The Structure and Biological Activity of Immunoglobulins and their Subunits*

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With 3 Figures

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I. Introduction

A. Definition of immunoglobulins

Immunoglobulins have been defined by a World Health Organization (WHO) committee on nomenclature in the following terms[1]: Immunoglobulins are proteins of animal origin endowed with known antibody activity, and certain proteins related to them by chemical structure and hence antigenic specificity. Related proteins for which antibody activity has not been demonstrated are included — for example, myeloma proteins, BENCE-JONES proteins and naturally occurring subunits of the immunoglobulins.

Immunoglobulins are not restricted to the plasma but may be found in other body fluids or tissues, such as urine, spinal fluid, lymph-nodes, spleen, etc. Proteins may occur which fulfill the above requirements but which have widely differing physicochemical properties such as electrophoretic mobility, sedimentation coefficient, and diffusion coefficient, and different chemical properties such as carbohydrate content, amino acid composition of polypeptide chains, etc. Immunoglobulins do not include the components of the complement system.

B. Terminology and abbreviated notation

Advances in biological and chemical studies of immunoglobulins have emphasized the need for a nomenclature and classification to meet new requirements. Terminology should be based on the identification of the polypeptide chains which by complementation make up the molecule. In addition, broader terms are needed for grouping classes of molecules with common physicochemi-

[1] A memorandum on the nomenclature of immunoglobulins was drafted by CEPELLINI, R., S. DRAY, G. EDELMAN, J. FAHEY, F. FRANEK, E. FRANKLIN, H. C. GOODMAN, P. GRABAR, A. E. GURVICH, J. F. HEREMANS, H. ISLIKER, F. KARUSH, E. PRESS, Z. TRNKA and was published in Bull. Wld Hlth Org. **30**, 447 (1964). Requests for reprints should be addressde to Immunology, World Health Organization, Palais des Nations, Geneva.

cal and biological characteristics. This can be achieved by minor modifications of current terminologies.

Two symbols for immunoglobulins are considered to be appropriate. The logical abbreviation for the term immunoglobulin would be the symbol Ig. Since the symbol Ig may present some difficulties in verbal communication and, furthermore, since the symbol γ has been commonly employed to designate the immunoglobulins, both symbols γ and Ig are recognized as appropriate. These symbols should be accompanied by a capital letter designating a specific class of immunoglobulin.

Three major classes of immunoglobulins have been recognized which — according to the old terminology — have been termed γ-, β_2A- and β_2M-globulins. The inadequacy of these symbols to meet the above requirements has led to the introduction of other names which have created some confusion among non-specialists. The following symbols proposed are based on differences associated with the heavy chains of immunoglobulins.

Present usage	*Proposed usage*
γ, 7 Sγ, 6.6 Sγ, γ_2, γSS	γG or IgG
β_2A, γ_1A	γA or IgA
γ_1M, β_2M, 19 Sγ, γ-macroglobulin	γM or IgM

In the following we shall use the notations γG, γA and γM. The structural and biological aspects of the γG- and γM-immunoglobulins will be dealt with in separate sections. The little which is known on the substructure of γA-immunoglobulins will be included in the corresponding subheadings of these sections.

II. The γG-immunoglobulins

The group of proteins which move most slowly when plasma is submitted to electrophoresis at pH 8.6 make up the major part of the γG-immunoglobulins but small amounts of the same class of immunoglobulins extend as far as the α_2-electrophoretic region. As a rule, γG-immunoglobulins contain antibodies but they may also contain pathological proteins in proliferative disorders of lymphocytic or plasmocytic cells.

Before any structural work is attempted it is essential to separate the γG-immunoglobulins from the γA- and γM-immunoglobulins. The procedures to purify γG-immunoglobulins have been described in other reviews (ISLIKER 1957; CANN 1959; KABAT and MAYER 1962). By using the cold ethanol method (ONCLEY, MELIN, RICHERT, CAMERON and GROSS 1949), varying amounts of aggregated immunoglobulin may be formed during fractionation. It is therefore crucial to use the most gentle methods such as chromatography on DEAE-cellulose (SOBER, GUTTER, WYCOFF and PETERSON 1956; STRAUSS, KEMP, VANNIER and GOODMAN 1964) and gel filtration on Sephadex G200 (KILLANDER and FLODIN 1962). Immunoelectrophoresis is a most sensitive method to detect contaminations.

The degradation of γG-immunoglobulins may be effected by different means such as reductive cleavage and proteolysis. Although structural changes result from thiol treatment, with regard to shape and diffusion constant, no reduction in molecular size is apparent in the absence of enzymes and denaturing agents. The avidity of combination with antigen may decrease slightly, depending on the amount of thiol used, but no reduction of antibody titer is apparent if sufficient time is left for the antigen-antibody reaction to occur. As outlined in section II.C.1, γG-immunoglobulins are composed of chains which are linked together by disulfide bonds as well as by regions of noncovalent bonding. In order to obtain a cleavage into subunits, reduction of interchain disulfide bonds must be followed by exposure to denaturing agents such as urea or acid to break the noncovalent bonds. The subunits are then referred to as *chains*.

The other possibility for degradation of γG-immunoglobulins takes advantage of the action of proteolytic enzymes such as papain or pepsin combined with reducing agents. Limited proteolysis will cause the breaking of peptide bonds. The subunits obtained after reduction are then referred to as *fragments*.

A. The structure of fragments derived from γG-immunoglobulins

1. Size. The fragmentation of γG-immunoglobulins by proteolytic enzymes was studied by WEIL, PARFENTIEV and BOWMANN (1938) during attempts to reduce the viscosity of antibody preparations and to accelerate the diffusion rates of antibodies *in vivo*. PETERMANN (1946) found that the action of pepsin and bromelin did not suppress the ability of the antibody to fix to the antigen. Subsequent investigation showed that the fixation to antigen did not require the total surface of the antibody molecule which is of the order of 26,000 Å^2. The combining site has been estimated at 100 to 200 Å^2 which represents only about 0.4 to 0.7% of the antibody surface (KABAT 1956; KARUSH 1962). PORTER (1959) has carried out enzymatic cleavage of γG-immunoglobulins under controlled conditions. He showed that rabbit γG-immunoglobulins with a molecular weight of about 150,000 can be cleaved by mercury-papain — activated with cysteine and EDTA — into three large fragments, each representing about one third of the size of the original molecule. These subunits can be separated by chromatography on CM-cellulose and have been termed fragment I, II, and III according to the order of their elution with sodium acetate buffer at pH 5.5. The first two fragments (I and II) have sedimentation constants of 3.5 S and molecular weights of the order of 45,000. Each contains a single antigen-binding site which does not seem to have been altered by the enzymatic cleavage, since it has the same specificity and combining power as the original molecule. The WHO committee on nomenclature has proposed the term Fab (*a*ntigen *b*inding *f*ragment) for this subunit. The third fragment (III) is somewhat larger with a molecular weight of the order of 50,000 (MARLER, NELSON

and TANFORD 1964). The solubility of fragment III is considerably smaller than that of fragments I and II. Fragment III may be crystallized by dialysis of a 0.02 N acetic acid solution against 0.1 M phosphate buffer at pH 6.8. The WHO committee on nomenclature (1964) has proposed the term Fc for this *c*rystallizable *f*ragment. Fc has no binding site for the antigen but possesses other biological activities such as skin-reactive and complement-fixing properties (section II.B).

2. The electrophoretic mobility of rabbit immunoglobulin fragments has been determined on starch-gel in 0.1 M acetate buffer pH 5.5. It is in accord with the order of elution from CM-cellulose: Fab-fragment I (according to PORTER) is slightly anionic at this pH, Fab-fragment II somewhat cationic, and fragment III even more cationic than the native γG-immunoglobulin. Using vertical starch-gel electrophoresis, PUTNAM, TAN, LYNN, EASLEY and MIGITA (1962) have shown fragment Fc to be composed of 3 electrophoretically distinct subfractions which varied in amount depending on the pH and time of incubation during proteolysis. The heterogeneity of immunoglobulins and the fragments derived thereof will be more amply discussed in section II.A.6. The size, charge and chemical composition of the fragments largely depend on the nature of the proteolytic enzyme used for cleavage (section II.A.4). Following the decisive contribution of PORTER, mercury-papain has been most widely used for enzymatic cleavage. If not otherwise stated, the fragments described in the following sections have been obtained from *rabbit* γG-immunoglobulins, according to the method of PORTER (1959).

In other species the physicochemical properties of antibody fragments may differ considerably. Electrophoretic mobilities of certain Fab- and Fc-fragments may even be inverted as compared to fragments derived from rabbit γG-immunoglobulin (section II.A.7).

3. Chemical composition. *Amino acids.* The fragments obtained by digestion with mercury-papain do not appear to be susceptible to further proteolysis after prolonged enzymatic treatment. The fragments are not homogeneous although prolonged digestion of the Fab-fragments (I and II) with papain does not yield fragments with sedimentation coefficients smaller than 3.5 S. However, further digestion of the Fab-fragments with chymotrypsin yields approximately 35 peptides, and 50 to 55 peptides derived from the Fc-fragment (GITLIN and MERLER 1961). Chromatography revealed between 55 to 75 peptides after hydrolysis with subtilisin. The peptides derived from both Fab-fragments were nearly identical and differed considerably from those derived from the Fc-fragment. GIVOL and SELA (1964) — using high voltage electrophoresis and chromatographic resolution of peptides — confirmed these findings and found in Fab-fragment II a strongly basic peptide absent in the peptide map of Fab-fragment I. Similar differences have been found for the amino acid analysis obtained after exhaustive hydrolysis. The Fc-fragment

displayed considerable differences especially with regard to Thr, Pro, Gly, Ala, Met, Tyr, and the basic amino acids (table 1).

PORTER (1950) determined the N-terminal peptide sequence of rabbit antiovalbumin by the method of SANGER. Since ovalbumin has no N-terminal amino groups, a specific ovalbumin-antiovalbumin precipitate was used directly for end-group analysis. The N-terminal pentapeptide was composed of Ala-Leu-Val-Asp-Glu. Ala would be the principal N-terminal amino acid for fragment I and II (PORTER 1959). The sequence for Fab-fragment I is alanyl-aspartyl. Fab-fragment II contains a leucyl instead of an aspartyl residue in the second position. PUTNAM, TAN, LYNN, EASLEY and MIGITA (1962) indicate leucine as N-terminal residue of the Fc-fragment. The C-terminal amino acids have been determined by SILMAN, CEBRA and GIVOL (1962) by hydrazinolysis. Rabbit γG-immunoglobulin contains four C-terminal amino acids: glycine, serine, threonine, alanine in molar ratios of 2:1:0.5:0.5. The Fab-fragments have the same groups, whereas the Fc-fragment contains only two C-terminal glycyl residues per mole of fragment and only traces of serine, threonine and alanine.

Table 1. *Amino acid analysis of fragments from non-immune serum*[1]

Amino acid	Fab		Fc
	I	II	III
	N (% of total N per fragment)		
Aspartic acid .	5.66	6.01	6.18
Threonine . .	10.42	11.56	5.61
Serine	8.77	9.57	7.34
Glutamic acid	5.99	6.18	7.83
Proline . . .	5.08	5.08	6.68
Glycine . . .	7.57	6.93	3.18
Alanine . . .	5.65	6.12	2.82
Valine . . .	7.38	7.58	5.96
Methionine . .	0.64	0.75	1.48
Isoleucine . .	2.20	2.26	3.43
Leucine . . .	5.17	5.39	5.19
Tyrosine . .	3.44	3.58	2.33
Phenylalanine	2.53	2.65	2.76
Histidine . .	1.59	1.87	5.23
Lysine . . .	6.03	6.84	9.95
Arginine . . .	5.94	6.22	13.93
Tryptophan	not estimated		
$^1/_2$ Cystine . .	2.80	2.22	1.18

[1] From PORTER (1959).

Carbohydrates. γG-immunoglobulins of most animal species contain 2 to 3 % carbohydrates. Two thirds of the carbohydrates are associated with the Fc-fragment. According to NOLAN and SMITH (1962) the polysaccharide fraction of rabbit γG-immunoglobulin is constituted of 2 units of mannose, 4 units of galactose, 8 units of N-acetyl-glucosamine, 1 unit of fucose and 1 unit of sialic acid per molecule. The carbohydrate is linked to the protein through the β-carboxyl group of the C-terminal aspartic acid.

In human γG-immunoglobulin, ROSEVEAR and SMITH (1961) isolated an oligosaccharide with a molecular weight of 4,000 containing 5 mannose, 3 galactose, approximately 10 N-acetyl-glucosamine, 2 fucose and 1 sialic acid residue. As in the case of rabbit γG-immunoglobulin the carbohydrate is associated with the crystalline Fc-fragment and is also linked to the C-terminal aspartic acid. It has not been clearly established if the presence of this oligosaccharide on fragment Fc has a relation to its biological activity.

