per asymmetric unit has been found, which proves that the γG molecule has a two-fold axis of symmetry. The unit cell dimensions are 195 Å x 93 Å x 51 Å. The molecule cannot be strictly spherical because an equivalent sphere would not fit into the unit cell. The unit cell dimensions are consistent with extended models of the molecule (Edelman and Gally, 1964; Kratky et al, 1955), as well as Y-shaped models. Preliminary data have also been obtained on the crystalline Fc fragment from rabbit immunoglobulins and human myeloma proteins (Poljak et al., 1967; Humphrey, 1967; Goldstein et al., 1968). As suggested by the known primary structure, there was a two-fold axis of symmetry in the Fc fragment. The crystalline Fc fragment can be enclosed in a parallepiped 50 Å x 40 Å x 70 Å. If these maximum dimensions are corrected for the water content of the crystal, they are consistent (Goldstein et al., 1968) with those calculated from electron micrographs (Valentine and Green, 1967).

Recently the Fab fragments of several human myeloma proteins have been crystallized (Rossi and Nisonoff, 1968), and preliminary crystallographic data on this fragment have been reported. There is one Fab fragment per asymmetric unit, and the crystal has monoclinic symmetry. Further details of the structure of this region are of particular interest, because the Fab fragment contains the combining site of an antibody.

5. The antigen combining site. Despite the fact that detailed knowledge of the tertiary structure of an immunoglobulin is lacking, several properties of the antigen combining site have been established. The two antigen binding sites of γG immunoglobulins are probably located at ends of the molecule, as suggested by electron micrographs of a variety of antigen-antibody complexes (Feinstein and Rowe, 1965; Valentine and Green, 1967). Although it has not been established, it seems likely that the binding sites for various antigens are in the same position in the tertiary structure. The size of an antigen binding site has been estimated to be about 25-30 Å x 10 Å x 6 Å (see Kabat (1966) for a review), indicating that it is small compared to the size of the Fab fragment. Why, then, is the diversity of amino acid sequence distributed in such a large region of light and heavy chains? The answer may lie in the requirements for a great variety of sites. Three types of amino acid residues might contribute to the three-dimensional structure of the antigen-binding site. First, there are "contact residues" which are directly involved in the binding of antigen, in a manner similar to the binding of substrates by enzymes. As in the teritary structure of enzyme-substrate complexes (see, for example, Phillips, 1967), these contact residues may not be close together in the amino acid sequence of the molecule. This is supported by the observation (Cathou and Haber, 1967) that antibodies with bound hapten appear to be stablized towards denaturation by guanidine. In addition to contact residues, there may be "modulating residues" (Edelman et al., 1963; Edelman and Gally, 1964) which could vary to change the folding of the polypeptide chain near the site so that the most efficient contact residues would be appropriate positions to bind the antigen. Third, there might be "compensating residues" which could vary in order to maintain the necessary overall folding pattern in the presence of variation in the contact or modulating residues. The various kinds of residues might be distributed over a relatively long stretch in a linear sequence.

Several kinds of experiments have indicated that both heavy and light chains contribute to the antigen-binding site (Wofsy et al., 1962; Edelman et al., 1963; Roholt et al., 1964). Although most light chain preparations alone are not active, carefully purified heavy chains (Porter and Weir, 1966) may retain some antigen binding activity. It has been demonstrated that immunoglobulin molecules may be reconstituted from their chains (Olins and Edelman, 1964), and combining activity and specificity are usually increased when specific light chains are recombined with the specific heavy chains (Edelman et al., 1963; Roholt et al., 1964).

Direct evidence that both chains participate in antigen-binding has come from affinity labeling experiments (Singer et al., 1967). The method of affinity labeling (Wofsy et al., 1962) utilizes the specificity of purified antibody to bind a hapten carrying a functional group which will react to form a covalent bond with certain amino acids in the region of the site. Contact residues within the antigen-binding site appear to have been labeled and the labels were attached to both light and heavy chains isolated from antibodies produced by rabbits, sheep, guinea pigs, and mice (Good et al., 1968). Hypothetically, light chains and heavy chains could interact to form a shared site, in which residues from both chains would participate directly in binding the antigen, or to form a modulated site (Edelman and Gally, 1964; Edelman et al., 1963) in which the principal residues which bind the antigen would be located on one chain, and the conformation of the site would be modified by changes in the conformation of the other chain. Affinity labeling experiments suggest that, at least in antibodies to the several haptens studied, the sites are shared. In view of the heterogeneity of antibodies, however, some molecules may have had only light chains or heavy chains labeled.

Peptides containing the affinity label have recently been isolated from the heavy and light chains of mouse and rabbit anti-2,

4-dinitrophenyl γG antibodies (Singer and Thorpe, 1968). It was shown that labeled tyrosine-containing dipeptides from the light chain were derived from the V region, confirming the inference that this region of the molecule is directly involved in antigen binding.

The disulfide bonds in immunoglobulins may have a role in stabilizing the antigen-combining sites in the molecule, as will be discussed below. Before presenting the experimental data which lead to this suggestion, it is appropriate to briefly review certain aspects of the chemistry of disulfide bonds in proteins, as well as some earlier work on the disulfide bonds of immunoglobulins.

C. The Chemistry of Disulfide Bonds

Disulfide bonds are the most labile of the covalent bonds found in proteins. (For a general review, see Cecil and McPhee (1959), Boyer (1959), Benesch et al., (1959), Benesch and Benesch (1962), and Cecil (1963).) They are especially susceptible to interchange reactions of the type shown in (1),

$$R_1\text{-S-S-}R_2 + R_3\text{-SS-}R_4 \rightleftharpoons R_1\text{-SS-}R_3 + R_2\text{-S-S-}R_4 \qquad (1)$$

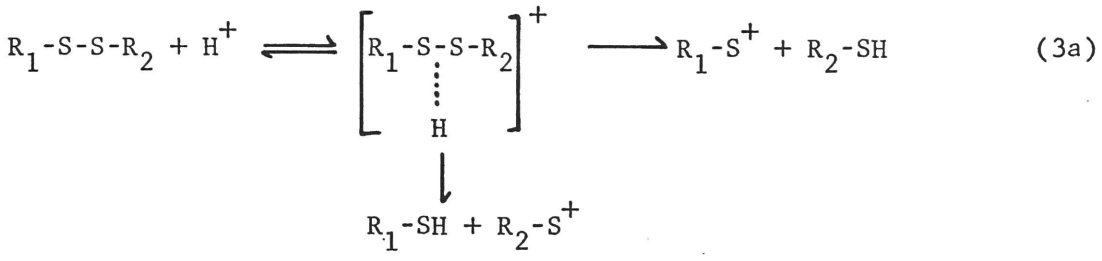
which can take place either in alkaline or strongly acid solution (Ryle and Sanger, 1955). Disulfide bonds are least susceptible to interchange in mildly acid solutions. In alkaline solutions, interchange probably takes place through an $R\text{-}S^-$ intermediate formed by hydrolytic cleavage of the disulfide bond, as shown in reaction (2). This reaction is catalyzed by trace amounts of a thiol.

$$R_1\text{-S-S-}R_2 + R_3\text{-}S^- \rightleftharpoons R_1\text{-S-S-}R_3 + R_2\text{-}S^- \qquad (2a)$$

$$R_1\text{-S-S-}R_2 + R_3\text{-}S^- \rightleftharpoons R_3\text{-S-S-}R_2 + R_1\text{-}S^- \qquad (2b)$$

Disulfide interchange can also take place in strongly acid solution, and the reaction probably proceeds through a sulfenium ion intermediate

(Benesch and Benesch, 1958) as shown in (3). In contrast to the reaction in alkaline solution, the interchange reaction is inhibited by thiols.



$$R_1\text{-}S\text{-}S\text{-}R_2 + H^+ \rightleftharpoons \left[R_1\text{-}S\text{-}S\text{-}R_2\right]^+ \longrightarrow R_1\text{-}S^+ + R_2\text{-}SH \qquad (3a)$$

$$\left[R_1\text{-}S\text{-}S\text{-}R_2\right]^+ \longrightarrow R_1\text{-}SH + R_2\text{-}S^+$$

$$R_1\text{-}S\text{-}S\text{-}R_2 + R_1\text{-}S^+ \longrightarrow R_1\text{-}S\text{-}S\text{-}R_1 \qquad (3b)$$

$$R_1\text{-}S\text{-}S\text{-}R_2 + R_2\text{-}S^+ \longrightarrow R_2\text{-}S\text{-}S\text{-}R_2 \qquad (3c)$$

An asymmetric disulfide, R_1-SS-R_2, has been used to emphasize that the products of the interchange reaction are not unique; for example, in a protein containing 5 disulfide bonds, 945 different arrangements are possible. It is thus obvious why great care must be taken to avoid disulfide interchange in the determination of the arrangement of the disulfide bonds in a protein.

Because of their lability, it is usually advantageous to convert any disulfides or sulfhydryl groups to more stable derivatives when studying the primary structure of proteins. Several methods may be used for this purpose. Performic acid (Toennies and Homiller, 1942) was used to oxidize the disulfide bonds of insulin to the stable cysteic acid derivatives (Sanger, 1949). In addition to cysteine and cystine, performic acid oxidizes tryptophanyl residues and thus is not generally suitable for use with tryptophan-containing proteins. Treatment with an excess of performic acid also results in the destruction of histidine and tyrosine (Schram et al., 1954; Moore, 1963), although this may be avoided by the use of a minimum of performic acid (Hirs, 1956).

The method of choice for the preparation of stable cysteine derivatives is reduction under conditions which are specific for disulfide bonds followed by alkylation to protect the sulfhydryl groups from air oxidation. Numerous reducing agents and alkylating agents have been used for this purpose, and each has its particular advantage and disadvantage.

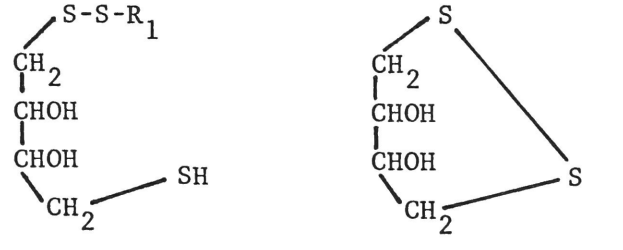
The most commonly used reducing agents are thiols, such as mercaptoethanol and mercaptoethylamine. The reaction is carried out at pH 8-9 and proceeds according to equation (4). Although the pK of a thiol is between 8 and 9, the thiols are indicated as R-SH, rather than as $R\text{-}S^-$, in these equations. Because the equilibrium constants of reactions 4a and 4b are near 1, a large molar excess of the reducing agent is required to drive the reaction to favor the complete reduction of the disulfide.

$$X\text{-}SH + R_1\text{-}S\text{-}S\text{-}R_2 \rightleftharpoons X\text{-}S\text{-}S\text{-}R_2 + R_1\text{-}SH \qquad (4a)$$

$$X\text{-}SH + X\text{-}S\text{-}S\text{-}R_2 \rightleftharpoons X\text{-}S\text{-}S\text{-}X + R_2\text{-}SH \qquad (4b)$$

Recently the dithiols dithiothreitol and dithioerythritol have been introduced (Cleland, 1964). These reducing agents react by a somewhat different mechanism and allow the use of much lower reducing agent concentrations. The effectiveness of these reagents is due to the fact that reaction 4b can be intramolecular and is facilitated by the stereochemistry of the reducing agent as illustrated in (5).

$$HS\text{-}CH_2(CHOH)_2CH_2\text{-}SH + R_1\text{-}S\text{-}S\text{-}R_1 \rightleftharpoons HS\text{-}CH_2(CHOH)_2CH_2\text{-}S\text{-}S\text{-}R_2 + R_1\text{-}SH \qquad (5a)$$



(5b)

The equilibrium constant of reaction 5b favors the cyclization and the production of R_1SH. Both dithiothreitol and dithioerythritol are now commonly used for the specific reduction of disulfide bonds.

Non-thiol reducing agents, such as metal hydrides, have also been used to reduce disulfide bonds (Edman and Diehl, 1952; Moore et al., 1958). The possibility of side reactions with these reducing agents, including peptide bond cleavage, makes the thiols, in particular dithiothreitol and dithioerythritol, the reagents of choice.

The oxidation of thiols to form disulfides (6) in the presence of air is catalyzed by trace amounts of metal ions, particularly copper.

$$R_1\text{-SH} + R_2\text{-SH} + 1/2O_2 \rightleftharpoons R_1\text{-S-S-}R_2 + H_2O \qquad (6)$$

For this reason, thiols produced by the reduction of disulfide bonds are usually converted to more stable derivatives. Because of the highly nucleophilic nature of the thiol group, several kinds of alkylating agents may be used for this purpose. The most commonly used alkylating agents are alkyl halides, such as iodoacetic acid and iodoacetamide which react to form the S-carboxymethyl- and S-carboxamidomethyl-cysteine derivative, respectively. The major disadvantage of the alkyl halide reagents is the possibility of side reactions with other amino acid residues, notably methionine, histidine, and lysine. The reaction of iodoacetic acid with ribonuclease has been thoroughly investigated (Gundlach et al., 1959; Crestfield et al., 1963). The reaction of cysteinyl residues with iodoacetic acid is relatively rapid, and by careful control of pH and reaction time carboxymethylation is quite specific for these residues (Crestfield et al., 1963).

The side reactions of the alkyl halides can also be avoided by using alkylating agents containing a carbon-carbon double bond, such as N-ethyl maleimide (Alexander, 1958; Roberts and Rouser, 1958) or acrylonitrile (Weil and Seibles, 1961).

Ethylenimine has also been used to alkylate cysteinyl residues, resulting in the formation of S-amino-ethyl cysteine (Raferty and Cole, 1966). This derivative, in addition to being stable to acid hydrolysis, has the added property of being susceptible to cleavage by trypsin (cf. Lindley, 1956). This may be an advantage in certain structural studies.

Reduction and alkylation of disulfide bonds yields stable derivatives, but this irreversibility may not always be desirable. Formation of S-sulfocysteine derivatives is sufficient for many purposes. The reaction of sulfite with disulfides (Cecil and McPhee, 1955; Bailey and Cole, 1959) is shown in (7).

$$R\text{-}S\text{-}S\text{-}R + SO_3^{=} \underset{\text{air or } Cu^{++}}{\rightleftharpoons} R_1\text{-}S\text{-}SO_3^{-} + R\text{-}S^{-} \qquad (7)$$

By including an oxidizing agent (such as air or cupric ions), the reaction may be cycled until all of the cystines have been converted to S-sulfocysteine (Bailey and Cole, 1959). From (7) it may also be seen that treatment of the S-sulfoprotein with an excess of thiol will result in the regeneration of disulfide bonds. A very important consideration in this process is the lack of specificity in the bond reformation. Under certain reaction conditions, e.g. very low protein concentration, the specificity of the reformation may be controlled by the conformation of the proteins and a product resembling the native protein may be obtained. In most cases, however, this is difficult to accomplish. The importance of the S-sulfocysteine derivatives in structural studies lies in the opportunity to introduce a negative charge into a protein where performic acid oxidation is not usable. This technique has been used to advantage in the separation of the chains of chymotrypsin (Dinh Van Hoang *et al.*, 1963).

The formation of stable derivatives of cysteine and cystine residues is of great value in the determination of the amino acid

sequence of proteins. These methods may also be used to advantage in the determination of the arrangement of the disulfide bonds.

D. A Strategy for the Determination of the Arrangement of Disulfide Bonds in Proteins

Although knowledge of the complete amino acid sequence of a protein is necessary to determine the exact location of the disulfide bonds in the molecule, information on the arrangement of these bonds may be obtained without this knowledge. Particular features of the overall structure of the protein must be determined, however, before beginning experiments on the arrangement of the disulfide bonds.

The total number of half-cystinyl residues in the protein must first be determined. (The term "half-cystinyl" is used when referring to cysteinyl or cystinyl residues in a protein without regard to the oxidation state of the sulfur.) The method of choice for this purpose (Moore, 1963) is based upon oxidation of the protein with an excess of performic acid, removal of the excess performic acid by the addition of HBr to prevent over-oxidation, and determination of the cysteic acid produced by amino acid analysis following acid hydrolysis. If substances which interfere with the determination of cysteic acid by amino acid analysis are present in the hydrolyzate of the unoxidized proteins, cysteic acid may also be determined by ion exchange on Dowex 1 (Moore, 1963). This method is widely applicable and has proven useful in the study of several proteins.

Once the total half-cystine content is known, the amounts of cystine and cysteine must be determined. The usual practice is to measure the number of free sulfhydryl groups per mole of protein and to assume that the remainder of the half-cystine is in the form of cystine. Many methods have been developed for sulfhydryl determinations; for general reviews see Cecil (1963) and Benesch and Benesch (1962). Several methods involve potentiometric or amperometric titrations in the presence of heavy metals which will complex with free sulfhydryl

groups. These methods require relatively large amounts of protein, and the results may often be equivocal because of the possibilities that metal ions may form complexes with other residues in the protein. More sensitive methods have been developed using spectrophotometric techniques.

The spectrophotometric method of Boyer (1954) utilizes the difference in absorption between p-hydroxymercuribenzoate and its corresponding mercaptide in the region from 250 to 255 mμ. The reagent used is p-chloromercuribenzoate (PCMB), but this compound is rapidly converted to the p-hydroxy analog in aqueous solution at pH 8-9, the conditions of the assay. A standard procedure for this assay has been described (Benesch and Benesch, 1962). A similar spectrophotometric method utilizes titration with 5,5'-dithiobis(2-nitrobenzoic acid), or Ellman's reagent (Ellman, 1959). Thiols may also be determined by amino acid analysis after alkylation of the protein with iodoacetamide, N-ethyl maleimide, etc. Cysteinyl residues in proteins may have widely different rates of reaction with various reagents, and for analytical purposes it is necessary to carry out the assay under denaturing conditions to expose any relatively unreactive sulfhydryl groups.

It should be noted that the total half-cystine content of proteins can be measured by titration of the sulfhydryl groups formed by cleavage of all of the disulfide bonds. One method (Zahler and Cleland, 1968) is based on reduction with dithiothreitol or dithioerythritol determination of the resulting thiols with Ellman's reagent. The assay is carried out in the presence of arsenite, which forms a tight complex with the dithiol reducing agents, but not with the monothiols.

An extremely sensitive method (Karush et al., 1964) is based on the observation that the fluorescence of fluorescein mercuric acetate is quenched in the presence of thiols. The assay is carried out in strong base to promote hydrolytic cleavage of the disulfides and the

subsequent formation of the mercurial-thiol complex.

Half-cystines in native proteins are usually present either as cysteinyl residues or as part of a disulfide bond. However, other chemical states are possible. It has been shown that in the proenzyme of streptococcal proteinase (Liu et al., 1963), a cysteinyl residue is blocked with a volatile mercaptan (Ferdinand et al., 1965), which has not yet been identified. Possibilities such as this also must be considered when determining the disulfide bond structure of a protein.

The presence of both free sulfhydryl groups and disulfide bonds in a protein makes the determination of the arrangement of the disulfide bonds more complicated. In most cases, the free sulfhydryls may be selectively blocked, for example, by alkylation. The problems of possible disulfide interchange, initiated by the thiol, are thus eliminated. Selective alkylation also provides a method for the identification of the exact half-cystinyl residue which is in the form of a free sulfhydryl group. Because there are no free sulfhydryl groups in the immunoglobulin I will be discussing, this problem will not be considered here in further detail.

Once the number of disulfide bonds present in the molecule has been determined, the general strategy for determining the arrangement of these bonds is to obtain a peptide containing the intact disulfide bond, cleave the disulfide bond, and identify the half-cystines forming this bond by further analysis of the resulting peptides. The ideal method is to isolate a pure peptide with an intact disulfide bond, reduce and alkylate or oxidize the bond, and separate the resulting peptides. The amino acid composition and sequence of these peptides can then be determined and compared with the amino acid sequence of the protein. For various reasons, it is often laborious and difficult to isolate a pure peptide containing a disulfide bond, and fortunately this need not be accomplished in every case. For example, a fraction with a single disulfide-containing peptide may be isolated by ion-exchange chromatography, although other non-disulfide peptides may be present in

the mixture. The chemical properties of the disulfide peptide may be selectively changed, e.g. by oxidation or by reduction and alkylation, and the mixture can be chromatographed again under the same conditions. The half-cystine-containing peptides, modified by the intervening treatment, will now have different properties and may be separated from the unaffected peptides. When only one disulfide-containing peptide was present in the original mixture, the interpretation of the results is unequivocal.

A method of this type using high voltage paper electrophoresis to separate the peptides has been developed by Brown and Hartley (1966). The advantage of this method lies in the high resolving power of high voltage paper electrophoresis. This so-called "diagonal method" will be used extensively in my experiments. Details of the method will be described below.

During the preparation and isolation of cystine-containing peptides, the possibility of disulfide interchange must be carefully considered. Because interchange is least likely in the pH range from 2 to 7, all of the steps involved in production and isolation of peptides should be carried out in this pH range. For this reason, pepsin is the enzyme of choice for preliminary digestion of the protein. Tryptic digestion at pH 6.5 to 7 has also been used with satisfactory results.

Attempts have been made to reduce the possibility of disulfide interchange by carrying out the digestion and isolation procedures in the presence of low concentrations of alkylating agents (cf. Spackman et al., 1960). These efforts have not always been successful (Ryle and Anfinsen, 1957), and in general the control of pH is the best method to reduce interchange. If more cystine-containing peptides are found than a unique arrangement of disulfides would permit, disulfide interchange has probably taken place during the preparation and isolation of peptides. Other methods to reduce interchange must be considered.

In proteins made up of more than polypeptide chain, there may be disulfide bonds between two chains (interchain bonds) as well as disulfide bonds between two portions of the same chain (intrachain bonds). In some cases, the interchain bonds may be selectively broken, allowing the determination of the number and location of these bonds without the additional complications of the intrachain bonds.

In summary, after the determination of the number of disulfide bonds in the protein, peptides containing each of these bonds must be isolated. Cleavage of the disulfide bond followed by isolation and analysis of the resulting peptides allows the identification of the half-cystinyl residues which form this bond in the native molecule. Comparison of these data with the amino acid sequence of the protein, if known, enables the exact location and arrangement of the bonds to be determined.

E. Early Work on the Disulfide Bonds of Immunoglobulins

Most of the early studies on immunoglobulins were done on pooled, normal γ-globulins. The heterogeneity of these proteins made detailed interpretations of the results difficult, although several general conclusions did emerge. Amino acid analyses of immunoglobulins from various species showed that these proteins have a relatively large number of half-cystine residues, ranging from 34-50 per mole (Crumpton and Wilkinson, 1963; Smith et al., 1955). Determinations of the free sulfhydryl content of normal, pooled immunoglobulins gave varying results, although most measurements indicated that there was less than one free sulfhydryl group per mole, if any (Edelman and Poulik, 1961; Cunningham and Nuenke, 1959; Katchalski et al., 1957; Franek and Lankas, 1963).

The first indication that immunoglobulins were made up of polypeptide chains linked by disulfide bonds came from studies (Deutsch and Morton, 1958) on pathological γM immunoglobulins. The 19S native molecule could be dissociated by treatment with thiols into 6.3 S

"monomers". However, these 6.3S units were no smaller than normal γG immunoglobulins.

The demonstration that γG immunoglobulins were multichain proteins (Edelman, 1959) was based on the dissociation of the molecule into subunits after sulfitolysis or reduction in urea solutions. Pathological γM immunoglobulins could also be dissociated into components smaller than the 6.3S "monomer" by these means (Edelman, 1959; Edelman and Poulik, 1961). Treatment with reagents which would not cause cleavage of disulfide bonds, e.g. hydroxylamine, did not lead to dissociation of the molecule, and it was concluded that the polypeptide chains were linked through disulfide bonds (Edelman and Poulik, 1961).

