STUDIES ON THE MECHANISM OF COMPLEMENT ACTIVATION BY MURINE IMMUNOGLOBULIN G

Thesis by

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ABSTRACT

UPC-10 myeloma protein, a murine immunoglobulin of the IgG class, and its carbohydrate antigen, levan, have been used to define the nature of classical complement activation by IgG. A four step purification procedure was developed for the myeloma protein to minimize albumin contamination and to insure the structural integrity of the purified antibody. In addition, methods were developed for the isolation of levan from perennial ryegrass and Aerobacter levanicum; a novel technique employing water-soluble sulfonated polystyrene was used to prepare levan oligosaccharides which were then fractionated according to molecular weight. Association constants were determined for the binding of various levan preparations to UPC-10 myeloma protein, thereby allowing an initial understanding of the antigen-antibody interactions observed in this system.

Further experiments explored the role of antigen-antibody complexes in classical complement activation. An extremely sensitve assay of hemolytic complement activity employing radiolabeled erythrocytes was developed to allow the use of limited quantities of antigen in these studies. Mixtures of antibody and bacterial levan were found to fix complement only in strictly defined concentration ratios, while no complement fixation was observed when conditions of antigen-excess were present, thereby establishing the aggregation of antibody molecules as a necessary, if not sufficient, step for this activity. A more detailed analysis with sized levan oligosaccharides confirmed this requirement for aggregation and indicated that the molecular weight of the levan oligosaccharides is an important determinant of its ability to assemble antibody molecules into active complexes. Direct observations of such

antigen-antibody complexes were made through the use of analytical ultracentrifugation, establishing that all of the levan oligosaccharides capable of inducing complement fixation by IgG molecules were also able to assemble the antibody molecules into multimeric antibody complexes. Thus, these studies find no evidence for a purely conformational mechanism for the expression of complement activation by murine IgG, and instead confirm that aggregation of antibody molecules is required for this effector function.

ABSTRACTS OF PROPOSITIONS

Proposition I:

Both *in vitro* and *in vivo* experiments employing UPC-10 myeloma protein and bacterial levan are proposed to analyze the clearance of immune complexes from the circulation.

Proposition II:

A reexamination of the role of the complement peptide C3a in the development of increased vascular permeability during an inflammatory process is proposed with experiments focused on possible cellular interactions.

Proposition III:

Tyrosine phosphorylation has been found to occur in a membrane receptor system and certain RNA tumor viruses. Experiments utilizing a peptide sequence known to undergo tyrosine phosphorylation *in vitro* are proposed to identify other cellular tyrosine kinases and to investigate the possible significance of tyrosine phosphorylation in the transformation process.

Proposition IV:

The destructive changes which occur at the neuromuscular junction in myasthenia gravis have been shown to result from the production of anti-acetylcholine receptor antibodies. Experiments are proposed to investigate the reversal of this degenerative process using antibody fragments.

Proposition V:

Experiments are proposed to investigate the role of the tra J gene product in the regulation of the transfer operon for conjugation in E. coli.

ABBREVIATIONS

activated erythrocytes	EA
amino acids:	
arginine	Arg
asparagine	Asn
glutamine	Gln
lysine	Lys
tryptophan	Trp
tyrosine	Tyr
bovine serum albumin	BSA
buffers:	
EDTA-GVB	gelatin veronal buffer containing EDTA
GVB^{2+}	gelatin veronal buffer containing Ca^{2+} and Mg^{2+}
PBS	phosphate-buffered saline
dinitrophenyl	DNP
EDTA	ethylene-diamine-tetra-acetic acid
SDS	sodium dodecyl sulfate

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Chapter I: Introduction

Structure of Antibody Molecules

The immune system of vertebrates possesses the distinctive property of being able to synthesize humoral antibodies which combine specifically with foreign antigens (Kubo et al., 1973). Starting from rudimentary immunoglobulin-like molecules found in the earliest vertebrates, a rich variety of soluble proteins have subsequently evolved, which together potentiate a broad range of immunologic activities in man.

The basic structural element of an immunoglobulin molecule consists of a dimer of two polypeptide chains, designated "heavy" and "light". Each heavy chain is associated with a light chain through covalent and/or non-covalent interactions; two of these heavy-light pairs are covalently linked by one or more interheavy chain disulfide bonds to form a dimer consisting of 4 polypeptide chains, this molecular structure being the common element for all immunoglobulins (Fleischman et al., 1963; reviewed in Cathou and Dorrington 1974; Nisonoff et al., 1975).

Heavy chains can be differentiated into several classes based upon primary sequence, biologic activity, and immunochemical variations. The isolated heavy chains are designated α, γ, δ, ε and μ, and can be assembled into the basic immunoglobulin structure to form the classes IgA, IgG, IgD, IgE, and IgM, respectively. Depending upon the class, antibody molecules occur either as a monomer of the basic 4-chain structure or as a polymer of this form. For example, serum IgM occurs as a pentamer of 4-chain subunits linked by disulfide bonds in conjunction with an additional short chain, the J chain. Serum IgA in man consists of approximately 80% 4-chain monomers, with the remainder

as dimers, trimers, and higher oligomers of this 4-chain structure. IgA is also found in external secretions as secretory IgA, consisting primarily of two 4-chain units of IgA linked together in a complex with one J chain and an additional chain, the secretory component. Thus, variations in heavy chain class can translate into alterations of immunoglobulin architecture.

Heavy chains within a given class can be further broken down into subclasses which arise as characteristic variations in the primary sequence of these chains. Human IgG has been divided into 4 subclasses, with each class (IgG1, IgG2, IgG3, and IgG4) having a characteristic serum concentration (Nisonoff et al., 1975). Differences in heavy chain subclass confer upon the antibody molecule specific biologic properties. In this way, human IgG1, IgG2, and IgG3 can activate complement while IgG4 cannot, whereas only IgG1 and IgG3 have the ability to attach to monocytes (Nisonoff et al., 1975). Thus, the dependence of biologic activity upon heavy chain subclass allows the immune system great selectivity in the types of responses possible. Indeed, it has been shown that specific antigenic determinants are able to elicit an antibody response that is preferential not only for class, but also for subclass as well (Furuichi and Koyama, 1975).

Light chains also fall into one of two antigenically distinct classes, κ and λ (Korngold and Lipair, 1956). The ratio of κ:λ chains varies from species to species, and from heavy chain subclass to subclass. Each chain has characteristic portions of primary sequence, while the degree of homology between them is about 40%, suggesting a common ancestral precursor (Nisonoff et al., 1975).

All antibody classes contain carbohydrate which is linked to the heavy chain (Turner, 1977). Although different sugar sequences have been found associated with a number of IgG molecules, there is at this time no evidence that would link oligosaccharide structure to antibody subclass.

The analysis of amino acid sequences of light and heavy chains has yielded a striking pattern. Between different light chains, the N-terminal halves (approximately 110 residues) vary considerably in sequence, while the C-terminal halves (again, approximately 110 residues) are extremely similar, thus giving rise to the terminology of variable (V_L) and constant (C_L) regions. Similar results were obtained by comparison of heavy chains of the same class from different antibody molecules. A variable region (V_H) was identified at each N-terminus consisting of approximately 110 residues, while the remainders of the chains (C_H) were found to be markedly similar.

In addition, it is possible to divide the antibody molecule into a series of homologous units which bear considerable structural similarity to one another. The variable regions of heavy and light chains and the constant region of light chains each constitute such a unit, which has the general characteristics of being approximately 110 amino acid residues in length and having a centrally positioned disulfide bond. The constant region of heavy chains can likewise be subdivided into a series of similar segments. A γ chain, for example, consists of 3 constant regions (C_H1, C_H2, and C_H3), all of which display considerable sequence homology. This homology suggests that the constant portion of the heavy chain arose by duplication and divergence

of a single ancestral gene that coded for a protein of approximately 110 residues (Edelman, 1970) (Fig. 1).

In a classic experiment, Porter treated rabbit IgG with papain to produce two types of fragments: one which retained the ability to bind but could no longer crosslink antigen, termed the F_{ab} fragment, and another which crystallized at low temperatures, termed the F_c fragment (Porter, 1959). It is now known that the F_{ab} fragment consists of the complete light chain in association with the V_H and C_H1 segments of the heavy chain, and that the F_c fragment consists of a dimer of C_H2 and C_H3 units. Thus, each IgG molecule contains two F_{ab} and one F_c fragment (Fig. 1).

Although attempts to observe antibody molecules by electron microscopy were initially frustrated, clear micrographs were obtained when anti-DNP antibody was allowed to react with a bivalent DNP-hapten (Valentine and Green, 1967). The majority of antigen-antibody complexes had formed closed ring structures from which the overall Y-shaped geometry of the antibody molecule was apparent; molecular dimensions obtained from an examination of these complexes indicated that each F_{ab} arm was 60Å in length with a width of 35Å, while the F_c portion was 45Å long and 40Å wide. In addition, X-ray diffraction patterns of antibody crystals have provided a distance for antigen-binding site separation of 140Å in a T-shaped molecule (Sarma et al., 1971) agreeing well with an F_{ab} length of 60-70Å.

These measurements have been contradicted by studies which have been undertaken to describe the solution structure of IgG molecules. Using small-angle X-ray scattering techniques, early estimates of the distance between antigen-binding sites on antibodies in solution

provided a separation of 240Å with the F_{ab} arms fully extended to form a T-shaped structure (Pilz et al., 1974); these observations have been confirmed by recent studies using both X-ray and neutron small-angle scattering, yielding distances between antigen-binding sites of approximately 280Å (Cser et al., 1978; Cser et al., 1980). The large discrepancy existing between crystal and electron microscopic measurements and those obtained by X-ray and neutron scattering methods has not yet been explained.

The question of the possible flexibility of the F_{ab} segments with respect to the rest of the molecule has been addressed by fluorescence polarization measurements of IgG in solution. These studies have suggested nearly complete rotational freedom of the F_{ab} segments (Nezlin et al., 1970). This finding, in addition to electric birefringence studies, indicates that the antibody molecule may be able to assume a range of structures in order to best bind to an antigenic surface (Cathou et al., 1974).

Determinations of antibody molecular structure by X-ray crystallography have provided several basic observations (reviewed in Davies et al., 1975). An IgG molecule consists of globular domains which are linked together by short stretches of polypeptide. These domains consist of a pair of homology units which are folded together, so that V_L and V_H form the variable domain; C_L and C_H1 form the C_H1 domain; and the C_H2 and C_H3 domains consist of a dimer of C_H2 or C_H3 homology units, respectively. In this way, each IgG molecule contains 6 globular domains. Direct evidence for folding of the heavy and light chains into such domains is found in the ability of proteolytic enzymes to break the molecule into individual domains by

attacking the short intervening sequences of polypeptide which connect the domains (Chapter 3).