4. Mechanism of enzymatic cleavage. Using an enzyme-substrate ratio of 1 to 100, papain will cleave 66% of rabbit γG-immunoglobulin within 15 min (in the presence of 0.001 M cysteine and 0.002 M EDTA and at its pH optimum of 6.0). Cleavage is complete after 3 h incubation at 37° C (PUTNAM, TAN, LYNN, EASLEY and MIGITA 1962).

Using a water-insoluble papain-derivative, CEBRA, GIVOL, SILMAN and KATCHALSKI (1961) have shown the cleavage of γG-immunoglobulin to occur in two consecutive stages. A limited proteolysis is followed by a reduction with 0.01 M cysteine. In the first stage, 3 to 5 peptide bonds are split without affecting the sedimentation coefficient and without liberation of small-molecular weight peptides. The limited proteolysis does not affect the immunologic properties of the globulin and is completed within 5 min. Disulfide bonds are exposed which may then be cleaved during the second stage by the action of cysteine: the latter brings about a decrease of the sedimentation coefficient from 6.2 to 3.5 S and a complete loss of the precipitating capacity of the antibody. However, the 3.5 S fragments are able to specifically inhibit the precipitation of antigen by the native antibody. A two-stage cleavage of rabbit γG-immunoglobulins has been shown to occur with pepsin by NISONOFF, WISSLER and WOERNLEY (1959) and with trypsin by ISLIKER (1961) followed by the reducing action of 2-mercaptoethylamine. At a pepsin-substrate ratio of 1 to 100 and pH 4.5, a large bivalent fragment with a sedimentation coefficient of approximately 5 S and a molecular weight of 106,000 is formed. It is capable of specifically precipitating antigen (NISONOFF, WISSLER, LIPMAN and WOERNLEY 1960; NISONOFF, MARKUS and WISSLER 1961; MANDY, RIVERS and NISONOFF 1961).

Pepsin liberates a small number of peptides which are derived from the Fc-fragment. The 5 S fragment may then be cleaved by 2-mercaptoethylamine into two fragments with sedimentation coefficients of 3.5 S and molecular weights of 56,000. NISONOFF, MARKUS and WISSLER (1961) showed that this last step was brought about by the reductive cleavage of one highly labile disulfide bond. Each of the univalent 3.5 S fragments contained one free sulfhydryl group and had a similar amino acid composition as the Fab-fragments obtained after papain digestion (NISONOFF, WISSLER and WOERNLEY 1959). However, the fragments obtained according to the method of PORTER (1959) differed slightly from those obtained by NISONOFF. If the free sulfhydryl groups of the latter fragments are not blocked by the action of iodoacetamide they will reoxidize to form a disulfide bond giving the initial divalent 5 S component. According to the new nomenclature the *pepsin* 3.5 S fragments are referred to as Fab′. For 5 S fragments obtained by pepsin digestion or by reassociation of two Fab′-fragments the notation would be $F(ab')_2$.

The reversibility of the reductive cleavage suggested to NISONOFF and RIVERS (1961) the possibility of preparing antibodies of mixed specificity:

5 S fragments were prepared from two specifically purified antibodies, anti-ovalbumin (A) and anti-bovine γ-globulin (B), which were mixed and reduced in the same container with 0.01 M 2-mercaptoethylamine. This procedure resulted in an essentially complete breakdown to 3.5 S fragments, which were then reoxidized to form 5 S molecules. For random recombination of equal amounts of the two antibodies, the ratios of antibodies A-A, B-B, and A-B would be 1:1:2. The 5 S hybrid molecules precipitated neither ovalbumin nor bovine γ-globulin, but only a mixture of the two antigens.

5. Disulfide bonds of γG-immunoglobulins. The disulfide bonds not only play a major role in holding the different chains of the immunoglobulin molecules together; they are also thought to confer on the γG-immunoglobulin molecule the necessary rigidity to maintain the configuration of the antigen-binding site.

Amino acid analysis indicates a total number of 44 half-cystine residues, corresponding to a number of 22 disulfide bonds per native molecule. When using reducing agents such as 2-mercaptoethylamine (at concentrations of 0.4 M, pH 7.5, 37° C during 30 min) about one third of the disulfide bonds of rabbit γG-immunoglobulins may be cleaved, corresponding to 7 disulfide bonds. This number may be increased to 11 in the presence of 1 % sodium dodecyl sulfate and to 22 in the presence of 10 M urea. No reduction of molecular weight has been observed in the absence of denaturing agents. The same experiment has been carried out using 5 S fragments obtained after peptic digestion of γG-immunoglobulins. 4.2 disulfide bonds have been opened by reductive cleavage with 2-mercaptoethylamine, whereas only 1.7 disulfide bonds can be reduced when a Fab-"papain" fragment is used as a starting material. The total number of disulfide bonds which may be cleaved in the two Fab-"papain" fragments, is thus 3.4, as opposed to 4.2 disulfide bonds which may be cleaved in the 5 S pepsin fragment. The difference of 0.8 corresponds to the highly labile disulfide bond which is cleaved during the reduction of a 5 S fragment to Fab-fragments. Other reagents such as sulfite and performic acid may also be used for the cleavage of disulfide bonds. In the presence of 8 M urea FRANEK (1961) obtained, by S-sulfonation of rabbit γG-immunoglobulins, subunits with a sedimentation coefficient of 3.4 S corresponding to a molecular weight of 74,000. The treatment of human γG-immunoglobulins by the same procedure produced subunits with a sedimentation coefficient of 1.9 S (molecular weight 46,000). These become strongly aggregated and quite insoluble in water suggesting a multi-chain structure for the γG-immunoglobulin.

6. Heterogeneity of γG-immunoglobulins and the fragments derived thereof. The differences between Fab-fragments I and II were thought to be due to distinct physicochemical features of each fragment within a given molecule. It then became evident that a γG-immunoglobulin preparation may contain different families of globulins, each being able to produce either fragment I or

fragment II. This hypothesis was substantiated by the finding that different γG-immunoglobulin preparations formed different quantities of fragment I and II, even though these preparations have been derived from specifically purified antibodies (STELOS, ROHOLT and PRESSMAN 1962).

PALMER, MANDY and NISONOFF (1962) fractionated rabbit γG-immunoglobulin by chromatography on CM-cellulose and eluted one portion with 0.06 M sodium chloride (95 mg), the other portion with 0.5 M sodium chloride (99 mg). Each of these portions was digested with papain and chromatographed under identical conditions. The following data give the yields of the 3 fragments obtained:

after elution with:	Fab-fragments I	Fab-fragments II	Fc-fragment III
0.06 M NaCl	20.8 mg	3.8 mg	11.5 mg
0.5 M NaCl	2.2 mg	11.5 mg	11.9 mg

The results show unequivocally that the Fab-fragments I and II are derived from different parent molecules. The quantities of Fc-fragment III derived from the two portions of rabbit γG-immunoglobulin are nearly identical and represent approximately one third of the total digest. The Fc-fragment appears to be common to γG-immunoglobulins, although it is heterogeneous because of its greater susceptibility to proteolysis.

Carrying out similar experiments with a mixture of purified antibodies of two specificities produced in a single rabbit, GROSSBERG, ROHOLT and PRESSMAN (1963) came to the same conclusion: antibody activity to different haptenic groups appeared in different fragments and in unequal quantities, indicating a marked heterogeneity of the γG-immunoglobulins, even in a single animal.

7. Enzymatic cleavage of antibodies from different species. The fragments derived from human, mouse, and guinea pig γG-immunoglobulins are in many respects similar to those derived from rabbit γG-immunoglobulins. Differences exist in respect to the kinetics of the cleavage of immunoglobulins from different species. As a rule, the susceptibility to cleavage decreases from rabbit, to human, bovine, equine and porcine γG-immunoglobulins (HSIAO and PUTNAM 1961). In the case of human γG-immunoglobulin, prolonged papain digestion produces a fourth fraction with a molecular weight below 5,000 representing from 2 to 5 % of the total protein (HSIAO and PUTNAM 1961; DEUTSCH, STIEHM and MORTON 1961). This fraction was found to be heterogeneous and probably results from random degradation of denatured protein by papain. Human γG-immunoglobulin fragments (EDELMAN, HEREMANS, HEREMANS and KUNKEL 1960; FRANKLIN 1960; HSIAO and PUTNAM 1961) and the corresponding mouse fragments (ASKONAS and FAHEY 1961; FAHEY and ASKONAS 1962) differ from rabbit fragments in their electrophoretic mobility: whereas Fab-fragments (I and II) of the rabbit migrate at the same rate or more rapidly than intact rabbit γG-immunoglobulins, the corresponding fragments of human and

mouse bearing the antigen-binding sites migrate at the same rate or more slowly than intact globulins. For this reason, Fab-fragments of human γG-immunoglobulins have been designated as S (slow) fragments. When first preparing human immunoglobulin fragments, FRANKLIN (1960) introduced the letters A and C for the antigen-binding fragments. The different nomenclatures lead to considerable confusion and the notation Fab proposed by the WHO-nomenclature committee (1964) should be useful.

Rabbit Fc-fragment migrates more slowly than the fragments containing the antigen-binding sites, whereas the corresponding fragments of human or mouse origin display an electrophoretic mobility which is greater than that of the Fab-fragments. The fragment of human origin devoid of antigen-binding sites has therefore been termed F-fragment by EDELMAN, HEREMANS, HEREMANS and KUNKEL (1960) or B-fragment according to FRANKLIN (1960). The following summary on the proposed abbreviated notations should help to establish a precise terminology for the antibody-fragments derived from different species.

Present usage		*Proposed usage*
papain fragments	A, C, S (I, II) *	Fab-fragment (antigen-binding)
	B, F (III) *	Fc-fragment (crystallizable)
	A piece	Fd-fragment
pepsin fragments corresponding to	A, C, S (I, II) *	Fab'-fragment
pepsin fragment	5 S	$F(ab')_2$-fragment

* The parentheses enclose terms used for fragments in the rabbit only.

B. The biological activity of fragments derived from γG-immunoglobulins

1. Antigen-binding properties. It has been clearly demonstrated that the antigen-binding sites of antibodies are located on the Fab-fragments (I and II). The estimation of the size of the binding site has shown that it comprises but a few per cent of the surface of the antigen-binding fragment (KABAT 1956, KARUSH 1962). However, in order to retain their specificity, the binding sites must be held together by portions of the fragments which by themselves display no affinity for the antigen. The involvement of chains which are not directly a part of the binding site will be dealt with in more detail in section II.D. The Fab-fragments (I and II) — although specifically inhibiting the reaction of native antibody with the antigen — are unable to form *specific precipitates*. This property is limited to native antibodies or to 5 S $F(ab')_2$-fragments which contain two antigen-binding sites.

ISCAKI and RAYNAUD (1961) have prepared 3 and 5 S fragments by peptic digestion of specific precipitates of diphtheria toxin and β-2-type horse antitoxin. The neutralizing activity was retained. In the case of the 3 S fragments the capacity to precipitate the antigen was lost. In the presence of 5 S fragments and with sufficiently large quantities of antigen the total amount of 3 S fragment was co-precipitated. This finding provides the possibility of

devising a quantitative test for measuring non-precipitating fragments of antibody (RAYNAUD and ISCAKI 1964).

The above considerations, which are valid for precipitation, also apply to *agglutination* reactions: red blood cells sensitized with rabbit 3.5 S antibody-fragments are not agglutinated. However, these Fab-fragments inhibit agglutination by the whole antibody against the red cell. The fixation of the fragments on the red cell surface may be demonstrated by hemagglutination following the addition of goat antibody specific for univalent fragments of rabbit γG-immunoglobulin (FUDENBERG and NISONOFF 1962).

Pretreatment of the red cells with proteolytic enzymes does not render the cells susceptible to agglutination by Fab-fragments. This is unlike the behavior of red cells in the presence of 7 S "incomplete" antibodies. In this case a pretreatment of the cells with proteolytic enzymes may render them agglutinable. These results are consistent with current theories that "incomplete" antibodies are not univalent.

FUDENBERG, DREWS and NISONOFF (1964) have prepared antibodies of mixed specificity against ovalbumin and bovine γG-immunoglobulin by oxidation of a mixture of the Fab-fragments of the two specifically purified antibodies. The hybrid 5 S $F(ab')_2$-fragments were demonstrated by agglutination of tanned erythrocytes of different species coated with the corresponding antigens. Mixed "agglutinates" were formed which could be dispersed by treatment with 0.1 M mercaptoethylamine.

Antibodies against motile microorganisms may show the property of causing *immobilization*. For immobilization of *Trichomonas foetus*, bivalent antibody molecules are required. 5 S $F(ab')_2$-fragments retain full immobilizing activity when compared to native γG-immunoglobulin. The activity of the 5 S $F(ab')_2$-fragments may be destroyed by subsequent addition of cysteine to the immobilized protozoa, giving a complete recovery of motility. In accord with these findings, papain Fab-fragments are devoid of immobilizing activity (MOLINARO, JATON, SCHOLER and ISLIKER 1964). The *neutralization* of viruse sand toxins by antibody-fragments is discussed in section IV.