Several studies on the number and location of these interchain bonds were carried out, primarily on rabbit γG immunoglobulins. Reduction of native rabbit γG immunoglobulins with 0.2 M mercaptoethanol resulted in the cleavage of five disulfides which presumably were interchain bonds (Fleischman et al., 1963). From the S-carboxymethylcysteine content of the isolated chains, one interchain bond was assigned between each light and heavy chain. The remaining three bonds were assigned to link the heavy chains, with one in the Fab region and two in the Fc region of the molecule. Interpretation of these results was complicated by the demonstration that pooled rabbit immunoglobulins were heterogeneous with respect to their content of inter-heavy chain bonds (Palmer and Nisonoff, 1964). After the demonstration that partial reduction followed by acidification led to the dissociation into half-molecules containing one light and one heavy chain (Palmer et al., 1963), this conversion could be studied as a function of reducing agent concentration (Palmer and Nisonoff, 1964). About half the molecules in the normal population could be split to produce half-molecules after reduction of one disulfide bond, although reduction of 2.5 to 3 bonds per molecule was necessary to convert 90% of the population to half-molecules.

The relationship of the interchain disulfide bonds to the fragments produced by limited proteolytic digestion of immunoglobulins was of particular interest. Interpretation of the fragments produced by papain digestion was complicated by the reducing agents added to the digestion mixture to activate the papain (Porter, 1958, 1959). Treatment with pre-activated insoluble papain (Jaquet et al., 1964) resulted in separation of the Fab and Fc antigenic determinants of the heavy chain.

Digestion with pepsin yielded a 5S fragment which could be dissociated into two Fab fragments by reduction of a single disulfide bond (Nisonoff et al., 1961). This disulfide bond was presumed to link the heavy chains, forming an $F(ab')_2$ dimer. The site of cleavage by pepsin was probably carboxyl terminal to the inter-heavy chain bond, while the site of cleavage by papain was amino-terminal to this bond (Utsumi and Karush, 1967). Recent studies on the amino acid sequence of rabbit immunoglobulins in the region of the molecule which is most susceptible to digestion have indicated that this is the case (Smyth and Utsumi, 1967; Givol and DeLorenzo, 1968).

Similar studies showed that the interchain disulfide bonds of human immunoglobulins were analagous to those of rabbit immunoglobulins, although not in every detail. Cohen (1963) and Milstein (1965) showed that there was a single disulfide bond linking the light chain to the heavy chain, and sulfitolysis experiments suggested that there were four interchain bonds in human γG immunoglobulin (Christian and Schur, 1965). Recent studies have provided amino acid sequence data in the region of the interchain bonds of the four subclasses of human γG immunoglobulins (Frangione and Milstein, 1967; Pink and Milstein, 1967b; Steiner and Porter, 1967; Gall et al., 1968; Frangione and Milstein, 1968). These results will be discussed in detail below.

Several experiments involving the disulfide bonds of immunoglobulins have had important implications for the understanding of immunity. Karush (1962) suggested that the disulfide bonds of the

antibody molecule played an essential role in the acquisition of the antigen-binding specificity of antibodies. Based upon experiments correlating the loss of antigen binding activity with the reduction of disulfide bonds, Karush proposed that the pairing of at least some of the disulfides in immunoglobulins was not predetermined by the amino acid sequence of the molecule. If the pattern of pairing could somehow be related to the nature of the antigenic determinant, then the chemical basis of antigen binding specificity would be explained. The proposed variable pairing could also explain the observed heterogeneity of the immunoglobulins, because different pairing patterns might bring different charged groups to the surface of the molecule.

This elaborate theory was discarded when it became clear that antigen binding specificity depends on the amino acid sequence of the protein. One important kind of evidence for this conclusion came from the refolding experiments of Haber (1964) and Whitney and Tanford (1965). Fab fragments prepared from active antibody were treated with thiol reducing agents in solutions of guanidine hydrochloride under these conditions, the protein was completely unfolded. By slow removal of the denaturing and reducing agents, the protein could be refolded and reoxidized to form active fragments. Similar experiments with non-specific Fab fragments did not result in the formation of active fragments. Many other lines of evidence also lead to the conclusion that antibody specificity depends on amino acid sequence (cf. Haber, 1968).

Although the hypothesis of Karush can no longer be considered valid, some of the disulfide bonds in the molecule may be important for the antigen binding function. This possibility will be considered in the Discussion.

II. EXPERIMENTAL

A. Purpose of the Experiments

Several fundamental problems of immunity would be clarified if we understood more details of the structure and function of antibody molecules. Previous studies had suggested that there was something unusual about the arrangement of the disulfide bonds in immunoglobulins, and that this arrangement might be important for the function of the molecule. The purpose of the following experiments was to determine the arrangement and exact location of the disulfide bonds in the γG1 immunoglobulin Eu.

B. Outline of the Experiments

The preliminary characterization of a protein with respect to purity, molecular weight, amino acid composition, etc., is essential before any more detailed structural studies are undertaken. For this reason experiments on the isolation and characterization of the protein Eu, its constituent light and heavy chains, and the fragments produced by limited tryptic digestion were first carried out. The number and location of the interchain disulfide bonds was determined by selective reduction and alkylation of these bonds. Peptides which contained the intact interchain bonds were then isolated in pure form, and their structure was determined. The arrangement of the intrachain bonds was then proven using the technique of diagonal electrophoresis. The exact location of these disulfide bonds in the amino acid sequence of the molecule was possible because of studies on the complete amino acid sequence of this protein carried out concurrently in the same laboratory.

C. Materials and Methods

1. Preparation of purified Eu myeloma protein. Plasma collected in sterile polyethylene bags was brought to 34 percent saturation with ammonium sulfate at 4°. The mixture was immediately centrifuged at 400 x g for 15 minutes to remove the precipitate, which was dissolved in water, again made 34 percent saturated with ammonium sulfate, and

centrifuged. These precipitates were enriched in myeloma protein and could be conveniently stored at 4°. Moderate amounts of Eu were prepared from the precipitate by zone electrophoresis on starch (Kunkel, 1954), but gram quantities were more easily prepared by ion exchange chromatography on DEAE-cellulose (Peterson and Sober, 1956; Fahey and Horbett, 1959). Cellex D (Bio-Rad Laboratories, Richmond, Calif., exchange capacity 0.98 meq/g) was equilibrated with 0.01 M potassium phosphate buffer, pH 8.4, and poured into a 5.5 x 150 cm column. About 250 ml of a 5% solution of the ammonium sulfate precipitate which had been dialyzed against the phosphate buffer was loaded on the column and eluted with the same buffer. Eu emerged from the column in high yield, while the other components of the precipitate were retained on the ion exchanger. About 70% of the protein in the precipitate was recovered as myeloma protein.

2. Partial reduction and alkylation. Eu was dissolved in 0.15 M Tris hydrochloride buffer, pH 8.0, which was 0.15 M in NaCl and 0.002 M in EDTA; the final protein concentration varied from 22 to 40 mg/ml. Dithiothreitol was added, and the solution was allowed to stand at room temperature. After various times, iodoacetamide (K and K Laboratories, Plainview, N.Y., twice recrystallized from water) was added, and the solution was allowed to stand in the dark. Amounts of dithiothreitol and iodoacetamide were varied from experiment to experiment, as described below. Iodoacetamide-1-^{14}C (New England Nuclear, Boston, Mass.), was used in some experiments. The optimal reaction conditions for partial reduction and alkylation before separation of light and heavy chains were: 0.005 M dithiothreitol, 40 minutes, 0.010 M iodoacetamide, 20 minutes.

Reagents were removed by dialysis against water in the dark at 4°, or by gel filtration on Sephadex G-25. In order to separate chains directly, the reaction mixture was applied to a Sephadex column which was wrapped in aluminum foil to shield the sample from light.

3. Complete reduction and alkylation. A 1-2% solution of protein in 6 M guanidine hydrochloride, 0.5 M in Tris and 0.002 M in EDTA, pH 8.1, was placed in a screw cap vial. The tube was flushed with nitrogen, capped, and placed in a 50° water bath for 30 minutes to denature the protein fully. Dithiothreitol (50 moles per mole of disulfide in the protein) was added; the tube was flushed briefly with nitrogen and maintained at 50° for four hours. The solution was then cooled to room temperature and iodoacetamide (100 moles per mole of disulfide) was added with addition of NH_4OH as necessary to maintain constant pH.

Disulfide-linked peptides were reduced and alkylated in guanidine using radioactive iodoacetamide. For complete reduction and aminoethylation prior to tryptic digestion, Fab(t) (10 mg/ml) was dissolved in 8 M urea, 1.5 M in Tris, 0.003 M in EDTA, and 0.1 M in 2-mercaptoethanol, pH 8.5. After four hours the solution was made 0.8 M in ethylenimine. Twenty minutes after addition of the ethylenimine, the reagents were removed by gel filtration on Sephadex G-50.

4. Alkylation without prior reduction. Eu was dissolved (20 mg/ml) in 6 M guanidine hydrochloride, 0.5 M in Tris and 0.002 M in EDTA, apparent pH 8.1, previously flushed with nitrogen to remove oxygen, and the solution was placed in a water bath at 50°. At zero time, iodoacetamide-1-^{14}C was added in a 200-fold molar excess over Eu, or about a 6-fold molar excess over each half-cystine residue. After 4 hours, the protein was separated from reagents by gel filtration on Sephadex G-25 in 50% acetic acid.

5. Preparation of light and heavy chains. Heavy and light chains were prepared from partially reduced and alkylated Eu by gel filtration on Sephadex G-100 in 1 M propionic acid (Fleischman et al., 1962). For analytical purposes, 5 to 20 mg of partially reduced and alkylated protein was loaded on a 1 x 100 cm column. Larger amounts of chains were prepared on 4.5 x 150 cm columns with loads of 0.5 to

1 g. Heavy and light chain fractions were further purified by repeated gel filtration under the same conditions.

For the determination of total half-cystine and methionine content, chains were prepared by reduction in 0.01 M dithiothreitol and gel filtration in 1 M propionic acid which was 0.002 M in dithiothreitol.

6. Preparation of tryptic fragments. Five hundred milligrams of protein Eu was dissolved in 10 ml of 0.15 M NaCl, 0.05 M in $CaCl_2$. The solution was placed in a water-jacketed vessel at 25°, and the pH was adjusted to 8.0. Ten milligrams of trypsin treated with L-1-tosylamino-2-phenylethylchloromethylketone (TPCK-trypsin, Calbiochem, Los Angeles, Calif., lot 65345) was added, and the pH was maintained at 8.0 by the automatic addition of 0.1 N NaOH (Radiometer TTT-1 titrator). After one hour of digestion, 20 mg of soybean trypsin inhibitor (Nutritional Biochemicals Corp., Cleveland, Ohio, lot 8822, 5X recrystallized) was added. The sample was chilled at 4°. Starch zone electrophoresis (Kunkel, 1954) was used to separate the fragments.

Tryptic fragments were also separated by ion exchange chromatography on DEAE-cellulose (Cellex D, Bio Rad Laboratories, Richmond, Calif., exchange capacity 0.98 meq/g). In a typical fractionation, 5.5 g of digest was dialyzed against 0.005 M sodium phosphate, pH 8.5, and applied to a 3 x 35 cm column of Cellex D equilibrated with the same buffer. Fab(t) was not retarded by the column, and Fc(t) was eluted with a linear gradient from one liter of the initial buffer to one liter of the same buffer made 0.15 M in NaCl. In initial experiments, partially reduced and alkylated Eu was used. For later experiments on the arrangement of the disulfide bonds, Eu was digested without prior reduction and alkylation.

7. Preparation of papain fragments. Digestion with papain (Worthington Biochemical Corp., Freehold, New Jersey, lot PAP 5593, 2X crystallized) was performed as described by Porter (1958), except

that digestion was carried out for five minutes. The fragments were separated by zone electrophoresis on starch (Kunkel, 1954).

8. Cyanogen bromide cleavage. The protein was dissolved to a final concentration of 50 mg/ml in 70% formic acid. A 1.5 fold (w/w) amount of CNBr was added (Gross and Witkop, 1962) and the reaction was allowed to proceed at room temperature for four hours. The reaction was terminated by the addition of ten volumes of distilled water, followed immediately by lyophilization.

9. Enzymatic digestion of large fragments. Material to be digested with pepsin was dissolved in 5% formic acid at a final concentration of 10 mg/ml and placed in water bath at 37°. Pepsin (Worthington Biochemical Corp., 2X crystallized, lot No. PM 709) was added in three aliquots (each of 1% by weight), the first at zero time, the second after three hours, and the third after nine hours. After digestion for a total of 18 hours, the digest was lyophilized.

Material to be digested with trypsin was dissolved in 0.1 M ammonium acetate, pH 7.0, at a final concentration of 10 mg/ml and placed in a water bath at 37°. Trypsin treated with L-1-tosylamino-2-phenylethylchloromethylketone (Calbiochem, Los Angeles, Calif., lot 73325) equal to 2% of the substrate by weight was added, and the digest was lyophilized after 4 hours.

Tryptic digestion of the H_1-H_4-L_3 CNBr fragment complex was carried out as follows. Twelve milligrams of trypsin treated with L-1-tosylamino-2-phenylethylchloromethylketone (TPCK-trypsin, Calbiochem, Los Angeles, Calif., lot 65345) was added to a solution of 615 mg of the fragment complex in 60 ml of distilled water, pH 4, in a jacketed cell maintained at 37°. The solution was brought to pH 7.2 by the dropwise addition of 2 M NH_4OH, and the pH was maintained at 7.2 with a Radiometer TTT1b pH stat. After 4 hours, the turbid solution was brought to pH 3 by the dropwise addition of glacial acetic acid. The resulting clear solution was lyophilized.

10. Molecular weight determinations. A Spinco model E ultracentrifuge equipped with interference and phase plate schlieren optics and automatic temperature control was used. Sedimentation velocity experiments were done at 52,000 rpm in double sector cells with sapphire windows. Molecular weights of Eu, heavy and light chains, and Fab(t) and Fc(t) fragments were determined by the high speed equilibrium method (meniscus depletion) described by Yphantis (1964). Molecular weight data are presented as graphs of the logarithm of the concentration expressed in microns of fringe deviation versus the radius (r) squared divided by two, and the weight average molecular weights were calculated from the least squares slope of the line through the points. Molecular weight heterogeneity is reflected by consistent deviations from linearity in the graph. Partial specific volumes, calculated from the amino acid composition (McMeekin et al., 1949) were 0.73 cc/g for Eu, its constituent chains, and the tryptic fragments.

Uncertainty in the molecular weight due to errors in the determination of the concentration distribution in the cell was calculated to be 5%, using the procedure described by Yphantis (1964). Uncertainty in the calculated partial specific volume would introduce further errors, estimated to be less than 5%.

The molecular weight of peptide H-H-1 was determined in 6.3 M guanidine HCl, 0.1 M in Tris, pH 8.4, using the short column method of Yphantis (1960); in some experiments, the solvent was also 0.1 M in 2-mercaptoethanol. Insulin (bovine pancreas, recrystallized, Mann Research Laboratories, New York, N.Y., lot N2054) was used as a standard. The partial specific volume of peptide H-H-1 was estimated from its amino acid composition and corrected for the presence of guanidine (Tanford et al., 1967). A partial specific volume of 0.73 cc/g was used for insulin. Because of the uncertainty in the partial specific volume and the high density of the guanidine-Tris solvent, the uncertainty in the molecular weight was approximately 10%.

11. Amino acid analysis. Quantitative analyses were performed by the technique of Spackman, Stein and Moore (1958) with minor modifications, using a Beckman model 120C amino acid analyzer equipped with 6.6 mm flow cells, a 1 mv range recorder, and an Infotronix (Houston, Texas) model CRS-11AB integrator. Acidic and neutral amino acids were determined on a 57 cm column of Beckman AA-15 resin, with a buffer flow rate of 93 ml/hr using the standard 0.2 N sodium citrate buffers at pH 3.23 and 4.25. Basic amino acids were determined on a 4.8 cm column of Beckman PA-35 resin using 0.35 N sodium citrate buffer containing 5% (v/v) n-propanol (Hubbard, 1965) with a flow rate of 93 ml/hr. The columns were maintained at 50°. The analyzer was modified by adding a second ninhydrin pump and reaction coil so that the short column could be used continuously. The original suppressed 570 mμ channel, equipped with a 6.6 mm flow cell, was used exclusively for the short column. Analyses at the 10 nmole level were reproducible within 5%. The reported values for homoserine (Hsr) are the sums of homoserine and homoserine lactone. The total half-cystine content of Eu, heavy and light chains, and Fab(t) and Fc(t) fragments was determined on unalkylated samples by performic acid oxidation as described by Moore (1963). Cysteic acid was determined by amino acid analysis using glutathione as a standard. Tryptophan content was estimated from the tyrosine to tryptophan ratio determined by the spectrophotometric method of Goodwin and Morton (1946).

Hydrolysis was carried out at 110° in evacuated and sealed Pyrex tubes with metal free 6 N HCl prepared from glass distilled water saturated with HCl gas. Analyses were made on 20, 48, and 72 hour hydrolyzates of the whole protein, the constituent chains, and the Fab(t) and Fc(t) fragments. Corrections were made for the destruction of threonine and serine and the slow release of isoleucine and valine. Routine analyses of peptides were made on 18 to 22 hour hydrolysates and are uncorrected.

12. Separation of large fragments and peptides. Gel filtration was carried out in glass columns using Sephadex G-100, G-75, G-50 (fine) and G-25 (fine). Details of column size, solvent, and volume per fraction are given in the legends to the figures.

Mixtures of peptides were chromatographed on Bio-Rad AG50W x 4, 30-35 microns (Bio-Rad Laboratories, Richmond, Calif., lot No. 3934) in jacketed columns maintained at 40°. Linear gradients of pyridine acetate from 0.2 M pyridine brought to pH 3.1 with acetic acid to 2.0 M pyridine adjusted to pH 5.5 with acetic acid were employed. Redistilled spectral grade pyridine (Mallinckrodt Chemical Works, St. Louis, Mo.) was used to prepare all buffers. Flow rates were maintained at 30 ml/hr by means of a miniPump (Milton Roy Co., Philadelphia, Pa.) and fractions of 1-2 ml were collected. Further details are given in the legends to the figures.

13. Analysis of effluent fractions. The ultraviolet absorbance at 280 mμ and 230 mμ of the effluent fractions from Sephadex columns was measured with a Zeiss PMQII spectrophotometer. Ninhydrin analysis of the effluent fractions from some Sephadex columns and all ion exchange columns was carried out manually (Moore and Stein, 1954) or automatically using an AutoAnalyzer (Technicon Corporation, Ardsley, N.Y.) equipped with an automatic sampler attachment fitted to a Research Specialties Co. (Richmond, Calif.) model 1205A fraction collector. In the latter case, the signal from the colorimeter served as a single input to a linear recorder. Consequently the observed signal was proportional to transmittance rather than absorbance. An arbitrary scale of 0-3.0 was adopted, and all data from this instrument are reported as A^{*}_{570}. In some experiments, cystine containing fractions were identified by spotting 50 or 100 μl aliquots on paper and staining with nitroprusside (Toennies and Kolb, 1951). In other experiments, the fluorescence quenching of fluorescein mercuric acetate as described by Karush et al. (1964) was used. The cystine content of the effluent is given on an arbitrary scale.

14. Amino terminal analysis. Quantitative amino-terminal analysis on the protein Eu was performed by the carbamylation method of Stark and Smyth (1963). Qualitative amino-terminal analyses were carried out by the dansyl method as described by Cunningham et al. (1968).

15. Amino acid sequence determination. The amino acid sequence of peptides was determined by the dansyl-Edman method of Gray (1967a,b) as modified by Cunningham et al., (1968).

16. Preparation of diagonal maps. Diagonal electrophoresis (Brown and Hartley, 1966) was carried out in pyridine acetate buffer at pH 4.7 (pyridine: acetic acid: water, 25:25:950) in tanks under Varsol (Schwartz and Edelman, 1963). Electrophoresis in the first dimension was done for 1 hour and 20 minutes with a potential gradient of 50 v/cm. After drying, marker strips were cut from the paper and the remaining strip was oxidized over performic acid (40 ml of 97-100% formic acid and 2 ml of 30% H_2O_2) in a desiccator for 2 1/2 hours and then hung up to dry in a hood. Marker strips were stained for cystine with the fluorescein mercuric acetate reagent of Karush et al. (1964). The portion of the oxidized strip containing disulfide peptides as judged from the marker strips was sewn onto a 27 x 92 cm sheet of paper, and electrophoresis was done in the second dimension for 3 to 3 1/2 hours with a potential gradient of 28 v/cm. A mixture of ε-dinitrophenyl lysine, aspartic acid, and lysine was used to provide marker substances with neutral, acidic, and basic mobilities. In some maps, the lysine marker had run off the paper. After drying at room temperature, the maps were sprayed with dilute ninhydrin (Bennett, 1967) and the color was developed at room temperature. Peptides appearing off the diagonal in the same vertical zone were presumably linked by a disulfide bond before oxidation. These peptides were cut out and eluted with 50% pyridine. The eluates were dried by rotatory evaporation and hydrolyzed in 6 N HCl for 18 hours at 110^{o} prior to amino acid analysis.

17. Other methods. Immunoelectrophoresis was done using the technique of Scheidegger (1955). Immune diffusion was carried out as described by Olins and Edelman (1964). Starch gel electrophoresis in formate-urea was performed as described by Edelman and Poulik (1961). The protein concentration of starch zone eluates was determined by a modified Folin-Ciocalteu method (Lowry et al., 1951). Hexose was determined with anthrone reagent using a mixed standard of 4.5 parts galactose, 4.5 parts mannose, and 1 part of fucose (Muller-Eberhard and Kunkel, 1956).

Samples for liquid scintillation counting were added to 20 ml of a scintillation fluid containing 500 ml p-dioxane, 70 ml Liquifluor (Pilot Chemicals, Watertown, Mass.), 50 g naphthalene and enough toluene to make a final volume of 1 liter. Some samples were streaked on a 5 x 20 mm cellulose acetate strip, dried under an infrared lamp, and immersed in the same scintillation fluid for counting. A Packard (Donners Grove, Ill.) model 574 scintillation counter was used.

D. The Structure of Myeloma Protein Eu, its Constituent Chains, and the Enzymatic Fragments

Studies on the characterization of the myeloma protein Eu, its heavy and light chains, and enzymatic fragments were carried out to provide data for more detailed studies on the covalent structure of the protein. Results of these studies are presented first, inasmuch as they are necessary for the interpretation of the experiments on the disulfide bonds which follow.

1. Characterization of Eu myeloma protein. Protein Eu obtained from the ammonium sulfate precipitate of plasma by starch zone electrophoresis or DEAE-cellulose chromatography was free of other plasma proteins, as determined by antigenic analysis. Immunoelectrophoresis of purified Eu, using rabbit antiserum against whole human serum, showed a sharp line of precipitate characteristic of a myeloma protein,

rather than the broad arc of normal γG-immunoglobulin. No contamination with other serum proteins was observed. Genetic typing of Eu, carried out by Dr. H.H. Fudenberg, showed it to be Gm (a-, f+, b-) and Inv (a-). On the basis of this antigenic typing, it was concluded that the Eu heavy chain belongs to the γ1 subclass.

In the analytical ultracentrifuge, purified Eu sedimented as a single, symmetrical peak with an $s^{o}_{20,w}$ of 6.6S, in 0.05 M Tris, 0.15 M NaCl, pH 8.0. The molecular weight of Eu, determined by the high-speed sedimentation equilibrium method of Yphantis (1964), is shown in Figure 3 in terms of a plot of the logarithm of the protein concentration versus the radius squared divided by two. The data points fit a straight line and no curvature is apparent, suggesting that the preparation is homogeneous with respect to molecular weight. The weight average molecular weight of the preparation was 154,000 ± 8,000 daltons.

Hexose determinations showed that the carbohydrate content of Eu was similar to that of pooled human γG immunoglobulin (Clamp and Putnam, 1964). On this basis, the molecular weight of the carbohydrate component was estimated to be 4,000, yielding a molecular weight of 150,000 for the polypeptide component.