Evidence has accumulated to suggest that each constant domain of the heavy chain may have evolved to carry out different functions. These functions include complement fixation, opsonization, placental transfer, and binding to cellular receptors. The assignment of these various functions to individual domains of the heavy chain has been made possible by the isolation of various heavy chain fragments (Chapter 3).

More detailed diffraction analysis has shown that each homology unit consists of a basic framework, the "immunoglobulin fold", which contains two β-pleated sheets which are roughly parallel and surround a tightly packed core of hydrophobic side chains (Poljak et al., 1974). This structure serves as a foundation upon which additional stretches of polypeptide are anchored. The V_L and V_H units, for example, include an additional length of polypeptide which forms a loop that lies against the framework structure (Poljak et al., 1973).

As has been discussed, pairs of homology units associate to form globular domains. Non-covalent interchain forces are sufficiently strong in the C_H1 and C_H3 domains to maintain the two chains in close apposition, as evidenced by the stable isolation of these domains as dimers of their constituent homology units (Ellerson et al., 1972; 1976). In contrast, X-ray diffraction studies have suggested that the C_H2 domain is relatively unstable compared to C_H1 or C_H3, as the two C_H2 homology units fail to form a tight dimer and tend to separate from each other (Diesenhofer et al., 1976).

The F_{ab} segments are connected to the F_c segment of the IgG molecule through a stretch of polypeptide called the "hinge region".

This stretch of amino acids contains the unique sequence ProProPro which is thought to disrupt any secondary structure and create a bend in the polypeptide chain which corresponds to the junction of the arms in the Y shape (Smyth and Utsumi, 1967). This sequence also contains the interheavy chain disulfide, which is unusually susceptible to reduction, as well as the initial cleavage points for both papain and pepsin, suggesting that this hinge region is highly exposed.

One of the primary functions of an antibody molecule is antigen binding. Since F_{ab} fragments retain this activity, it was deduced that the antigen binding site was located at the N-termini of the light and heavy chains (Porter, 1959); confirmation was obtained by an additional proteolytic cleavage which showed that the isolated V domain (F_v fragment) retained antigen-binding properties (Inbar et al., 1972). Thus, each IgG molecule contains two identical antigen binding sites.

X-ray crystallography has shown that the antigen binding site exists as a shallow groove or crevice between the light and heavy chains (Amzel et al., 1974). Antibody specificity arises as a result of non-covalent interactions between the antigen and amino acid residues in the binding pocket. The size of the binding site has been found to vary considerably depending upon the amino acid residues which line the site; the binding site on McPC 603 for phosphorylcholine resides in a cavity of dimensions 20Å x 15Å x 12Å (Segal et al., 1974), while the binding site for vitamin K-OH on the human IgG New consists of a much smaller cleft of dimensions 15Å x 6Å x 6Å (Wu and Kabat, 1970).

In addition to the general variability of primary sequence in the V_L and V_H regions, there are 3 segments in V_L and 4 segments in

V_H that have sequences which are highly variable from one antibody molecule to another (Wu and Kabat, 1970; Kabat and Wu, 1971). At least five out of seven of these "hypervariable regions" have been found to line the surface of the antigen binding site. Also, affinity labels used to tag amino acid residues in the antigen binding site have been found to react with amino acids contained in the hypervariable regions (reviewed by Givol, 1974). This suggests that antibody specificity is determined largely by the primary sequence of these hypervariable segments and that the remainder of the molecule serves as both a framework upon which the antigen binding site is built and as an apparatus for carrying out secondary functions.

Myeloma Proteins

The ability to carry out quantitative biochemical characterizations of pure antibody preparations is in large part due to the occurrence of immunoglobulin-producing tumors in mice, the most widely utilized class of which is the plasmacytoma. Each of these tumors has been found to be derived from a single clone of cells, this conclusion being based upon the observation that each secretes a single molecular species. Thus, plasmacytomas can be used to prepare large quantities of homogeneous immunoglobulin (myeloma protein) that can be studied with a precision not possible with heterogeneous antibody preparations (for recent reviews see Potter, 1972; Potter, 1975; Potter, 1977).

Proof that myeloma proteins are structurally identical to antibodies induced by traditional immunization techniques has come largely as a failure to find differences between the antibodies produced via these two routes (Nisonoff et al., 1975). Such an analysis has

included the finding of identical genetic markers in both groups of proteins, and has been extended to the occurrence of large amounts of primary sequence homology between normal and myeloma immunoglobulins (Hood et al., 1978). Thus, the myeloma protein is considered to be a representative antibody molecule, rather than an aberrant product of a neoplastic cell.

The enormous number of plasmacytomas which have been studied has been due to the ability to induce such tumors in mice by the extremely simple procedure of intraperitoneal injections of mineral oil (Potter and Boyce, 1962). The influence of genetic factors is apparent in the fact that only two inbred strains of mice, BALB/c and NZB, have been found to produce induced plasmacytomas with any frequency (Merwin and Redmon, 1963). The incidence of various antibody classes produced by all the plasmacytomas studied has shown a marked predominance of the IgA class (74%; Potter, 1972); rather than reflecting an unusually high susceptibility of IgA-producing plasma cells to neoplastic transformation, it has usually been inferred that the representation of various antibody classes from the known pool of plasmacytomas reflects the normal population distribution of plasma cells in the peritoneal cavity where such tumors are derived (Nisonoff et al., 1975; Potter, 1972).

Through relatively random screening procedures, it has been found that approximately 5% of the myeloma proteins produced have a specificity against one of the antigens tested (Potter, 1970; Potter, 1971). These sytems of antigen and specific myeloma protein therefore allow studies which can simulate the <u>in vivo</u> interactions between antibody and antigen under carefully controlled experimental conditions.

Unfortunately, since myeloma proteins arise spontaneously and not as a result of an immunization procedure, it is virtually impossible to induce a plasmacytoma that secretes antibody with a specificity for a desired antigen.

More recently, a technique has been developed that involves the fusion of cells from a non-secreting plasmacytoma with a population of normal spleen cells which includes antigen-specific lymphocytes whose number has been increased by immunization procedures (Kohler and Milstein, 1975; Kohler and Milstein, 1976). Such methods now allow the realization of the exciting ability to raise single clones of the decendants of these fused cells which synthesize antibody with an antigenic specificity of choice.

Carbohydrate-binding Antibodies

Current understanding of the nature of carbohydrate-binding antibodies has come almost exclusively from the study of myeloma proteins, especially those possessing anti-dextran, anti-fructosan, and anti-galactan activity. It is not surprising that vertebrates display anticarbohydrate activity in the repertoire of their immune system since bacteria and other microorganisms present complex and distinctive polysaccharide coatings to the outside world (Potter, 1970). In addition, no myeloma proteins with antipolysaccharide specificity were found to be induced in germ-free BALC/c mice (McIntire and Princler, 1969), suggesting that exposure to enteric microorganisms makes possible the production of these artifically-induced plasmacytomas. By the comparison of myeloma proteins with various specificities, general features of the protein-carbohydrate interactions can be discerned;

characterizing the dual nature of this mutual interaction, such features can be seen to exist as properties either of the sugar or of the three-dimensional structure of the antibody combining site.

The immunochemistry of a polysaccharide is dictated by the sequence of its constituent sugar monomers and their linkages. Through the use of homopolymers as antigens containing only one type of sugar monomer, the corresponding myeloma proteins have been shown to exhibit strict specificity with regard to the sugar and the stereochemistry of the linkages; this has been shown in the dextran series (Lundblat et al., 1972; Cisar et al., 1974; Cisar et al., 1975; Sugii et al., 1979), the levan series (Lundblat et al., 1972; Cisar et al., 1974; Lieberman et al., 1975), and the insulin series (Cisar et al., 1974; Streefkerk and Glaudemans, 1977). For example, the myeloma protein J558(IgA) binds with high affinity to destrans containing a high proprotion of $\alpha 1 \rightarrow 4$ and $\alpha 1 \rightarrow 6$ glycosidic bonds (Lundblat et al., 1972).

Antibody binding to carbohydrate antigens can also be characterized by the size of the oligosaccharide which exhibits maximum complementary (and therefore maximum binding) with the antibody molecule. By measuring the binding constants of variously sized oligosaccharides to the appropriate myeloma proteins, it has been suggested that the antigen binding pocket can optimally accommodate a structure as small as a trisaccharide (Cisar et al., 1974) or as large as a hexasaccharide (Cisar et al., 1975). This would provide a rough estimate that the average size of a binding site is 34 x 12 x 7 Å (Kabat, 1966). In spite of the difficulties encountered in measuring association constants in this low range (10^3-10^4 M^{-1}), the resulting maximum dimensions

correspond well with those suggested by various protein antigens (Kabat, 1968).

From the binding data obtained by the interaction of oligosaccharides and myeloma proteins, it has been observed that such binding exhibits one of two possible patterns (Cisar et al., 1975; Takeo and Kabat, 1978). W3129, an IgA myeloma protein with activity against $\alpha 1 \rightarrow 6$ linked dextran, shows a maximum complementarity to a pentasaccharide with an association constant of 2×10^5 M^{-1}; when the contribution of each sugar monomer was calculated by obtaining binding constants for monomer, dimer, etc. up to pentamer, it was seen that the monomer, methyl-α-D-glucoside, contributed about 60% of the total free energy of binding. Such a pattern of binding clearly showed a specificity of W3129 for the non-reducing terminus of the dextran molecule. Alternatively, the IgA myeloma protein QUPC 52, also with activity against $\alpha 1 \rightarrow 6$ linked dextran, was found to have a central specificity, as methyl-α-D-glucoside possessed less than 5% of the maximum binding energy exhibited by the hexasaccharide. From the classification of antibody molecules as having terminal or central specificities, it was inferred that the binding pocket of an antibody molecule with a terminal specificity exists as a cavity in the three-dimensional protein surface, while an antibody with a central specificity binds the antigen in a groove on the protein surface (Wu et al., 1978). Such a definition in protein architecture has not been confirmed by X-ray diffraction methods.

Complement

The term "complement" is used to describe a complex system of humoral proteins consisting of eleven separate components and at least seven inhibitors. This integrated system of proteins is aptly named, for it is responsible for much of the biologic activity possessed by the humoral immune system, and carries out these secondary effector functions in association with antigen recognition units--the antibody molecules. As a reflection of the interdependence of recognition and effector activities, the phylogenetic development of the complement system has been found to closely parallel the appearance of immunoglobulin-like molecules (Gigli and Austen, 1971). (For recent reviews of the complement system, see Porter, 1979; Opferkuch and Segerling, 1977; Muller-Eberhard, 1975).