2. Complement-fixing properties. The biological activities of antibodies are not confined to the binding of antigens: antibodies exhibit distinct skin- and tissue-reactive properties, as well as complement-fixing properties, which manifest themselves when the antibody molecules are brought into apposition either by the antigen or by other conditions. The work of ISHIZAKA (1963) provides evidence that the Fc-fragment plays an important part in the induction of these biological activities. Although fragment Fc does not fix complement by itself, it does so when it is heated to 63° C or when it is aggregated by other means, such as by coupling with bis-diazotized benzidine. The lack of activity of the two aggregated Fab-fragments is compatible with the finding that complexes of either fragment with the corresponding antigen are devoid of

complement-fixing activity. Thus, fragment Fc appears to contain a structural unit essential for the induction of complement fixation. It is a prerequisite that at least two of these fragments be tied together either by physical or chemical treatment, or — when one is dealing with the native antibody system — by the corresponding antigen.

Some of these findings have been reinvestigated by SCHUR and BECKER (1963). They showed that the 5 S $F(ab')_2$ rabbit and sheep fragments obtained after digestion of antibody with pepsin retain some complement-fixing properties in presence of antigen. However, the 5 S $F(ab')_2$-fragments differ from 7 S antibodies since preformed antigen-antibody complexes produced with 5 S fragments fixed 20 to 40 % of the total amount of complement which could be fixed with 7 S antibody. The portion of complement fixed by the insoluble aggregates derived from 5 S antibody appeared different from the residual complement in the supernatant solution. This finding is surprising since 5 S fragments are thought to be devoid of fragment Fc. REISS and PLESCIA (1963) have expanded these studies by digesting specific precipitates of ovalbumin and its rabbit-serum antibody treated with human complement. After digestion with papain, complement was not bound to fragment Fc but rather to the Fab-fragments. These conflicting data with regard to the portion of the antibody molecule which is responsible for complement fixation have not yet been reconciled.

Preparations of γG-immunoglobulins are known to fix complement in the absence of antigen. In the case of human γG-immunoglobulin prepared by the cold ethanol method (ONCLEY, MELIN, RICHERT, CAMERON and GROSS 1949) anticomplementary activity is essentially due to the presence of trace amounts of 30—100 S components. According to SCHULTZE, HAUPT, HEIDE, MÖSCHLIN, SCHMIDTBERGER and SCHWICK (1962) these macroglobulins are native serum constituents, according to others they are artifacts formed during fractionation. The aggregates may be removed by limited proteolysis or ultracentrifugation for 7 h at 40,000 g, which largely reduces anticomplementary activity (BARANDUN, KISTLER, JEUNET and ISLIKER 1962).

The residual activity is due to 8—9 S components which can be eliminated if ultracentrifugation is carried out at 100,000 g for 7 h. ISLIKER, JACOT-GUILLARMOD and THOENI (1962) have shown that anticomplementary activity could also be removed by pH 4 treatment at 37° C or by treatment with reducing agents (see table 2, section II. B. 3). The addition of 0.1 M mercaptoethylamine to human γG-immunoglobulins did not produce a significant decrease of anticomplementary activity. However, if the reduced globulin was immediately treated with 0.1 M iodoacetamide, anticomplementary activity disappeared. This experiment shows that the dissociation of disulfide bonds is reversible unless the newly formed sulfhydryl groups are blocked. Iodoacetamide alone at a concentration of 0.1 M had little or no effect on anticomplementary

activity. WIEDERMANN, MIESCHER and FRANKLIN (1963) have confirmed these findings and showed that γG-immunoglobulins treated with 0.1 M mercaptoethanol and iodoacetamide still bind antigen without fixing complement. In contrast, pH 4 treated 7 S hyperimmune globulins are able to fix complement in the presence of antigen. Other reagents, such as oxidizing agents, ultraviolet irradiation and ultrasonication did not abolish anticomplementary activity of human immunoglobulins. MIESCHER, SPIEGELBERG and BENACERRAF (1963) showed that complementfixation was required to enhance opsonic activity of immune sera. Consequently, antibodies treated with mercaptans and Fab-fragments are not capable of opsonizing red cells and bacteria. ROWLEY, ISLIKER and THOENI (1964) confirmed that specific Fab-fragments were without significant influence on the clearance of *Salmonella adelaïde* from the circulation of infected mice.

3. Skin-reactive properties. The skin-reactive properties of soluble antigen-antibody complexes are dependent on their composition with respect to antigen-antibody ratio and to the species of origin of the antibody. The work of ISHIZAKA (1963) showed that human and rabbit antibodies become skin-reactive when two or more antibody molecules are brought into apposition, as described in the case of complementfixation. These reactions are of fundamental importance for the induction of allergic and anaphylactic reactions. OVARY and KARUSH (1961) showed that fragment Fc was capable of sensitizing guinea pig skin for reversed passive cutaneous anaphylaxis (PCA). On the other hand a site injected with Fab-fragments (I and II) did not show any skin response when the guinea pigs were challenged by an injection of horse antiserum to rabbit γG-immunoglobulin. Similarly, the antigen-binding fragments did not block passive sensitization of guinea pig skin with rabbit antibody when they were injected with sensitizing antibody. OVARY and TARANTA (1963) showed that pepsin-digested rabbit 5 S $F(ab')_2$-fragments provoked reversed PCA in the guinea pig, but were less effective than the native antibody. Cleavage of the 5 S fragment into two monovalent fragments markedly reduced the capacity to induce reversed PCA. Thus, the valence or the molecular weight of the antibody or both, appear to be of critical importance in the mechanism of reversed PCA. It remains to be seen if the activity of 5 S fragments is due to contamination with residual Fc-fragment, which was shown to be active in producing reversed PCA.

The above data suggest that fragment Fc plays at least an important part in inducing the complement- and skin-reactive properties of antibodies. However, it has not been established if the sites responsible for these properties are identical. Attempts have been made to destroy one of these activities without affecting the other. ISLIKER, JACOT-GUILLARMOD and THOENI (1962) removed anticomplementary activity of human γG-immunoglobulins (Cohn fraction II) by incubation at pH 4 and 37° C in the presence or absence of

pepsin. The same treatment also abolishes the anticomplementary activity of γG-immunoglobulin aggregated at 63° C. The third column in table 2 illustrates these findings.

Table 2. *Fixation of human γG-immunoglobulins on human liver tissue; comparison with anticomplementary activity*[1]

γG-immunoglobulin preparation	Per cent fixation of γG-immunoglobulins on liver tissue (average values)[2]	Anticomplementary activity[3]
COHN fraction II (batch 68)	23.9	16
incubated at pH 4[4]	35.5	<2
incubated at pH 4[4] with pepsin (1:10000) . .	18.2	<2
aggregated at 63° C	53.6	1024
aggregated at 63° C incubated at pH 4[4] . .	60	<2
COHN fraction II (batch 66)	21.5	96
incubated with MEA and IAA[5]	22.9	<2

[1] From ISLIKER and THOENI (1962).

[2] One to 30 μg of I^{131} labelled γG-immunoglobulins were incubated with 100 mg human liver tissue for 5 min at 37° C and washed 3 times (5 to 7 measurements).

[3] For 1% solution; expressed according to KABAT and MAYER (1962).

[4] Incubation was performed for 24 hours at 37° C; the pH was readjusted to 7.0.

[5] The samples were incubated with 0.1 M MEA (mercaptoethylamine) and dialysed against 0.1 M IAA (iodoacetamide).

ISLIKER and THOENI (1962) have tested the above preparations for their ability to fix to human liver tissue. No parallelism was observed between tissue and complement fixation. On the contrary, pH 4 treated γG-immunoglobulins were found to be more sticky than untreated globulins. Aggregated immunoglobulins fixed 2—3 times more to human liver tissue — whether they were left at pH 7 or incubated at pH 4.

γG-immunoglobulins reduced and alkylated with mercaptoethylamine and iodoacetamide were also tested for their ability to fix on human liver cells (BARANDUN, KISTLER, JEUNET and ISLIKER 1962). The lower part of table 2 indicates no difference between the native and reduced immunoglobulins. In contrast, the reduced and alkylated fraction displayed a strong decrease of anticomplementary activity as compared to untreated COHN fraction II. Thus, complement and tissue fixation of immunoglobulins appear to be mediated by different sites.

ISHIZAKA and ISHIZAKA (1964) have measured tissue affinity and complement fixation of Fc-fragments and their aggregates: When fragment Fc was reduced and alkylated in 2 M urea, the activity of eliciting reversed PCA was nearly lost, and remained at the same low level upon aggregation. In contrast, aggregates produced in the same way were able to inactivate complement *in vitro*, indicating that this treatment degrades the site essential for tissue affinity, but does not affect the sites responsible for complement fixa-

tion. The latter were only degraded by complete reduction of the fragment in 6 M urea.

It has been assumed that the sites responsible for the properties of antibodies discussed above were located on the same molecule. Recently, BENACERRAF, OVARY, BLOCH and FRANKLIN (1963) described two families of antibodies in guinea pig serum directed against the same antigen, which are endowed with different biological properties. These antibodies differ in their electrophoretic mobilities and may be separated by various techniques, such as starch-block electrophoresis and chromatography on DEAE-cellulose. These two antibody types differed antigenically only in their fragment Fc (THORBECKE, BENACERRAF and OVARY 1963). OVARY, BENACERRAF and BLOCH (1963) showed that the faster migrating antibodies (termed γ_1) are responsible for anaphylactic sensitivity. The slower antibodies (γ_2) mediate such phenomena which depend upon the fixation of hemolytic complement, such as cell lysis and the Arthus reaction (BLOCH, KOURILSKY, OVARY and BENACERRAF 1963). NUSSENZWEIG, MERRYMAN and BENACERRAF (1964) have also identified two antibody types in the mouse. As in the case of the guinea pig, mouse γ_1-antibodies transfer anaphylactic sensitivity, while mouse γ_2-antibodies appear to be responsible for lytic phenomena in the presence of complement. Thus, it is conceivable that the loci responsible for skin-reactive and complement-fixing properties are located on different antibody molecules. Further studies are required to determine if these properties can be separated in the γG-immunoglobulins of other species. According to BINAGHI and BENACERRAF (1964), the anaphylactic antibodies of the rat migrate more rapidly than the main γ_2-components, but have not been isolated to allow identification either as a γA- or γ_1-immunoglobulin. In man, skin-sensitizing antibody, although not isolated, has been shown to be a γA-immunoglobulin (FIREMAN, VANNIER and GOODMAN 1963). Thus, it is possible that various animal species — guinea pig, mouse, rat, human, dog and monkey — produce antibody types capable of binding to their own tissue receptors to mediate anaphylactic sensitivity. Studies with antibodies from other species capable of sensitizing guinea pigs for PCA have shown that these antibody types are all γ_2-complement-fixing antibodies which are not able to transfer anaphylactic sensitivity in their own species (OVARY, BLOCH and BENACERRAF). In view of data which indicate that the γA-immunoglobulin in human sera may not be the homologue of the γ_1-globulin in guinea pig sera, it is possible that the PCA activity shown by the guinea pig γ_1-globulin represents a different class of "skin-sensitizing" antibody from that reported in human, rat and mouse sera.

The phenomena outlined above may be of major biological importance. The fact that sensitized cells — which are otherwise susceptible to lysis — are not lysed by complement under certain conditions may be due to competition of complement-fixing with anaphylactic antibodies. The as yet unexplained

phenomenon of tumor enhancement may also be related to a specific protective effect of tissue-active antibodies against complement-fixing antibodies (NUSSENZWEIG, MERRYMAN and BENACERRAF 1964). The same applies for allergic phenomena, where the presence of "blocking" antibodies has been demonstrated in addition to reaginic antibodies. The protective effect of blocking antibodies may well be due to their ability to combine with allergens more avidly than the reaginic antibodies, thus preventing the harmful effects of the latter.

4. Reaginic antibodies. Antibodies in allergic individuals can be subdivided into two categories: skin-sensitizing antibodies and blocking antibodies. They differ in their physicochemical properties and their affinity for the homologous allergen.

Human skin-sensitizing antibodies are associated with fast γ-globulins and the similarities with anaphylactic antibodies have already been discussed (BENACERRAF, OVARY, BLOCH and FRANKLIN 1963). However, reaginic antibodies from man differ from the γ_1-globulins of guinea pig in their sedimentation coefficient and in their susceptibility to mercaptans. LEDDY, FREEMAN, LUZ and TODD (1962) calculated sedimentation coefficients of reaginic antibodies ranging from 9.5 to 20.7 S. ROCKEY and KUNKEL (1962) found values of 8 to 11 S for reaginic antibodies reacting with highly purified glucagon. Similar data were found for the skin-sensitizing sera of ragweed-sensitive patients by FIREMAN, VANNIER and GOODMAN (1963), TERR and BENTZ (1964) and ANDERSEN and VANNIER (1964).