It is now possible to compare these results with the molecular weight calculated from the amino acid sequence (Table II). The molecular weights are in reasonable agreement, although the comparison suggests that the molecular weight of the carbohydrate moiety may be somewhat higher than 4,000 daltons. The amino acid compositions determined for the whole protein, its constituent chains, and tryptic fragments have been computed on the basis of the calculated, rather than the observed, molecular weights; this difference in normalization in no way affects the interpretation of these experiments.

The amino acid compositions of 20, 48, and 72 hour hydrolyzates of protein Eu are given in Table III. For amino acids which are stable

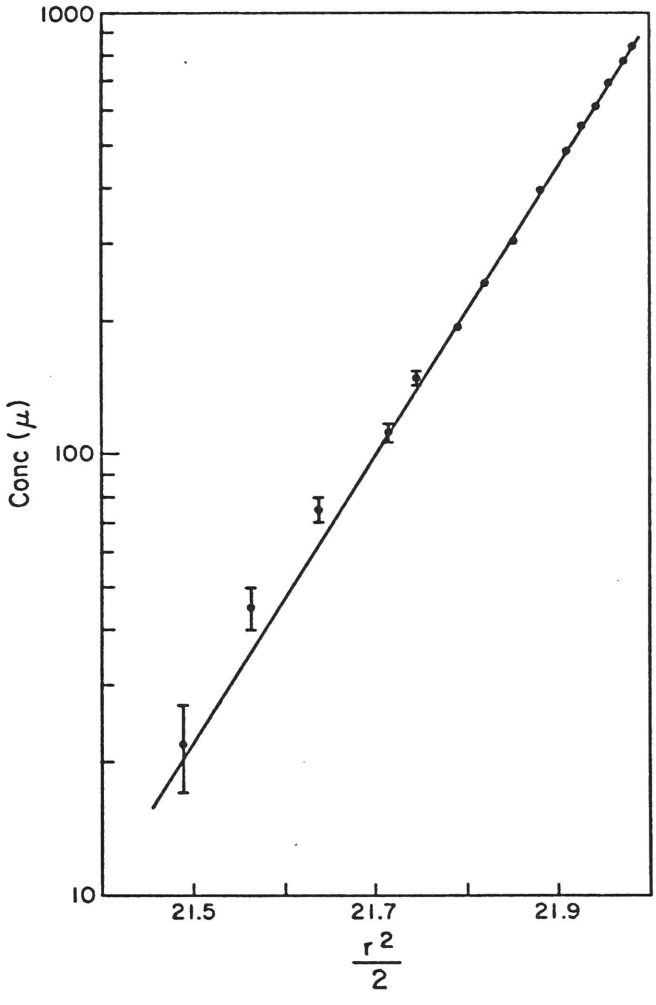


Figure 3. Plot of the logarithm of the concentration vs. (radius)^2/2 used for the determination of the molecular weight of Eu. Initial concentration 0.12 mg/ml in 0.05 M Tris, 0.15 M in NaCl, pH 8.0; equilibrium speed 20,000 rpm. Concentrations are expressed in microns of fringe deviation (μ). Bars indicate the estimated standard deviation (5μ) of each point.

TABLE II

Molecular Weights of Eu, Its Constituent Chains, and Enzymatic Fragments

	Measured	Calculated[a]	Protein Moiety[b]
Eu	154,000 ± 8,000	147,700	143,700
H	51,600 ± 2,600	50,500	48,500
L	23,500 ± 1,200	23,400	23,400
Fab(t)	46,000 ± 2,300	46,700	46,700
Fc(t)	48,000 ± 2,400[c]	54,400	50,400

[a] Calculated from molecular weight of protein moiety assuming that the total molecular weight of the carbohydrate is 4,000. All of the carbohydrate is attached to the Fc portion of the heavy chain (Waxdal *et al.*, 1968b).

[b] Calculated from the known amino acid sequence and rounded to the nearest 100 daltons.

[c] As discussed in the text, this molecular weight is known to be an underestimate.

TABLE III

Amino Acid Composition of Eu Myeloma Protein

	Residues per 143,700 g			
	20 hr	48 hr	72 hr	Average[a]
Lys	88.3	88.9	86.8	88.1
His	23.3	22.0	22.5	22.6
Arg	32.4	33.3	33.0	32.9
Asp	95.1	94.2	94.8	94.7
Thr	101	102	94.3	107
Ser	148	125	126	159
Glu	140	139	140	140
Pro	97.7	101	98.7	98.7
Gly	90.1	91.0	93.2	91.4
Ala	68.3	69.5	71.6	69.8
Cys	32.4			32.4
Val	113	118	124	124
Met	17.0			17.0
Ile	29.5	32.8	30.8	31.8
Leu	89.5	90.6	90.6	90.2
Tyr	49.6	51.9	52.1	51.2
Phe	44.4	44.6	45.7	44.9
Trp	21.4			21.4

[a] Threonine values are based on a linear extrapolation to zero hydrolysis time. Serine values are based on a linear extrapolation of 20 and 48 hr values to zero time. Valine values are from the 72 hr hydrolyzate. Half-cystine and methionine values are from 20 hr hydrolyzates of oxidized samples. The 20 hr value for isoleucine is not included in the average.

to acid hydrolysis, the average of the three values was taken. Threonine values are based on a linear extrapolation to zero hydrolysis time. The serine content decreased between 20 and 48 hours, but not between 48 and 72 hours. The value given is an estimate obtained by a linear extrapolation of the 20 and 48 hour values to zero hydrolysis time.

2. Characterization of Eu light and heavy chains. Light and heavy chains were prepared from protein Eu by partial reduction and alkylation under mild conditions followed by separation on Sephadex G-100 in 1 M propionic acid; a typical analytical chromatogram is shown in Figure 4. The small peak eluted first from the column appeared to contain aggregated material, as tested by starch gel electrophoresis in formate-urea. The second peak, designated H, accounted for 65% of the material loaded and contained the heavy chains. The final peak (L), accounted for 30% of the material loaded and contained the light chains. In most cases, the heavy and light chain fractions were further purified by repeated gel filtration under the same conditions. Light and heavy chains prepared by this method were free of cross-contamination as determined by starch gel electrophoresis in formate-urea.

The molecular weights of the chains were measured in 20% acetic acid containing 0.5% sucrose to provide a stabilizing density gradient (see Yphantis, 1964). A graph of concentration versus the radius squared divided by two for Eu heavy chain is shown in Figure 5. The line is straight, consistent with homogeneity of the preparation, and the weight average molecular weight of the preparation was 51,600 $\pm$ 2,600. A similar plot for the light chain is shown in Figure 6. The line is slightly curved, and the point weight average molecular weights (see Yphantis, 1964) tend to decrease as the concentration increases. The reciprocal of the reduced point weight average molecular weights plotted against the concentration at each point is shown in Figure 6 (insert). The plot is linear, and the least squares line through the points has an intercept corresponding to a molecular weight of 23,500.

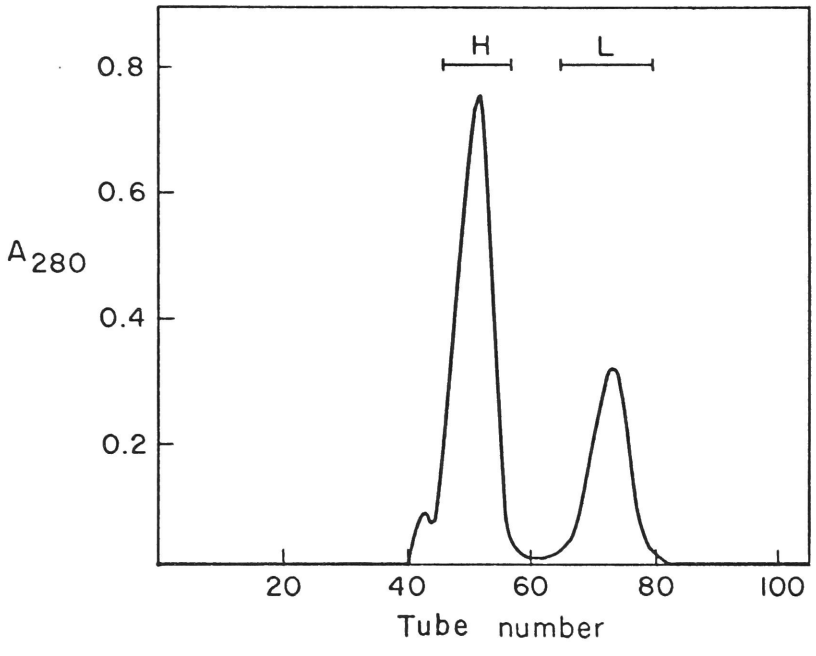


Figure 4. Separation of light and heavy chains from partially reduced and alkylated Eu (5 mg) by gel filtration. Column: Sephadex G-100 in 1 M propionic acid, 1 x 100 cm. Volume per tube, 0.83 ml; H = heavy chains, L = light chains, A_{280} = absorbance of effluent at 280 mμ.

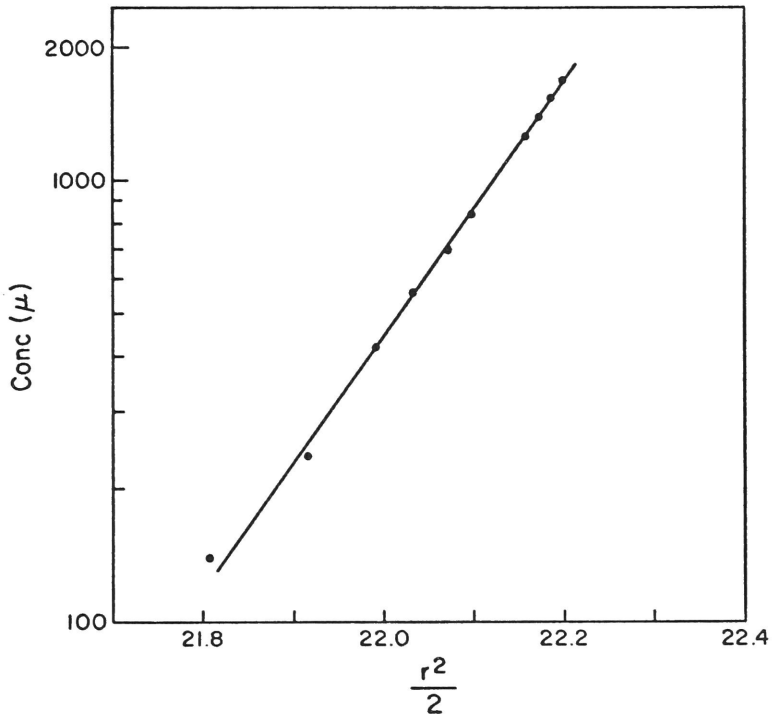


Figure 5. Plot of the logarithm of the concentration vs. (radius)^2/2 used for the determination of the molecular weight of Eu heavy chain. Initial concentration, 0.67 mg/ml in 20% acetic acid, 0.5% sucrose; equilibrium speed 32,000 rpm. Concentrations are expressed in microns of fringe deviation (μ).

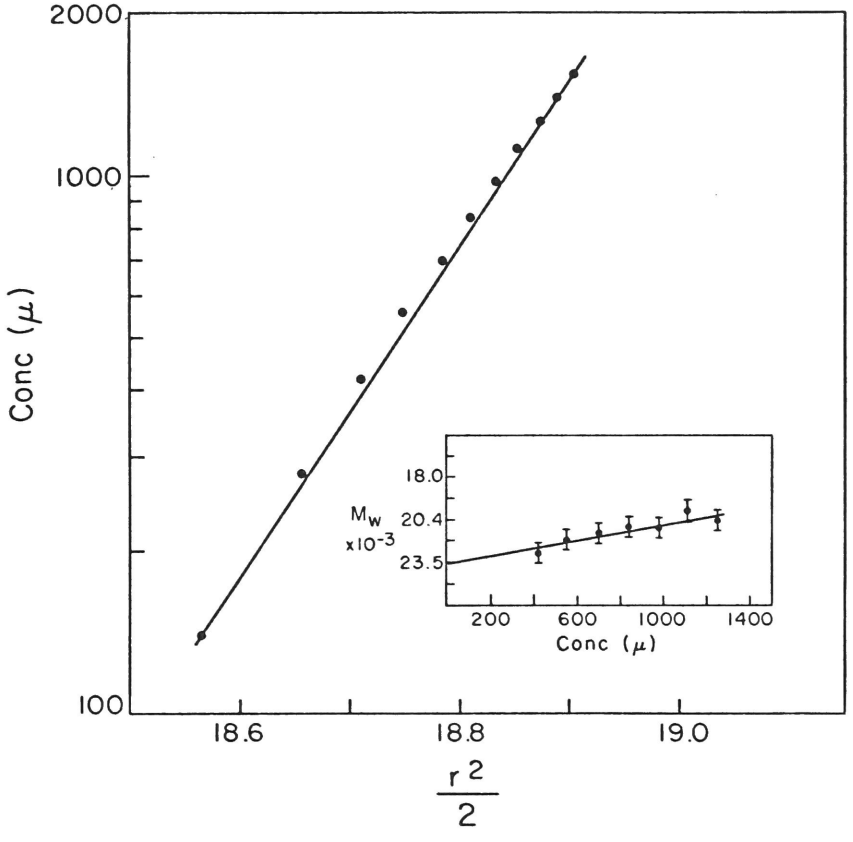


Figure 6. Plot of the logarithm of the concentration vs. (radius)^2/2 used for the determination of the molecular weight of Eu light chains. Initial concentration, 0.75 mg/ml in 20% acetic acid, 0.5% sucrose; equilibrium speed 52,000 rpm. Insert: reciprocal point weight average molecular weights of light chains as a function of concentration. Concentrations are expressed in microns of fringe deviation (μ).

The molecular weight of the light chain was taken to be 23,500 ± 1,200.

The amino acid compositions of the heavy and light chains are given in Tables IV and V. Total half-cystine and methionine values were measured by performic acid oxidation of chains prepared without alkylation.

3. Amino-terminal analysis of Eu and the isolated chains. End group analysis of Eu by the carbamylation method gave 1.7 moles of aspartic acid per mole of protein. Qualitative end group analysis of the isolated light chain by the dansyl method gave only aspartic acid; an amino terminal residue was not detected for the heavy chain.

The above data suggest that there are two light and two heavy chains per molecule of the protein Eu. The yields of heavy and light chains after reduction and alkylation are consistent with this hypothesis, and the observed molecular weight of the protein (154,000) is within 3% of twice the sum of the molecular weights of the chains (150,200). The amino-terminal analyses are consistent with the presence in the molecule of two heavy chains with blocked amino termini and two light chains with an amino terminal aspartic acid (or asparagine) residue, yielding two moles of amino-terminal aspartic acid per mole of myeloma protein. The presence of a blocked amino terminus in the heavy chain is consistent with the report of Press et al. (1966), who found an amino-terminal pyrrolidone carboxylic acid residue in the human γG-immunoglobulin Daw. The amino acid composition of Eu agress reasonably well with twice the sum of the compositions of the light and heavy chains (Table VI). For most amino acids, the sum differs from the observed composition by less than 4%. The largest difference (8%) occurs for threonine. The four-chain structure of Eu has been proven by further studies on the number and location of the cyanogen bromide fragments of the molecule and the arrangement of its disulfide bonds (see below).

TABLE IV

Amino Acid Composition of Eu Heavy Chain

	Residues per 48,500 g			
	20 hr	48 hr	72 hr	Average[a]
Lys	29.7	31.3	30.1	30.4
His	8.0	8.7	9.1	8.6
Arg	10.0	10.7	10.0	10.3
Asp	30.9	31.2	31.0	31.0
Thr	31.8	31.9	30.6	32.6
Ser	43.7	28.6	37.1	54.8
Glu	43.2	43.8	43.0	43.3
Pro	37.4	37.6	38.2	37.7
Gly	31.3	32.6	32.2	32.1
Ala	21.5	21.9	21.5	21.6
Cys	11.5			11.5
Val	40.7	44.0	45.5	45.4
Met	5.8			5.8
Ile	8.0	9.4	9.3	9.3
Leu	29.1	29.3	30.2	29.5
Tyr	18.1	18.4	16.8	18.2
Phe	15.8	16.1	15.2	15.7
Trp	8.1			8.1

[a] Threonine values are based on a linear extrapolation to zero hydrolysis time. Serine values are based on a linear extrapolation of 20 and 48 hr values to zero time. Isoleucine and valine values are from the 72 hr hydrolyzate. Half-cystine and methionine values are from 20 hr hydrolyzates of oxidized samples of heavy chains prepared without alkylation. The 72 hr value for tyrosine is not included in the average.

TABLE V

Amino Acid Composition of Eu Light Chain

	Residues per 23,400 g			
	20 hr	48 hr	72 hr	Average[a]
Lys	14.9	14.7	14.1	14.6
His	3.1	3.0	2.4	3.0
Arg	5.2	5.0	5.3	5.2
Asp	16.1	16.3	16.5	16.3
Thr	16.8	16.4	16.2	17.1
Ser	27.2	24.5	22.6	29.0
Glu	23.9	24.5	25.0	24.5
Pro	11.3	11.6	12.3	11.7
Gly	14.1	14.2	14.1	14.1
Ala	13.1	12.9	12.9	13.0
Cys	5.2			5.2
Val	15.2	16.3	16.8	16.8
Met	2.9			2.9
Ile	5.8	5.9	6.0	6.0
Leu	14.8	14.9	15.0	14.9
Tyr	8.4	8.7	8.9	8.7
Phe	7.7	8.0	8.1	7.9
Trp	3.0			3.0

[a] Threonine and serine values are based on a linear extrapolation to zero hydrolysis time. Isoleucine and valine values are from the 72 hr hydrolyzate. Half-cystine and methionine values are from 20 hr hydrolyzates of oxidized samples of light chains prepared without alkylation.

TABLE VI

Comparison of the Amino Acid Composition of Eu to that Calculated by Addition of the Compositions of Two Heavy and Two Light Chains

	Light Chain Residues per 23,400 g	Heavy Chain Residues per 48,500 g	2(Heavy + Light)	Eu Residues per 143,700 g
Lys	14.6	30.4	90.0	88.1
His	3.0	8.6	23.2	22.6
Arg	5.2	10.3	31.0	32.9
Asp	16.3	31.0	94.6	94.7
Thr	17.1	32.6	99.4	107
Ser	29.0	54.8	168	159
Glu	24.5	43.3	136	140
Pro	11.7	37.7	98.8	98.7
Gly	14.1	32.1	92.4	91.4
Ala	13.0	21.6	69.2	69.8
Cys	5.2	11.5	33.4	32.4
Val	16.8	45.4	124	124
Met	2.9	5.8	17.4	17.0
Ile	6.0	9.3	30.6	31.8
Leu	14.9	29.5	88.8	90.2
Tyr	8.7	18.2	53.8	51.2
Phe	7.9	15.7	47.2	44.9
Trp	3.0	8.1	22.2	21.4

4. Characterization of the Fab(t) and Fc(t) fragments. Because of its restricted specificity, trypsin, rather than papain, was used for the preparation of fragments from Eu. The highest yields of fragments were obtained by digesting partially reduced and alkylated Eu in 0.15 M NaCl, 0.05 M in $CaCl_2$ at pH 8.0. After the addition of trypsin (Figure 7) about four moles of base per mole of protein were taken up in the first five minutes. The rate of base uptake dropped steadily, and after 60 minutes of digestion about seven moles of base had been taken up per mole of protein.

Electrophoresis of a one-hour digest on starch (Figure 8) showed three components. The slowest moving (most cathodal) component, accounting for 63% of the protein, was immunologically identical to the Fab fragment produced by papain digestion as shown in Figure 9. The fastest moving component, accounting for 34% of the protein, was immunologically identical to the Fc fragment (Figure 9). The tryptic fragments were designated Fab(t) and Fc(t) respectively, to distinguish them from the similar products of papain digestion. A minor component which remained near the origin (Figure 8) accounted for about 3% of the digested protein. Its electrophoretic behavior on cellulose acetate was identical to that of undigested myeloma protein.

The molecular weights of Fab(t) and Fc(t) fragments were determined in 0.05 M Tris, 0.15 M in NaCl, pH 8.0. The graph of concentration versus the radius squared divided by two for Fab(t) is shown in Figure 10. The weight average molecular weight for the whole preparation was 46,000 $\pm$ 2,300. A similar plot for Fc(t) (Figure 11) showed consistent deviation of the points at lower radii, suggesting the presence of lower molecular weight components. The weight average molecular weight of the largest component was estimated to be 48,000 $\pm$ 2,400; this value may be low because of the heterogeneity (cf. Table II). The preparation may be composed of a population of Fc(t) fragments which are similar but not identical and which arise because of lack of strict specificity in the limited tryptic cleavage.

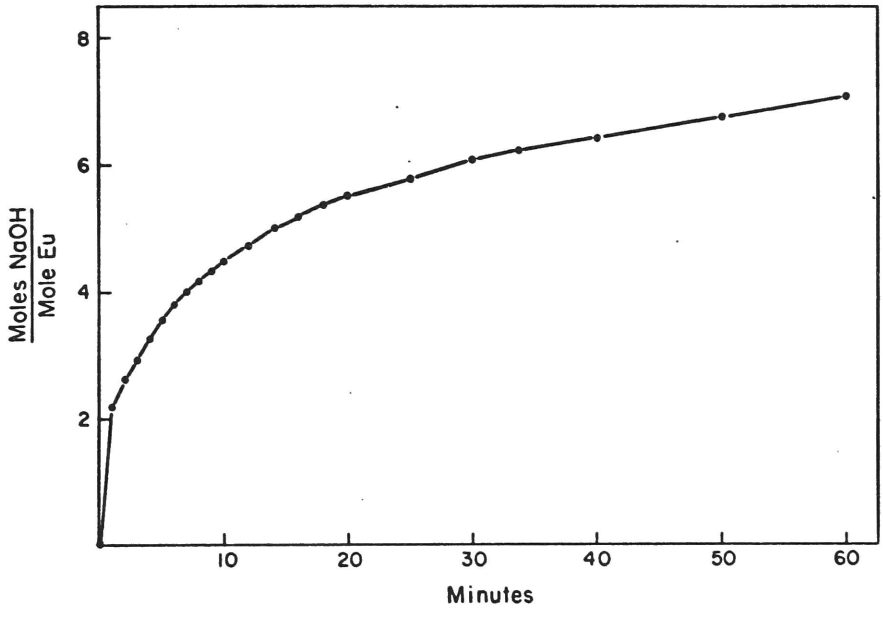


Figure 7. Base uptake during tryptic digestion of partially reduced and alkylated Eu. The protein (500 mg) was dissolved in 10 ml of 0.15 M NaCl, 0.05 M in $CaCl_2$ at 25°, and the pH was maintained at 8.0 by the addition of 0.1 N NaOH.

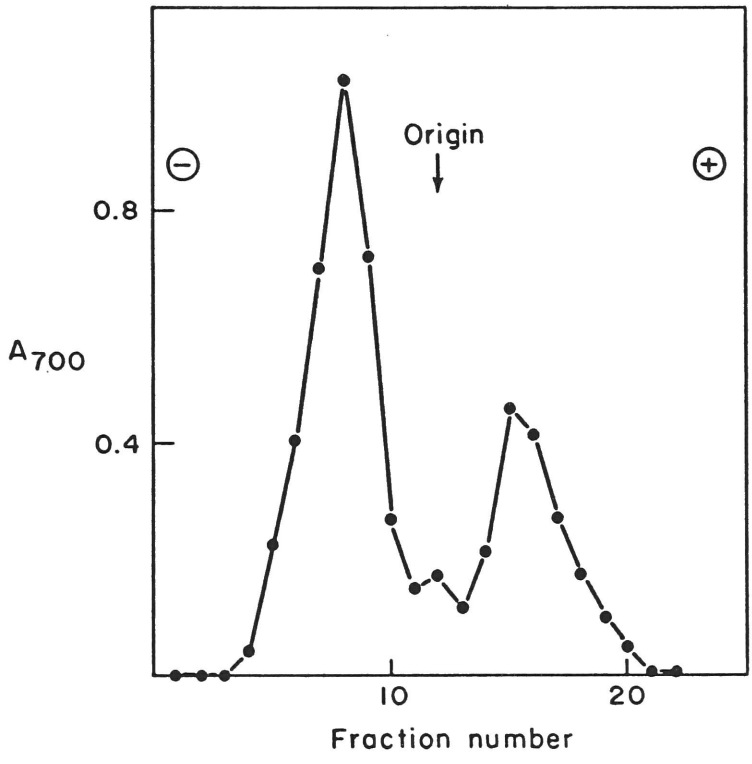


Figure 8. Zone electrophoresis on starch of a tryptic digest of partially reduced and alkylated Eu. Ordinate: Protein concentration as determined with Folin's reagent, expressed as absorbance of the reaction mixture at 700 mμ (A_{700}). Abscissa: fraction number; (-) = cathode, (+) = anode.