Complement mediates two primary biologic activities--the assemblage of a protein complex which penetrates a membrane surface and can result in cell lysis, and the generation of vasoactive agents which participate in inflammatory processes. While the complement system has been thought of as humoral, much of the current understanding of complement activity depends upon specific interactions between the various soluble protein components and a cell surface, whether it be a gram-negative bacterium or the endothelial lining of a capillary. Thus, it can be seen that complement activity can be advantageous (through the destruction of foreign microorganisms) or deleterious (as a result of inappropriate activation and vascular damage).

The importance of the complement system to the immune defense of man is underscored by the consequences of a deficiency of one or more components. These abnormalities can be associated with the expected

failure to eliminate microorganisms, as evidenced by recurrent bacterial infections, as well as by more complex patterns shown in systemic lupus erythematosus and rheumatic diseases (Snyderman, 1980). The integrity of a normal complement system is therefore a requirement for proper immune function.

The complement system has been shown to consist of a central sequence of reactions which can be initiated by one of two pathways. These pathways are arranged to operate as a cascade of enzymatic steps, with each component being activated in order to carry out the activation of the next protein in the sequence. This architecture of repeated activation lends two primary advantages to this process. One of these is that since each step is enzymatic in nature, sequential activation affords a tremendous opportunity for the amplification of an initiating stimulus. A second advantage lies in the fact that multiple levels of control are possible with the division of the sequence into many discrete steps. This allows for the elaboration of several related biologic activities, all of which can be mediated by the complement system and controlled at several levels to provide for the differential expression of these functions.

The classical pathway of complement consists of nine proteins. The first of these, C1, is composed of three subunits, C1q, C1r, and C1s. (A fourth subunit, C1t, was described (Assimeh and Painter, 1975) and later identified as the plasma amyloid P-component which is not involved

*The nine proteins are numbered by order of activation except for C4, which was later found to be second in the sequence. A bar indicates the active form of the enzyme. In some cases a component is split into two chains which separate; these are designated by small case letters, such as C3a and C3b, both of which are cleavage products of C3. This is in accordance with the recommendations of the W.H.O. Committee on Complement Nomenclature (1968).

in the complement sequence (Painter, 1977)). C1q interacts with immune complexes containing IgG or IgM antibodies by a mechanism that is still unknown; this subject is discussed in detail in Chapter 3. Interaction of C1q with immune complexes causes the conversion of C1r to an active form $\overline{C1r}$, consisting of two disulfide-bonded fragments which enzymatically cleave C1s to give the active protease $\overline{C1s}$ (Sakai and Stroud, 1974; Takahashi et al., 1975).

The activated subunit $\overline{C1s}$ has been found to have enzymatic activity against both C2 and C4 (Lepow et al., 1956). Activation of C4 is accomplished through enzymatic cleavage by $\overline{C1}$ into a small fragment C4a and a larger fragment C4b (Patrick et al., 1970). This cleavage reveals a site on C4b which is able to bind C2 (Muller-Eberhard et al., 1967); no catalytic activity is known for C4b (Porter, 1979). Activation of C2 occurs also as a result of cleavage by C1 into two non-bonded fragments; the larger fragment, C2a, remains bound to C4b (Polley and Muller-Eberhard, 1968) and has serine protease activity (Medicus et al., 1976a).

The complex $\overline{C4b,2a}$ in the presence of Mg^{+2} has the ability to split C3 into two unlinked fragments (Muller-Eberhard et al., 1967). The larger fragment C3b associates with $\overline{C4b,2a}$ to form a trimeric complex $\overline{C4b,2a,3b}$ which becomes bound to the cell membrane (Muller-Eberhard, 1966). A smaller fragment, C3a, is released into the circulation to mediate an inflammatory response through its actions on mast cells, endothelial cells, smooth muscle and leukocytes (Hugli, 1978).

The macromolecular enzyme $\overline{C4b,2a,3b}$ cleaves C5 to yield a polypeptide C5a and a larger fragment C5b (Cochrane and Muller-Eberhard,

1968; Shin et al., 1968); this is the last known enzymatic step in the complement cascade. C5a is a very biologically active molecule, possessing both anaphylactic and chemotactic properties (Hugli, 1978; Orr et al., 1979). C5b is able to associate in the fluid phase with C6 and C7 to form a termolecular complex (Arroyave and Muller-Eberhard, 1973) which is then absorbed onto the surface of a cell membrane; this membrane binding can be either to the surface of the antigen-bearing cell or another local cell not involved in this activation process (Rother et al., 1974). The membrane attack complex is completed by the addition of one molecule of C8 and six molecules of C9 to form a decameric complex ($C5b \cdot C6 \cdot C7 \cdot C8 \cdot C9_6$) on the membrane surface consisting of all five proteins (Kolb et al., 1972) (the classical complement pathway is illustrated in Fig. 4).

The details of the mechanism whereby the C5b-9 complex produces cell lysis are still unknown. Two models were originally proposed: 1) an enzymatic degradation of membrane lipid by C5b-9 to produce a membrane lesion, and 2) an insertion of some portion of C5b-9 into the membrane to provide a transmembrane channel or "doughnut". Current evidence has ruled out the enzymatic mechanism (Kinoshita et al., 1977), while many studies, including the observation by electron microscopy of circular structures in erythrocyte membranes treated with complement (Iles et al., 1973), the release of intact phospholipid from complement-treated liposomes (Kinoshita et al., 1977; Shin et al., 1977), and the apparent behavior of C5b, C7, C8, and C9 as integral membrane proteins when assembled into the multimolecular attack complexes (Hammer et al., 1975; Hammer et al., 1977; Boyle et al., 1978), all provide evidence for the membrane insertion model.

Due to the damaging effects of complement on intact cells, it is critically important in such an immune reaction that the lytic response be narrowly localized to the intruding microorganisms. It appears that the complement system has evolved a two-fold level of control, one aspect of which exists as an intrinsic time limit imposed upon many of the complement components due to the instability of their activated forms. In this way, complement activity is shortened by the lability of C2a in the $\overline{C4b,2a}$ complex (Mayer et al., 1954; Stroud et al., 1966; Hoffman, 1969; Opferkuch, 1971a), by the fluid-phase instability of the C5b,6,7 complex (Porter, 1979), and by the rapid decay of free C4b (Muller-Eberhard et al., 1966) and cell-bound C5b (Cooper and Muller-Eberhard, 1970). These inherent instabilities in conjunction with the affinity of two complexes (C5b,6,7 and $\overline{C4b,2a,3b}$) for membrane attachment insure that the activation process will be localized to a small area and cannot be spread throughout the serum or tissue fluid to result in a massive overresponse to a minor immunologic insult.

To further limit the activation of complement, a number of inhibitors exist in serum to inactivate specific components of the cascade. The $\overline{C1}$ inhibitor ($\overline{C1}$ INH) is a glycoprotein which binds the $\overline{C1}$ molecule stoichiometrically to yield an inactive complex (Gigli, 1974). The necessity of forming a covalent bond between $\overline{C1}$ and the inhibitor in order to abolish the proteolytic activity of $\overline{C1}$ has not been established (Harpel and Cooper, 1975). In addition to its activity in modulating the complement system, $\overline{C1}$ INH also participates in the control of the coagulation and fibrolytic pathways through its effect on Hageman factor, plasma thromboplastin antecedent, and kallikrein (Austen, 1974).

Another way in which the complement system is controlled is through the action of the C3b inactivator (C3b INA). This serum enzyme cleaves C3b into inactive fragments and thereby abolishes all the known biologic properties of C3b, including its role in the complement cascade, immune adherence, and stimulation of phagocytosis by neutrophils (Tamura and Nelson, 1967). C3b INA also inactivates C4b (Pangburn et al., 1977) and has been shown to be identical to a C4b cleaving enzyme earlier described (Cooper, 1975; Shiraishi and Stroud, 1975). C3b INA requires the presence of another protein, β1H, for proteolytic activity (Pangburn et al., 1977); in addition, β1H has a direct inhibitory effect on C3b (Whaley and Ruddy, 1976) and accelerates the decay of an activated complex of the alternative pathway (Weiler et al., 1976).

In addition to these well-characterized proteins, other inhibitors of complement components have been observed. A substance has been found in human, rabbit, and guinea pig serum that inactivates C6 in the membrane complex but not in the fluid phase (Tamura and Nelson, 1967). Also, many substances are known to interact with C1, including fraction A (Conradie et al., 1975) and many anionic substances, such as RNA (Agnello et al., 1969) and DNA (Cooper, 1973), although the significance of these interactions to the control of the complement cascade is unclear.

As well as inhibitors which control the development of the membrane attack complex C5b-9, there are also inactivators of the biologically potent peptides which are generated as a consequence of complement activation. The anaphylactic activity of both C3a and C5a is abolished by the removal of a single C-terminal Arg residue through the action of a serum carboxypeptidase with specificity similar to that exhibited by

pancreatic carboxypeptidase B (Hugli, 1978). In this way, the potentially damaging effects of these potent anaphylatoxins are limited to the immediate focus of activation.

Along with the classical pathway of complement activation, there exists a second, alternate pathway which bypasses the first three classical components C1, C4, and C2 and generates other activated enzymes which are able to produce C3b and thereby a complex with C5-activating properties (Gewurz and Lint, 1977; Muller-Eberhard and Schreiber, 1980). Although elements of the alternate pathway were described as early as 1954 (Pillemer et al., 1954; Pillemer et al., 1955), many details remain at this time to be elucidated. The primary difference between the classical and alternate pathways is that the alternate pathway does not require the participation of antigen-specific immunoglobulin; inasmuch as the alternate pathway is notably activated by bacterial and fungal cell walls (Medicus et al., 1976b; Goetze and Muller-Eberhard, 1976), a possible benefit to the organism resulting from the development of the alternate pathway would be an immediate immune response to microorganisms that did not depend upon the relatively slow induction of antibody synthesis. Thus, it has been shown that both pathways of the complement system serve to carry out many effector functions of the immune system that follow recognition events.

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Figure 1

The basic primary structure of an IgG molecule is depicted, showing both the heavy and light chains, homology units, interchain disulfide bond, and the cleavage products of papain treatment.