An association of skin-sensitizing antibodies with the γA-immunoglobulins of serum was suggested by HEREMANS and VAERMAN (1962) and conclusively shown by FIREMAN, VANNIER and GOODMAN (1963). All γA-immunoglobulins, however, are not skin-sensitizing antibodies.

Degradation of γA-immunoglobulins with proteolytic enzymes brings about a complete disappearance of skin-sensitizing activity. Reductive cleavage, acid treatment and alkylation yield "heavy" and "light" chains similar to those obtained with γG-immunoglobulins. HEREMANS, CARBONARA, MANCINI and LONTIE (1964) have found the antigenic specificity of γA-immunoglobulins to be associated with the heavy chains. These are heterogeneous and contain nearly all the carbohydrates of the native molecule. The "light" chains are immunologically undistinguishable from those of the other major classes of immunoglobulins. They occur in two different serological types (section II. C. 1).

One of the criteria to rapidly distinguish γG- from γA-immunoglobulin is the susceptibility of the latter to 0.1 M mercaptoethanol (LEDDY, FREEMAN, LUZ and TODD 1962). This property will be more fully discussed in section III. A. 3 in the case of γM-immunoglobulins. It may offer a possibility of destroying skin-sensitizing activity *in vivo* by intravenous administration of thiols to allergic patients.

The other category of antibodies present in allergic individuals — the "blocking" antibodies — differ from reaginic antibodies in their sedimentation coefficient and mobility which correspond in every respect to the parameters found for γG-immunoglobulins. They give a strong passive hemagglutination which is inhibited by univalent antibody fragments (GYENES, SEHON, FREEDMAN and OVARY 1964). They are absorbed on immunosorbents but to a lesser extent than skin-sensitizing antibodies. It is of major biological importance that skin-sensitizing antibodies can be displaced by the addition of sera possessing blocking antibodies (SEHON 1964). Contrary to reaginic antibodies, the blocking antibodies are not destroyed by 0.1 M mercaptoethanol.

A comparison of the anaphylactic, electrophoretically fast γ-globulins from different animal species (guinea pig, mouse) shows marked differences with the reaginic γA-immunoglobulins of man. The only animal species studied which have anaphylactic antibodies with skin-sensitizing characteristics similar to human reagins are the dog and the rat. Anaphylactic antibodies of the rat are present in trace amounts and attach to tissues for long periods of time without loss of sensitizing activity. Like reagins, they are heat-labile and have sedimentation coefficients between 7 S and 19 S (BINAGHI, BENACERRAF, BLOCH and KOURILSKY 1964).

Reagins may be regarded as one kind of "cytophilic" antibody. It was demonstrated that rabbit antisera contain antibodies with cytophilic properties (BOYDEN and SORKIN 1961). However, suggestive evidence was obtained that this particular cytophilic antibody is not important in anaphylaxis (SORKIN 1963). It will be important to determine if the attachment of these antibodies to epithelial tissues, guinea pig skin and other cells types is mediated through the Fc fragment (III), which is devoid of antigen-binding properties.

Sera of guinea pigs immunized with sheep red cells mixed with FREUND'S complete adjuvant contained "cytophilic" antibodies and, significantly enough, these animals were strongly delayed hypersensitive (BOYDEN 1964). The possibility that "cytophilic" antibody might play a role in delayed hypersensitivity deserves further attention.

5. Antigenic and immunogenic properties. The capacity of rabbit antibody fragments to react with antibodies produced against native rabbit γG-immunoglobulins appeared to be smaller than that of the native rabbit γG-immunoglobulins. This decrease in affinity was pronounced with the antigen-binding fragments, but fragment Fc (III) still precipitated about 70% of the antibody precipitated by the undigested γG-immunoglobulin (PORTER 1959).

The immunogenic properties of immunoglobulin fragments, that is, their ability to produce anti-antibodies, is similar to that of native γG-immunoglobulin (PORTER 1959). Antisera against the different fragments form sharp

lines of precipitation in agar diffusion tests. On immunoelectrophoresis, the lines produced by Fab- and Fc-fragments cross in all animal species tested.

6. Transfer through membranes. There are different pathways by which maternal antibodies reach the offspring: transfer *in utero* and transfer by milk. The relative importance of these pathways varies with the animal species and depends mainly on the type of placenta. In man, γG-immunoglobulins are transferred mainly across the placenta to appear in the fetal circulation at 4 to 5 months and to reach the maternal level at term. On the other hand, γA- and γM-immunoglobulins do not cross the placental barrier, a fact which has been explained on account of their elevated molecular weight. The following observations render this last assumption questionable. While antitoxins prepared in guinea pigs and injected into pregnant guinea pigs are transmitted in significant amounts to the young guinea pig, pepsin-digested antitoxins are transmitted to young guinea pigs only in trace amounts (BRAMBELL, HEMMINGS, OAKLEY and PORTER 1959). Similar findings were reported for rabbits and it was assumed that a part of the antitoxin molecule, which is lost by peptic digestion, is essential for placental transmission. The portion of the antibody molecule which is most rapidly destroyed by peptic digestion, comprises essentially fragment Fc. These findings are not conclusive by themselves, since pepsin-digestion at pH 4 — although reducing the molecular weight by $^1/_3$ — may change the shape of the molecule so as to render it less diffusible. For this reason, fragments were prepared according to the method of PORTER (1959). The Fc fragment (III) was transmitted nearly as readily as the whole γG-immunoglobulin, whereas the antigen-binding fragments (I and II) were transmitted only $^1/_5$ and $^1/_{10}$ as readily as fragment Fc. BRAMBELL, HEMMINGS, OAKLEY and PORTER (1959) suggest that fragment Fc contains the configuration by which the cells recognize homologous γG-immunoglobulins.

It was interesting to test whether the anaphylactic γ_1-immunoglobulins of guinea pigs behave similarly to human γA-immunoglobulins. As outlined in section II. B. 3, the differences between γ_1- and γ_2-globulins reside on the heavy chains and particularly on fragment Fc (BENACERRAF, OVARY, BLOCH and FRANKLIN 1963). In the case of guinea pigs, the γ_1- and γ_2-antibodies present in the serum of actively or passively immunized pregnant guinea pigs were both transmitted to the young in significant amounts. Thus, γ_1- and γA-immunoglobulins — although similar in many respects — display fundamental differences among species.

To summarize, placental transfer of antibodies appears to be mediated by specific portions of the molecules. Factors, such as size and shape, appear to play an accessory role. A transfer may occur only if the molecule or one of its fragments is fixed to the membranes in the first place.

The level of antibodies in the milk varies from one species to another. When transmission of antibody occurs mainly *in utero*, the transfer by milk is relatively unimportant. In ruminants, where placental transmission is nonexistent, the transfer by milk and colostrum is vital. The mechanism by which antibodies get into the milk is little understood. Milk contains predominantly γA-immunoglobulins (HANSON 1961, BLANC 1962).

Antibodies from colostrum or milk may be almost completely absorbed from the gut in new-born calves. No previous digestion is required for absorption to take place. One week after birth, non-digested antibodies do not pass the intestinal mucosa in the species examined and they cannot be detected in the circulation (DIXON 1959).

7. Catabolism. The biological half-life of human γG-immunoglobulins is of the order of 20 days (section III. B. 5). The corresponding figures for rabbit γG-immunoglobulins are of the order of 7 to 8 days (KELLER, ISLIKER and AEBI 1957). It has been demonstrated that the structure responsible for the catabolism of γG-immunoglobulins is present in papain fragment Fc. Passive administration of mouse γG-immunoglobulin or fragment Fc, but not Fab-fragments results in an increased turnover of γG-immunoglobulin in mice (FAHEY and ROBINSON 1963). The elimination from the circulation of I^{131}-labelled papain fragment Fc is closely related to the elimination of intact γG-immunoglobulin, whereas pepsin 5 S $F(ab')_2$-fragment and papain Fab-fragments are rapidly catabolized (SPIEGELBERG and WEIGLE 1964).

C. The polypeptide chains of γG-immunoglobulins

1. "Heavy" and "light" chains. The analysis of N-terminal amino acids has led to a complex picture of the structure of γG-immunoglobulins, suggesting marked differences in the number of polypeptide chains present in the γG-immunoglobulins of different species (MCFADDEN and SMITH 1955; PORTER 1959). EDELMAN and POULIK (1961) have shown that the reduction of human γG-immunoglobulins with mercaptoethanol in the presence of 8 M urea leads to a reduction of the sedimentation coefficient from 6 to about 2 S, corresponding to a decrease of molecular weight from 170,000 to 40,000. The subunits separated by electrophoresis on starch gel in 8 M urea at pH 3.5 revealed two groups of components which could be separated on CM-cellulose. One peak contained components with a molecular weight of the order of 20,000 which have been termed "light" (L) chains. The other peak, comprising about 70% of the γG-immunoglobulin, contained chains with molecular weights of the order of 50,000 which were designated as "heavy" (H) chains. They have a low solubility in aqueous solvents and exhibit a strong tendency to aggregate.

EDELMAN and BENACERRAF (1962) prepared antiovalbumin, anti-DNP (dinitrophenol) and anti-arsonate antibodies from guinea pig sera by specific

immunochemical methods. The heavy chains, as revealed by starch gel electrophoresis in 8 M urea at pH 3.5 had a low mobility which was similar or identical for each specific antibody. The light chains were more diffuse, had a higher electrophoretic mobility and displayed differences from one specific antibody to another. It was concluded that the specificity of antibodies was confined to the light chains and that the latter contained the antigen-binding site.

FLEISCHMAN, PAIN and PORTER (1962) have treated rabbit γG-immunoglobulins with mercaptoethanol in concentrations ranging from 0.02 to 0.8 M at pH 8.2. Five disulfide bonds were broken under these conditions. The reduced antibody still precipitated the homologous antigen, but the reaction was slower than with untreated antibody. No change in sedimentation coefficient was observed. However, if the reduced and alkylated globulins were dialyzed against N acetic acid at 4°C and then run through a Sephadex G 75 column, two components were obtained which corresponded to those described by EDELMAN and POULIK (1961). However, the components were soluble at neutral pH and retained some of their biological activity.

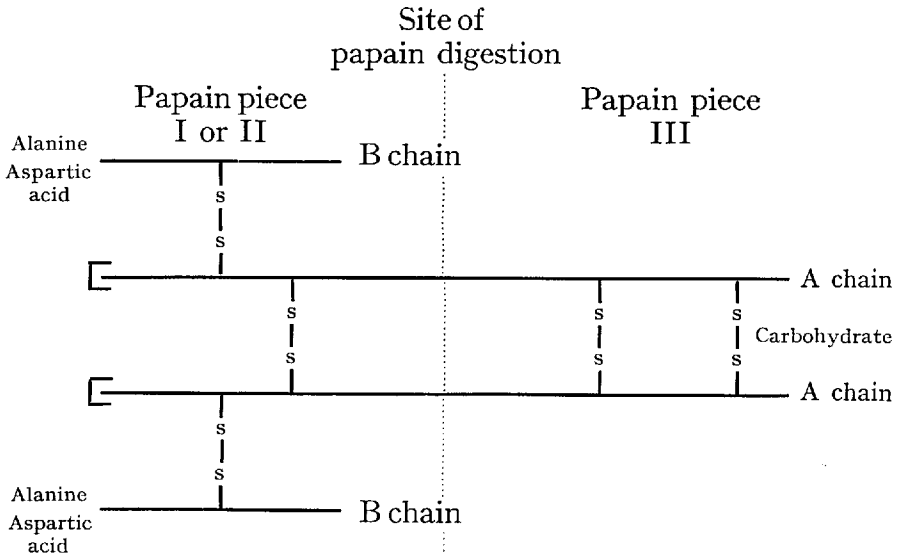

Fig. 1. Diagrammatic structure of rabbit γG-immunoglobulin. From COHEN (1963) and PORTER (1963)

Based on the available data, PORTER's group has postulated a structure of rabbit γG-immunoglobulin consisting of four chains: two heavy chains (A) corresponding to the H chains of human γG-immunoglobulins, each having a molecular weight of 50,000, and two light chains (B or L) with a molecular weight of 20,000 each (PAIN 1963). According to PORTER's scheme, the chains are linked together by 5 disulfide bonds (Fig. 1). The amino acid analysis is given in table 3. N-terminal amino acids are located on the light chains

Table 3. *Amino acid analysis of the heavy and light chains of rabbit γG-immunoglobulin*[1]

	Moles/Mole			
	Heavy chain (H or A)	Light chain (L or B)	2 H + 2 L	γG-Immuno-globulin
	Assumed mol. wt.			
	50000	20000		140000
Lysine	23	7.8	62	63
Histidine	6.5	1.3	16	18
Arginine	16	2.6	37	39
Aspartic acid	33	16	98	106
Threonine	49	24	146	144
Serine	49	19	136	135
Glutamic acid	40	18	116	120
Proline	36	10	92	99
Glycine	33	16	98	98
Alanine	24	13	74	73
Valine	41	18	118	118
Methionine	4.6	0.5	10	12
Isoleucine	15	6.2	42	43
Leucine	30	10	80	84
Tyrosine	17	9.6	53	54
Phenylalanine	14	5.2	38	39
Cystine (half)	9.3	5.5	29	36
S-carboxymethylcysteine	3.7	1.0	9.4	—
Tryptophan	7.8	1.9	20	22

[1] From PORTER (1963). Calculated from the data of CRUMPTON and WILKINSON (1963) and assuming the molecular weights from the horse γG-immunoglobulin peptide chains (PAIN 1963).