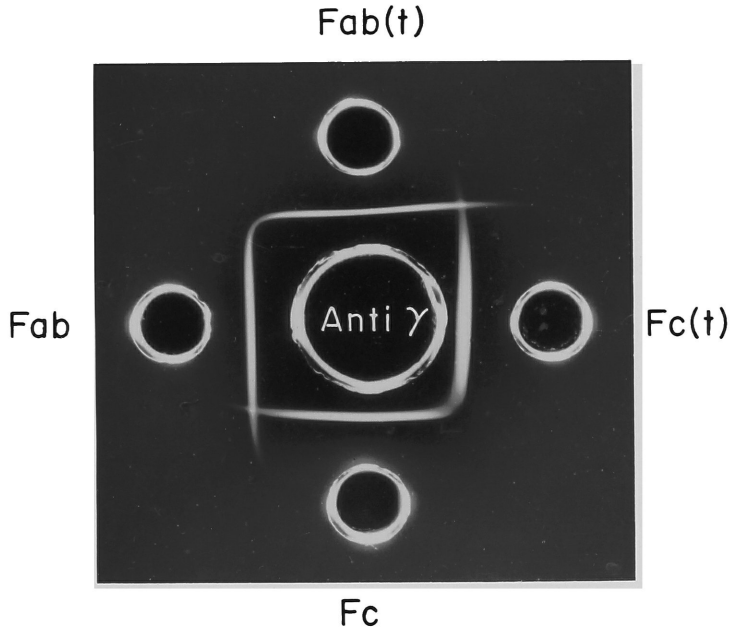


Figure 9. Comparison of tryptic and papain fragments of Eu by immuno-diffusion. Fab(t), Fc(t) = tryptic fragments; Fab, Fc = papain fragments. Anti γ = rabbit antiserum against human γ-globulin.

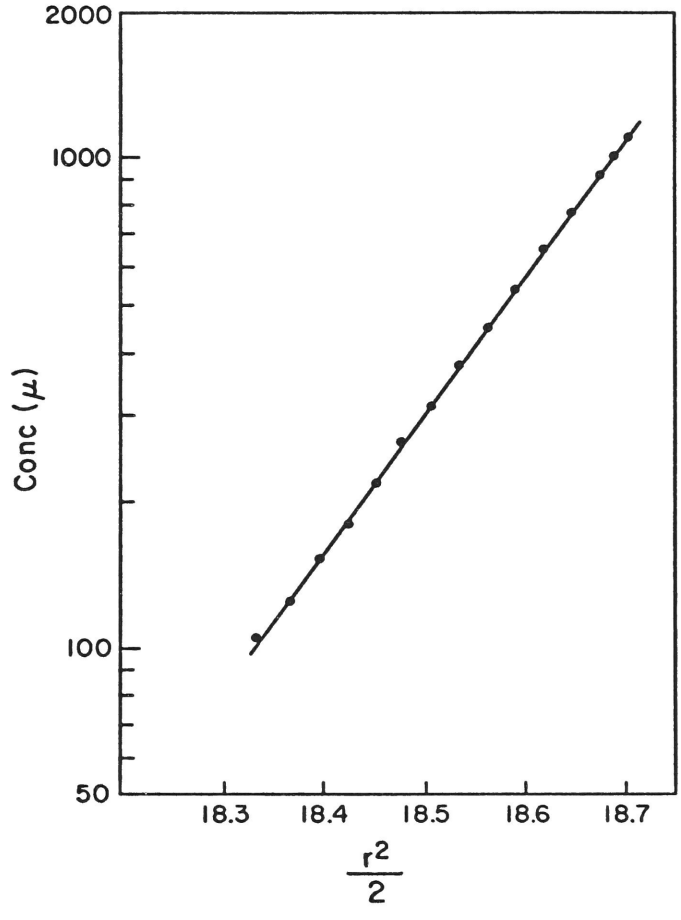


Figure 10. Plot of the logarithm of the concentration vs. $(\text{radius})^2/2$ used for the determination of the molecular weight of the Fab(t) fragment. Initial concentration, 0.15 mg/ml in 0.05 M Tris, 0.15 M in NaCl, pH 8.0; equilibrium speed, 34,000 rpm. Concentrations are expressed in microns of fringe deviation (μ).

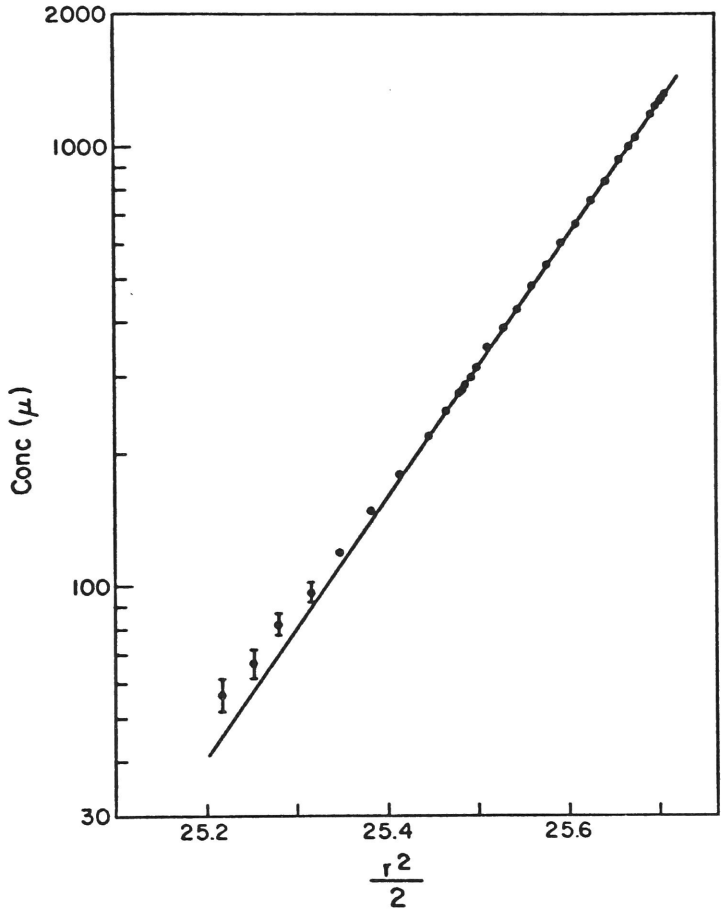


Figure 11. Plot of the logarithm of concentration vs. $(\text{radius})^2/2$ used for the determination of the molecular weight of the Fc(t) fragment. Initial concentration 0.26 mg/ml in 0.05 M Tris, 0.15 M in NaCl, pH 8.0; equilibrium speed 34,000 rpm. Concentrations are expressed in microns of fringe deviation (μ). Bars indicate the estimated standard deviation (5μ) of each point.

TABLE VII

Amino Acid Composition of Fab(t) and Fc(t) Fragments[a]

	Fab(t) Residues per 46,700 g	Fc(t) Residues per 50,400 g	2 Fab(t) + Fc(t)	Eu Residues per 150,000 g
Lys	29.6	34.8	94.0	88.1
His	4.1	14.4	22.6	22.6
Arg	9.2	11.9	30.3	32.9
Asp	31.0	41.3	103	94.7
Thr	38.0	31.4	107	107
Ser	60.1	38.2	158	159
Glu	46.4	53.4	146	140
Pro	27.2	46.2	101	98.7
Gly	39.4	22.7	102	91.4
Ala	30.0	17.2	77.2	69.8
Cys	10.1	12.0	32.2	32.4
Val	43.4	48.9	136	124
Met	6.0	6.0	18.0	17.0
Ile	12.1	9.1	33.3	31.8
Leu	29.7	35.7	95.1	90.2
Tyr	17.3	19.1	53.7	51.2
Phe	15.9	15.2	47.0	44.9
Trp	9.7	6.9	26.3	21.4

[a] Threonine and serine contents are based on a linear extrapolation of values from 20 and 72 hr hydrolyzates to zero time. Isoleucine and valine values are from 72 hr hydrolyzates. Half-cystine and methionine values are from 20 hr hydrolyzates of oxidized fragments prepared without reduction and alkylation.

The amino acid compositions of the tryptic fragments are given in Table VII together with the calculated composition of the whole protein based on the sum of one Fc(t) and two Fab(t) fragments per mole. The calculated composition agrees with the observed composition within 8%. These compositions and the yields after starch zone electrophoresis suggest that losses of large amounts of material due to extensive tryptic digestion do not occur in the preparation and isolation of the tryptic fragments.

Because of the possibility of disulfide interchange in the presence of reducing agent, Fab(t) and Fc(t) fragments used for the determination of the arrangement of the intrachain disulfide bonds were prepared by limited tryptic digestion of Eu without prior reduction and alkylation. Results essentially similar to those described for the partially reduced and alkylated protein were obtained.

5. The total half-cystine content of Eu. Amino acid analyses of performic acid oxidized Eu and its constituent chains and fragments (Tables III, IV, V, and VII) show that Eu contains 32 half-cystinyl residues per mole, 11 of which are in each heavy chain and 5 of which are in each light chain. The Fab(t) fragment contains 10 residues per mole, and the Fc(t) fragment contains 12.

The presence of free sulfhydryl groups in Eu was investigated by alkylation with iodoacetamide in a denaturing solvent to expose any relatively unreactive sulfhydryl groups as described in Materials and Methods. The incorporation of iodoacetamide into the protein measured both by radioactivity and amino acid analysis was less than 0.3 moles per mole of protein. A similar experiment performed after reduction with dithiothreitol led to the incorporation of 32 moles of iodoacetamide per mole of protein. On the basis of these data, we concluded that there are no free sulfhydryl groups in Eu. All of the half-cystines participate in bonds which are susceptible to reduction with dithiothreitol and thus are probably disulfide bonds.

6. The detailed structure of Eu. In order to facilitate the discussion of the experiments which prove the arrangement of the disulfide bonds of protein Eu, it is now necessary to summarize the results of studies on the CNBr fragments (Waxdal et al., 1968a,b) and the amino acid sequence of the light and heavy chains (Cunningham et al. 1968; Gottlieb et al., 1968; Rutishauser et al., 1968; Edelman et al., 1969) which have been carried out primarily by other workers in the same laboratory.

Cyanogen bromide cleavage of Eu yields ten unique fragments (Table VIII), three from each light chain (L_1 to L_3) and seven from each heavy chain (H_1 to H_7). Cleavage of the Fab(t) and Fc(t) fragments yields two new fragments, H_{4A} and H_{4B}, respectively. Fragment H_{4B} contains about 30 residues, indicating that the major point of cleavage to form Fab(t) and Fc(t) is near the carboxyl terminus of the region corresponding to fragment H_4. The order of the CNBr fragments in the molecule was proven by isolation of overlap peptides containing methionine (Waxdal et al., 1968a). These studies provide unequivocal proof of the four chain structure of γG immunoglobulins (Edelman and Gally, 1964).

A model of the Eu molecule based on the CNBr fragments is shown in Figure 12. The CNBr fragments are indicated by the bars and the length of each bar is proportional to the molecular weight of that fragment. The half-cystinyl residues in the protein are indicated by the vertical marks; the position of each mark represents the position of that residue in the sequence. For convenience in discussing the disulfide bonds of the molecule, the half-cystines have been numbered in order from L-I to L-V in the light chain and H-I to H-XI in the heavy chain, as shown in Figure 12.

The cyanogen bromide fragments fall into three classes with respect to the disulfide bonds. The first class includes fragments which contain no half-cystine and thus for our purposes can be

TABLE VIII

CNBr Fragments from Eu (Waxdal et al., 1968b)

Fragment	Yield (%)[a]	Molecular Weight x 10^{-3}	Obtained From				
			Light	Heavy	Fd(t)	Fc(t)	Fab(t)
L_1-L_2	70	10.5[b]	+,+				+,+
L_3	67	13.0[b]	+				+
H_1	56	5.1[b]		+	+		+
H_2	70	0.5[b]		+	+		+
H_3	100	3.0[b]		+	+		+
H_4	99	18.5[c]		+			
H_{4A}	-	15.5[c]			+		+
H_{4B}	-	3.2[b]				+	
H_5	75	14.8[c,d]		+		+	
H_6	60	8.6[c]		+		+	
H_7	100	2.0[b]		+		+	

[a] From Eu. [b] Calculated from animo acid composition. [c] Determined by sedimentation equilibrium. [d] Includes carbohydrate.

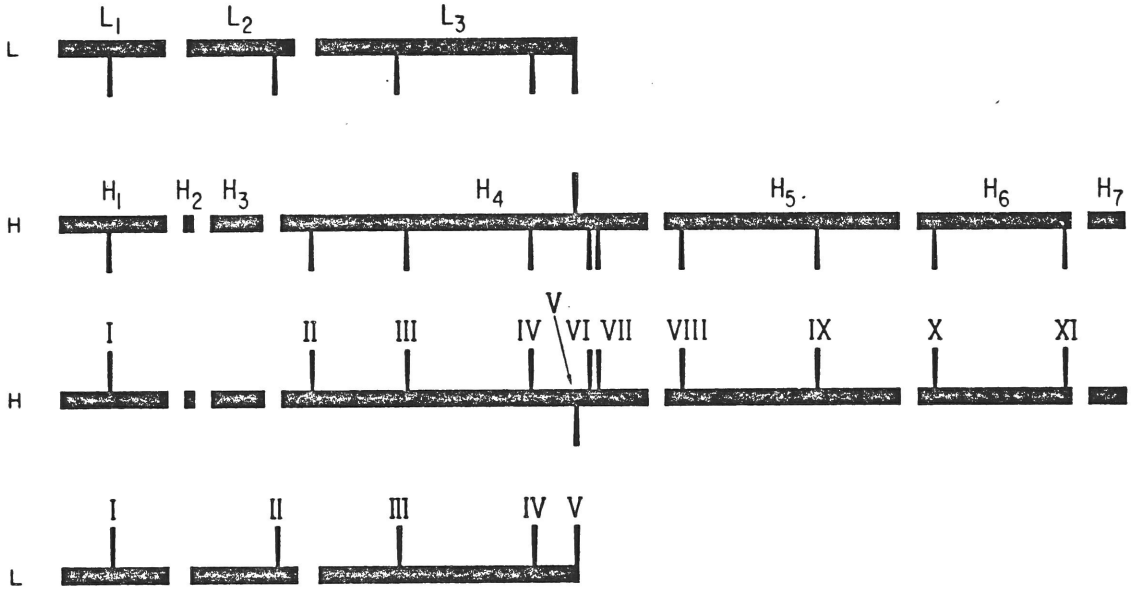


Figure 12. The structure of Eu based on studies of the CNBr fragments (Waxdal et al., 1968a,b). The length of the bar representing each fragment is proportional to the molecular weight of that fragment. The position of the vertical mark representing each half-cystinyl residue corresponds to the position of that residue in the amino acid sequence.

neglected. These are fragments H_2, H_3, and H_7. The second class consists of fragments which contain half-cystine but may be isolated free of other fragments without reduction and alkylation, indicating that they are not linked to other fragments by disulfide bonds. These are fragments H_5 and H_6. The third class of fragments contains half-cystine and can be isolated free of other fragments only after reduction and alkylation, suggesting that they contain disulfide bonds which link them to other fragments. Only two of these fragment complexes are isolated after cyanogen bromide cleavage of the protein Eu. One contains fragments L_1 and L_2, and is called the L_1-L_2 complex. The second is made up of fragments H_1, H_4, and L_3, and is called the H_1-H_4-L_3 complex. A similar fragment complex may be isolated by CNBr cleavage of Fab(t); this is the H_1-H_{4A}-L_3 complex. Because the H_1-H_4(or H_{4A})-L_3 complex is the only one which contains both light and heavy chain components, it must contain the disulfide bond linking the light and heavy chains. These fragment complexes provide starting material for the isolation of some of the disulfide containing peptides.

The amino acid sequence around each of the half-cystines in the molecule is different enough so that the amino acid compositions of small cystine-containing peptides isolated from extensive enzymatic digest were sufficient to identify unequivocally the individual half-cystinyl residues.

E. The Interchain Disulfide Bonds of Eu

1. The number of interchain bonds. Several lines of evidence have suggested that some of the disulfide bonds of immunoglobulins form covalent links between their polypeptide chains, as discussed previously. From earlier work (Fleischman et al., 1963; Christian and Schur, 1965; Cecil and Wake, 1962), it seemed likely that conditions could be found under which the interchain disulfide bonds of protein Eu could be reduced without significant reduction of intrachain disulfide bonds. The incorporation of iodoacetamide into the protein

could be used to determine the extent of reduction, and the number of interchain bonds could be counted.

A series of experiments was carried out to determine the extent of incorporation of iodoacetamide into Eu under different reducing agent concentrations, alkylating agent concentrations, and reaction times (Table IX). These data indicate that about eight moles of iodoacetamide are rapidly incorporated into the protein after exposure to the reducing agent. Under these conditions, alkylation without prior reduction resulted in the incorporation of about 0.5 moles of iodoacetamide per mole of protein. These data suggested that there may be four interchain disulfide bonds in the protein Eu.

2. Localization of the interchain bonds in the chains and fragments. In order to localize the bonds which were susceptible to reduction and alkylation under these mild conditions, a larger amount of partially reduced and alkylated protein was prepared by reduction with 0.005 M dithiothreitol for 40 minutes and alkylation with iodoacetamide-1-^{14}C (final concentration 0.010 M) for 20 minutes. After extensive dialysis against water to remove excess reagent, 8.0 moles of iodoacetamide had been incorporated into the protein (Table X). Eight moles of S-carboxymethylcysteine per mole of protein was recovered after acid hydrolysis, indicating that all of the label was present as S-carboxamidomethylcysteine.

Heavy and light chains were prepared in 95% yield from the reduced and alkylated protein, indicating that all of the interchain bonds had been broken. The specific activities of the chains are shown in Table X. The light chain contained one mole of S-carboxamidomethylcysteine per mole and the heavy chain contained three moles per mole. All of the radioactivity in the reduced, alkylated protein was recovered in the isolated chains.

TABLE IX

Incorporation of Iodoacetamide-1-^{14}C into Eu Under Different Conditions of Reduction and Alkylation[a]

Expt.	Dithiothreitol concentration (molar excess over protein)	Reduction time (min.)	Iodoacetamide concentration (molar excess over protein)	Alkylation time (min.)	Moles radio-activity per mole protein
1	none	-	0.060 M (400X)	20	0.5 ± .01
2	0.005 M (30X)	20	0.030 M (200X)	20	7.7 ± .1
		40	0.030 M	20	7.3 ± .2
		60	0.030 M	20	8.5 ± .2
3	0.015 M (100X)	20	0.060 M (400X)	20	7.8 ± .9
		60	0.060 M	20	8.3 ± .8
		80	0.060 M	40	8.9 ± .1
		120	0.060 M	20	8.9 ± .3

[a] The protein concentration was 22 mg/ml in 0.15 M Tris, 0.15 M NaCl, and 0.002 M in EDTA, pH 8.0. Reactions were carried out at room temperature. After alkylation, reagents were removed by gel filtration on Sephadex G-25, and aliquots of the protein fraction were streaked on cellulose acetate strips for counting. Protein concentration were determined with a modified Folin-Ciocalteu reagent (Lowry *et al.*, 1951). Results are reported as the mean ± the average deviation of duplicate or triplicate samples.

TABLE X

Content of S-Carboxamidomethylcysteine in Partially Reduced and Alkylated Eu and Isolated Chains and Fragments

	Moles Radioactivity/Mole
Eu, alkylated	0.5
Eu, partially reduced and alkylated	8.0
Eu light chain	0.9
Eu heavy chain	3.2
2(heavy + light) calc.	8.2
Eu Fab(t)	2.0
Eu Fc(t)	3.7*
2 Fab(t) + Fc(t) calc.	7.7

* Determined as S-carboxymethylcysteine after acid hydrolysis.

Fab(t) and Fc(t) fragments were prepared from the partially reduced and alkylated protein. The Fab(t) fragment contained two moles of S-carboxamidomethylcysteine per mole and the Fc(t) fragment contained four moles per mole (Table X). All of the radioactivity in the reduced, alkylated protein was recovered in the fragments.

The enzymatic fragments were analyzed to determined whether the iodoacetamide was broadly distributed or was attached to specific half-cystinyl residues. Fab(t) was fully reduced and aminoethylated in urea and digested with trypsin. The fraction of the digest soluble in 2% acetic acid (containing 94% of the radioactivity) was subjected to gel filtration on Sephadex G-25 (Figure 13). Two peaks of radioactivity were found which together accounted for 83% of the radioactivity loaded. Paper electrophoresis at pH 6.5 of fraction I gave a single, neutral, radioactive fraction. Fraction II yielded a single, acidic, radioactive fraction. These data suggested that greater than 80% of the radioactivity in Fab(t) was present in two tryptic peptides.

Fc(t) was cleaved with cyanogen bromide and the fragments were separated by gel filtration (Figure 14). Eighty percent of the radioactivity loaded on the column was recovered, and the indicated fraction contained 75% of the recovered radioactivity. Subsequent purification of this fraction by gel filtration on Sephadex G-50 in 2% acetic acid (Figure 14, bottom) yielded a fragment which was identified as H_{4B} by amino acid composition and end group analysis (Waxdal *et al.*, 1968b). Fragment H_{4B} contains two moles of S-carboxamidomethylcysteine; because there are two moles of H_{4B} per mole of Fc(t) (Waxdal *et al.*, 1968a), all of the radioactivity in the Fc(t) fragment (Table X) is accounted for by this CNBr fragment.

The above data suggest that alkylation of partially reduced Eu takes place at specific half-cystinyl residues, one of which is located in each light chain, and three of which are in each heavy chain. Two of these half-cystines in the heavy chain are contained in cyanogen bromide fragment H_{4B}.

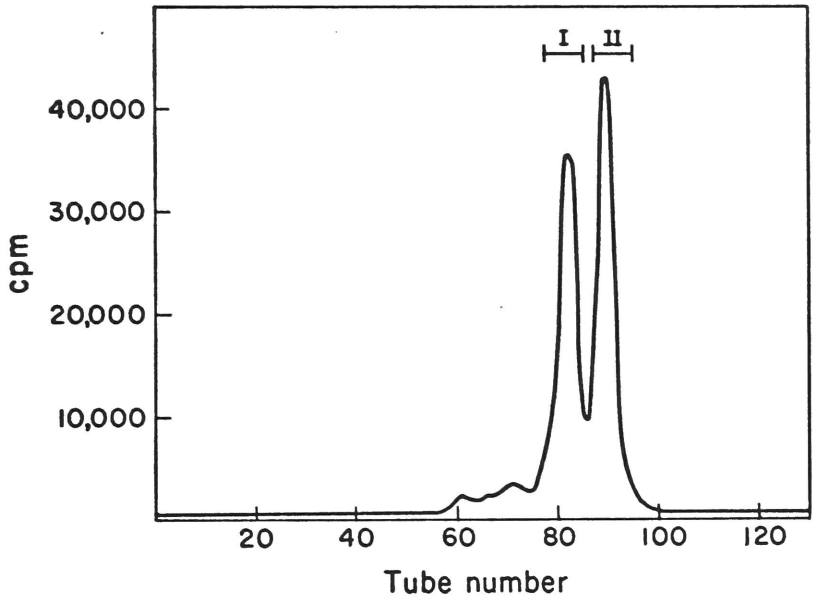


Figure 13. Gel filtration of a tryptic digest of Fab(t). The Fab(t) was prepared from partially reduced Eu which was alkylated with iodoacetamide-1-^{14}C and then was fully reduced and aminoethylated before digestion. Column: Sephadex G-25 fine in 2% acetic acid, 1.0 x 150 cm. Volume per tube, 1.0 ml; cpm, counts per minute per tube.