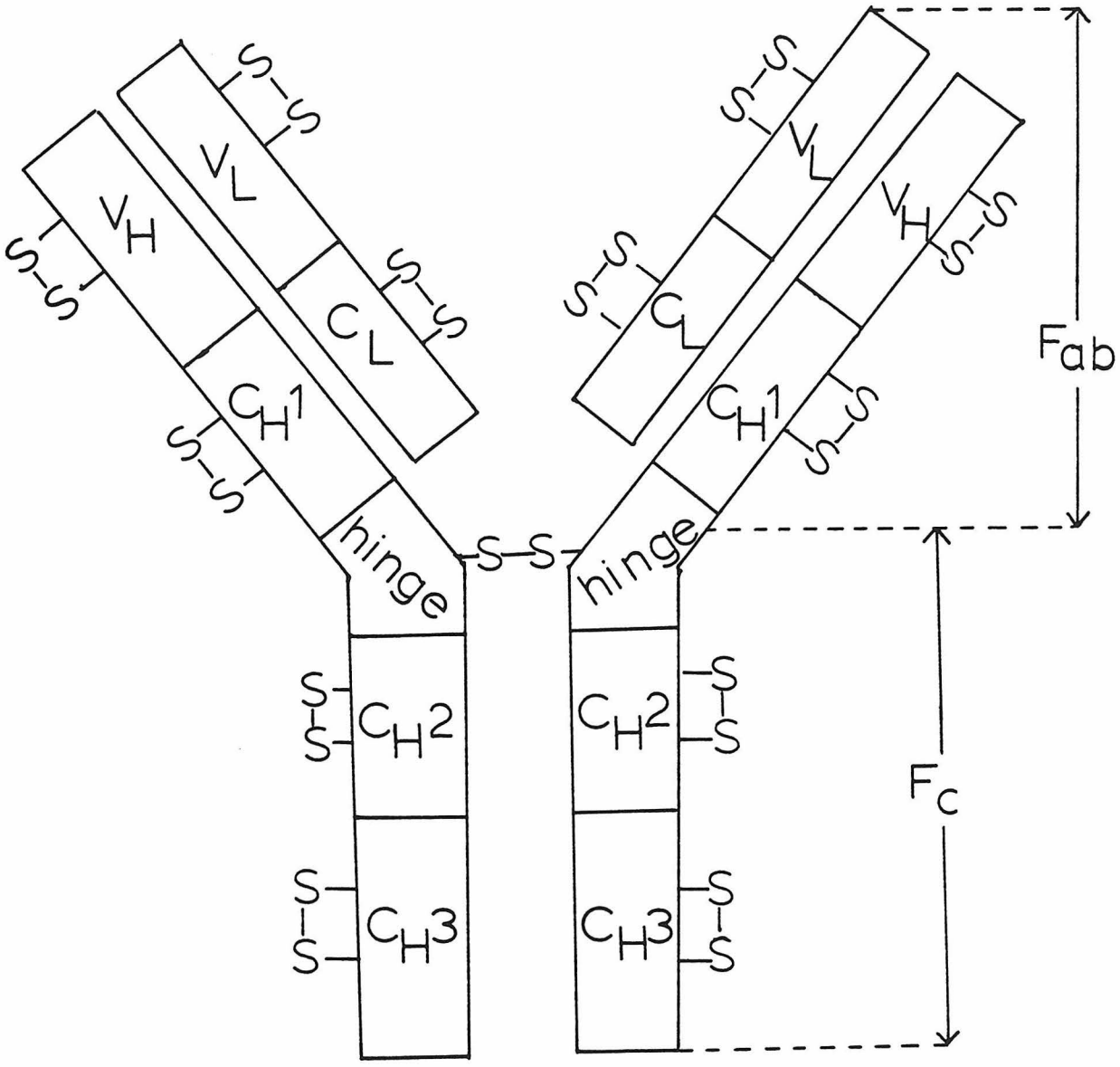
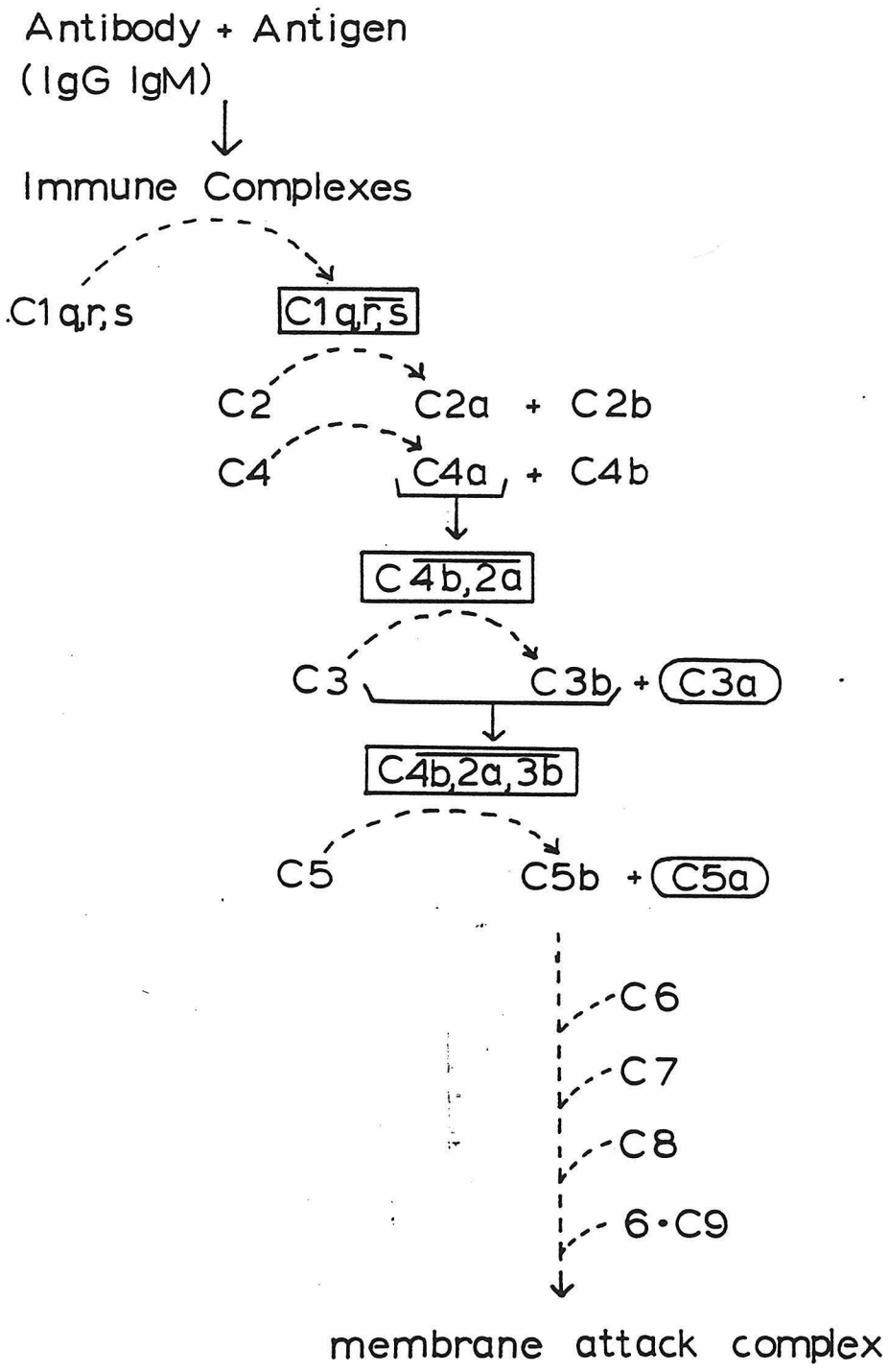

S-S
VL
VH
CL
CH1
hinge
S-S
CH2
CH3
Fab
Fc

Figure 2

This diagram outlines the pathway of classical complement activation initiated by immune complexes and leading to the formation of a membrane attack complex. Boxed complexes represent active enzymatic species. Circled components represent small, biologically active proteins released during the course of activation.


Antibody + Antigen
(IgG IgM)
Immune Complexes
C1q,r,s
C1q,r,s
C2
C2a + C2b
C4
C4a + C4b
C4b,2a
C3
C3b + C3a
C4b,2a,3b
C5
C5b + C5a
C6
C7
C8
6·C9
membrane attack complex

Chapter II: Characterization of the Antigen-binding Properties of UPC-10 Myeloma Protein

INTRODUCTION

Levans

Levans are homopolymers of fructose containing predominantly β2→6 linkages and β2→1 branches. Common polysaccharides in nature, various forms of levan have been isolated from grasses such as leafy cocksfoot, italian ryegrass (Bell and Palmer, 1949), and perennial ryegrass (Laidlaw and Reid, 1951) and from numerous bacterial species including Claviceps (Arcamone et al., 1970), Pseudomonas (Bell and Dedonder, 1954), Bacillus (Bell and Dedonder, 1954; Murphy, 1952), Corynebacterium (Hennis and Aschner, 1954), and Aerobacter (Hestrin et al., 1943). These levans vary in their overall molecular weight and degree of branching.

One of the earliest studied levans was that isolated from ryegrass (Laidlaw and Reid, 1951). Methylation analyses have been carried out to determine the degree of β2→1 branching present; these studies indicate that exhaustive methylation yields 6-7% of 3,4-dimethyl fructose upon hydrolysis, establishing the average β2→6 linked chain length between branch points as 14 sugar residues (Bell and Palmer, 1949; Lindberg et al., 1973). These studies also suggested that the non-reducing properties of the levan molecule were due to a terminal glucose linked to the fructose chain as in sucrose (Laidlaw and Reid, 1951). A more recent analysis of ryegrass levan has indicated that two forms of levan can be isolated, the major one of which is unbranched (Tomasic et al., 1978). Molecular weight estimates have ranged from 5000 daltons (determined by sedimentation and diffusion constants) (Bell and Palmer, 1949) to 3300 daltons (determined by periodate oxidation of the terminal

glucopyranose residue) (Laidlaw and Reid, 1951). These molecular weights place the number of fructose monomers per ryegrass levan molecule in the range of 20-30 residues.

Cultures of Aerobacter levanicum (ATTC 15552) have served as a well-characterized source of levan. This organism, which was originally isolated from a fruit tree root in Jerusalem, has been shown to produce large amounts of levan as a capsular material when grown in the presence of sucrose or raffinose (Hestrin et al., 1943). The enzyme responsible for this polymerase activity, levansucrase, has been extensively studied as isolated from Aerobacter (Hestrin and Avineri-Shapiro, 1944; Avineri-Shapiro and Hestrin, 1945; Hestrin et al., 1956; Feingold et al., 1956) and from Bacillus strains (Peaud-Lenoel, 1957; Rapoport and Dedonder, 1963a,b,c; Rapoport and Dedonder, 1966; Berthou et al., 1974; Chambert et al., 1974; Gonzy-Treboul et al., 1975; Tanaka et al., 1979). These studies have shown that levansucrase hydrolyzes sucrose into glucose and an enzyme-fructose complex in a reversible step, followed by the irreversible transfer of fructose to an acceptor molecule, usually a growing levan chain. In cultures containing sucrose, the initial acceptor is a sucrose molecule, so that all levan chains terminate in a non-reducing glucopyranose residue.