(FLEISCHMAN, PORTER and PRESS 1963). They appear to be blocked by acyl groups (PORTER 1963). 95% of the carbohydrates are associated with the heavy chains.

The different abbreviated notations used for the polypeptide chains of various animal species have led the WHO committee on nomenclature (1964) to introduce a more precise terminology:

a) The "*heavy*" (H or A) chains have been shown to determine the distinctive properties of each major class of immunoglobulins (γG, γA, γM). It was proposed to designate the chains by small Greek letters corresponding to the Roman capital letters used for the immunoglobulin classes.

Immunoglobulin class	*Heavy chain*
γG	γ
γA	α
γM	μ

Use of the term γ-chain (rather than another Greek letter) to designate the heavy chain of the γG-immunoglobulin molecule preserves an association with the distinctive properties of this major component within the immunoglobulin system.

b) The *"light"* (L or B) chains are at this time the only subunits known to be common to the three major classes of immunoglobulins. Two types of light chains (Type I and Type II) are recognized as occurring in man (FAHEY 1962). To conform with the use of Greek letters for distinctive polypeptide chains, the following symbols are proposed:

Present usage	*Proposed usage*	
	Abbreviated notation for immunoglobulins	Notation for light chains
Type I	Type K	$\varkappa$
Type II	Type L	λ

c) *Molecular formulae.* The proposed system is similar to that in use for haemoglobin. Some typical formulae would be:

Present usage	*Abbreviated notation for immunoglobulins*	*Molecular formulae*
7 Sγ — type I	γGK	$\gamma_2\varkappa_2$
7 Sγ — type II	γGL	$\gamma_2\lambda_2$
γ_1A — type I	γAK	$\alpha_2\varkappa_2$
γ_1A — type II	γAL	$\alpha_2\lambda_2$
γ_1M — type I	γMK	$(\mu_2\varkappa_2)n$ *
γ_1M — type II	γML	$(\mu_2\lambda_2)n$ *
Urinary globulin composed of light chains (BENCE-JONES) **		$\varkappa_2$ and λ_2 (if in the dimer form)

* n in this case may be 6.
** FRANKLIN (1959); EDELMAN and GALLY (1962).

2. Interrelationships between chains and fragments. FLEISCHMAN, PAIN and PORTER (1962) have shown that goat antisera directed against rabbit Fab-fragment reacted with the heavy as well as with the light chains, whereas fragment Fc contains the residual part of two heavy chains only. Reduction of a papain Fab-fragment released two "pieces", A and B, the former containing the portion of the heavy chain with the blocked N-terminal amino acid, the latter resembling the light chain very closely (FLEISCHMAN, PORTER and PRESS 1963).

Fig. 1 represents a rough scheme without attempting to fit the known dimension of the γG-immunoglobulin molecule. EDELMAN and GALLY (1964) have proposed a cylindric model which corresponds more closely to the physicochemical measurements obtained for human γG-immunoglobulin (Fig. 2). The combining sites have been placed at the two ends and the dimensions correspond to those defined by KABAT (1960) in his studies on antidextran antibodies. The volume of the light chain was based on measurements carried out on Bence-Jones proteins. These are usually composed of two light chains disposed lengthwise which are tied together by one disulfide bond (EDELMAN and GALLY 1964).

The L and H chains are linked together by noncovalent bonds and a single disulfide bond. The former are responsible for the fact that the chains only dissociate if reduction of the latter is followed by the action of denaturing agents. If alkylated L and H chains are mixed under appropriate conditions, 7 S molecules are reconstituted in yields exceeding 30%, with an antigenic structure similar to that of the original γG-immunoglobulin. The chains of the reconstituted molecules are linked by noncovalent interactions since they could be dissociated readily by gel filtration in propionic acid. Random reassociation of rabbit L chains and human H chains, and of human L chains and rabbit H chains gave rise to hybrid molecules. This indicates that the regions between L and H chains and between L-H pairs may be similar in γG-immunoglobulins from different species (EDELMAN and GALLY 1964).

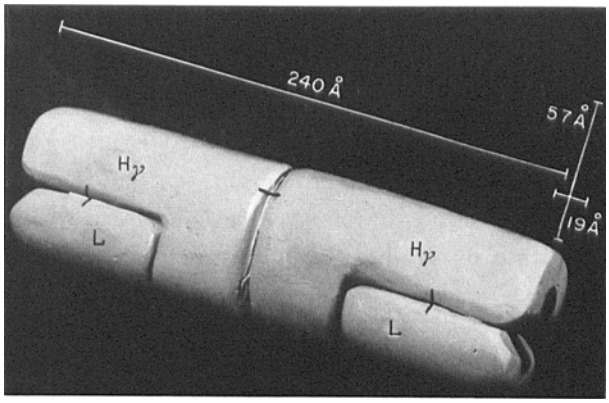

Fig. 2. Model of γG-immunoglobulin molecule according to EDELMAN and GALLY (1964). Hγ: heavy polypeptide chain; L: light polypeptide chain. The single lines drawn on the model represent interchain disulfide bonds

PORTER's model suggested that each H-L pair is linked to the other by 3 disulfide bonds. Recently, PALMER and NISONOFF (1964) presented evidence that two H-L pairs may be obtained by cleavage of one disulfide bond only, if the γG-immunoglobulin is subsequently exposed to a pH of 2.5. The alkylated, identical half-molecules could be reassociated after neutralization to form molecules with the same sedimentation coefficient as the original globulin. Since the sulfhydryl groups had been alkylated after reduction, the subunits of the reaggregated γG-immunoglobulin appear to be linked by noncovalent bonds (NISONOFF and PALMER 1964). By using this technique, it is also possible to form hybrid molecules with active binding sites derived from different antibodies. Antibodies of mixed specificity have been obtained from anti-ovalbumin and normal rabbit γG-immunoglobulins.

Fig. 3, taken from EDELMAN and GALLY (1964), illustrates the four ways in which γG-immunoglobulins may be degraded to form either single chains (I), half-molecules (II), pepsin (III), or papain fragments (IV). The above scheme is oversimplified and does not take into account the heterogeneity of both heavy and light chains. The heterogeneity of heavy chains is apparent from their antigenic determinants, antigen-binding sites and their N-terminal amino acids. Furthermore the heavy chains are associated with different allelic forms of the human Gm allotype (see section II. C. 3).

COHEN and PORTER (1964) analyzed *heavy* chains of normal human γG-immunoglobulins in urea-acrylamide gel and found a broad electrophoretic spread, while myeloma heavy chains were relatively homogeneous.

The *light* chains of all species examined could be resolved into about ten distinct components by electrophoresis in urea-glycine starch-gel. Analysis of the light chains isolated from human pathological globulins revealed only one or two electrophoretic components with mobilities that correspond to one or another of the ten components of normal human light chains. According to COHEN and PORTER (1964) the complexity of human light chains may result from the synthesis of chemically distinct light chains by different cell types.

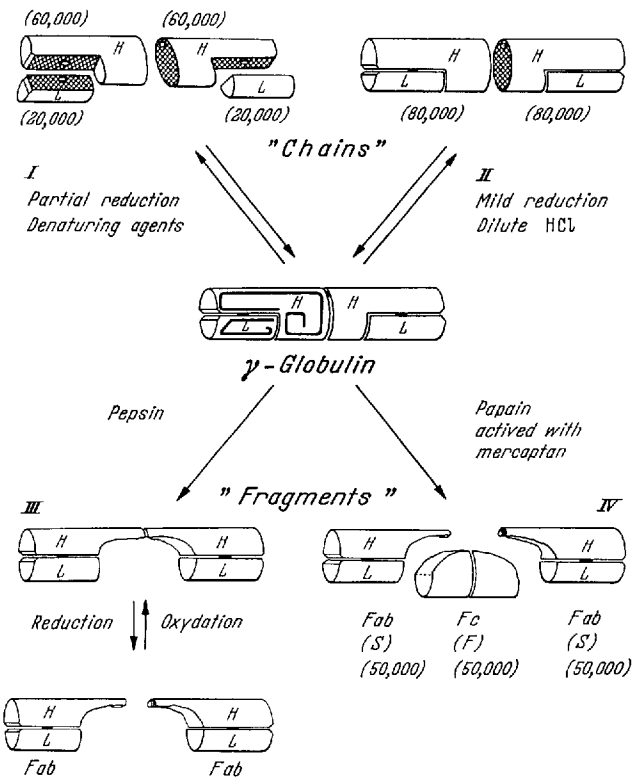

Fig. 3. Schematic diagram illustrating the ways in which the γG-immunoglobulin molecule may be degraded (EDELMAN and GALLY 1964). Numbers in parentheses are approximate molecular weights. Hatched areas indicate regions of noncovalent interchain bonding. Disulfide bonds or half-cystines are indicated in these regions

3. Structural basis for genetic variations. The knowledge of the structure and the interrelationships between chains and fragments has proved useful in elucidating the genetic control of the synthesis of immunoglobulins. The existence of genetic variations in immunoglobulins of different members of the same species is well established and has been reviewed by FUDENBERG (1963). Allotypes have been described in immunoglobulins of the rabbit, guinea pig and mouse. In man, the Gm groups of immunoglobulins are regulated by two distinct loci, probably located on different chromosomes. Gm activity is found only on γG-immunoglobulins while the second locus, the Inv, expresses itself on γG-, γM- and γA-immunoglobulins. This is in accord with the findings of HARBOE, OSTERLAND and KUNKEL (1962) and FRANKLIN, FUDENBERG, MELTZER and STANWORTH (1962) that Gm activity may be found solely on the Fc-fragment and probably on the heavy chains of human γG-immunoglobulins. In contrast, Inv activity resides in the part

of the molecule which is common to the three immunoglobulin classes (light chains). Consequently, Inv activity is always associated with the Fab-fragments. Inv activity may also reside in Bence-Jones proteins belonging to Type K (I), and has so far not been demonstrated in Type L (II).

The suggestion that the different chains and fragments might be under separate genetic control has far-reaching implications (FRANKLIN 1964). Bence-Jones proteins — usually composed of two light chains — are formed in patients synthesizing light chains in excess of heavy chains. It is likely that the light chains appear transiently in the blood before being excreted in the urine. The counterpart to this situation has been found in a patient with large amounts of heavy chains in serum and urine having none of the thermal and antigenic properties of Bence-Jones proteins (FRANKLIN, MELTZER, GUGGENHEIM and LOWENSTEIN 1963). While most normal immunoglobulins are heterozygous for Gm or Inv activity, Bence-Jones proteins, Waldenstrøm-macroglobulins and myeloma proteins are always homozygous (HARBOE, OSTERLAND, MANNIK and KUNKEL 1962).

In a recent review, FRANKLIN (1964) speculates on the kind of mutation in the sex linked recessive congenital form of agammaglobulinemia. In these cases with a virtual absence of each class of immunoglobulins the mutation is likely to have occurred at the Inv locus.

D. Antigen-binding sites on the polypeptide chains

Since antigen-binding is associated with the Fab-fragments (I and II), the specific binding site should be located on the light chain or on the part of the heavy chain contained in these fragments, or on both. One of the difficulties in resolving this problem consists of the loss of activity after dialysis of reduced rabbit antibody against propionic acid. By using equine antibodies, which are more resistent to acidification, FLEISCHMAN, PAIN and PORTER (1962) were able to show the binding site to be associated with the heavy chain. Studying horse antitoxins, FRANEK and NEZLIN (1963) showed the main part of the specific combining site to reside on the heavy chains. However, the presence of light chains was required to restore the full activity of the binding site. EDELMAN, OLINS, GALLY and ZINDER (1963) confirmed that the activity of an antigen-binding site depended on interactions of the heavy and of the light chains: using the chains of two different specific antibodies, activity to a given antigen was recovered upon mixing of homologous heavy chains with heterologous light chains. However, mixing of the homologous light chains with heterologous heavy chains did not restore antibody activity, although 7 S molecules were reconstituted (EDELMAN 1964).