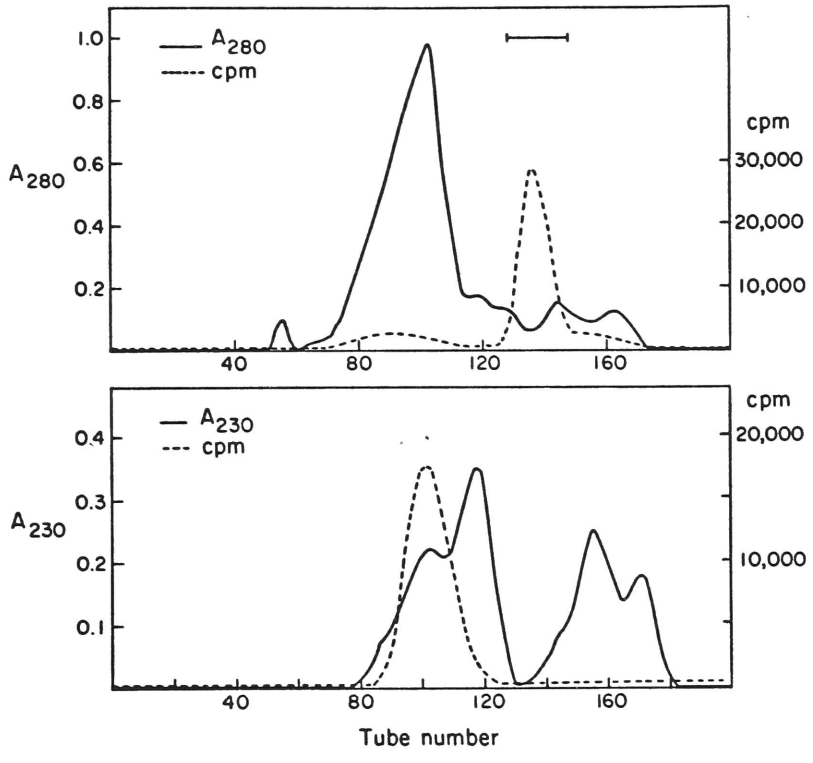


Figure 14. Gel filtration of CNBr cleaved Fc(t) (26 mg, top). The Fc(t) was prepared from partially reduced Eu which was alkylated with iodoacetamide-1-^{14}C. Column: Sephadex G-100 in 1 M propionic acid, 1 x 110 cm. Volume per tube, 0.5 ml; A_{280}, absorbance of effluent at 280 mμ; cpm, counts per minute per tube. The fraction indicated by the bar was further purified by gel filtration on Sephadex G-50 in 2% acetic acid (bottom). Column: 1.2 x 60 cm. Volume per tube, 0.5 ml; A_{230}, absorbance of effluent at 230 mμ; cpm, counts per minute per tube. Tubes containing significant radioactivity were pooled and lyophilized.

3. Isolation of peptides containing the intact interchain disulfide bonds. As has been shown above, all of the interchain disulfide bonds are contained in the H_1-H_4-L_3 fragment complex obtained by the cyanogen bromide cleavage of Eu (Waxdal et al., 1968a,b). In order to isolate peptides containing these bonds, this fragment complex (523 mg) was digested with trypsin at pH 7.2, and the digest was fractionated by gel filtration on Sephadex G-50 (Figure 15). Effluent fractions were pooled as indicated and lyophilized. The tryptic peptides containing the intact interchain disulfide bonds were isolated from these three fractions by a combination of gel filtration and ion-exchange chromatography.

To test the possibility that extensive disulfide interchange might occur under the conditions of the tryptic digest, a control digest of fraction I was prepared in the presence of 0.01 M iodoacetamide-1-^{14}C (approximately a five-fold molar excess over the total half-cystine). Separation of the peptides on Sephadex G-50 showed that less than 10% of the half-cystine had been alkylated, as measured by the incorporation of radioactivity. This radioactivity was diffusely distributed in the peptide fractions. If certain disulfide bonds were exceptionally susceptible to interchange, peaks of radioactivity corresponding to highly labeled peptides would have been observed. These data suggest that extensive disulfide interchange did not occur under the digestion conditions.

a. The light chain-heavy chain disulfide bond. Preliminary experiments indicated that the tryptic peptide containing the intact light chain-heavy chain disulfide bond contained less than ten residues. For this reason, fraction C (Figure 15) was used as starting material for the isolation of this peptide. It was anticipated (Pink and Milstein, 1967a; Cunningham et al., 1968) that the half-cystinyl residue contributed to this disulfide bond by the light chain is included in the carboxyl-terminal tripeptide, Gly-Glu-Cys. Therefore

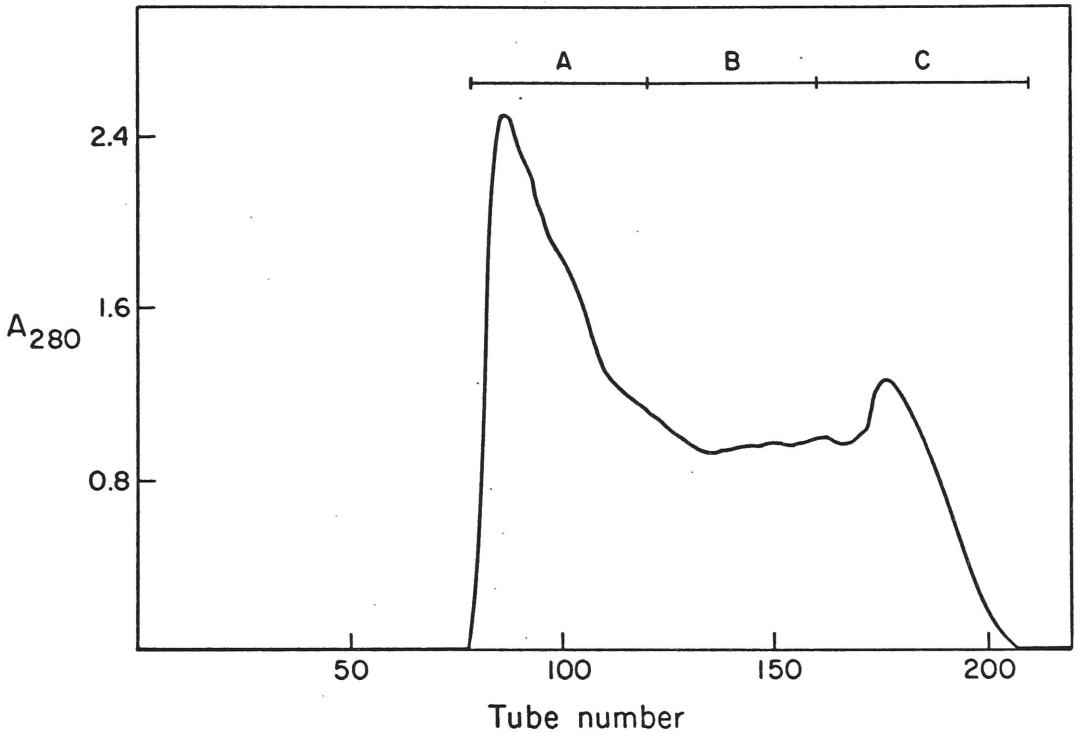


Figure 15. Gel filtration of the tryptic digest of fraction I obtained by CNBr cleavage of Eu. Column: Sephadex G-50 (fine) in n-propyl/alcohol : acetic acid: water (1:1:3, v/v/v), 2.5 x 190 cm. Volume per tube, 4.2 ml; A_{280}, absorbance of effluent at 280 mμ.

peptide fractions having a high ratio of cystine to lysine or arginine and containing glutamic acid and glycine were sought. The nitroprusside stain (Toennies and Kolb, 1951) was used to test column effluent fractions for cystine.

Gel filtration of fraction C on Sephadex G-25 in 5% formic acid gave 65 mg of nitroprusside-positive material which was filtered again on the same column (Figure 16) yielding 35 mg of a cystine containing peptide mixture. After a third gel filtration on the same column, this mixture was chromatographed on AG50X4 (Figure 17). A single nitroprusside-positive fraction was obtained from which peptide L-H-1 (Table XI) was isolated by chromatography with a more shallow gradient on AG50X4 (Figure 17). The yield of L-H-1 after the six steps in the purification was 16%.

Amino terminal analysis of L-H-1 indicated the presence of two end groups, glycine and serine. High voltage paper electrophoresis of L-H-1 at pH 4.7 demonstrated the presence of a single, ninhydrin-positive component with a small net negative charge. Following oxidation with performic acid, two negatively charged, ninhydrin-positive components were observed.

Reduction of L-H-1 with dithiothreitol and alkylation with iodoacetamide-1-^{14}C followed by gel filtration on Sephadex G-25 in 2% acetic acid gave two radioactive components, L-H-2 and L-H-3 (Table XI), in addition to reagents. The amino acid sequence of L-H-2 was Ser-CMCys-Asp-Lys. This peptide was also isolated from tryptic digests of H_4 and Fd(t), and contains half-cystine residue H-V. The sequence of L-H-3 was Gly-Glu-CMCys, the carboxyl-terminal tryptic peptide of the light chain (Cunningham *et al.*, 1968), containing half-cystine residue L-V. These data show that L-H-1 is the tryptic peptide containing the intact light chain-heavy chain disulfide bond and that this bond links half-cystine residues L-V and H-V.

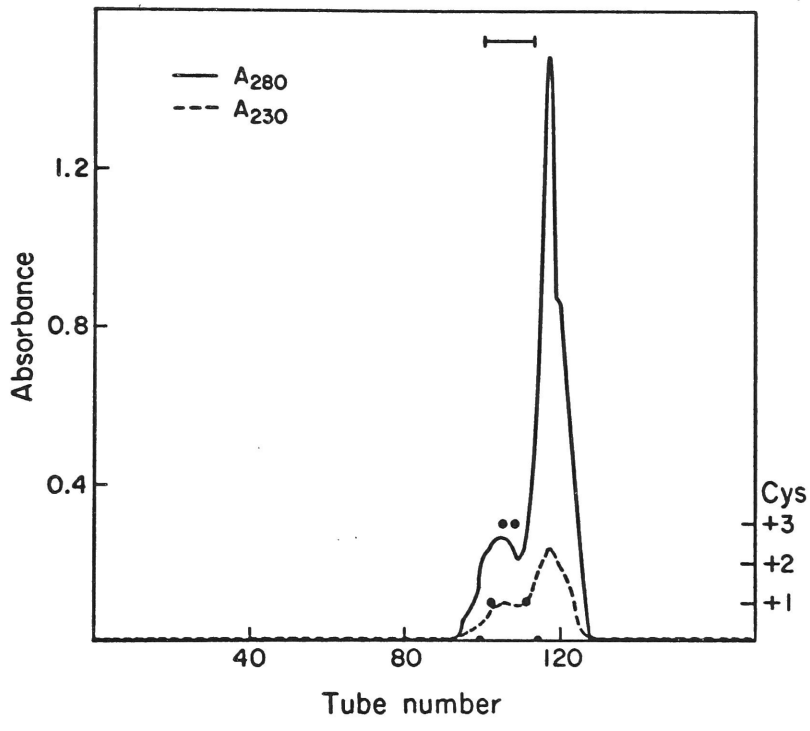


Figure 16. Gel filtration of nitroprusside-positive material from fraction C (Fig. 15). Column: Sephadex G-25 fine in 5% formic acid, 2.1 x 98 cm. Volume per tube, 2.5 ml; (-) absorbance of effluent at 230 mμ; Cys (·) Estimated intensity of nitroprusside reaction with 50 μl aliquots.

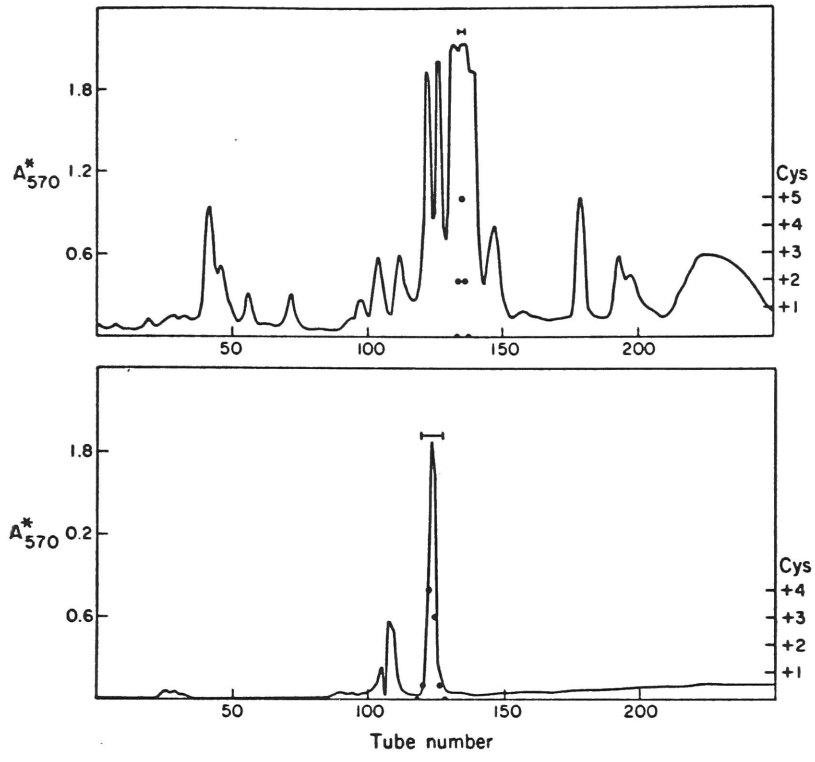


Figure 17. Ion exchange chromatography of nitroprusside-positive material from Fig. 16. Top: AG50X4, 0.9 x 15 cm at 37°, equilibrated with 0.05 M pyridine acetate, pH 3.1. Volume per tube, 2 ml. Gradient: tubes 1-19 were eluted with initial buffer. A linear gradient from 50 ml of initial buffer to 50 ml of 0.2 M pyridine acetate, pH 3.1, was begun at tube 20. Tubes 55 to 77 were eluted with 0.2 M pyridine acetate buffer, pH 3.1. A linear gradient from 150 ml of 0.2 M pyridine acetate, pH 3.1, to 150 ml of 1 M pyridine acetate, pH 5.5, was begun at tube 78. Tubes 212 to 229 were eluted with 1 M pyridine acetate, pH 5.5. A linear gradient from 50 ml of 1 M to 50 ml of 2 M pyridine acetate, pH 5.5, was begun at tube 230. A^*_{570}: ninhydrin color value on an arbitrary scale. Cys (·): estimated intensity of nitroprusside reaction with 50 µl aliquots. The fraction indicated by the bar was further purified (bottom) by ion exchange chromatography on AG50X4, 0.9 x 7.5 cm at 37°, equilibrated with 0.2 M pyridine acetate, pH 3.1. Volume per tube, 2 ml. Gradient: tubes 1 to 69 were eluted with initial buffer. A linear gradient from 170 ml of initial buffer to 170 ml of 1 M pyridine acetate, pH 5.5, was begun at tube 70. Tubes 218 to 250 were eluted with 1 M pyridine acetate, pH 5.5. A^*_{570}: ninhydrin color value on an arbitrary scale. (·): Estimated intensity of nitroprusside reaction of 50 µl aliquots.

TABLE XI

Amino Acid Composition of Peptides Containing the Light Chain-Heavy Chain Disulfide Bond

	L-H-1	L-H-2	L-H-3
Lys	1.0	1.1	
Asp	1.0	1.0	
Ser	0.9	0.9	
Glu	1.0		1.1
Gly	1.0		1.0
Cys	2.1		
CMCys		1.0	1.0
Total Residues	7	4	3

b. The heavy chain-heavy chain disulfide bonds. Preliminary experiments indicated that the two disulfide bonds between the heavy chains were located within a large tryptic peptide having a high proline to cystine ratio and derived from cyanogen bromide fragment H_{4B}. Fractions A and B (Figure 15) were used as starting material for the isolation of this peptide, and peptide fractions were evaluated on the basis of their proline and cystine content. Column effluents were monitored for cystine using the nitroprusside stain.

Chromatography of 216 mg of fraction A and 57 mg of fraction B on Sephadex G-50 in n-propyl alcohol:acetic acid:water (1:1:3, v/v/v) yielded 28 mg of a peptide fraction having a high proline to cystine ratio. Gel filtration of this material on Sephadex G-25 (Figure 18A) yielded a single nitroprusside-positive fraction which was again filtered on Sephadex G-25 and then filtered on Sephadex G-50 (Figure 18B). The cystine-containing portion of this peptide mixture was chromatographed on AG50X4 (Figure 19) to yield peptide H-H-1 (Table XII). The yield after the five steps of purification was 20%.

Amino terminal analysis of H-H-1 gave only threonine, and the amino acid composition of H-H-1 (Table XII) suggested that it was a single peptide containing two half-cystines per histidine residue. Alkylation of H-H-1 with iodoacetamide-1-^{14}C in guanidine in the absence of reducing agent did not lead to incorporation of radioactivity into the peptide. This indicates that H-H-1 contained no sulfhydryl groups and that the half-cystinyl residues were present in a disulfide linkage.

In order to show that H-H-1 is composed of two identical polypeptide chains linked by two disulfide bonds rather than a single peptide chain containing an internal disulfide bond, the molecular weight of H-H-1 was compared to the minimal molecular weight calculated from the amino acid composition. The molecular weight of H-H-1 (Table XIII) was measured in guanidine in order to dissociate

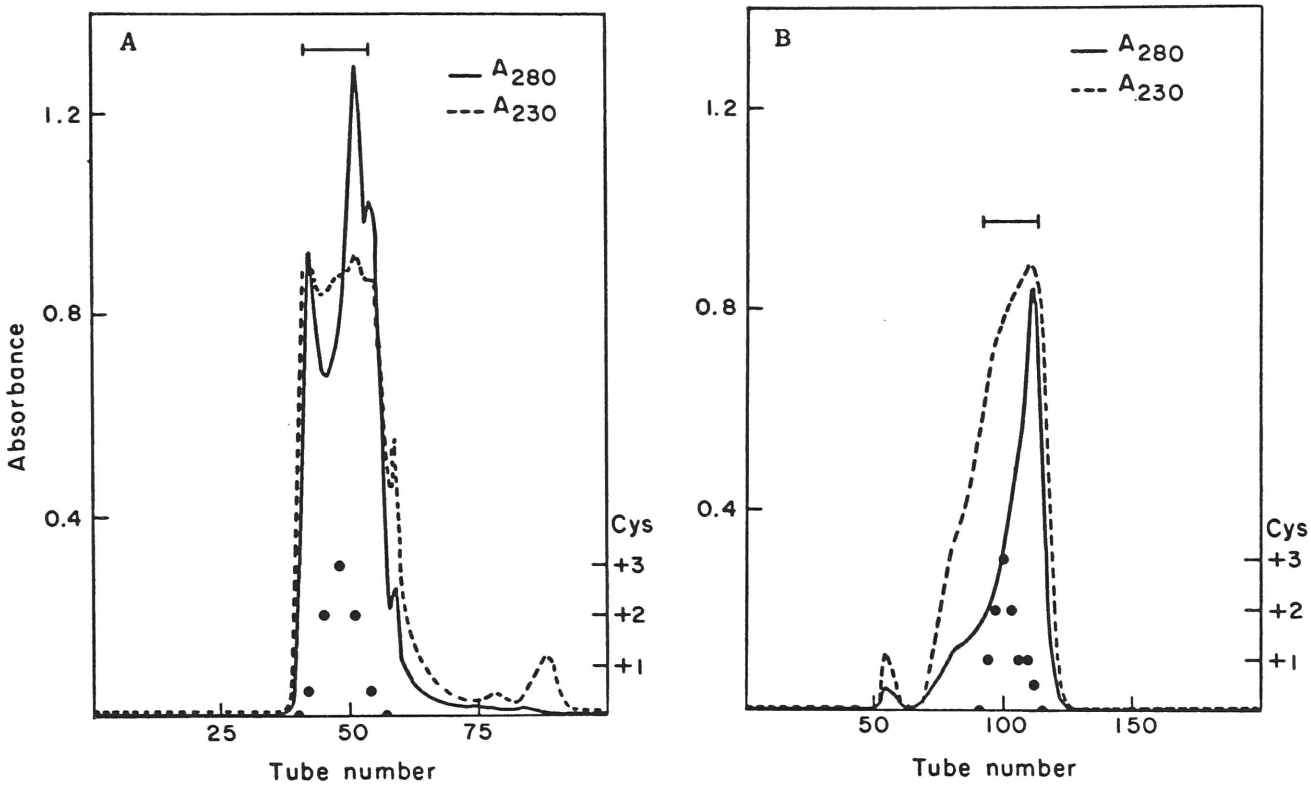


Figure 18. (A) Gel filtration of nitroprusside positive material from fractions A and B, (Fig. 15). Column: Sephadex G-25 (fine) in 2% acetic acid, 1.5 x 150 cm. Volume per tube, 3 ml. (-) absorbance at 280 mμ, (- - -) absorbance at 230 mμ. (·) Estimated intensity of nitroprusside reaction with 100 μl aliquots. The fraction indicated by the bar was lyophilized and filtered again on the same column before further purification by gel filtration on Sephadex G-50. (B) Column: Sephadex G-50 in 2% acetic acid, 1.5 x 150 cm. Volume per tube, 1.75 ml. (-) absorbance at 280 mμ; (- - -) absorbance at 230 mμ; Cys (·) estimated intensity of nitroprusside reaction with 50 μl aliquots.

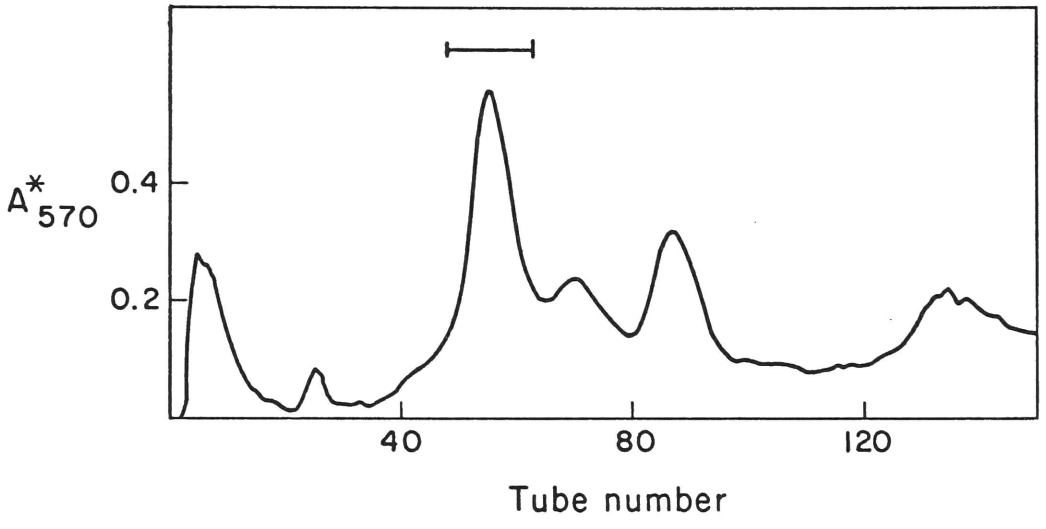


Figure 19. Ion exchange chromatography of nitroprusside positive material from Figure 18B. Column: AG50X4, 0.9 x 7.5 cm at 37° equilibrated with 0.2 M pyridine acetate, pH 3.1. Volume per tube, 2 ml. Gradient: tubes 1-8 were eluted with initial buffer. A linear gradient from 170 ml of initial buffer to 170 ml of 1 M pyridine acetate, pH 5.5 was started at tube 9. The fraction indicated by the bar contained peptide H-H-1. A^*_{570}: ninhydrin color value in arbitrary units.