Aerobacter levan has been shown to contain β2→6 linkages with β2→1 branches by methylation analysis; the degree of branching, however, has been variously determined, from a value of 11% (Hestrin et al., 1956; Feingold, 1955) to 18% (Lindberg et al., 1973). These percentages correspond to an average chain distance between branches of between 5 and 9 residues. Thus, Aerobacter levan is more highly branched than the ryegrass levan previously discussed.

An estimate of the molecular weight of Aerobacter levan has suggested a value of 1.75×10^7 daltons (Avigad and Feingold, 1957). This value makes bacterial levan 3,500 times larger than the levan found in ryegrass.

In addition to levan, another fructose polymer often found in nature is inulin, which has been isolated from a number of plant sources (Bell and Palmer, 1949). Methylation analysis has shown inulin, however, to be a exclusively β2→1 linked in a highly unbranched structure (Lindberg et al., 1973). This difference in linkage results in a radical dissimilarity in the three-dimensional structures of oligosaccharides prepared from levan and inulin (Cisar et al., 1974).

UPC-10 Myeloma Protein

UPC-10 is a murine plasmacytoma that was isolated at NIH; developed in BALB/c mice, it was induced by intraperitoneal injections of mineral oil (Lieberman et al., 1975) and the myeloma protein produced by the tumor cell line was serotyped as belonging to the 2a subclass of murine IgG (Cisar et al., 1974). Screening for antibody activity indicated that UPC-10 myeloma protein was specific for some determinant on bacterial levan (Cisar et al., 1974). These properties made UPC-10 myeloma protein the first homogeneous mouse antibody with a known specificity and of a heavy chain class that is able to activate the classical pathway of complement.

Early studies were carried out using highly impure preparations consisting of ascites fluid drained from the peritoneal cavities of mice carrying the tumor cell line (Cisar et al., 1974). Other investigators improved the purity of the UPC-10 myeloma protein slightly by performing

a 37-50% saturated ammonium sulfate precipitation on the ascitic fluid (Lieberman et al., 1975). Attempts to purify levan-binding proteins, including UPC-10 myeloma protein, by affinity chromatography using various levans as ligands immobilized on a Sepharose support met with little success, as an extremely harsh treatment with 5 M guanidine hydrochloride was required to elute bound protein from the column in appreciable yield (Vrana et al., 1976).

Analysis of the binding characteristics of UPC-10 myeloma protein showed that although the antibody was precipitated by bacterial levan, which contains both β2→6 and β2→1 linkages, it did not react with inulin, suggesting that the protein bound only to fructose sequences that were β2→6 linked (Cisar et al., 1974). Although oligosaccharides were not available from the β2→6 series, the inferred antigenic specificity of UPC-10 myeloma protein was supported by the observation that all of the oligosaccharides with β2→1 linkages tested failed to inhibit the interaction between the antibody and levan preparations.

MATERIALS AND METHODS

General

Ring tests for the detection of antibody-antigen precipitates were performed by the method of Garvey et al. (1977). Immunodiffusion assays were conducted according to Ouchterlony (1949); goat anti-mouse IgG was obtained from Cappell.

The extinction coefficient of UPC-10 myeloma protein at 280 nanometers was determined by measuring the absorbance of a solution of salt-free antibody of known concentration--a value of 1.32 OD units for a mg/ml solution was obtained.

Isoelectric focusing in polyacrylamide gels was performed using the materials and apparatus manufactured by LKB.

Buffers

Phosphate-buffered saline (PBS) consisted of 20 mM sodium phosphate, 0.15 M sodium chloride pH 7.4.

SDS Gel Electrophoresis

Disk gels: 7.5% acrylamide disk gels and SDS buffer were purchased from BioRad.

Slab gels: Analytical slab gels of dimensions 13 x 11 x 0.1 cm were prepared by the method of Laemmli (1970). An 8→18% exponential gradient of acrylamide concentration was poured using a standard gradient mixer; this separating gel was overlayed by a 3% acrylamide stacking gel. Gels were stained with Coomassie Brilliant Blue. A mixture of standards which were used to calibrate each gel consisted of

cytochrome c, bovine β-lactoglobin, myoglobin, soybean trypsin inhibitor, and bovine serum albumin, all from Sigma.

Tumor Maintenance and Ascites Production

UPC-10 plasmacytoma was obtained from Litton Bionetics and was maintained by serial transmission in female BALB/c mice (TIMCO). Subcutaneous solid tumors of UPC-10 were removed from mice under sterile conditions, rinsed with Locke's solution, and minced into small (1 mm) pieces of tissue which could be injected subcutaneously into a new host using a 13 G thoracocentesis needle (Potter and Glaudemans, 1972). Tumor transfer was performed as often as was necessary in order to prevent the tumors from rupturing through the skin or killing the mouse.

Ascites was produced in CDF hybrid female mice (BALB/c x DBA/2; Cumberland), each of which was first primed by an intraperitoneal injection of 0.5 ml mineral oil. After a period of at least 3 weeks, primed mice received an intraperitoneal injection of 0.5 ml of a tumor cell suspension--one or more tumors were minced and forced through a 200-mesh wire screen into Locke's solution to produce a suspension that could pass through a 21 G needle. An appreciable quantity of ascites usually took 2-3 weeks to develop and these accumulations of fluid were drained from the peritoneal cavities of each mouse using an 18 G needle twice weekly. This procedure was continued until the mice had expired, usually allowing 7-8 tappings per mouse. Any cells present were removed by centrifugation and the ascites was stored at -20°C until use.

UPC-10 Myeloma Protein Purification

Approximately 200 mls of frozen ascites were thawed and filtered through a plug of glass wool to remove any gelatinaceous material. A solution of saturated ammonium sulfate at neutral pH was added dropwise with stirring at room temperature to a final concentration of 45% saturation. (All of the following steps were carried out at 4°C). This suspension was stirred for 2 hours, followed by centrifugation at 16,000 g for 30 minutes to sediment the precipitated protein. The pellet was resuspended in 150 mls PBS and gently rocked until it had dissolved. This protein solution was then dialyzed against PBS to remove any remaining ammonium sulfate, clarified by centrifugation at 16,000 g for 30 minutes, concentrated on a PM-10 ultrafiltration membrane (Amicon) to a volume of 100 mls, and reclarified by centrifugation at 35,000 g for 30 minutes.

Next, gel filtration of the protein solution was performed on a column (5 cm dia. x 30 cm) of Cibacron Blue-Sepharose 4B (Pharmacia) equilibrated and washed in PBS. Fractions containing eluted protein were pooled and concentrated on a PM-10 membrane to 100 mls. This solution was then applied to a column (5 cm dia. x 60 cm) of DE-52 cellulose (Whatman) which had been equilibrated with 30 mM potassium phosphate, pH 8.0; the IgG was present in the initial protein peak eluted with the same buffer. This peak was pooled, concentrated on a PM-10 membrane, clarified by centrifugation at 27,000 g for 30 minutes, dialyzed against 50 mM sodium acetate, pH 5.0 overnight, and recentrifuged at 27,000 g for 30 minutes. This acidified solution was then applied to a column (5 cm dia. x 30 cm) of CM-52 cellulose (Whatman) equilibrated and washed with 50 mM sodium acetate, pH 5.0.

The IgG-containing fractions were pooled, concentrated on a PM-10 membrane, and dialyzed against PBS. Purified protein was stored at -20°C in PBS containing 10^{-5} M Thimerosal (Sigma); refreezing was avoided and all protein solutions were centrifuged at 27,000 g for 30 minutes before use.

Preparation of F_{ab} Fragments

This procedure is a modification of the methods of Porter (1959) and Putnam et al. (1962). Enough mercuri-papain (Sigma) was added to a solution of 15 mg/ml UPC-10 myeloma protein in a buffer containing 0.1 M sodium acetate, 1 mM cysteine and 2 mM EDTA, pH 5.5 to give a protein:enzyme weight ratio of 100:1. This digestion mixture was then incubated at 37°C for 5 hours. The reaction was quenched by the addition of the sodium salt of p-hydroxymercuirbenzoate (Aldrich) to a concentration of 1 mM. (All of the following steps were carried out at room temperature, unless indicated). This reaction mixture was dialyzed overnight against 50 mM sodium phosphate, pH 6.6, and then applied to a column (2.5 cm dia. x 40 cm) of DE-52 cellulose (Whatman) which had been equilibrated with 50 mM sodium phosphate, pH 6.6. The column was washed with approximately 600 mls of this buffer, and then the eluting buffer was switched to a linear gradient from 50 mM sodium phosphate, pH 6.6 to 500 mM sodium phosphate, pH 6.6 in a total volume of 400 mls. The F_{ab} fragment eluted first from the column followed by the F_c fragments. Appropriate fractions were pooled, concentrated on a PM-10 ultrafiltration membrane, dialyzed against PBS, and stored at -20°C.

^{125}I-labeling of Staphylococcal Protein A

This procedure follows the protein iodination method of Hunter and Greenwood (1962). Twenty-five µl of a buffer containing 0.4 Tris, 4 mM EDTA, pH 7.4 (Tris-EDTA) was added to 25 µl of protein A (1 mg/ml) (Pharmacia) in Tris-EDTA buffer in a polyethylene tube. To this solution was added 10 µl of Na^{125}I (100 mCi/ml; Amersham). Coupling was begun by the addition of 10 µl chloramine-T (2.5 mg/ml in Tris-EDTA buffer) (Eastman); the tube was shaken gently for 60 seconds at room temperature. The reaction was quenched by adding 25 µl sodium metabisulfite (2.5 mg/ml in Tris-EDTA buffer) and allowing the mixture to stand for 5 minutes. Protein losses due to absorption during desalting were minimized by adding 100 µl 1% BSA (Sigma) in Tris-EDTA buffer to the reaction mixture. Desalting was accomplished on a column (4 ml total volume) Sephadex G-25 superfine (Pharmacia) equilibrated in Tris-EDTA buffer; the gel was poured into a 5 ml plastic syringe fitted with a cotton plug at the outlet. The syringe was then supported in a centrifuge tube and spun in a desk-top centrifuge fitted with a swinging-bucket rotor at 2000 rpm for 5 minutes to spin out all of the buffer from the column. The reaction mixture was then applied to the top of the dried column, allowed to absorb for 1 minute, and the column was respun at 2000 rpm for 5 minutes. The eluant from the column containing ^{125}I-protein A was counted on a gamma counter and stored frozen at -70°C; the dried column containing unreacted ^{125}I was discarded.