Recently, METZGER, WOFSY and SINGER (1963, 1964) have proposed methods for attaching a chemical label to the active site of antibodies. After subsequent enzymatic degradation of the antibody the peptide regions involved

in the active sites can be isolated and analyzed. In this method of "affinity labelling", the labelling reagent must have the characteristics of a hapten; in other words, it must combine specifically with the antigen-binding site of the antibody. In addition, it must contain a functional group capable of reacting with amino acid residues to form irreversible covalent linkages. By using diazonium derivatives of various haptens, the tyrosine residues of the binding sites of rabbit antibodies were specifically labelled. When the chains were prepared, between 65 to 80% of the label was associated with the heavy chains and 20 to 35% with the light chains of the antibodies (SINGER, WOFSY and GOOD 1964). This elegant method should lead to a precise map of the antigen binding sites. It does not, however, resolve the conflicting data on the contribution of each chain to the binding sites of γG-immunoglobulins.

EDELMAN and BENACERRAF (1962), JAQUET, BLOOM and CEBRA (1964) and ROHOLT, RADZIMSKI and PRESSMAN (1963) suggest that only light chains are involved in the binding site; FLEISCHMAN, PAIN and PORTER (1962), GIVOL and SELA (1964) and KARUSH (1964) implicate heavy chains to determine antibody specificity. FRANEK, KOTINEK, SIMEK and ZIKAN (1964) suggest that both chains contribute to the binding site and more recently ROHOLT, ONOUE and PRESSMAN (1964) claim that both light and heavy chains retain a weak but distinct capacity to bind specific hapten.

In view of FRANEK'S, SINGER'S and EDELMAN'S most recent contributions (1964) the site appears to be shared by both the heavy and light chains. It requires further investigation, however, to determine to which extent specific amino acid sequences of both chains are involved. According to EDELMAN, the heavy chains might have the specific sequence, while the light chains cause the heavy chains to fold into the proper configuration (*"modulated site"*). The light chains might enhance the binding capacity in a relatively nonspecific manner (*"unsymmetrical shared site"* according to SINGER). A third possibility favored by PRESSMAN would be a *"cooperatively shared site"*, in which both types of chains are intrinsically capable of binding the antigen very weakly. Only the combination of the chains results in an "activation", providing the high binding capacity observed in native antibodies (FRANEK, KOTINEK, SIMEK and ZIKAN 1964).

III. The γM-immunoglobulins

A. Structure

The macroglobulins were first identified in the sera of horses which had been immunized against pneumococcal polysaccharides. The sedimentation coefficients of these macroglobulins were of the order of 19 S corresponding to a molecular weight of approximately 1,000,000. They have been called β_2M- or γ_1M-globulins. The WHO committee on nomenclature (1964) has

introduced the term γM-immunoglobulins for these "heavy" immune globulins. γM-immunoglobulins have also been found in rabbits, mice, chicken, pigs and man (FAHEY 1962). Human macroglobulins have been most extensively studied. Their purification has been difficult because of the relatively small quantities present in normal sera (approximately 1 % of the total serum proteins). In Waldenstrøm macroglobulinemia, the purification is easier to carry out. Evidence that the pathological proteins have antigenic determinants that are not present on normal macroglobulins has been presented by HABICH and HÄSSIG (1953). Specific antisera are therefore of great clinical value for the detection of Waldenstrøm macroglobulins. FAHEY (1962) favors the concept that "pathological" proteins are in fact normal proteins formed in a clone of plasma cells present in abnormally large numbers because of mutations in a gene-determining cell proliferation. For the diagnostic identification of "paraproteins" the observations of HABICH and HÄSSIG (1953) retain their full validity.

1. Purification. The classical methods of plasma protein fractionation (cold ethanol method, precipitation by salting out, electrophoresis, etc.) have not proved satisfactory for the isolation of macroglobulins. Until recently, the method of choice was ultracentrifugation at 100,000 g for several hours, during which the pellets were repeatedly resuspended in order to remove small molecular contaminants. Since the latter could not be eliminated completely, density gradient ultracentrifugation has been applied by KUNKEL (1960). This procedure yields a mixture of α_2- and γM-globulins which can be purified by dialysis against buffer of pH 7.1 and low ionic strength (0.002). Under these conditions, the γM-immunoglobulins behave as euglobulins and precipitate (FILITTI-WURMSER and HARTMANN 1962). A disadvantage of this method is the formation of high molecular aggregates with sedimentation coefficients of 30—100 S. More recently, KILLANDER and FLODIN (1962) have introduced gel filtration on Sephadex G 200. The first fractions eluted are practically devoid of low-molecular weight contaminants.

2. Physicochemical properties. The particles with molecular weights of one million are approximately 6 times as large as γG-immunoglobulins. The intrinsic viscosity is 6.7. KOVACS and DAUNE (1961) have calculated the diameter from electron microscopic data to be of the order of 150—250 Å.

DEUTSCH and MORTON (1957) showed that the γM-immunoglobulins may be depolymerized by certain reducing agents such as thiols and sulfites. Their subunits have sedimentation coefficients of the order of 6 to 7 S but are not identical to γG-immunoglobulins of the 7 S type (FRANKLIN and STANWORTH 1961). If the reducing agent is removed, the subunits are capable of reaggregating leading to the formation of heavy components. These, however, have not the same sedimentation characteristics as the original native macroglobulins (13 to 19 S) (DEUTSCH and MORTON 1958). The spontaneous

reaggregation of macroglobulins may be inhibited if iodoacetate or iodoacetamide is added immediately after reduction. The system behaves as if γM-immunoglobulins were composed of 6 subunits (monomers) which are linked together by highly labile disulfide bonds. From the marked effect of concentration on the sedimentation coefficients DEUTSCH and MORTON (1958) concluded the γM-immunoglobulin molecule to be highly asymmetric.

3. Mechanism of the reductive cleavage. ISLIKER (1961) and WILBRANDT (1963) have studied the dissociation of normal and pathological γM-immunoglobulins. Ultracentrifugation and viscosity measurements showed that thiol concentrations of the order of 10^{-2} M were required to achieve a complete cleavage of a 10^{-4} M solution of macroglobulins. There were small variations in the lowest concentration of the different thiols required, mercaptoethylamine and thioglycolate being efficient at lower concentrations than cysteine and glutathione. ISLIKER (1961) showed that the oxidized form of mercaptoethylamine (cystamine) at a concentration of 0.05 M, was also capable of cleaving macroglobulins, provided that trace amounts of the reduced form were present to initiate the process (0.005 M mercaptoethylamine, which by itself has no cleaving effect). These two reagents operate by promoting disulfide interchange reactions leading to the formation of mixed disulfides between protein subunits and thiol. The existence of mixed disulfides was demonstrated by using mercaptoethylamine labelled with S^{35}.

The decrease of viscosity induced by thiol cleavage of macroglobulins has been used for a semiquantitative determination of the latter in pathological sera. The addition of 0.1 M mercaptoethylamine induces an immediate decrease of the viscosity — as measured in an Oswald viscosimeter — which is proportional to the content of pathological macroglobulins in the serum. This procedure renders possible a rapid screening of sera for Waldenstrøm macroglobulins. Positive results must then be confirmed by ultracentrifugation.

4. Structure of the 6 S subunits. The monomers obtained by reduction of human γM-immunoglobulins display physicochemical and immunochemical properties which differ from those of γG-immunoglobulins (DEUTSCH and MORTON 1958; ISLIKER 1961). REISNER and FRANKLIN (1961) have fractionated the subunits into three components by chromatography on DEAE-cellulose. Two components have sedimentation coefficients of the order of 8 S, whereas the third sediments more slowly. The first two fractions differed from each other and from native macroglobulin in electrophoretic behavior. MCDOUGALL and DEUTSCH (1964) have prepared monomers from pathological macroglobulins which had a greater anodic mobility than the native protein. The difference in mobility is probably due to anion-binding by the monomer, but the existence of hidden acid groups in the native protein has not been excluded. Upon exposure to acid the monomer is degraded irreversibly into two components which can be separated by electrophoresis.

The amino acid composition of the monomers varies from that of γG-immunoglobulins. In addition, the monomers contain ten times more hexose than γG-immunoglobulins.

Differences between the two proteins have also been shown by analysis of antigenic determinants. These are due to the distinct properties of their heavy chains, whereas cross reactions are based on the common structure of their light chains. HITZIG and ISLIKER (1959) found more pronounced cross-reactions of γG-immunoglobulins with the macroglobulin monomers than with the native macroglobulin. The first two monomer subfractions obtained by REISNER and FRANKLIN (1961) were antigenically identical and shared antigenic groups with γG-immunoglobulins. The third subfraction could not be identified. HILL (1964) demonstrated unique sequences on horse macroglobulins by gel diffusion techniques. The γM-immunoglobulins produced a precipitin band which coalesced with the one produced by γG-immunoglobulins, as well as an additional band that was unique to the γM-immunoglobulin. Absorption of the anti-γM-immunoglobulin serum with γG-immunoglobulin removed the band of identity but did not affect the other band; hence the unabsorbed antibody was specific for the γM antigenic sites and did not crossreact with the γG-immunoglobulin.

B. Biological activity

1. Distribution of activity between γG- and γM-immunoglobulins. Antibody activities may be found in all three categories of immunoglobulins: γG, γA and γM. The distribution of antibody activities between these globulins may depend on the following factors:

a) the *nature of the antigen:* particulate antigens (bacteria, red cells) produce in a first phase predominantly 19 S antibodies (FAHEY 1962), although small amounts of early γM-antibodies may also be formed against soluble proteins;

b) the *animal species:* fish, turtles, produce predominantly γM-antibodies (GREY 1963);

c) the *age* of the immunized animal: young animals produce larger quantities of γM-antibodies than adult animals;

d) the *quantity of antigen* and the *time of immunization* are the main determinant factors as shown by UHR and FINKELSTEIN (1963) with bacteriophage and by SVEHAG and MANDEL (1964) with poliomyelitis and coxsackie virus: Antigen doses of the order of 10^6 virus particles produce only γM-immunoglobulins with a maximum peak after 3 to 4 days and disappearance after 3 to 4 weeks. If an equivalent antigen dose is injected at this moment, no booster effect is obtained. The antibody response is similar to that obtained after the first injection. Higher doses of antigen (10^{10} virus particles) give rise to the formation of γM-immunoglobulins which start to decline at the

moment when γG-immunoglobulins begin to appear. The γG-antibodies will persist longer than the γM-antibodies.

If the antigen dose is sufficiently high to elicit the formation of γG-antibodies, a later injection of antigens will produce a pronounced booster effect on γG-antibodies. On the other hand, the response of γM-antibodies will be similar after each new injection of antigen.

These facts suggest that the anamnestic response is dependent on the formation of γG-antibodies. While γG-antibodies are produced for a prolonged period of time, the formation of γM-antibodies stops rapidly. It has therefore been claimed that γM-antibodies have no "immunological memory". The treatment of animals with X-rays 20 hours before the injection of an antigen nearly suppresses the formation of γG-antibodies whereas the synthesis of γM-antibodies is only slightly affected under similar conditions.

2. Valency of γM-immunoglobulins. The question arises as to the valency of the γM-immunoglobulins and their subunits. From 6 to 20 combining sites per molecule have been suggested. So far, sufficient amounts of purified antibody of the macroglobulin type have not been obtained to permit a precise determination of immunologic valency. Using isotope techniques, JACOT-GUILLARMOD and ISLIKER (1964) have provided evidence that each subunit has at least one specific combining site. Anti-B isoagglutinins of the 19 S type and their monomer subunits have been labelled with I^{131} and incubated with type B erythrocytes. The fixation was of the same order of magnitude in both cases. Although nonspecific adsorption was shown to occur by using controls with type A erythrocytes or non-immune γM-immunoglobulins, calculations indicated the existence of at least one combining site per monomer subunit. The isoagglutinins adsorbed to the homologous red cells displayed a strong agglutination when treated with anti-globulin sera as opposed to the subunits fixed on the heterologous red cells. Similar results were obtained by displacing labelled native 19 S isoagglutinins and hemolysins by the non-labelled subunits (JACOT-GUILLARMOD 1964).

3. Reversibility of the reductive cleavage. It has been shown in section III. A. 4 that reaggregated subunits of γM-immunoglobulins do not possess all the physicochemical characteristics of the native protein (sedimentation coefficients, electrophoretic mobility, solubility). Until recently, it has been admitted that the biological activity of the γM-immunoglobulins was not reconstituted after reaggregation of their subunits.