TABLE XII

Amino Acid Composition of Peptides Containing the Inter-heavy Chain Disulfide Bonds

	H-H-1	H-H-2
His	1.0	1.0
Thr	1.9	1.8
Ser	1.1	1.1
Glu	1.1	1.1
Pro	4.7	5.0
Gly	2.0	2.2
Ala	1.0	1.1
Cys	1.8	-
CMCys	-	2.0
Val	1.0	1.0
Leu	2.0	2.1
Phe	1.0	1.0
Total Residues	(38)[a]	19

[a]The composition has been reduced to a minimal molecular weight corresponding to 19 residues.

non-covalently bonded aggregates and was found to be twice the minimal molecular weight (Table XIII), suggesting that the peptide contains 38 residues (Table XII). Control experiments with bovine insulin (Table XIII) indicated that 6.3 M guanidine-HCl and 0.1 M mercaptoethanol were sufficient to reduce and dissociate insulin into its constituent chains. Under these conditions, the observed molecular weight of H-H-1 decreased by about half. Peptide H-H-1 was reduced and carboxamidomethylated in guanidine, and reagents were removed by gel filtration on Sephadex G-25 in 2% acetic acid. The amino acid composition of the reduced and alkylated peptide (H-H-2) was identical to that of H-H-1, except that two residues of S-carboxymethylcysteine were found instead of two residues of half-cystine (Table XII). The data indicate that H-H-1 is a symmetrical dimer linked by two disulfide bonds.

The amino acid sequence of H-H-2, determined by the dansyl-Edman procedure was: Thr-His-Thr-CMCys-Pro-Pro-CMCys-Pro-Ala-Pro-Glx-Leu-Leu-Gly-Gly-Pro-Ser-Val-Phe. This sequence is identical to that around half-cystines VI and VII in the heavy chain. In addition a radioactive peptide was isolated from a chymotryptic digest of heavy chain prepared from partially reduced Eu which had been alkylated with iodoacetamide-1-^{14}C; the partial sequence of this peptide was Thr-CMCys-Pro-Pro-CMCys-Pro-Ala(Pro, Glx, Leu_2).

These data indicate that the half-cystinyl residues H-VI and H-VII participate in the two inter-heavy chain disulfide bonds.

The amino acid sequence of protein Eu in the region of the inter-chain bonds is shown in Figure 20. The disulfide bonds linking the heavy chains are shown in a parallel, rather than an anti-parallel, arrangement. Proof of this linkage requires cleavage of the peptide between half-cystines H-VI and H-VII under conditions which do not allow disulfide interchange. The -Cys-Pro-Pro-Cys- sequence makes this rather difficult. Evidence for a parallel arrangement of these bonds has been obtained in γG3 proteins (Frangione and Milstein, 1968), and, by analogy, the bonds in Eu have been assumed to be parallel.

TABLE XIII

Molecular Weight of Peptide H-H-1 Before and After Reduction[a]

Sample	Guanidine	Guanidine-SH
H-H-1		
Observed	3,870 ± 80	2,180 ± 140
Predicted	3,840	1,920
Insulin		
Observed	5,800 ± 20	3,190 ± 20
Predicted	5,734	2,970

[a] Guanidine = 6.3 M guanidine HCl, 0.1 M in Tris, pH 8.4; Guanidine-SH = 6.3 M guanidine HCl, 0.1 M in Tris and 0.1 M in 2-mercaptoethanol, pH 8.4. Observed molecular weights are weight average molecular weights ± average deviation of two channels with a 2-fold difference in initial concentration. Because of the uncertainty in the partial specific volume and the high solvent density, results are considered reliable only within 10%. Predicted molecular weights for insulin are based on the known sequence. The minimal molecular weight of peptide H-H-1, based on its amino acid composition, is 1,920.

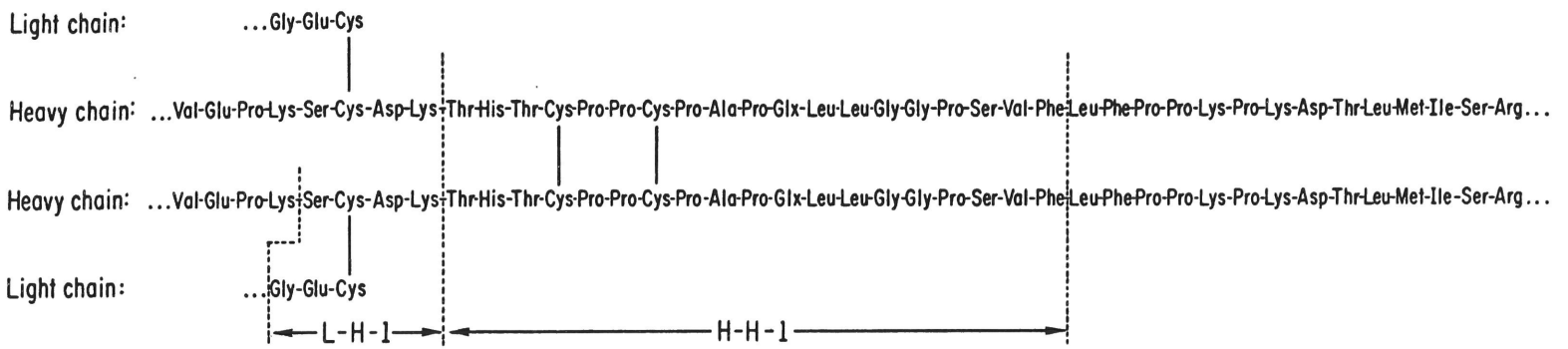


Figure 20. Amino acid sequence of Eu in the region of the interchain disulfide bonds (Gall, et al., 1968). L-H-1 and H-H-1 indicate the peptides containing the light chain-heavy chain and inter-heavy chains disulfide bonds, respectively, which were isolated from a tryptic digest of the H_1-H_4-L_3 fragment complex.

Peptide L-H-2 is immediately amino-terminal to peptide H-H-2 in the sequence of the heavy chain (Figure 20). Thus all of the half-cystines forming interchain bonds lie within ten residues in the heavy chain. Because the light chain-heavy chain disulfide bond was shown to be in the Fab(t) fragment, and the inter-heavy chain bonds were shown to be in the Fc(t) fragment, the major point of cleavage to form these fragments must be at the lysyl residue in peptide LH-1.

F. The Intrachain Disulfide Bonds of Eu

1. The Fab region. In addition to the disulfide bond linking the light chain to the heavy chain, the Fab(t) fragment contains the four half-cystinyl residues in the light chain and the four half-cystinyl residues in the Fd portion of the heavy chain which form intrachain disulfide bonds (cf. Figure 12). In order to obtain evidence for the correct pairing of these half-cystines, Fab(t) fragment was cleaved with cyanogen bromide, and the products were separated by gel filtration (Figure 21). As was expected from previous studies on the cyanogen bromide fragments of the Eu Fab(t) fragment (Waxdal et al., 1968a,b), two major fractions containing half-cystine were obtained. Fraction II (Figure 21) contained the H_1-H_{4A}-L_3 fragment complex, which contains all of the disulfide bonds in the Fab region of the molecule except one. The remaining bond is contained in the L_1-L_2 fragment complex (Fraction III, Figure 21). Fraction IV (Figure 21) contains the smaller fragments H_2 and H_3, which do not contain half-cystine. Fraction I (Figure 21) is composed of aggregated or uncleaved material, and was not studied further.

Fragments L_1 and L_2 each contain only one half-cystine, and previous studies (Waxdal et al., 1968a,b) suggested that these two residues formed an intrachain disulfide bond. To provide proof of this linkage, the L_1-L_2 complex (Fraction III, Figure 21) was extensively digested with pepsin, and the products were further digested with trypsin. A diagonal map of this digest is shown in Figure 22. The

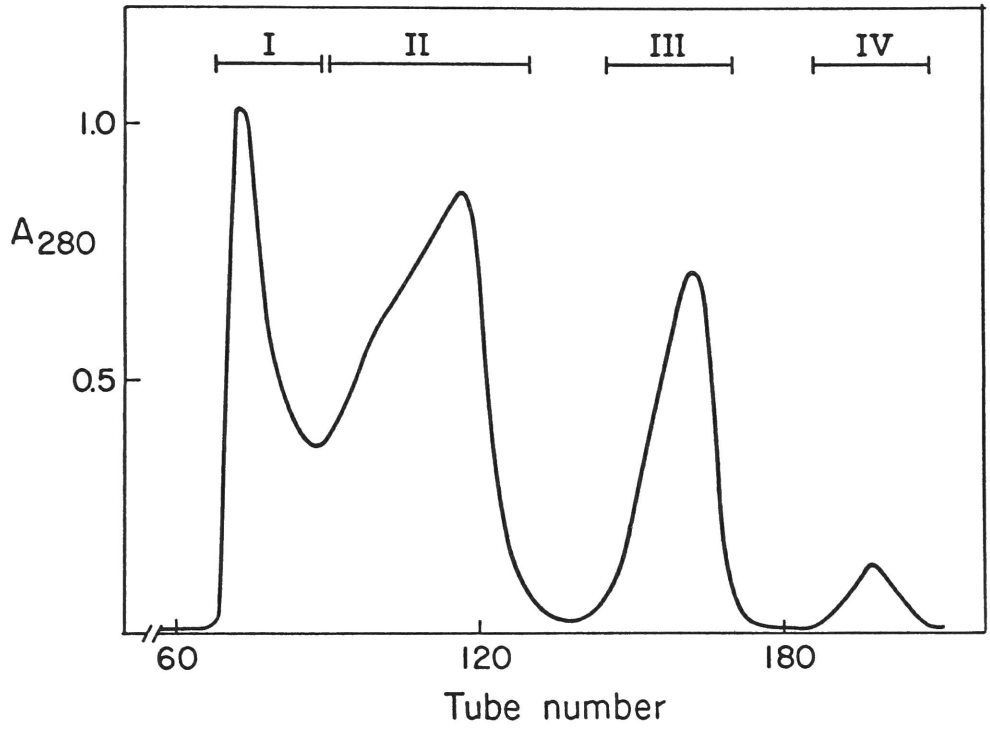


Figure 21. Gel filtration of products from cyanogen bromide cleavage of Eu Fab(t) fragment (163 mg) prepared without reduction and alkylation. Column: Sephadex G-100 in 1 M propionic acid, 2.5 x 190 cm. Volume per tube, 4.5 ml; A_{280}, absorbance of effluent at 280 mμ. Roman numerals indicate the fractions taken for further analysis.

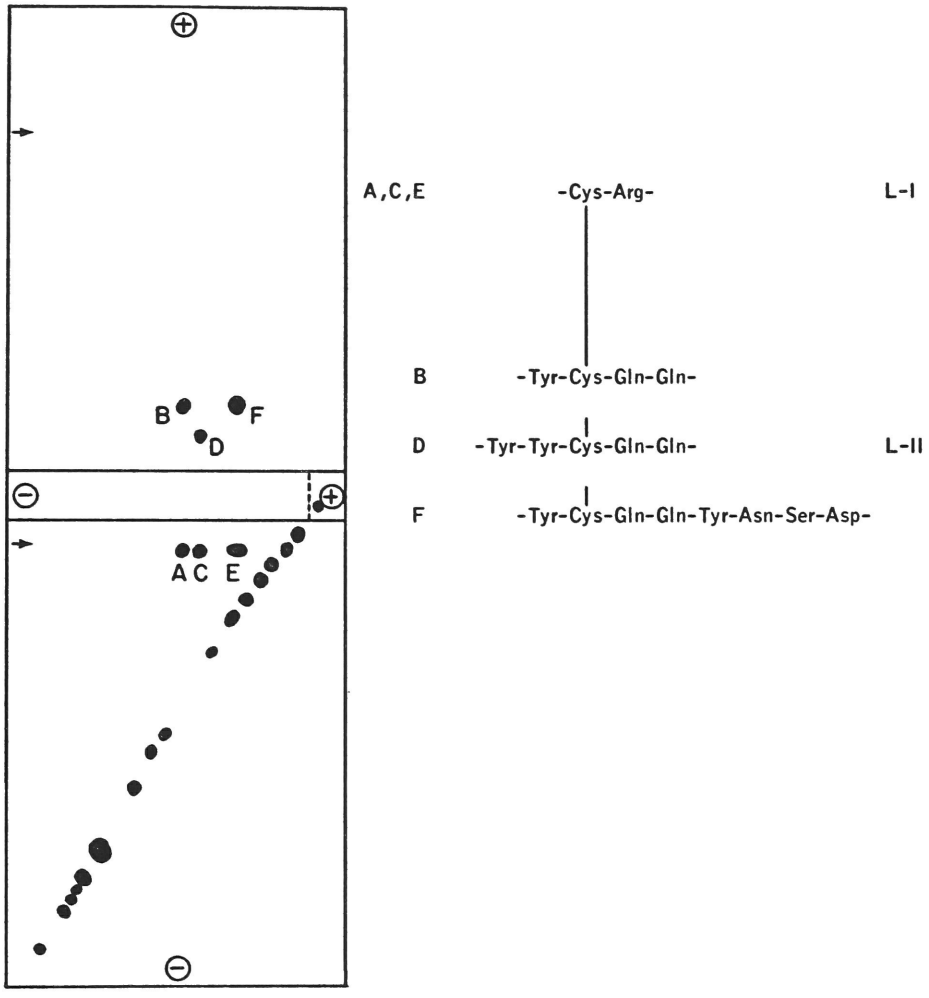


Figure 22. Diagonal map of a peptic-tryptic digest of the L_1-L_2 fragment complex. +, anode; -, cathode. Small arrows indicate the position of marker substances in the second dimension. The sequence of the peptides which lie off the diagonal, deduced from their composition (Table XIV) and the known sequence of Eu, is shown at the right of the figure. The half-cystine residue contained in each peptide is indicated at the far right.

amino acid compositions of peptides A through F are given in Table XIV. Peptides A, C, and E all had the same amino acid composition, and contain half-cystine L-I. The compositions of peptides B, D, and F differ from each other, but all are consistent with the amino acid sequence around half-cystine L-II. The yield (Table XIV) of both peptides in each pair, i.e. A and B, C and D, and E and F, was nearly the same, indicating that both components of the original disulfide-linked peptide had been recovered. These data provide direct proof of the disulfide bond between half-cystinyl residues L-I and L-II.

The isolation of three different peptides containing half-cystine L-II is due to a relative lack of specificity in the initial peptic digest. Similar results were obtained in several of the diagonal maps which will be shown below. For this reason, only the major peptides which provide proof for the linkage of the half-cystinyl residues will be presented in detail.

The H_1-H_{4A}-L_3 fragment complex (Fraction II, Figure 21) was used to determine the arrangement of the remaining intrachain disulfide bonds in the Fab region of the molecule. A peptic digest of this complex was filtered on Sephadex G-50, yielding three fractions (Figure 23). Each fraction was digested with trypsin, and diagonal maps were prepared (Figures 24 and 25). The diagonal map of the digest of fraction I was not significantly different from that of fraction II, and is not shown. The amino acid compositions of the peptides which migrated away from the diagonal are given in Table XV. The composition of peptide A (Figure 24) corresponds to the amino acid sequence around half-cystine H-IV, and the compositions of peptides B and C correspond to the sequence around half-cystine H-III. Half-cystines III and IV of the heavy chain are therefore linked in a disulfide bond. Peptides D and E (Figure 24) contain half-cystines H-V and L-V, in agreement with the previous conclusion that these residues form an interchain disulfide bond.

TABLE XIV

Amino Acid Compositions of Peptides Isolated from A Diagonal Map of a Peptic-tryptic Digest of Fragment Complex L_1-L_2 (Figure 22)[a]

Peptide	A	B	C	D	E	F
Arg	1.0		1.0		1.0	
$CySO_3H$	0.8	0.8	0.7	0.7	0.5	0.8
Asp						2.0
Ser						0.9
Glu		2.0		2.0		2.0
Tyr		1.0		1.7		1.9
Total Residues	2	4	2	5	2	8
Yield (moles)	1.5	2.5	4.5	4.0	4.9	6.7
(%)	0.8	1.2	2.2	2.0	2.5	3.3

[a] In this and the following tables, amino acids present in amounts corresponding to 0.1 residue per mole of peptide or less are omitted.

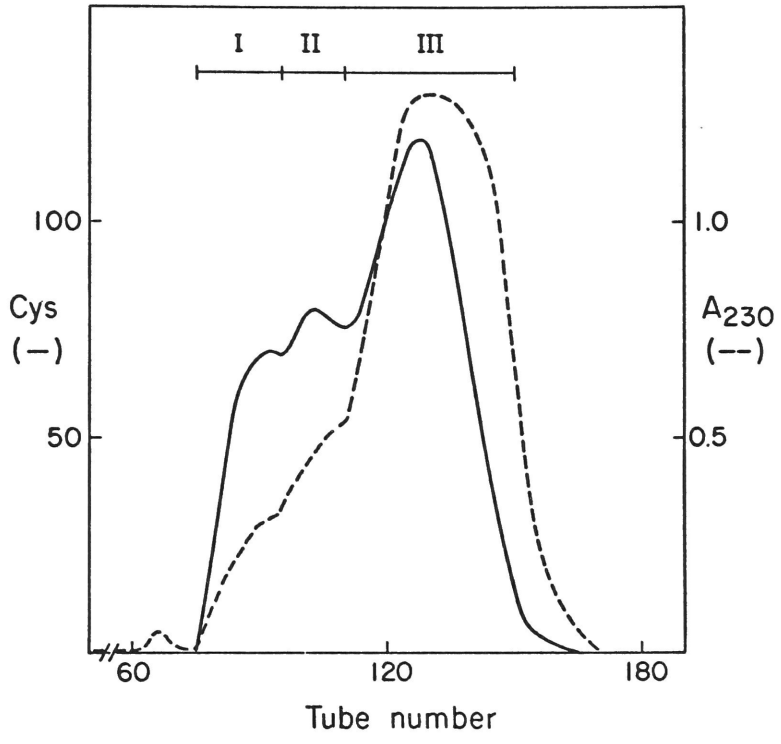


Figure 23. Gel filtration of a peptic digest of the H_1-H_{4A}-L_3 fragment complex (85 mg). Column: Sephadex G-50 (fine) in acetic acid: n-propyl alcohol: water (2:10:88 v/v), 1.9 x 110 cm. Volume per tube, 1.6 ml; (-) half-cystine content of the effluent, on an arbitrary scale; (- - -) absorbance of the effluent at 230 mμ. Roman numerals indicate fractions taken for further analysis.

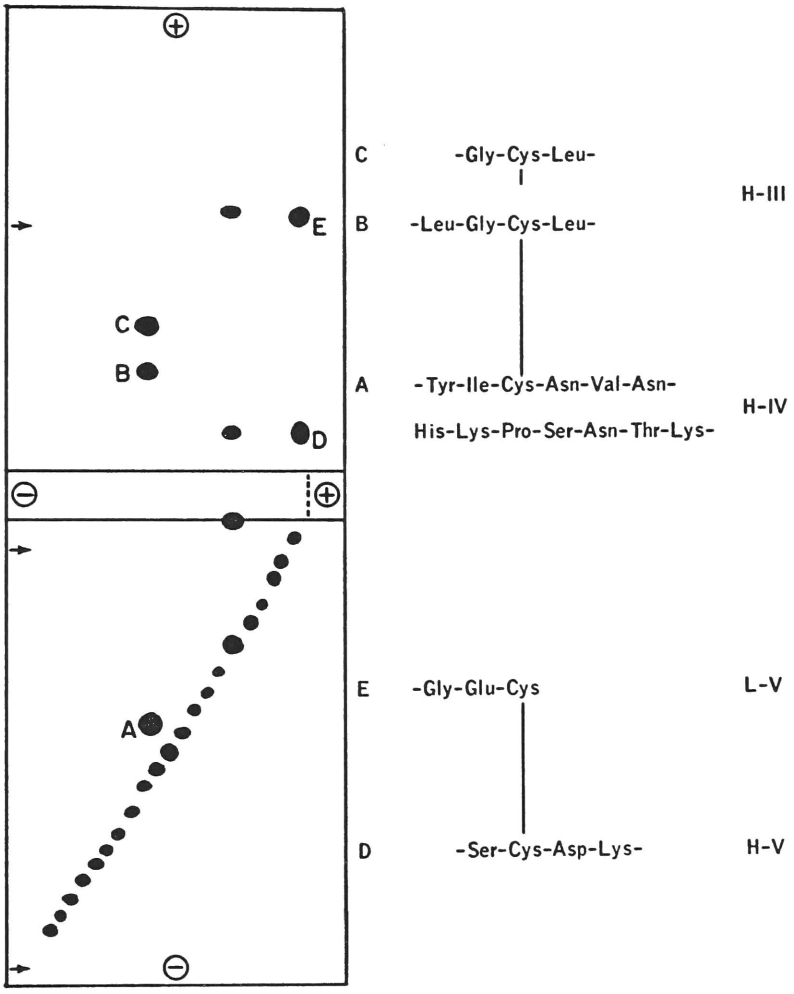


Figure 24. Diagonal map of a tryptic digest of Fration II from a peptic digest of the H_1-H_{4A}-L_3 fragment complex (Fig. 23). See text and legend of Figure 22 for details.

TABLE XV

Amino Acid Compositions of Peptides Isolated from Diagonal Maps of Enzymatic Digests of the H_1-H_{4A}-L_3 Fragment Complex

Map	Fraction II (Fig. 24)					Fraction III (Fig. 25)			
Peptide	A	B	C	D	E	A	B	C[a]	D[a]
Lys	1.9			0.9		0.5			
His	0.8								
$CySO_3H$	0.8	0.9	1.0	0.9	0.9	1.0	0.7	0.8	0.8
Asp	2.9			1.0					
Thr	0.9								
Ser	0.9			0.8		1.1			
Glu					1.0				1.0
Pro	1.0								
Gly		0.8	0.7		0.8		2.0		
Ala							1.0		0.9
Val	0.8					0.9		1.8	
Ile	0.9								
Leu		1.9	1.1					1.0	
Tyr	0.8						1.0		
Phe							0.8		
Yield (nmoles)	84	28	50	61	48	15	12	8	7
(%)[b]	3.5	1.2	2.1	2.5	2.0	2.1	1.7	1.1	1.0

[a] Isolated from a diagonal map of Fraction III without prior tryptic digestion.

[b] Based on amount of H_1-H_{4A}-L_3 complex digested with pepsin.