Isolation of Perennial Ryegrass Levan

This procedure is a modification of the method of Laidlaw and Reid (1951). Freshly cut (Manhattan) perennial ryegrass was oven-dried at 60°C until dry to the touch. The dried material (450 grams) was then packed into a Soxhlet apparatus and extracted with ether for 48 hours; the solvent was changed to 80% ethanol, 20% water and the extraction procedure continued for another 60 hours. The grass residue was then removed, air-dried, and shaken with 5 liters of water at room temperature for 5 hours. The water extract was filtered and concentrated on a rotary evaporator to 2 liters. This solution was heated to 95°C and 100 mls of 10% $CdSO_4$ and 50 mls of 0.5 N NaOH were added with stirring. After cooling, the precipitate was removed by filtration through "Filter-Cel" (Johns-Manville). The clear filtrate was deionized on a mixed-bed ion-exchange resin MB-3 (Mallincrodt). The eluant was evaporated to 200 mls and decolorized with charcoal, yielding a syrupy solution which was added dropwise to 4 liters of ethanol with stirring. The resultant precipitate was cooled to 4°C, centrifuged at 16,000 g for 30 minutes and the pellets were dissolved in water and lyophilized to yield a fluffy white product.

Isolation of Levan from Aerobacter levanicum

Serial cultures of Aerobacter levanicum (American Type Culture Collection #15552) were grown at 27°C on 1% agar slants containing 0.8% nutrient broth (Difco) and 6% sucrose (Lieberman et al., 1975). For large scale broth cultures, bacteria were first grown in 250 ml aliquots of media (0.8% nutrient broth, 6% sucrose) for 48 hours at 27°C and these were then used to inoculate 18 liter flasks of media which were

incubated for 8 days at 27°C. Bacteria were removed by centrifugation from the supernatant which was then stored at 4°C after the addition of 0.02% NaN_3.

Isolation of the bacterial levan was accomplished by heating 2 liters of the spent growth medium to 95°C, adding 100 mls 10% $CdSO_4$ and 50 mls 0.5 N NaOH, and allowing the suspension to cool. The precipitate was removed by centrifugation at 16,000 g for 30 minutes, and the supernatant was deionized by passage over a column of MB-3 (Mallincrodt). The column eluant was concentrated by rotary evaporation to 500 mls, decolorized with charcoal, and evaporated again to 300 mls. This syrupy solution was added dropwise to a vigorously stirred 6 liter flask containing 4 liters ethanol. This suspension was stirred for 2 hours and then cooled to 4°C overnight. The precipitate was removed by centrifugation at 16,000 g for 10 minutes and the pellet was dissolved in water and lyophilized to yield a white, fluffy product.

Preparation of Polystyrene-sulfonic Acid

This procedure follows the method of Painter (1960). Polystyrene (4.75 g; Aldrich, M_w = 321,000 daltons) was dissolved in 80 mls chloroform and refluxed with stirring at 60°C. Chlorosulfonic acid (30 mls) was added cautiously to 1,4-dioxane (60 mls) with ice-cooling; this acid solution was added dropwise to the polystyrene solution over a period of 1 hour. The reaction temperature was raised to 70°C and allowed to continue for 12 hours. An additional aliquot of 20 mls chlorosulfonic acid in 20 mls dioxane was added and allowed to react for 12 hours, at which time this addition was repeated. After a total reaction time of 48 hours the mixture was cooled and volatile material

was removed by evaporation under reduced pressure. The tarry residue was dissolved in 2 liters of water and stirred for 30 minutes. The solution was neutralized with aqueous NaOH, filtered and dialyzed in 12,000 dalton molecular weight cutoff tubing for 4 days against running tap water. The tubing contents were then refiltered and applied to a column (5 cm dia. x 45 cm) of MB-3 mixed-bed exchange resin (Mallincrodt). The resin was washed with several column volumes of distilled water and the eluant was dialyzed against distilled water and lyophilized to yield a dark brown, spongy solid. The acid was stored in a vacuum dessicator at room temperature. Standardization was accomplished by titration with 0.1 N KOH and yielded a value of 187.5 grams sulfonated polystyrene/gram H^+.

Levan Fragmentation and Oligosaccharide Fractionation

This procedure is a modification of the method of Painter (1960). Polystyrene-sulfonic acid (0.06 g) was suspended with vigorous shaking in 25 mls water (0.0128 N H^+) and mixed with 25 mls of an aqueous solution containing 1 gram of purified bacterial levan. This hydrolysis mixture was tied into 5-6 individual small lengths of Spectrapor 6 dialysis tubing, molecular weight cutoff 25,000 daltons with a flat width of 32 mm (Spectrum Medical). These dialysis bags were suspended in a 1 liter beaker of distilled water maintained at 60°C and were gently stirred to provide thorough agitation. The dialysate was replaced every hour with 1 liter of heated distilled water until a total hydrolysis time of 14 hours was reached. The combined dialysates (14 liters) were concentrated to 200 mls on a rotary evaporator and lyophilized, yielding 600 mgs of crude hydrolysate.

To isolate individual oligosaccharides by molecular weight, 250 mgs of the crude hydrolysate were dissolved in 1 ml water and applied to a column (2.5 cm dia. x 170 cm) of BioGel P-2, -400 mesh (BioRad) packed and washed in distilled water at a flow rate of approximately 0.7 mls/minute; 12 minute fractions were collected. Fractions were assayed for carbohydrate using the orcinol assay.

Paper Chromatography of Oligosaccharides

This procedure follows that of Jolley and Glaudemans (1974). Oligosaccharides in aqueous solution were spotted onto Whatman 3 MM paper and thoroughly air-dried before development in 10:4:3 ethyl acetate/pyridine/water. After development, the sugars were visualized using $AgNO_3$ (Trevelyan et al., 1950). An aliquot (0.1 ml) of an aqueous saturated solution of $AgNO_3$ was mixed with 20 mls acetone, followed by the dropwise addition of water until the precipitate had redissolved. After air-drying, the chromatography sheet was pulled rapidly through a trough containing the silver solution. The sheet was then air-dried in the dark and sprayed with a solution of 2 grams NaOH dissolved in a minimum volume of water and then diluted to 100 mls with methanol. To enhance contrast, the sheet was briefly heated in a 100°C oven for less than 10 seconds if necessary.

Orcinol Assay for Ketohexoses

(For a review of colorimetric assays for ketohexoses, see Dische, 1962). Samples of levan oligosaccharides in a volume of aqueous buffer less than 0.5 ml were mixed with 3 mls of aqueous 0.17% (w/v) orcinol (3,5-dihydroxytoluene; Aldrich). Concentrated sulfuric acid (5 mls) was

carefully added to each tube and thoroughly mixed; the reaction mixtures were allowed to cool to room temperature and again mixed thoroughly before the absorbance was measured at 420 nm. A curve using fructose as the standard was constructed for quantitative assays (Fig. 1) and showed that the absorbance was linear with respect to fructose concentration over a wide range. The sensitivity of this assay allowed the measurement of as little as 1 microgram of fructose.

^{3}H-labeling of Oligosaccharides

Five mgs of oligosaccharides were dissolved in 500 µl water and to this solution was added 25 mCi of $NaB^{3}H_4$ (555 mCi/mmole; Amersham). The reaction mixture was incubated at 4°C for 48 hours, at which time a 20-fold molar excess of cold solid $NaBH_4$ was added to the oligosaccharide mixture for another 48 hours incubation at 4°C. Excess $NaBH_4$ was then destroyed by the sequential addition of 25 µl aliquots of 25% acetic acid which was continued until the reaction mixture no longer bubbled upon the addition of acid. This quenched solution was applied to a column (2.5 cm dia. x 160 cm) of BioGel P-2, -400 mesh (BioRad) packed and washed in distilled water at a flow rate of approximately 0.7 ml/minute. Fractions were collected at 12 minute intervals; 20 µl aliquots were removed from each fraction, mixed with 10 mls Aquasol-2 (New England Nuclear) and counted in a liquid scintillation counter. The radioactive peak corresponding to the oligosaccharide was pooled, lyophilized, and stored at -20°C.

Reduction of Oligosaccharides

Nine milligrams of fraction 59-79 dissolved in 1 ml water was treated with a 12-fold molar excess of solid $NaBH_4$ and allowed to react at 4°C for 48 hours. Excess BH_4 was destroyed by the dropwise addition of 25% acetic acid until all foaming had stopped. The reduced sugars were desalted on a column (2.5 cm dia. x 170 cm) of BioGel P-2, -400 mesh (BioRad) packed and washed in distilled water. Fractions containing carbohydrate, as assayed by the orcinol method, were pooled and lyophilized.

Equilibrium Dialysis

Experimental procedure: The measurement of the observed binding constants between various levan fractions and UPC-10 myeloma protein was carried out through the analysis of many individual binding determinations. Each binding determination was performed in pairs of 100 µl volume Lucite chambers separated by a Spectrapor 6 dialysis membrane with a molecular weight cutoff of 25,000 daltons (Spectrum Medical). One chamber, designated H, initially received only buffer and ligand, while the other, designated P, received a constant amount of antibody; all solutions of antibody and antigen were prepared in PBS containing 10^{-5} M Thimerosal (Sigma) to inhibit bacterial growth. Each binding determination was carried out in triplicate to reduce the impact of pipetting errors on any one pair of chambers. Eleven binding determinations were performed to calculate each binding constant. A typical arrangement is shown in Table 6A. In addition to the sample chambers used to calculate binding parameters, a set of control chambers was used to assess the approach to equilibrium. These consisted of

either antigen and buffer alone, or of antigen and antibody which were added to the same or different wells (Table 6B). One set of control wells indicated whether antigen alone had equilibrated across the dialysis membrane. Another set of control wells was used to assess whether the same equilibrium distribution of antigen had been obtained across the membrane regardless of whether the dialysis was begun with antibody and antigen together on the same side of the membrane or on opposite sides; this criterion was in accord with recommendations suggested for Scatchard analysis (Williams and Lefkowitz, 1978).

Once loaded with sample, the chambers were sealed with cellophane tape to prevent evaporation and rotated gently at the desired temperature until equilibrium was judged to have been reached by analysis of the control wells. The chambers were unsealed and 10 µl aliquots were removed in triplicate from each chamber, mixed with 10 ml AquaSol-2 (New England Nuclear) and counted.

Errors in removing and counting each 10 µl aliquot were minimized by averaging the three aliquots from each chamber. Errors in pipetting the antibody, antigen, and buffer into the chambers prior to equilibration were minimized by averaging the mean counts from each binding determination which was performed in triplicate.