JACOT-GUILLARMOD and ISLIKER (1962) have confirmed the results of GRUBB and SWAHN (1958) according to which the biological activity of 19 S isoagglutinins virtually disappeared after reductive cleavage with mercaptans and did not reappear upon reaggregation. On the other hand, if γM-immunoglobulins are treated with sodium borohydride rather than with thiols, biological activity reappears upon oxidative reaggregation. Care must be taken to

adjust borohydride to its optimum concentration (0.007 M to 0.01 M for protein concentrations ranging from 0.05 to 2.0 %) and to keep the pH between 8.4 and 8.8. The extent of the reductive cleavage was controlled by ultracentrifugation and by measurements of the free sulfhydryl groups. The native macroglobulins contained between 1 to 2 sulfhydryl groups per molecule. 45 sulfhydryl groups were released during reduction corresponding to the opening of 22 disulfide bonds. The reaggregated γM-immunoglobulins displayed full biological activity, indicating that the latter is not dependent on the full integrity of the native macroglobulin.

McDOUGALL and DEUTSCH (1964) have estimated the number of half-cystine residues from amino acid analysis. Eight out of 27 half-cystine residues in each subunit are reduced on production of the monomer from the macroglobulin. Since γM-immunoglobulins contain 6 subunits, reductive cleavage would yield 48 sulfhydryl groups, a number which is in fair agreement with the data of JACOT-GUILLARMOD and ISLIKER (1962). The released sulfhydryl groups could be due either to reduction of inter- or intra-subunit disulfide bonds. Since there are no methods available for differentiating these two types of bonds, it is not possible to conclude how many of the disulfide bonds are concerned in linking the subunits together.

4. Site of formation. The marked differences in the behavior of γG- and γM-immunoglobulins suggest that they are formed by different cells or at least by distinct biochemical processes. γG-immunoglobulins have been shown to be synthesized in plasma cells. However, new-born children may manufacture γM-immunoglobulins in the absence of plasma cells and cases of agammaglobulinemia have been described with a decreased number of plasma cells in the presence of an increased level of γM-immunoglobulins. GITLIN, GROSS and JANEWAY (1959) described a patient who had immunologically competent cells but who was lacking plasma cells as well as γG-immunoglobulins. Immunofluorescence revealed the red pulp of the spleen to contain γM-immunoglobulins. After splenectomy, the production of macroglobulins came to a full stop.

5. Turnover. The absolute turnover (synthetic) rate of γM-immunoglobulins in human adults varies from 0.2 to 0.7 g per 24 h corresponding to a mean value of approximately 0.4 g per day. FAHEY, BARTH, WOCHNER and WALDMANN (1964) showed that these values varied with the serum γM-immunoglobulin levels which are of the order of 100 mg % in normal patients. Whereas γG-immunoglobulins were found to be evenly distributed between intravascular and extravascular compartments, the γM-immunoglobulins remained predominantly in the intravascular, with only 20 % in the extravascular compartment. The total amount of macroglobulins in human adults has been estimated to be about 8 g. 15 % of this quantity is transferred each day from the intravascular to the extravascular compartment, a figure which is similar

to the transfer of fibrinogen, but much smaller than the transfer-rates of γG-immunoglobulin and albumin.

In addition to its dependence on turnover (synthetic) rate, the distribution of serum γM-immunoglobulins depends on their degradation rates. The site of degradation is in the intravascular compartment or in some site which is in rapid distribution equilibrium with plasma. The biological half-life of γM-immunoglobulins in normal adults is of the order of 5 days. Approximately 20% of the circulating macroglobulins are catabolized per day. This rapid turnover is in marked opposition to the turnover of γG-immunoglobulins, which has a half-life of some 20 days.

The low γM-immunoglobulin levels in agammaglobulinemia and multiple myeloma are due essentially to a deficient γM-immunoglobulin synthesis. In protein-losing gastroenteropathy the low γM-immunoglobulin levels are evidently due to an increased catabolism. No compensatory increase in γM-immunoglobulin biosynthesis has been shown to occur as a result of protein-loss.

The turnover studies done by COHEN and FREEMAN (1960), OLESEN (1963) and FAHEY, BARTH, WOCHNER and WALDMANN (1964) are difficult to carry out because of the tendency of iodinated macroglobulins to aggregate, causing diminished transfer-rates between compartments and increased clearance through the reticulo-endothelial system.

6. Properdin. Few proteins have caused so much controversy as the macroglobulin described by PILLEMER, BLUM, LEPOW, ROSS, TODD and WARDLAW (1954) under the name properdin. Many authors thought properdin to be a new entity, different from classical antibodies (ISLIKER 1958). They based their statement on the fact that properdin exhibited a pronounced bactericidal or bacteriolytic effect on a large variety of *gram*-negative bacteria. Properdin was thought to require cofactors resembling complement in order to react with these bacteria. In addition, properdin was found to be heat-labile, as opposed to the well-established heat-stability of classical antibodies. Further arguments which favored the concept that properdin represents a separate entity are its turnover, which is twice that of γG-type antibodies (KELLER, ISLIKER and AEBI 1957), and the finding that properdin is present in cases of agammaglobulinemia (BARANDUN and ISLIKER 1956).

ROWLEY (1957) and NELSON (1958) were among the first to challenge PILLEMER's concept. Properdin interacts with zymosan, an insoluble polysaccharide preparation of yeast membranes. This reaction requires the presence of magnesium ions and enables the complex to fix the third component of complement. NELSON (1958) showed that a "properdin-zymosan" complex contained in addition to "properdin" certain serum proteins which had reacted in the presence of calcium and magnesium ions, namely the first, fourth and second component of complement. Only when these components are fixed

is the complex capable of inactivating the third component of complement. Thus, properdin does not differ from classical antibodies with respect to its interaction with the complement system.

ROWLEY (1957) has opposed the widely accepted concept that natural immunity is mainly due to the properdin level. Injection of a few μg of lipopolysaccharides to animals increases the properdin level and protects the treated animals against the lethal effect of otherwise highly virulent strains (LANDY and PILLEMER 1956). However, no direct causal relationship could be established between properdin level and the changes of resistance induced by lipopolysaccharides (reviewed by ISLIKER 1959). Injection of lipopolysaccharides has also a stimulating effect on serum lysozyme, phosphatases, endotoxin detoxifying factor and the reticulo-endothelial system.

The concept that properdin does not represent an entity different from antibodies has been confirmed by recent observations on γM-immunoglobulins. ROWLEY and TURNER (1964) have observed a rapid increase of γM-immunoglobulins after injection of lipopolysaccharides to pigs. This increase was accompanied by a concomitant enhancement of opsonic activity towards certain salmonellas. The γM-immunoglobulins were found to be indistinguishable from the "natural" antibodies present in normal pig serum. The macroglobulin concentration and the opsonic activity were shown to increase for several days but returned to normal by the 15th day after injection of lipopolysaccharides.

The biological half-life of these γM-immunoglobulins is similar to that determined for properdin by KELLER, ISLIKER and AEBI (1957). These findings are compatible with the assumption that properdin represents a mixture of a large number of crossreacting γM-antibodies. The fact that properdin may be found in patients with agammaglobulinemia (BARANDUN and ISLIKER 1956) requires further investigation. Properdin represents less than 0.05 % of the total serum proteins. The absence of antibodies in agammaglobulinemia has rarely been shown to be complete, and it appears that the biological properdin test is so sensitive that even minute quantities may be detected. A thorough investigation of the "properdin" level in relation to the degree of antibody deficiency is required. The low serum properdin level, as compared to the macroglobulin levels, is due to the fact that all γM-immunoglobulins do not necessarily react with zymosan. Properdin activity may be due to a relatively limited number of γM-immunoglobulins of low specificity giving cross reactions between zymosan and *gram*-negative bacteria.

RYTEL (1959) and ISLIKER (1961) have proposed an alternative hypothesis to account for the large variety of systems which are susceptible to properdin. They have shown that properdin and γM-immunoglobulins, such as 19 S isoagglutinins may be reversibly cleaved by sodium borohydride. JACOT-GUILLARMOD and ISLIKER (1962) obtained a practically complete restoration

of biological activity upon oxidative reaggregation. Starting with a mixture of isoagglutinins anti-A and anti-B, it has been possible to reaggregate the 7 S subunits and to recover a fraction of antibodies with double specificity, bearing on the same molecule anti-A as well as anti-B activity. These "mixed" antibodies were brought into evidence by absorption and elution methods and also by mixed agglutination between type A buccal epithelial cells and type B erythrocytes. It is possible that properdin may be a "mixed" antibody derived from the 7 S subunits of 19 S bacterial and viral antibodies. This hypothesis assumes the existence of an enzymatic system capable of splitting and synthesizing disulfide bonds *in vivo*. MICHELI, JACOT-GUILLARMOD and ISLIKER (1963) have studied such systems in erythrocytes, which are capable of cleaving the highly labile disulfide bonds of γM-immunoglobulins. The possibility that this reducing system is identical with glutathione-reductase is open to question. The latter enzyme may act by regenerating the reduced form of oxidized glutathione so as to maintain a sulfhydryl concentration sufficient to cleave macroglobulins. The lowest thiol concentrations required for reductive cleavage are of the same order of magnitude as those maintained by glutathione-reductase.

As a whole, the controversy on properdin has been beneficial to the study of natural immunity. All questions are not yet settled. Recently LEPOW (1964) has presented evidence that properdin was a 5 S globulin, whereas ROTHSTEIN (1962) found properdin activity in a fraction obtained by density gradient ultracentrifugation, corresponding to a sedimentation coefficient of 11 S. One of the critical aspects of this work is the determination of properdin which is highly cumbersome and for which too many modifications have been proposed (see ISLIKER and LINDER 1958).

IV. Therapeutic applications of immunoglobulin subunits

1. Animal immunoglobulins. Repeated passive immunizations of patients with animal sera has the disadvantage of causing anaphylactic reactions. Proteolytic breakdown of antibodies to 3.5 S fragments does not significantly decrease immunogenicity. Although the antigen-binding properties are maintained, the complement-fixing properties are destroyed and bacteriolysis and phagocytosis will be impaired (see section II. B. 2).

The neutralization of viruses and toxins by fragments of immunoglobulins is difficult to evaluate. In the case of adenovirus, KJELLÉN (1964) has shown that cleavage of antibodies to univalent Fab-fragments brought about a loss of their capacity to neutralize virus infectivity. However, the fragments had retained their full ability to inhibit hemagglutination. Unfortunately, no experiments have been carried out with 5 S $F(ab')_2$-fragments.

The neutralization of diphtheria and tetanus toxins by the corresponding T-components of horse antitoxins and their fragments has been studied by

SCHULTZE, HAUPT, HEIDE, HEIMBURGER and SCHWICK (1964). In these cases F(ab)-fragments were still effective as toxin-neutralizing antibodies, but displayed a lower specific activity. On the other hand, 5 S $F(ab')_2$-fragments showed a higher antibody titre than the native T-component. In the case of Fab-fragments directed against *Cl. welchii* toxin PARKE and AVIS (1964) made similar observations. They found that reaction with antiglobulin sera increased the antitoxic activity of the Fab-fragments.

2. Human immunoglobulins. The administration of human immunoglobulins to patients with antibody deficiencies and recurring infection has received widespread attention. There are numerous reasons why the administration of human immunoglobulin by the intravenous route is to be preferred to the intramuscular application which is widely practised today: the immunoglobulin acts more rapidly; there is no loss due to local proteolysis; the greater efficiency of an intravenous administration reduces the cost of treatment; finally, it causes no painful local irritation if large quantities are given. As a routine method, however, intravenous administration of immunoglobulin is still not free of risk: untoward reactions may occur with intravenous use of standard γG-immunoglobulin. This is due to at least one endogenous and an exogenous factor. The "endogenous" factor is revealed by the finding that certain subjects only — especially patients with an antibody deficiency syndrome — react to intravenous infusions of immunoglobulins administered under standard conditions. This "endogenous" factor can be temporarily eliminated by "desensitizing" the patients (BARANDUN, KISTLER, JEUNET and ISLIKER 1962).

The "exogenous" factor resides in the immunoglobulin preparation itself and is related to its anticomplementary activity (ISLIKER, JACOT-GUILLARMOD and THOENI 1962). As outlined in section II. B. 2, anticomplementary activity is due to the presence of aggregated γ-globulins (30—100 S).

Since the removal of these aggregates by ultracentrifugation is not practicable on a large scale, peptic digestion has been used to remove anticomplementary activity. SCHULTZE and SCHWICK (1962) have developed a preparation ("Gammavenin, Behringwerke") containing about 80 % of a 5.1 S, 15 % of a 2.4 S and 5 % of a 7 S component. The main disadvantage of this preparation for intravenous use consists in the fact that fragments are rapidly eliminated through the kidney. ISLIKER, JACOT-GUILLARMOD and THOENI (1962) have therefore studied procedures which would modify human γG-immunoglobulin without degradation. The ratio of enzyme to globulin was first changed from 1/100 to 1/1,000, 1/10,000, and 1/100,000. It was found that the highest pepsin dilution would still decrease anticomplementary activity to negligible values. More important was the finding that the control tubes in which γG-immunoglobulin had been incubated at pH 4 for 17 hours at 37° C without addition of pepsin were deprived of anticomplementary activity. A decrease of

anticomplementary activity was also observed at pH values between 4.5 and 5.5, but a pH as low as 4 and an incubation time of 6 hours at 37° C was required to deprive immunoglobulins entirely of their complement-fixing properties. Below pH 3.8 a complete removal of anticomplementary properties was obtained within 3 hours. However, upon readjusting the pH from values below pH 3.8 to 7 distinct opalescence was observed and a slight precipitate was formed after standing for several weeks. It must be emphasized that pH 4 treatment does not affect the molecular weight of immunoglobulins. The pH 4 treated γG-immunoglobulin may be administered safely intravenously. However, it is imperative to test each new lot for anticomplementary activity before it is released for clinical use.