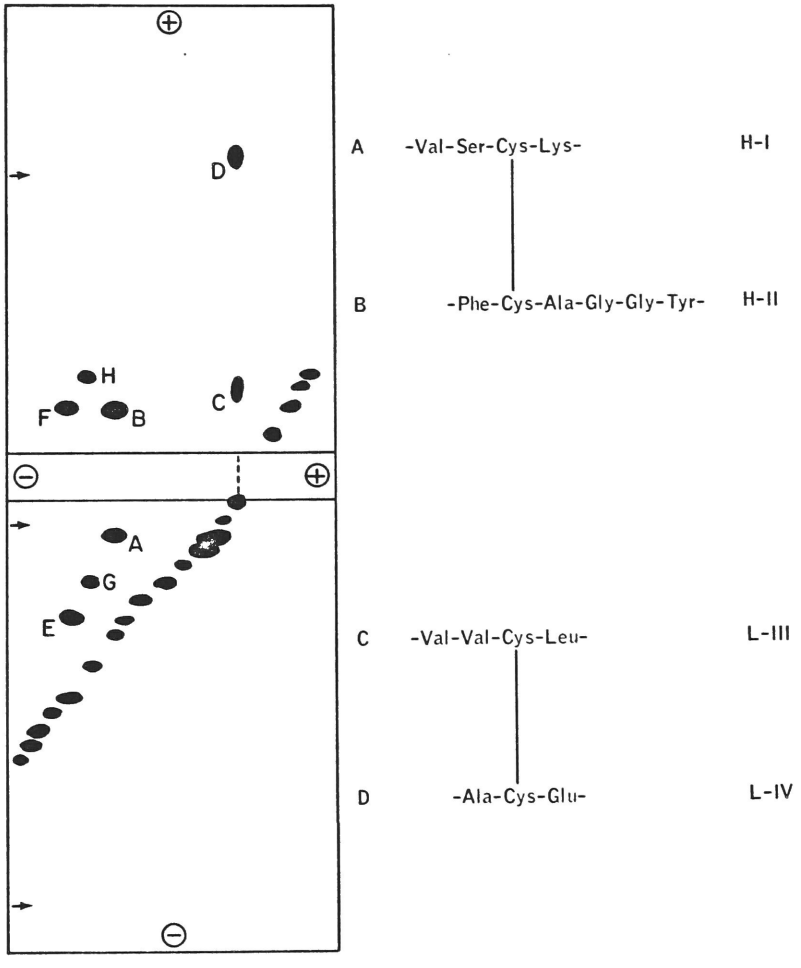


Figure 25. Diagonal map of a tryptic digest of Fraction III from a peptic digest of the H_1-H_{4A}-L_3 fragment complex (Figure 23). See text and legend of Figure 22 for details.

Peptides A and B (Figure 25, Table XV) establish that half-cystines H-I and H-II form the disulfide bond that links fragment H_1 to H_4 (cf. Figure 12). The amino acid compositions of peptides C and D (Figure 25) correspond to the amino acid sequence around half-cystines L-III and L-IV, respectively. Although these peptides were contaminated with peptides containing half-cystines L-V and H-V (compare with peptides E and D, Figure 24), this contamination was not observed in peptides corresponding to C and D which were isolated from a diagonal map of Fraction III (Figure 23) which had not been digested with trypsin. These results establish that half-cystines L-III and L-IV form a disulfide bond. The other peptides which migrated away from the diagonal (Figure 25) contain half-cystines H-III (peptides E and G) and H-IV (peptides F and H).

The above data demonstrate that the arrangement of the intrachain disulfide bonds in the light chain is linear, i.e. half-cystine L-I is linked to L-II, and L-III to L-IV. Similarly, half-cystine H-I is linked to H-II, and H-III to H-IV in the Fd region of the heavy chain. In addition, diagonal maps prepared from digests of the Fab(t) fragment, the H_1-H_4-L_3 fragment complex obtained by treating the intact protein with cyanogen bromide, and the Eu light chain confirm this arrangement. No conflicting data were obtained.

2. <u>The Fc region</u>. Eu Fc(t) fragment prepared without prior reduction was digested extensively with pepsin. Gel filtration of this digest yielded two major cystine-containing fractions (Figure 26). Diagonal maps of each fraction are shown in Figures 27 and 28, and the amino acid compositions of peptides which migrated away from the diagonal are given in Table XVI.

Peptides A and C (Figure 27) were identical and corresponded to the sequence around half-cystine H-IX. Peptides B and D contain half-cystine H-VIII, indicating that there is a disulfide bond between these residues. The composition of peptide E indicates that it contains half-

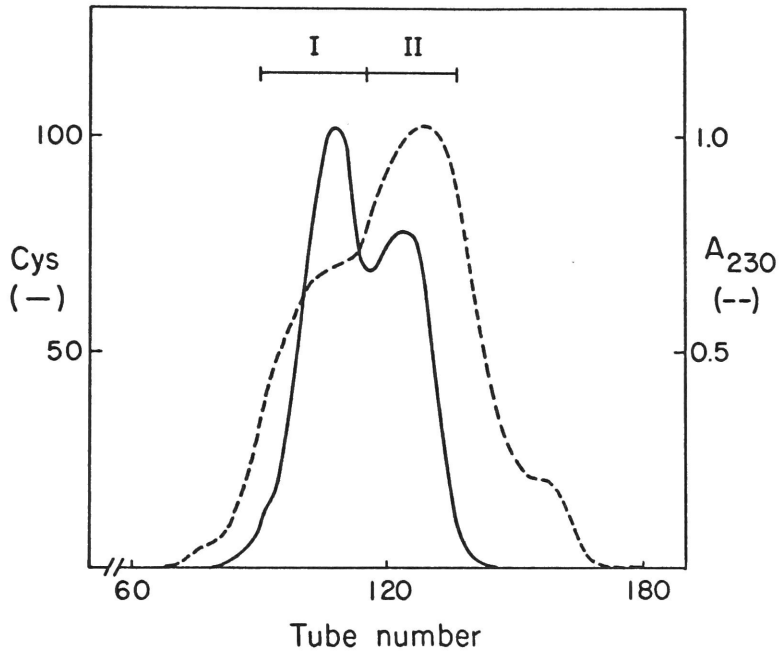


Figure 26. Gel filtration of a peptic digest of the Fc(t) fragment (130 mg). Column: Sephadex G-50 (fine) in acetic acid: n-propyl alcohol: water (2:10:88 v/v), 1.9 x 110 cm. Volume per tube, 1.6 ml; (-) half-cystine content of the effluent, determined by the fluorescence quenching method of Karush et al. (1964), on an arbitrary scale; (- - -) absorbance of the effluent at 230 mμ. Roman numerals indicate fractions taken for further analysis.

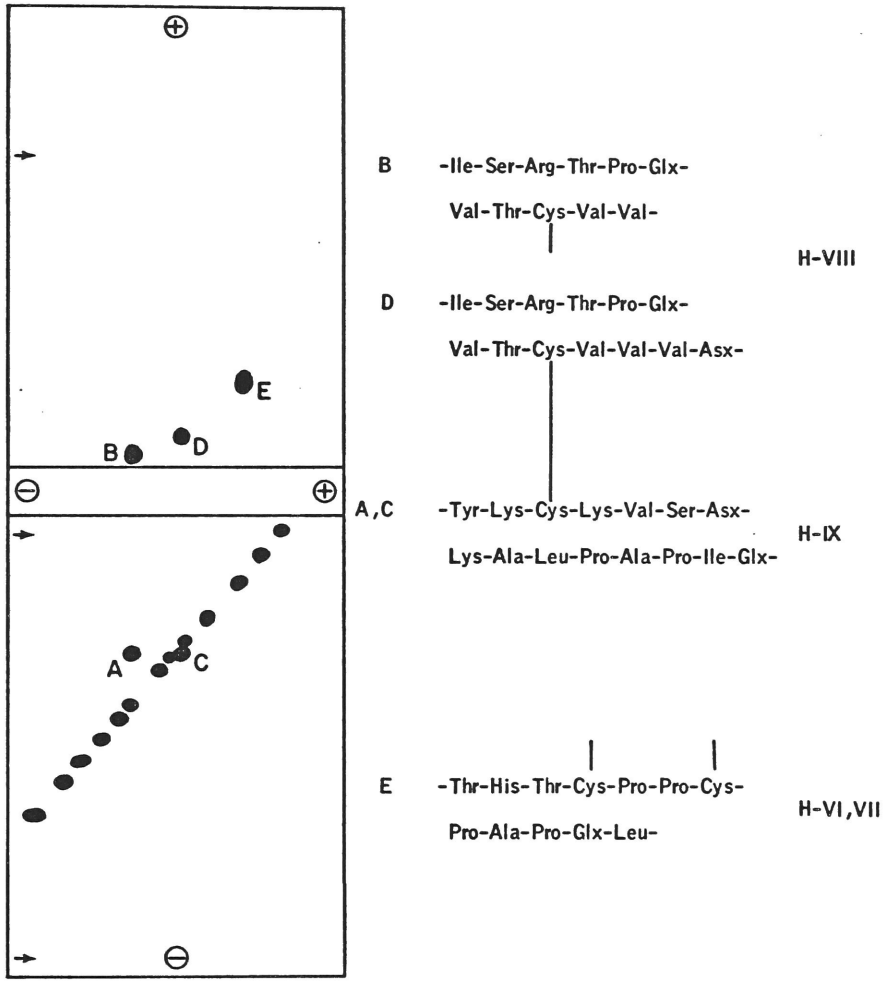


Figure 27. Diagonal map of Fraction I from a peptic digest of Eu Fc(t) fragment (Figure 26). See text and legend of Figure 22 for details.

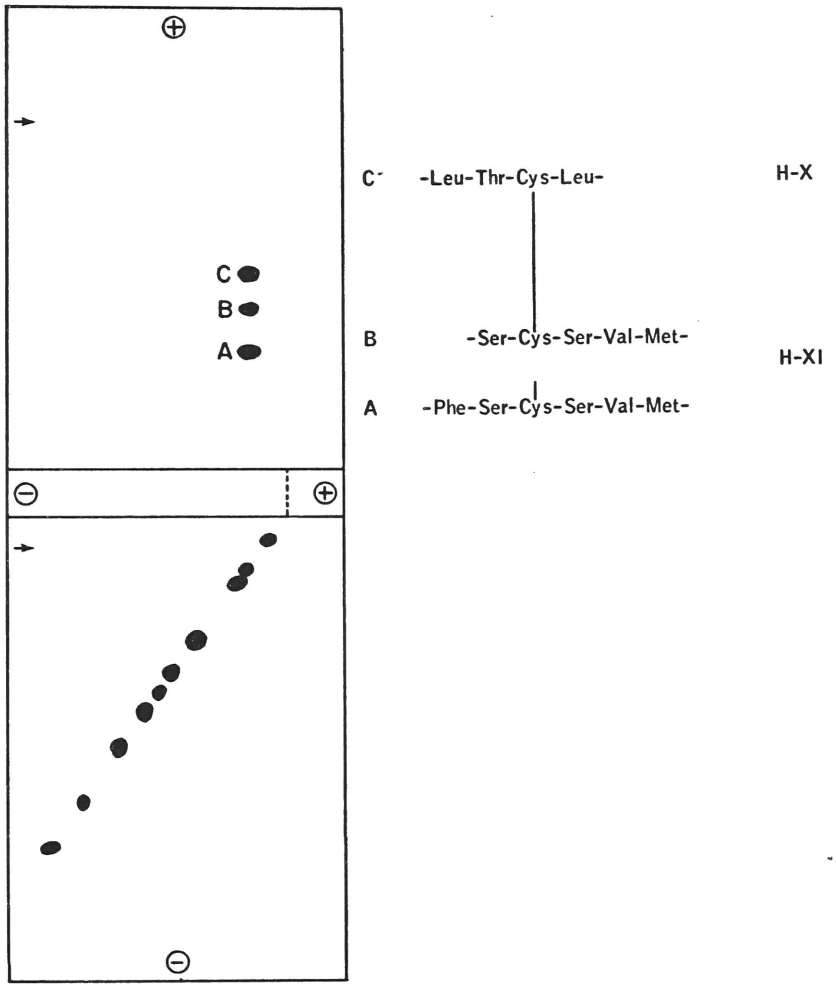


Figure 28. Diagonal map of Fraction II from a peptic digest of Eu Fc(t) fragment (Figure 26). See text and legend of Figure 22 for details.

TABLE XVI

Amino Acid Compositions of Peptides from Diagonal Maps of a Peptic Digest of the Fc Fragment[a]

Map	Fraction I (Fig. 27)					Fraction II (Fig. 28)		
Peptide	A	B	C	D	E	A	B	C
Lys	2.2		2.7					
His					0.8			
Arg		0.9		0.7				
$CySO_3H$	0.6	0.5	0.7	0.3	1.9	0.9	0.9	0.8
Asp	1.3		1.0	1.0				
Thr		2.3		2.0	1.5			1.0
Ser	1.0	1.2	1.0	1.0		1.9	1.9	
Glu	1.4	1.2	1.0	1.3	1.0			
Pro	2.1	0.7	1.8	0.8	3.7			
Gly								
Ala	2.0		2.0		1.0			
Val	1.1	2.8	1.0	3.8		1.0	1.0	
Met[b]						1.0	1.0	
Ile	0.9	0.9	1.0	0.8				
Leu	1.1		1.0		1.0			1.4
Tyr	0.4		0.4					
Phe						1.0		
Yield (nmoles	65	62	40	30	40	12	5	16
(%)[c]	5.5	5.2	3.4	2.5	3.4	1.4	0.6	1.8

[a] Amino acid analyses are of 18 hour hydrolyzates.

[b] as methionine sulfone.

[c] based on the amount of Fc(t) digested.

cystines H-VI and H-VII, which form the inter-heavy chain disulfide bonds. Because these bonds are symmetrical, oxidation yields only a single peptide.

The diagonal map of Fraction II (Figure 28) provides evidence that half-cystines H-X and H-XI form a disulfide bond. Peptides A and B contain half-cystine H-XI, and peptide C contains half-cystine H-X.

These results indicate that the arrangement of the intrachain disulfide bonds in the Fc region of the molecule is also linear, i.e. that half-cystine H-VIII is linked to H-IX, and H-X to H-XI. This agrees with the observation that cyanogen bromide fragments H_5 and H_6 (Figure 12) can be isolated without prior reduction and alkylation (Waxdal et al., 1968b).

G. The Arrangement and Location of the Disulfide Bonds of Eu

The arrangement and exact location of the disulfide bonds of Eu are shown on a linear model of the molecule in Figure 29. There are four interchain disulfide bonds; one between each light chain and its corresponding heavy chain and two between the heavy chain. There are 12 intrachain disulfide bonds; two in each light chain and two in both the Fd and Fc regions of each heavy chain. Four important features of the arrangement and location of the disulfide bonds are illustrated by this figure. First, the interchain bonds are grouped in a small region of the molecule, about in the middle of the heavy chain. Second, the arrangement of the intrachain bonds is linear, i.e. half-cystine I is linked to II, III to IV, etc. Third, the corresponding half-cystinyl residues are located in nearly parallel positions in the amino acid sequence of the light and heavy chains. For example, half-cystine L-I is residue 23, and H-I is residue 22. Fourth, each disulfide loop contains about 60 residues (Figure 29), although the amino-terminal loop in each heavy chain is somewhat longer.

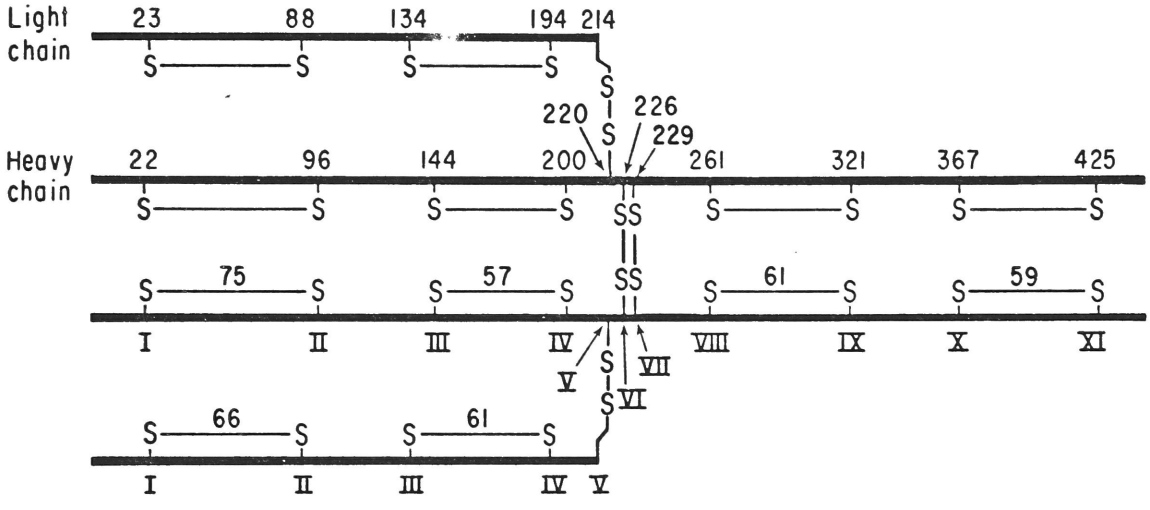


Figure 29. The disulfide bonds of the human γG1 myeloma protein Eu. The Arabic numbers above each half-cystine in the top half of the figure indicate the position of that residue in the amino acid sequence of the molecule. The number of residues contained in each disulfide loop is indicated by Arabic numerals in the appropriate positions in the lower half of the figure.

The implications of this linear and periodic arrangement of the disulfide bonds of Eu for the structure, function, and evolution of the immunoglobulins are discussed in the next section.

III. DISCUSSION

A. Comparison of the Disulfide Bonds of Eu with those of Other Immunoglobulins

Disulfide-containing peptides have been isolated from several other immunoglobulins, primarily by Milstein and co-workers. The complete determination of the arrangement and the exact location of these bonds was not possible because of the lack of complete amino acid sequences for these proteins. These peptides may be compared with the complete sequence of Eu, however, and the generality of the arrangement of the disulfide bonds in immunoglobulins can be determined.

1. Interchain disulfide bonds. Peptides forming the light chain part of the light chain-heavy chain disulfide bond were first described by Milstein (1965; Pink and Milstein, 1967a). Further studies on the interchain bonds of the various subclasses of γ chains have been reported (Pink and Milstein, 1967b, Frangione and Milstein, 1967, 1968). These results are summarized in Table XVII.

In all immunoglobulins studied, the carboxyl-terminal half-cystinyl residue of κ chains or the penultimate half-cystinyl residue of λ chains links these light chains to a half-cystine in the heavy chain. This is half-cystine residue H-V in Eu, and peptides consistent with this sequence have been isolated from several other γ1 heavy chains. Only seven residues separate the light-heavy and heavy-heavy interchain bonds in γG1 proteins, but in the other subclasses the light-heavy interchain bond may be located further away from the inter-heavy chain bonds. In γ2, γ3, and γ4 heavy chains, the peptide Pro-Leu-Ala-Pro-Cys-Ser-Arg is part of the light-heavy interchain bond (Pink and Milstein, 1967a,b; Frangione and Milstein, 1968; Frangione *et al.*, 1969), but its position in the amino acid sequence is unknown. The similar peptide Pro-Leu-Ala-Pro-Ser-Ser-Lys occupies a position from residue 127 to 133 in the Eu γ1 heavy chain (Edelman *et al.*, 1969). The close resemblance suggests that the γ2, γ3, and γ4 peptides are in a similar location in their respective heavy chains. Moreover, a

TABLE XVII

The Interchain Disulfide Bonds of Human γG Immunoglobulins[a]

I. Light chain - heavy chain bonds

Light chain component		Heavy chain component			
				127	133
K chains	-Gly-Glu-Cys(H)-COOH	γG1	Eu	-Pro-Leu-Ala-Pro-Ser-Ser-Lys-	
λ chains	-Thr-Glu-Cys(H)-Ser-COOH	γG2	Sa	-Pro-Leu-Ala-Pro-Cys(L)-Ser-Arg-	
		γG3	Kup, Bru	-Pro-Leu-Ala-Pro-Cys(L)-Ser-Arg-	
		γG4	Vin	-Pro-Leu-Ala-Pro-Cys(L)-Ser-Arg-	

		219	L	222
γG1	Eu	- - - - - - -	-Ser-Cys-Asp-Lys-	

II. Inter-heavy chain bonds

		215	V	VI	VII	230
γG1	Eu,Car,Daw,Dee	-Arg-Val-Glu-Pro-Lys-Ser-Cys(L)-Asp-Lys-Thr-His-Thr-Cys(H)-Pro-Pro-Cys(H)-Pro-Ala-Pro-Glu-Leu-Leu-				
γG2	Sa	-Thr-Val-Glx-Arg-Lys-Cys(H)-Cys(H)-----Val-----Glx-----Cys(H)-Pro-Pro-Cys(H)-Pro-Ala-				
γG3	Kup, Bru	(Cys(H)-Pro-Glu-Pro-Lys)Ser-Cys(H)-Asp-Thr-Pro-Pro-Pro-Cys(H)-Pro-Arg-Cys(H)-Pro-Ala-Pro-Glu-Leu-				
γG4	Vin	-Tyr-Gly-Pro-Pro-Cys(H)-Pro-Pro-Cys(H)-Pro-Ala(Ser,Glu)Phe-Leu-				
(Normal rabbit γG)		-Thr-Cys(?)-Ser-Lys-----Pro-Thr-Cys(H?)-Pro-Pro----------Pro-Glu-Leu-Leu-				

[a]Data are from Gall et al., (1968) and Edelman et al. (1969) for Eu, from Steiner and Porter (1967) for Daw, and from Milstein's laboratory (Frangione et al., 1969) for the other human proteins. The rabbit sequence is from Cebra et al. (1968) and Hill et al. (1967). L and H indicate disulfide bonds to light and heavy chains, respectively.

homologous peptide, Pro-Leu-Ala-Pro-Cys-Cys-Gly is in a similar position in the amino acid sequence of the Fd region of pooled rabbit γG immunoglobulin (Cebra et al., 1968).

Many studies have been carried out on the disulfide bonds that link the heavy chains. The amino acid sequence in the vicinity of the interheavy chain disulfide bonds is identical in three human γ1 heavy chains; similar amino acid sequences occur in the other subclasses (Table XVII). Although it has not been possible to show whether the half-cystines of the inter-heavy chain bonds are linked in a parallel or an anti-parallel arrangement in γG1, γG2, and γG4 proteins, the -Cys-Pro-Arg-Cys- sequence in γ3 heavy chains can be cleaved by trypsin between the two disulfide bonds, and it has been shown that the arrangement is parallel (Frangione and Milstein, 1968). This result suggests that the inter-heavy chain disulfide bonds in the other subclasses are also arranged in a parallel fashion.

In contrast to the other subclasses, γG2 immunoglobulins have four inter-heavy chain disulfide bonds and γG3 proteins probably have five. In addition to two bonds similar to those in γ1 and γ4 proteins the γG2 and γG3 subclasses have a third interchain bond which occurs between half-cystinyl residues that are homologous to half-cystine V of Eu (Table XVII). In γG2 proteins, an extra half-cystine is present at the position immediately amino-terminal to the half-cystine corresponding to H-V in γG1 proteins. This residue forms the fourth inter-heavy chain bond (Frangione et al., 1969). In γG3 proteins, the two remaining inter-heavy chain bonds have not been located in the amino acid sequence, although it has been established that one is present in the Fab fragment (Frangione and Milstein, 1968).

These data demonstrate that there is some variation in the inter-heavy chain disulfide bonding pattern in human immunoglobulins. These variations are in the form of additional bonds, however, and the structure analagous to the inter-heavy chain bonds in γG1 proteins

is preserved in all of the subclasses. It should be noted that the amino acid sequence of rabbit γ chains in the region of the interchain disulfide bonds (Table XVII) is similar to that in human γG1 proteins (Cebra et al., 1968; Hill et al., 1967; Smyth and Utsumi, 1967). Only one inter-heavy chain disulfide bond is present in most of the normal rabbit γG immunoglobulin (Palmer and Misonoff, 1964; Hong and Nisonoff, 1965) and it probably consists of half-cystinyl residues homologous to half-cystines H-VI (Table XVII) which form an inter-heavy chain bond in human γG1 proteins.

2. Intrachain disulfide bonds. A number of peptides containing half-cystines that contribute to the intrachain disulfide bonds of human immunoglobulins have been isolated and studied. These peptides are compared with the structure of Eu in Tables XVIII and XIX.