Data analysis: Raw counts due to labeled ligand were converted into molar concentrations using a conversion factor which was calculated for each experiment. In accordance with the Scatchard analysis of protein binding (Scatchard, 1949), a least-squares fit of the Scatchard relation:

$$r/c = nK - Ksr$$

to the experimental data was made to provide values of the association constant K and the antigen valancy s. An antibody valency n = 2.0 was assumed for whole UPC-10 myeloma protein, while n = 1.0 was used for F_{ab} fragments.

Competition binding studies were also carried out to determine the binding constants of unlabeled oligosaccharides. Binding parameters were determined by a plot of $c(\frac{1-(s/n)r}{r})$ versus $[I]_{total}$ as outlined in the Appendix. Values of s = 1.85 for levan fraction 59-79 and n = 2.0 for whole UPC-10 myeloma protein were used.

Error analyses of the binding parameters were performed as discussed by Beers (1957).

RESULTS

Purification of UPC-10 Myeloma Protein

The procedure reported in Methods for the purification of UPC-10 myeloma protein represents a selection of various techniques which were employed in the effort to increase the purity of the antibody preparation. Two obstacles were encountered in this regard. First, ascites fluid, while a good source of secreted immunoglobulin, contains large concentrations of albumin. Second, the myeloma protein had to be treated as gently as possible to reduce the chance for aggregation; centrifugation was employed at many stages to eliminate such protein aggregates. This consideration was necessary in order to avoid non-specific complement activation, as will be discussed in Chapter 3.

In light of the concern over precipitating the antibody and thereby potentially altering its native conformation, an attempt was made to avoid a customary ammonium sulfate precipitation and apply filtered ascites directly to an ion-exchange gel (Fig. 2). Although this large amount of unpurified protein greatly exceeded the ion-exchange capacity of the first column, succeeding ion-exchange steps were performed with apparently good resolution between IgG and contaminants (Figs. 3,4). However, SDS gel electrophoresis which was performed on this material showed that approximately 70% of the protein that was contained in the IgG peak was albumin (Fig. 5). Thus, even repeated ion-exchange steps performed in order to separate IgG from albumin were ineffective unless preceeded by an ammonium sulfate precipitation.

Although immunoglobulins are more easily salted out of solution than is albumin, it was felt to be important to keep the salt

concentration as low as possible to prevent significant quantities of albumin from precipitating along with the desired immunoglobulin. Although an ammonium sulfate concentration of 33% saturation has been recommended for the precipitation of human IgG (Kendall, 1938), a strongly positive ring test could be obtained between bacterial levan and the supernatant from a 33% precipitation of UPC-10 ascites. The degree of saturation was therefore increased to 45% and only a slightly positive antigen-antibody precipitation reaction could be obtained using the supernatant under these conditions.

As an added step to reduce the concentration of contaminating albumin, the protein fraction precipitated by 45% saturated ammonium sulfate was then redissolved in PBS and filtered through a Cibacron Blue-Sepharose 4B column. This chromatographic separation, which was rapid and easily performed, removed a great deal of mouse serum ablumin from the preparation through interactions with the immobilized dye. The combination of salt fractionation and Cibacron-Blue absorption reduced the amount of protein present in ascites to a level low enough to be handled on ion-exchange columns of reasonable size.

A single step ion-exchange chromatography procedure following the Cibacron-Blue column was used for some time. As the pI of mouse IgG was known to be in the neighborhood of 6.6 pH units, a strong anion exchanger such as QAE-Sephadex was employed at pH 7.6 in low ionic strength buffer in order to bind both IgG and albumin and then allow selective desorption using a gradient of increasing salt concentration. However, this treatment exhibited a number of drawbacks. The high ionic strength buffer which was necessary to desorb the bound proteins also shrank the volume of the gel bed by about 50%, thereby reducing

resolution. In addition, thorough equilibration of this strong exchanger with the starting buffer was critical and lengthy, often resulting in improperly equilibrated gels and poor separations. Under optimum conditions an antibody preparation which was quite clean could be obtained, although such purity could rarely be duplicated (Fig. 6).

Thus, a procedure employing two weak ion-exchange gels without buffer gradients was employed. First, a DEAE-cellulose column run at pH 8.0 eluted IgG before the more tightly bound albumin. Second, a CM-cellulose column run at pH 5.0 eluted albumin before the IgG peak, allowing a clear separation (Fig. 7). SDS gel electrophoresis performed on the IgG fraction after each column showed that although some albumin and other high molecular weight proteins remained in the IgG fraction after the DEAE column, these contaminants were largely removed by the CM column treatment. Thus, this four-step procedure involving salt fractionation and three chromatographic steps allowed the conversion of ascites (in which UPC-10 myeloma protein is a minor contaminant) into a purified IgG preparation.

Purification of F_{ab} Fragments

Due to the fact that little structural work had ever been carried out on mouse IgG, initial attempts to produce and purify F_{ab} fragments from UPC-10 myeloma protein were patterned after the techniques developed for rabbit IgG (Porter, 1959; Putnam et al., 1962). However, the chromatographic separation of digestion products on carboxymethyl-cellulose did not proceed as expected. Rather than observing two or more distinct peaks, only one protein peak was observed (Fig. 8). SDS gel electrophoresis of the single peak from the

CM-cellulose column indicated that it contained both F_{ab} and F_c fragments (Fig. 9); the total absence of any intact heavy chain in the digested material suggested that the proteolytic step had been complete. The comigration of F_{ab} and F_c fragments on the carboxymethyl-cellulose column indicated that the isoelectric points for mouse IgG F_{ab} and F_c fragments were radically different from their rabbit counterparts.

In order to carry out an ion-exchange chromatographic separation of the mouse fragments, it was first necessary to identify their isoelectric points. An isoelectric focussing gel in the pH range 3.5-9.5 was run using the digestion mixture as a sample. After development, one half of the plate was stained for protein with Coomassie Brilliant Blue and the other half was fixed, washed and incubated with ^{125}I-protein A. The gel stained for protein indicated two sets of closely spaced bands (due to single charge differences caused by deamidation of Asn and Gln) (Awdeh et al., 1970), with one set having a pI around 6 and the other set having a pI around 7 (Fig. 10). The radiolabeled gel after autoradiography showed that the acidic set of bands (pI $\sim$ 6) bound ^{125}I-protein A, identifying these as the F_c fragment.

A chromatographic step on DE-52 cellulose at pH 6.6 was then employed to separate F_{ab} and F_c fragments. As expected, two peaks were obtained (Fig. 11) which were identified by SDS gel electrophoresis as F_{ab} fragments contaminated with a small amount of F_c fragments, and pure F_c fragments.

Identification of UPC-10 Myeloma Protein

The purified UPC-10 myeloma protein was identified as an IgG antibody by the following criteria. Reducing SDS gels showed the presence of both 25,000 and 50,000 dalton molecular weight bands, corresponding to the κ and γ chains, respectively (Fig. 9). Staphlococcus aureus cells bound the myeloma protein and removed it from solution, thereby classifying the protein as an antibody of subclass IgG2a, IgG2b or IgG3 (Fig. 9) (Goding, 1978). Finally, a positive Ouchterlony reaction was observed between UPC-10 myeloma protein and goat anti-mouse IgG.

A sedimentary analysis revealed that the myeloma protein migrated as a homogeneous species (Fig. 12). A sedimentation coefficient $S_{20,w} = 6.52 \pm .05$ was observed, in good agreement with that found for rabbit IgG ($S_{20,w} = 6.5$) (Porter, 1959). This indicated that the isolated antibody exists as a monomer in solution, and that the preparation did not contain observable amounts of aggregated protein.

Identification of Levans

The levans isolated from Aerobacter and ryegrass were identified on the basis of their reaction with UPC-10 myeloma protein. Ring tests which were performed indicated that the levans were specific antigens for the UPC-10 antibody, although the bacterial levan gave a much stronger reaction than did the grass levan on a weight basis, reflecting in part the large disparity in molecular weights between these two polymers. In addition, monomer fractions which were obtained by acid hydrolysis of bacterial levan showed a single species on paper chromatography that migrated coincident with fructose (Fig. 13).

Partial acid hydrolysis of bacterial levan using a sulfonated-polystyrene provided oligosaccharides which could be isolated by gel filtration on a 170 cm column of BioGel P-2 (Fig. 14). These fragments were identified as monomer, dimer, trimer, etc. on the basis of their mobility in paper chromatography (Fig. 13) and by their migration position on the BioGel column. Plots of elution volumn versus logarithm of molecular weight revealed a linear relationship, confirming the column peaks as sequential levan oligosaccharides (Fig. 15). Unfortunately, this purifiation could not be extended to eliminate those fragments containing β2→1 branch points, so that every fraction corresponding to dimer or larger contained both unbranched and branched fragments of that molecular weight.

In addition to oligosaccharides of discrete size, fractions of leavn fragments that were not separated into individual units were obtained from the BioGel P-2 column. One of these, designated 59-79, was eluted at the void volume of the P-2 column (Fig. 14). Using a G-50 Sephadex superfine column calibrated with levan oligosaccharides (Fig. 16), an average molecular weight M_n = 3450 could be calculated for the 59-79 fraction, corresponding to an average size of 21 sugar units. Preparative chromatography of fraction 59-79 on the same column was carried out and fractions were arbitrarily pooled as shown in Fig. 17; these fractions and their molecular weights as determined by the gel filtration analysis described above are shown in Table 1.

Determination of the Binding Parameters of UPC-10 Myeloma Protein for Levan Oligosaccharides

The association constants of UPC-10 myeloma protein for levan fraction 59-79 at 24°C and 37°C were determined directly by the method of equilibrium dialysis and are listed in Table 2. The oligosaccharide preparation which was used for these experiments consisted of a small amount of tritium-labeled fraction 59-79 diluted into unlabeled fraction 59-79. A representative Scatchard plot obtained from the binding of antibody to fraction 59-79 is shown in Fig. 18.

In a similar manner, the association constants of both intact antibody and F_{ab} fragments for reduced fraction 59-79 (designated 59-79-OH) at 37°C were determined and are also shown in Table 2. For these experiments a small amount of tritium-labeled fraction 59-79 was diluted into a preparation of fraction 59-79 which had been reduced with cold $NaBH_4$.

The association constants of UPC-10 myeloma protein for discrete levan oligosaccharides (tetramer, pentamer, hexamer, heptamer, and dodecamer) were measured by equilibrium dialysis utilizing competition between the unlabeled oligomer and tritium-labeled fraction 59-79. These values are listed in Table 2 and show a marked dependence of binding affinity upon molecular weight.