In a further attempt to reduce anticomplementary activity of human immunoglobulins, reducing agents have also been employed by BARANDUN, KISTLER, JEUNET and ISLIKER (1962). Although treatment with mercaptoethylamine and alkylating agents removes anticomplementary activity, the stability of the immunoglobulin preparation was not sufficient to fulfill the requirements of a safe preparation. Addition of iodoacetamide to prevent reaggregation would have toxic effects.

3. Therapeutic approaches with drugs coupled to immunoglobulin fragments. The untoward side-effects of many drugs are due to their toxic action on normal tissues. If it were possible to concentrate drugs electively on bacteria or other harmful antigens, toxic effects on the host could be greatly diminished. With this in mind, JATON, SCHOLER and ISLIKER (1964) have studied the coupling of drugs to antibodies, or fragments thereof, directed against various microorganisms. Such conjugates – when injected into infected animals – should direct the drug to the infecting microorganisms. Model experiments with antihistoplasma antibodies coupled to sulfonamides have shown that the drug will only be active if it is released from the antibody. For this reason the bonds chosen between drug and antibody must be susceptible to cleavage by enzymes present in the host organism (ISLIKER, CEROTTINI, JATON and MAGNENAT 1964).

The same authors have conducted studies with tumor-specific antibodies linked to cytostatic agents. In all these cases it is important to control the *in vitro* and *in vivo* fixation of antibodies labelled with I^{131} or fluorescein. Nonspecific fixation is greatly diminished if antibodies are digested with proteolytic enzymes. The fixation of Fab-fragments (I and II) derived from normal human γG-immunoglobulins to human liver tissue is negligible, whereas the Fc-fragments display a fair amount of nonspecific fixation (ISLIKER, CEROTTINI, JATON and MAGNENAT 1964). Conversely, the Fab-fragments of anti-sheep red cell antibodies are specifically fixed on the homologous antigen, whereas the Fc-fragment is fixed to the same extent as an Fc-fragment derived from nonspecific γG-immunoglobulins (JATON and ISLIKER 1962).

CEROTTINI, JATON, FROIDEVAUX and ISLIKER (1964) have studied the tissue-binding properties of fragments derived from anti-histoplasma capsulatum antibodies by indirect immuno-fluorescence. The advantages of using Fab-fragments, rather than native antibodies, have been discussed. No therapeutic results have yet been obtained with drugs coupled to antibody fragments.

Antibody fragments have also been conjugated to ferritin for the localization of antigens by electron-microscopic techniques. In an attempt to demonstrate viral constituents in tumors, ISLIKER, LE MAIRE and MORGAN (1964) have coupled ferritin to antibodies against SV 40, Rous sarcoma and vaccinia virus. The best tagging was obtained with ferritin conjugated to Fab-fragment II. The high iron content of these conjugates may be related to their relatively low protein content or to the formation of complexes containing two ferritin particles per antibody-fragment. The occurrence of closely associated ferritin particles ("dublets") is more frequent in the electron-micrographs obtained with the antibody-fragment conjugate. Besides the method of SINGER (SINGER and SCHICK 1961) ferritin has been coupled to Fab'-pepsin fragments with divalent organic mercurials (ISLIKER, LE MAIRE and MORGAN 1964).

4. Desensitization. The tendency to form skin-sensitizing antibodies in man appears to be genetically determined. It is open to question why certain antibodies become attached to tissues in such a way that interaction with antigen elicits anaphylactic or allergic phenomena, whereas other kinds of antibodies are inactive in this respect. The Fc-fragment of immunoglobulins appears to play a major part and much effort has been made to prevent tissue fixation of anaphylactic or skin-sensitizing antibodies. The uptake of such antibodies by tissues always takes place in competition with normal host immunoglobulins.

HALPERN and FRICK (1962) have shown that passive sensitization of guinea pigs by rabbit antibody can be prevented by administration of 100 times as much normal rabbit γG-immunoglobulins. The sensitization by rabbit antibody can be inhibited by immunoglobulins from various species in the following order of effectiveness: rabbit > man > dog > guinea pig > rat > horse > cattle > pig > chicken > goat (BINAGHI, LIACOPOULOS, HALPERN and LIACOPOULOS-BRIOT 1962). Normal globulins are effective when given between 18 hours before and one hour after the sensitizing antibody. Displacement of sensitizing antibodies with normal nonspecific immunoglobulins is difficult to visualize. There are different ways to increase the stickiness of immunoglobulins for tissues. Table 3 shows the tissue-uptake of γG-immunoglobulins treated at pH 4 and aggregated at 63° C. Incubation at pH 4 will destroy the anticomplementary activity of the aggregated immunoglobulins, whereas its tissue-binding properties are increased or remain unchanged (ISLIKER and THOENI 1962; BARANDUN, KISTLER, JEUNET and ISLIKER 1962). These observations

provide further evidence that the sites responsible for tissue affinity and complement fixation are not identical (see section II. B. 3).

ISHIZAKA, ISHIZAKA and HATHORN (1964) have been able to block PRAUSNITZ-KUESTNER sensitization by administration of the heavy chains of human γA-immunoglobulins. The possibilities of using other subunits, Fc-fragments or aggregated Fc-fragments incubated at pH 4 should be actively pursued.

5. Reduction of γM-immunoglobulins. The reductive cleavage of γM-immunoglobulins by mercaptans has received wide attention because of its use in rapidly distinguishing 19 S from 7 S antibodies (see section III. A. 3). The disappearance of antibody activity after treatment with mercaptoethanol is believed to be a sufficient criterion for the presence of 19 S antibody in the original material. Care must be taken with the addition of the mercaptan since high concentrations may also modify the reactivity of 7 S-antibodies. Besides, the complement-fixing ability of γG-immunoglobulins may be destroyed by 0.1 M mercaptans (ISLIKER, JACOT-GUILLARMOD and THOENI 1962; WIEDERMANN, MIESCHER and FRANKLIN 1963). Thiols may also inactivate γA-immunoglobulins, especially skin-sensitizing antibodies in human sera (LEDDY, FREEMAN, LUZ and TODD 1962). This observation may have considerable importance since it is conceivable that thiols may be administered to allergic patients refractory to other treatment.

Considerable attention has been paid to the possibility of reducing macroglobulins *in vivo*. The high viscosity of the blood in patients with macroglobulinemia or with cryoglobulinemia may have deleterious effects on the circulation. For this reason, KAPPELER and ISLIKER (1959) have administered 2 ml of a 10% solution of mercaptoethylamine ("Becaptan" LABAZ) to patients with Waldenstrøm macroglobulinemia (ISLIKER 1961). The cleavage has been far from complete and caused no major regression of macroglobulins. On the other hand, RITZMANN, COLEMAN and LEWIN (1960) have treated patients with cryoglobulinemia with a daily dose of 1 g of penicillamine during 20 days with considerable success. The possibilities of cleaving and inactivating cold agglutinins and autoimmune antibodies of the γM-type have been studied by different groups (HINZ 1958). Although thiols exhibit little toxicity and could be employed in relatively large amounts, no significantly positive results have yet been obtained. This may be due to a rapid reoxidation of subunits reconstituting disulfide bonds, and to the high turnover synthetic rates of the macroglobulins.

V. Conclusions and summary

1. There are three major groups of immunoglobulins endowed with antibody activity: the γG-, γA- and γM-immunoglobulins. Each of these classes of antibody molecules may be cleaved by limited proteolysis to give "*fragments*" or by reduction in the presence of denaturing agents to yield "*chains*".

2. γG-immunoglobulins possess various biological activities which can be localized on 2 groups of fragments obtained by papain digestion:

The *antigen-binding* properties are located on the Fab-fragments (I and II). Two such fragments linked to each other are responsible for divalence of the antibody molecule, for precipitation, agglutination or immobilization of antigens. If divalent antibodies or fragments are cleaved to give univalent fragments, they lose the above properties, but are still capable of specifically combining with antigen. The specific binding site comprises 10 to 20 amino acid residues and represents only a small portion of the univalent fragments.

The *complement-fixing* and skin-reactive properties of antibodies are located on a third fragment (Fc or III) which lies between the antigen-binding fragments. In order to fix complement, at least two Fc-fragments of antibody molecules must be brought into apposition by aggregation with antigen or by chemical or physical procedures. The exact nature of the modifications of the tertiary structure in the Fc-fragment which bring about complement fixation are not yet established.

Skin-reactive properties are mediated also by Fc-fragments but probably by a different site. Evidence is accumulating, suggesting that complement-fixing and skin-reactive properties are located on Fc-fragments of different molecules which in some species can be separated by physicochemical methods. As a rule, the slow γ_2-immunoglobulins mediate complement-fixing, the fast γ_1-immunoglobulins skin- and tissue-reactive properties. Certain γA-antibodies with skin-sensitizing activity (human reagins) may be fixed to tissue in such a way that interaction with antigen elicits anaphylactic and allergic phenomena. Normal human γG-immunoglobulins — although less reactive with tissues — may exert a protective effect if administered in large quantities.

3. If the cleavage of immunoglobulins is effected by reduction of disulfide bonds in the presence of denaturing agents the subunits are referred to as *chains*:

The *heavy* chains (in the γG-immunoglobulins 2 chains with molecular weights of the order of 50,000) determine the distinctive properties of each major class of immunoglobulins.

The *light* chains (in the γG-immunoglobulins 2 chains with molecular weights of the order of 20,000) are common to all immunoglobulins. In man two forms of light chains have been recognized (type K or I, and type L or II).

The isolated chains display little or no activity towards the homologous antigen. Mixing of the two types of chains restores the original molecule without formation of covalent bonds. This suggests that noncovalent bonds are as important as disulfide bonds to maintain the native structure of the γG-immunoglobulin; that the heavy, as well as the light chains contribute to the specificity of the antigen-binding sites; and that the *in vivo* synthesis

of γG-immunoglobulins may proceed by self-assembly, without additional special mechanisms.

4. The γM-immunoglobulins (molecular weights of about 1,000,000) may be dissociated by reducing agents to form six 7 S subunits, each containing heavy as well as light chains. The 7 S subunits differ from γG-immunoglobulins in their amino acid composition and by their higher carbohydrate content.

The subunits derived from γM-immunoglobulins do not precipitate nor agglutinate the homologous antigen, nor do they fix complement. This finding is of importance for a rapid distinction from γG-immunoglobulins which retain most of their precipitating and agglutinating activity after treatment with mild reducing agents. However, the γM-immunoglobulin subunits are able to fix specifically to homologous antigen and must therefore contain at least one antigen-binding site. Upon oxidative reaggregation the 7 S subunits may reconstitute active macroglobulins, an observation which was applied for the preparation of antibodies of "mixed" specificity.

The cells synthesizing γM- were believed to be different from those producing γG-immunoglobulins. They are still lymphoid cells but they may be derived from different clones or may be cells at different stages of development. The turnover (synthetic) rate and the catabolism of γM- are also greater than that of γG-immunoglobulins. On the other hand, the transfer rates of γM-immunoglobulins between different compartments of the body are relatively low. Cells forming γM-immunoglobulins have no immunological memory.

5. "*Properdin*" appears to be a mixture of γM-immunoglobulins. The recent data on the rapid turnover and the special anamnestic response of γM-immunoglobulins indicate why "properdin" was considered to be a separate entity. It is conceivable that "properdin" contains γM-immunoglobulins of "mixed" specificity formed *in vivo* by aggregation of 7 S subunits of different specificity. Such a conception would account for the broad range of specificity of what has been called "properdin".

6. *Aggregated immunoglobulins.* Certain authors postulate that normal sera contain 30—100 S γ-macroglobulins. It is probable that some of these components are formed from γG-immunoglobulins by polymerization during fractionation or ultracentrifugation of serum. These aggregated immunoglobulins have anticomplementary activity comparable to that of antigen-antibody complexes. They are responsible for the untoward reactions observed after intravenous administration of human immunoglobulins to patients with agammaglobulinemia. Similar aggregates may also be formed *in vivo* under pathological conditions and thus produce harmful effects. More research should be conducted to elucidate the different pathways used by nature to polymerize and depolymerize immunoglobulins.

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