All of the peptides which have been isolated from κ chains are homologous to the corresponding region of the Eu light chain sequence (Table XVIII). The complete sequence of several other κ chains has been determined, and their structures are similar to that of the Eu light chain. As would be expected for peptides from the variable region, the sequences around half-cystines L-I and L-II are different from protein to protein, although any two are quite similar. The sequences around the constant region half-cystines L-III and L-IV, however, do not show variation. Similar results have been obtained for λ chains. Peptides corresponding to the C region disulfide bridges also have been isolated from pooled human normal light chains (Milstein, 1966). Human light chains thus have two intrachain disulfide bridges, one in the V region and one in the C region. Both of these bridges form loops containing about 60 residues. Because of the diversity of the V regions, it might be expected that some light chains containing extra half-cystines may be found. The sequence of a λ chain with an extra half-cystine residue in the V region has been recently reported (Milstein et al., 1968). This half-cystine is present partly as a

TABLE XVIII

Intrachain Disulfide Bonds in Human Light Chains[a]

I. Variable Regions

K Chains

		18					I				27	83					II				92
I	Eu	-Arg-	Val-	Thr-	Ile-	Thr-	Cys-	Arg-	Ala-	Ser-	Gln-	-Phe-	Ala-	Thr-	Tyr-	Tyr-	Cys-	Gln-	Gln-	Tyr-	Asn-
			Ile			Ala		Gln					Ile							Phe	Asp Blu
II	HBJ 3	-Pro-	Ala-	Ser-	Ile-	Ser-	Cys-	Arg-	Ser-	Ser-	Gln-	-Val-	Gly-	Val-	Tyr-	Tyr-	Cys-	Met-	Gln-	Ala-	Leu-
					Thr													Gln	Met	Arg	
III	Rad	-Arg-	Ala-	Thr-	Leu-	Ser-	Cys-	Arg-	Ala-	Ser-	Gln-	-Phe-	Ala-	Val-	Tyr-	Tyr-	Cys-	Gln-	Gln-	Tyr-	Glu-

λ Chains

		17					I			26	82					II			91
I	New	-Lys-	Val-	Thr-	Ile-	Ser-	Cys-	Ser-	Gly-	Gly-	Ser-	-Glu-	Ala-	Asp-	Tyr-	Tyr-	Cys-	Ala-	Thr-	Trp-	Asp-
		Arg	Ala	Ile						Ser					His	His		Gln	Ala		
		Gly																			
		Ala																			
II	Bo	-Ser-	Val-	Thr-	Ile-	Ser-	Cys-	Thr-	Gly-	Thr-	Ser-	-Glx-	Ala-	Asx-	Tyr-	Tyr-	Cys-	Ser-	Ser-	Tyr-	Val-
		Thr	Ile																		
III	Sh	-Thr-	Val-	Arg-	Ile-	Thr-	Cys-	Gln-	Gly-	Asp-	Ser-	-Glu-	Ala-	Asp-	Tyr-	Tyr-	Cys-	Asn-	Ser-	Arg-	Asp-
IV	Kern	-Thr-	Ala-	Val-	Ile-	Thr-	Cys-	Ser-	Gly-	Asp-	Asn-	-Glu-	Ala-	Asp-	Tyr-	Phe	Cys-	Gln-	Thr-	Trp-	Asp-
			Ser					Gly		Lys					Tyr				Ala		
										Glu											

II. Constant Regions

K Chains

	129		III		138		189		IV		198
Eu	-Thr-Ala-Ser-Val-Val-	Cys	-Leu-Leu-Asn-				-His-Lys-Val-Tyr-Ala-	Cys	-Glu-Val-Thr-His-		

λ Chains

New	-Lys-Ala-Thr-Leu-Val-	Cys	-Leu-Ile-Ser-Asp-				-His-Arg-Ser-Tyr-Ser-	Cys	-Gln-Val-Thr-His-		

[a]For references, see Edelman and Gall (1969). For variable regions, the sequence of one protein from each subgroup is given, and variants which have been observed are shown in the appropriate positions.

TABLE XIX

Intrachain Disulfide Bonds in Human Heavy Chains[a]

I. Variable Regions

		18 I 27 92 II 100
γ1	Eu	-Val-Lys-Val-Ser-Cys-Lys-Ala-Ser-Gly-Gly- -Ala-Phe-Tyr-Phe-Cys-Ala-Gly-Gly-Try-
	Dee	-Val-Arg-Ile(Ser,Cys,Lys,Ala,Ser,Gly) -Tyr-Tyr-Cys-Thr-Gly-Arg-Gly-Met-
	Daw	18 -Leu-Thr-Leu-Thr-Cys-Thr-Phe-Ser-Gly-Phe ?
γ4	Vin	-Leu-Ser-Cys-Ala-Ala-Ser-Gly-Phe- -Ala-Val-Tyr-Tyr-Cys-Ala-Arg-
μ	Ou	18 93 -Leu-Thr-Leu-Thr-Cys-Thr-Phe-Ser-Gly-Phe- -Ala-Thr-Tyr-Tyr-Cys-Ala-Arg-

II. Constant Regions

		140 III 147 198 IV 205
γ1	Eu, Dee, Car	-Ala-Ala-Leu-Gly-Cys-Leu-Val-Lys- -Tyr-Ile-Cys-Asn-Val-Asn-His-Lys-
γ2	Sa	-Ala-Ala-Leu-Gly-Cys-Leu- -Tyr(Thr,Cys,Asx,Val,Asx,His,Lys)
γ3	Kup, Bru	(Ala,Ala,Leu)Gly-Cys-Leu- (Tyr,Thr,Cys,Asx,Val,Asx,His,Lys)
γ4	Vin	-Ala-Ala-Leu-Gly-Cys-Leu- -Tyr-Thr-Cys-Asn-Val-Asp-His-Lys-

II. Constant Regions (continued)

		258 VIII 265	319 IX 325
γ1	Eu, Cra	-Glu-Val-Thr-Cys-Val-Val-Val-Asp-	-Tyr-Lys-Cys-Lys-Val-Ser-Asn-
γ2	Sa	(Glu,Val,Thr,Cys,Val)	-Thr-Lys-Cys-Lys-Val-Ser-Asn-
γ3	Zuc	-Glu-Val-Thr-Cys-Val(Val,Val,Asp)	-Tyr-Lys-Cys-Lys-Val-Ser-Asn-
γ4	Vin	(Glu,Val,Thr,Cys,Val)	-Tyr-Lys-Cys-Lys-Val-Ser-Asn-

		364 X 370	423 XI 429
γ1	Eu, Cra	-Ser-Leu-Thr-Cys-Leu-Val-Lys-	-Phe-Ser-Cys-Ser-Val-Met-His-
γ2	Sa	-Thr(Cys,Leu)	-Phe-Ser(Cys,Ser,Val,Met)
γ3	Zuc	-Leu-Thr(Cys,Leu)	-Phe-Ser(Cys,Ser,Val,Met)
γ4	Vin	-Leu-Thr-Cys-Leu-	(Phe,Ser,Cys,Ser,Val,Met)

---

[a]Data for proteins other than Eu are from Frangione, et al. (1969), with the exception of Ou (Wikler, et al., 1969), and Daw (Press and Piggot, 1967).

free thiol and partly blocked with half-cystine. The general structure characteristic of light chains is preserved, however.

It is noteworthy that normal rabbit and chicken light chains contain seven half-cystinyl residues (Hood et al., 1967), the linkage of which is unknown. It is important to determine the disulfide pattern in these proteins, particularly in the V region of the chain.

Only a few half-cystine-containing peptides have been isolated from heavy chains (Table XIX), but they show the expected similarity with the sequence of Eu. In contrast to the interchain bonds, the pattern of intrachain bonding in the heavy chain of the four subclasses seems to be preserved. These are three disulfide loops in the C region of the heavy chain, and differences in the sequences around the half-cystines in this region probably are specific for each subclass. If all of the γG heavy chains are assumed to have nearly the same length, then each intrachain bond in the C region forms a loop of about 60 residues, which is the same as the light chain C region loop. The amino-terminal loop, in the V region, is somewhat longer in the Eu heavy chain, and may also be longer in other γ chain V regions.

It should be noted that the Daw heavy chain contains two more half-cystines than the Eu heavy chain (Press and Piggot, 1967). One of these extra residues is at position 35 in the Daw variable region. The location of the other is not yet known. It has also been suggested that the intrachain bonds in the Fd region may be between half-cystines I and IV and II and III of this protein (Press and Piggot, 1967). If this is confirmed, the linear arrangement of the intrachain bonds may not be a constant feature in the V regions of all γ chains.

The amino acid sequence of a portion of what is probably the V region of the human μ chain Ou has recently been reported; this sequence contains a single disulfide loop (Table XIX) of 76 residues, between Cys-22 and Cys-97 (Wikler et al., 1969).

All of the data on the intrachain disulfide bonds of both heavy and light chains, therefore, suggests that the linear and periodic arrangement of these bonds is a common structural feature of all γG immunoglobulins, and perhaps of other classes as well. It is now pertinent to ask if a similar arrangement is common in other kinds of proteins.

B. Comparison of the Arrangement of the Disulfide Bonds in Immunoglobulins with the Disulfide Bonds of Other Proteins

The amino acid sequence and the arrangement of the disulfide bonds has been determined for several different proteins; these data are summarized in Figure 30. The disulfide bonds of most of these proteins form overlapping or intercolated patterns. The tertiary structures of ribonuclease A (Kartha et al., 1967) and lysozyme (Phillips, 1967) are such that the polypeptide chain is coiled in an irregular pattern, and many residues which are far apart in the amino acid sequence are near each other in the coiled molecule. The complicated pattern of the disulfide bonds, which stabilize the tertiary structure, is reasonable in the light of this folding. The papain molecule (Drenth et al., 1968) is roughly made up of two general regions, each of which is folded in an irregular fashion. This is reflected in the disulfide bond pattern, which shows two overlapping disulfide bonds in the amino terminal portion of the sequence and a single bond in the carboxyl terminal portion. The linear and periodic arrangement of the intrachain disulfide bonds in γG1 immunoglobulin is thus unusual; only the two disulfide bonds of growth hormone have a similar arrangement. This arrangement may reflect both the evolution and tertiary structure of the molecule.

C. Disulfide Bonds and the Evolution of Immunoglobulins

Well-defined antibody responses have been found only in vertebrates, and cyclostomes are the lowest vertebrates that have been found to produce humoral antibodies (Marchalonis and Edelman, 1968; see Smith et al., 1966 for a review). Diversity and multichain

Figure 30. The arrangement of the disulfide bonds in proteins for which both the complete amino acid sequence and the exact arrangement of the disulfide bonds are known. The polypeptide chain is represented by the heavy bar, and the disulfide bonds are represented by the lighter lines. The position of each mark representing a half-cystine corresponds to the position of that residue in the sequence. Data are from: Kassell *et al.* (1965), trypsin inhibitor; Chance *et al.* (1968), proinsulin; Takahashi (1965), ribonuclease T_1; Spackman *et al.* (1960), ribonuclease A; Hill *et al.* (1969), α-lactalbumin; Canfield and Liu (1965), lysozyme; Li *et al.* (1966), growth hormone; Drenth *et al.* (1968), papain; Kauffman (1965), trypsinogen; and Brown and Hartley (1966), chymotrypsinogen A.

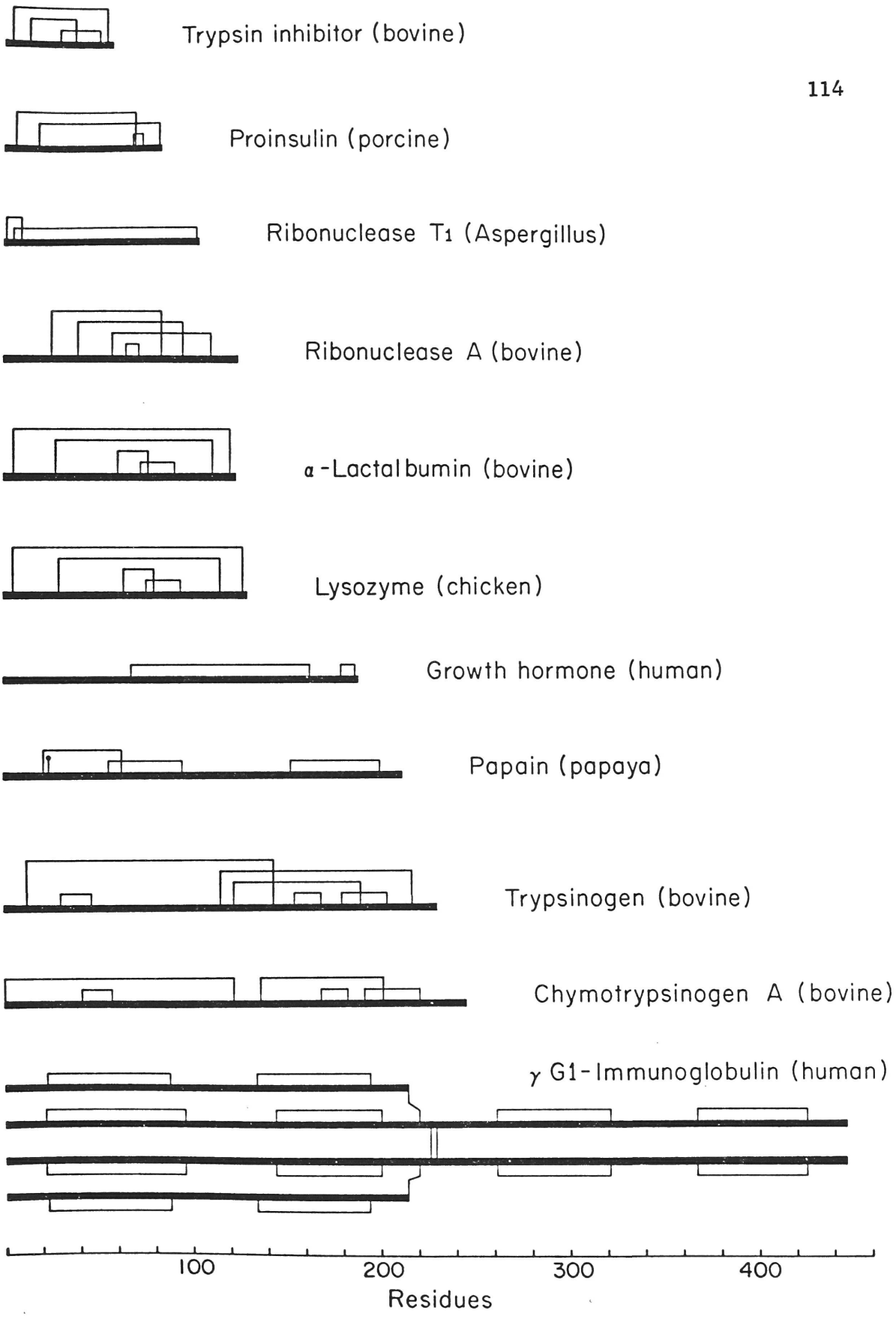

Trypsin inhibitor (bovine)
Proinsulin (porcine)
Ribonuclease T1 (Aspergillus)
Ribonuclease A (bovine)
α-Lactalbumin (bovine)
Lysozyme (chicken)
Growth hormone (human)
Papain (papaya)
Trypsinogen (bovine)
Chymotrypsinogen A (bovine)
γ G1-Immunoglobulin (human)
100
200
300
400
Residues

Figure 30

structure seem to be essential features of all immunoglobulins, although certain animal species may lack one or more of the immunoglobulin classes. For example, the predominant type of immunoglobulin in lampreys, sharks, and certain teleost fishes is very similar to the γM class found in mammals. The γ chain may have evolved at the time of or just prior to the evolutionary emergence of anuran amphibians; the Queensland lung fish is the most primitive vertebrate yet studied to have more than one class of immunoglobulins (Marchalonis and Nossal, 1969). Therefore, one of the major developments during the evolution of immunoglobulins may have been an increase in the number of different heavy chain classes so that antibodies could carry out more specialized effector functions.

Theories for the evolution of the immunoglobulin chains from a precursor gene coding for a protein about 110 residues long have been proposed (Hill et al., 1966; Singer and Doolittle, 1966) on the basis of comparisons of the sequences of different portions of various immunoglobulins. Successive duplications of this gene, perhaps combined with non-homologous crossing over, could have resulted in the production of proteins with the size of heavy chains. The periodicity of the disulfide bond structure is consistent with this hypothesis, and suggests that the precursor gene contained one intrachain disulfide bond. Internal homologies in the amino acid sequence of Eu (Edelman et al., 1969) provide strong evidence for this hypothesis, although as yet the data are not sufficient to draw a unique scheme for the emergence of each immunoglobulin class from a precursor.

Studies on the structure of immunoglobulins from lower animals have also suggested that some of these immunoglobulins may not have interchain disulfide bonds. The 6.7S immunoglobulin of lampreys can be dissociated into its polypeptide chains without reduction (Marchalonis and Edelman, 1968). This raises the possibility that interchain disulfide bonds emerged in later forms to provide increased

stability for the immunoglobulin molecule as it encountered different cellular and chemical environments.

D. Implications of the Covalent Structure of Eu for the Tertiary Structure of Immunoglobulins

1. The domain hypothesis. The main features of the covalent structure of Eu are summarized in Figure 31. This figure shows the location of the variable (V_L and V_H) and constant (C) regions of the molecule as well as the internal homology regions C_L, C_H1, C_H2, and C_H3. The linear and periodic arrangement of the intrachain disulfide bonds and the internal homologies in the molecule have suggested (Edelman and Gall, 1969) that each region of the molecule containing a single intrachain disulfide loop is folded in a compact domain (Figure 32). This would result in a structure consisting of linearly connected globular domains. Each Fab region would be composed of at most four and possibly just two such domains. Each chain in the Fc dimer would consist of two domains and each Fc fragment of four. If these globular domains exist, then there are eight different ways in which the heavy and light chains might interact. For example, the Fab region domains might be arranged in a parallel fashion, but the Fc region domains could be parallel or crossed.

Fragments composed of the carboxyl terminal half of the Fc fragment have been isolated in low yield from papain and pepsin digests of γG immunoglobulins (Turner and Bennich, 1968). These results indicate that there is a second enzyme-susceptible site near the middle of the Fc fragment, providing some support for the idea that the two halves of the Fc region of the molecule are compact and connected by an exposed region. In addition, the homology in the sequences of the two halves suggests that they may have similar conformations. This hypothesis also suggests that limited enzymatic digestion of the Fab fragment may yield analogous fragments.

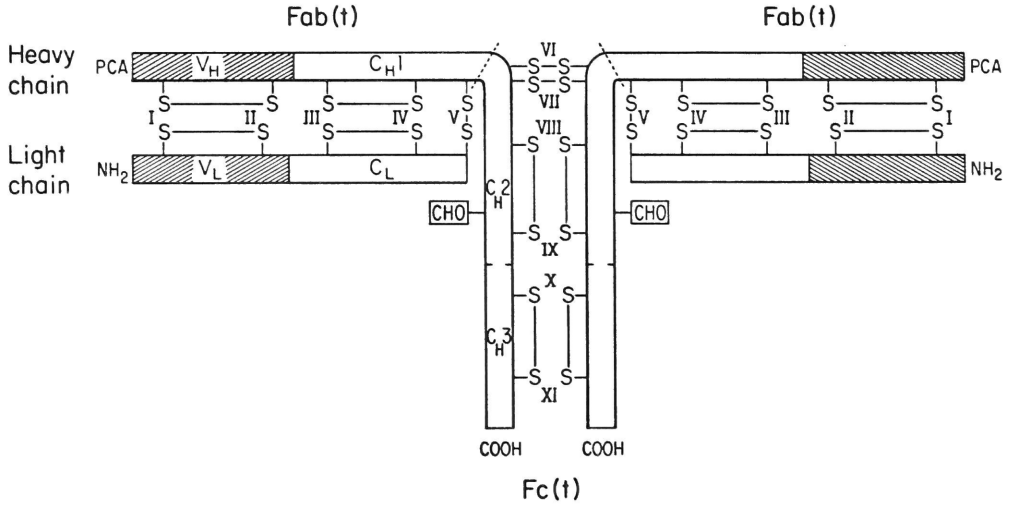


Figure 31. The structure of the human γG1 myeloma protein Eu (Edelman et al., 1969).

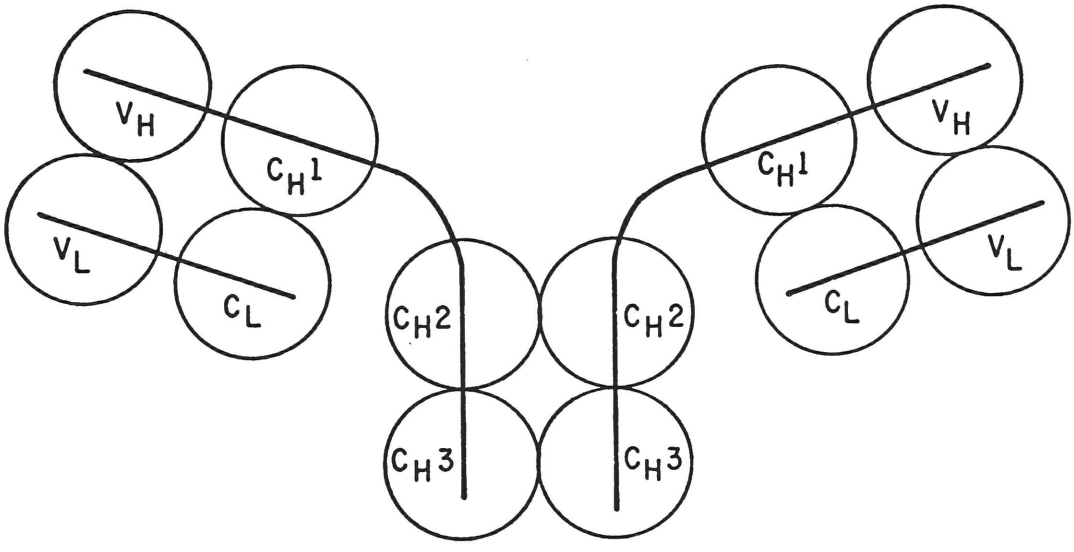


Figure 32. Hypothetical compact domains in γG immunoglobulin. Each domain is made up of one homology region (V_H, C_H1, etc.). One possible arrangement of these domains is shown.

The various classes of the immunoglobulins differ from one another in effector functions, and these functional differences may be reflected in structural differences in the kind of domains which make up the C regions. A model of a mouse α chain from a myeloma protein (Seki et al., 1968) suggests that this chain has the same linear pattern of intrachain disulfide bonds found in Eu, although an additional intrachain bond may be contained within one of the loops. This additional loop in no way affects the domain hypothesis, although an additional bridge between two domains might, however. More details of the structure of the constant regions of several immunoglobulins of different classes, as well as the complete three-dimensional structure of the molecule, will be needed to confirm or disprove this hypothesis.

2. The antigen combining site. One feature of the V regions which has been preserved is the disulfide loop. The two half-cystines forming this bond have been found in all of the light chains studied, and the limited data suggest that this is also true for heavy chain V regions. The antigen recognition function is common to all of the immunoglobulin classes, and there is some suggestion that heavy chain V regions may be shared by all of the classes (Wikler et al., 1969). Light c hains are known to be common to all of the classes. This disulfide loop in the V region of the chains may be necessary to provide a certain basic conformation necessary for the formation of antigen-binding sites. Variations in this structure as a result of the variations in amino acid sequence in the adjacent parts of the polypeptide chain could then modify the conformation of this region and give specificity to the site. Electron micrographs of different antigen-antibody complexes (Feinstein and Rowe, 1965; Valentine and Green, 1967) have suggested that the antigen combining sites are at the ends of the molecule, in a relatively fixed position. The disulfide bonds in the variable regions of the molecule may serve to fix the position of the site in just such a fashion.

E. Concluding Remarks

The studies presented in this thesis have suggested new ideas about the relationship of structure and function in the immunoglobulins. The overall linearity and periodicity of this structure makes it unique among the proteins for which structures are known. The γG immunoglobulins are relatively large proteins; in fact, Eu is the largest protein for which the complete covalent structure is known. It is not unreasonable to suggest that other large proteins may be constructed in a similar fashion. That is, large proteins may be the products of genes which have been formed by duplication in evolutionary time, and they may be organized into compact domains, each of which may have evolved to perform a different set of functions. Further structural studies on large proteins may show that this is the case.

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