Each competition binding experiment provided a measurement of the association constant of UPC-10 myeloma protein for labeled fraction 59-79 as well as for the unlabeled small oligosaccharide. These values are listed for comparison in Table 3. The value of the association constant of the intact antibody for fraction 59-79 at 4°C which is

listed in Table 2 was obtained in this manner for a competition between dedecamer and fraction 59-79.

The association constants of UPC-10 myeloma protein for fraction 59-79 were plotted as a function of temperature as shown in Fig. 19. In this way, the enthalpy change upon binding was calculated to be ΔH = -13.8 kcal/mole, while the entropy change of binding was ΔS = -24 cal/mole °K.

In addition to plotting binding data by the method of Scatchard (1949), Sips plots were used to assess the degree of homogeneity of antibody binding sites (Sips, 1948; Day, 1972). Using this method to analyze the binding of fraction 59-79 to UPC-10 myeloma protein (Fig. 20), a heterogeneity index (a) was measured to be 0.97; the index exists in the range $0 \leq a \leq 1$ with a value of 1 indicating all sites are identical (Eisen, 1964).

Along with the determination of association constants, it was possible to calculate a value of antigen valency (s) for fractions 59-79 in several experiments after assuming an antibody valency of n = 2.0 for intact myeloma protein or n = 1.0 for F_{ab} fragments; these values are shown in Table 2. The value of s = 1.85 for the valency of 59-79 at 37°C was used in the calculation of association constants for the oligosaccharides measured in competitive binding experiments.

DISCUSSION

For the purposes of this functional characterization of UPC-10 myeloma protein, it was critically important to obtain an antibody preparation which has not been exposed to harsh treatments and was free of contaminating albumin. Unfortunately, the direct method of affinity chromatography required the use of denaturing agents in order to achieve elution of the antibody from the column (Vrana et al., 1976). As discussed in Results, approaches originally designed for the isolation of more commonly used rabbit IgG through the use of ion-exchange chromatography had to be modified due to the lower isoelectric point of mouse IgG (pI = 6.6). This purification scheme yielded a highly purified IgG preparation while avoiding as much as possible agents which might denature or aggregate the protein molecules.

Since the isolation of defined levan oligosaccharides was lengthy, it was not possible to generate sufficient material in order to carry out equilibrium dialysis experiments in conventional apparatus containing 1-2 ml sample chambers. This problem was avoided through the use of smaller 100 µl chambers; accuracy of the results, therefore, was dependent upon the ability to minimize pipetting errors as much as possible through the use of microliter syringes and multiple determinations. In this way, each point on a Scatchard plot represents a total of 9 independent measurements from 3 separate but identical experiments. The use of small chamber volumes allowed antigen concentrations to be high enough to attain a broad range of r values in the Scatchard plot, thereby avoiding the necessity of having to extract binding parameters from a small cluster of data points (Fig. 18).

In addition to the error limits imposed upon the affinity constants as a result of counting and pipetting errors, an estimate of the accuracy of the calculated association constants of UPC-10 myeloma protein for fraction 59-79 can be obtained from a comparison of the values measured directly by equilibrium dialysis and indirectly by competition with oligosaccharide inhibitors (Table 3). It can be seen that there is reasonable agreement between the directly measured value and the estimates obtained from competition experiments with heptamer and hexamer. The failure of the pentamer and tetramer competitions to provide good estimates is not surprising in light of the difficulty in obtaining binding data for such weakly binding ligands.

As is noted in the Appendix, the assumption (necessary for the analysis of competitive binding experiments) that the concentration of bound inhibitor is approximately equal to the concentration of total inhibitor is usually true if the total number of antibody sites is less than the inhibitor dissociation constant K_i. As the greatest antibody concentration used in these studies was 10^{-4} M, this condition is easily met for tetramer, pentamer, and hexamer, and is just barely met for heptamer ([sites] = 1.98×10^{-4} M, $K_i = 2.08 \times 10^{-4}$ M). However, this limit is clearly exceeded for the dodecamer dissociation constant and it therefore may not be an accurate result; also, the association constant for fraction 59-79 at 4°C was obtained from the intercept of the competition plot for the dodecamer hapten and may be an overestimate of the true K_i.

Although an examination of the affinity constants of UPC-10 myeloma protein for levan oligosaccharides can provide important information concerning the interaction of antibody and antigen, this analysis is

complicated by the fact that the oligosaccharides were derived from a branched polymer. Thus, although the hexamer fraction, for instance, contains only molecules of molecular weight 990 daltons (corresponding to six fructose residues), this fraction is a mixture of oligosaccharides containing from 0 to 5 β2→1 branches. The proportion of each species can be calculated by considering all of the different isomeric forms, which has been done for monomer through heptamer and is shown in Table 4. Using the methylation data of Lindberg et al. (1973) a branch point occurs on the average every 5.5 residues. This means that the probability of a branch point at a given sugar residue is 0.18 and this value can be used to calculate the probabilities of species with one or more branches, at the same time keeping the total probability of all species of that molecular weight normalized to 1.0. These proportions are shown in Table 5.* These values indicate that the proportion of unbranched oligosaccharide decreases rapidly as the size of the oligosaccharide increases, so that for the smallest oligosaccharide for which an association constant can be measured (tetramer), the proportion of unbranched chains is only 38%. Since all evidence currently available suggests that UPC-10 myeloma protein binds only to β2→6 fructosan chains (Cisar et al., 1974), this means that the association constants measured for each oligosaccharide size are in fact underestimates of the affinity constants which would be measured for the unbranched haptens. A more detailed analysis is not possible without being able to measure binding constants of UPC-10 myeloma protein for defined branched oligosaccharides; such measurements would require the

*This analysis assumes that the probability of glycosidic bond cleavage is the same for β2→6 and β2→1 bonds. The rates of hydrolysis in either case will depend upon the stability of the same transition state, and will therefore be identical.

purification of oligosaccharides based upon the molecular weight and the number and position of branches, a separation which has not yet been possible.

In light of these considerations, a plot of ΔG values of binding at 37°C versus oligosaccharide size (Fig. 21) suggests a rapid initial increase in binding energy with increasing size to pentamer followed by a more gradual increase in binding energy up to at least that seen with a oligosaccharide preparation of average molecular weight corresponding to a 21-mer (fraction 59-79). This is in contrast to the binding energies observed upon association of dextran haptens with anti-dextran antibodies (Cisar et al., 1974); with these haptens, the binding energy increases with increasing hapten size until a plateau is reached, usually at pentamer or hexamer. Such a plateau signals that the maximum molecular complementarity has been reached between ligand and protein and that no further binding interactions can occur. (Indeed, one might expect a gradual decrease in negativity of ΔG of binding oligosaccharides of increasing size larger than those exhibiting maximum complementarity, due to the fact that ΔH, reflecting the binding interaction, is at a maximum constant value while ΔS becomes increasingly more negative with larger oligosaccharides that must lose transitional and rotational degrees of freedom upon binding.) That such a behavior is not exhibited by the UPC-10 myeloma protein-levan oligosaccharide system does not suggest that the antibody binding site can accommodate oligosaccharides larger than hexamer. The most likely explanation for the gradual increase in binding energy over the size range of hexamer and larger is that the proportion of branched fragments in the oligosaccharide preparation to which the antibody can bind

gradually increases with increasing oligosaccharide molecular weight. Due to the inability to calculate the relative contributions of various branched species to the observed binding energies, an estimate of the maximum complementary size of an unbranched oligosaccharide to UPC-10 myeloma protein cannot be made. Likewise, a determination of whether UPC-10 myeloma protein has a terminal or central specificity cannot be made without more detailed binding information, although the fact that the antibody has a comparable affinity for levan fraction 59-79 reduced and unreduced suggests that the oligosaccharide terminus does not have an appreciable contribution to the binding interactions.

In order to obtain binding data using levan fraction 59-79, a small amount of tritiated-labeled fraction 59-79 was diluted into unlabeled fraction 59-79 to provide a means of easily measuring the concentration of carbohydrate in each well. However, since the tritium labeling reaction requires reduction of the C2 carbon of the terminal fructose in the oligosaccharide, this terminal sugar is no longer able to cyclize to form the usual furanose ring. This presents the possibility that the labeled, reduced oligosaccharide, which is the only form of the oligosaccharides present that is measured, might bind differently to the antibody molecule than the unlabeled oligosaccharide. If so, this would mean that the binding constants which were obtained through the use of tritiated-labeled fraction 59-79 diluted into unreduced fraction 59-79 would not accurately represent the binding of antibody to unlabeled antigen. However, the similarity in association constants seen for both the reduced and unreduced oligosaccharide preparations supports this method.

The F_{ab} fragments can be seen to have an affinity for reduced fraction 59-79 that is comparable, although slightly less, than that shown by intact antibody. As will be discussed in Chapter 3, one hypothesis of effector function activation would require that a portion of the free energy released by antigen binding be used to perform energetically unfavorable conformational changes in the F_c portion of the molecule. This would mean that the intact antibody would be expected to have a lower observed association constant for antigen than would the F_{ab} fragment, since only the intact antibody would be able to transfer some binding energy to a distant point in the molecule. The data show, however, that this is not the case for UPC-10 myeloma protein; indeed, the intact antibody exhibits a greater affinity for antigen than does the F_{ab} fragment, with this difference translating into an additional -197 cal/mole of free energy of binding. Such an argument cannot rule out small conformational changes which may occur in the vicinity of the antigen binding site and would thus be present in both the F_{ab} fragment and in the intact molecule; such alterations have been inferred from kinetic data (Maeda et al., 1977) and may have been monitored in other antibody-antigen systems by a variety of techniques (Chapter 3).

The heterogeneity index of 0.96 obtained from a Sips plot constructed for the binding of levan fraction 59-79 to whole UPC-10 myeloma protein at 37°C indicates that the antibody preparation is extremely homogeneous. This confirms that the myeloma protein is functionally, as well as biochemically, pure and that all of the antibody binding sites are identical, thereby validating the assumption

of independence which was made in the analysis of the binding parameters from competition experiments.

The thermodynamic parameters obtained for the binding of fraction 59-79 to UPC-10 myeloma protein are quite reasonable in terms of the nature of the expected antigen-antibody interactions. Since the antigen is a carbohydrate, the predominant interactions between it and either the protein or solvent water will be hydrogen bonds, and thus the protein cannot exhibit large amounts of complex stabilization over the hydrated carbohydrate in solution. Since the association constant will reflect only the enhanced stabilization of the antigen by the antibody over that afforded by the solvent alone, one might expect lower association constants for carbohydrate-binding antibodies as opposed to antibodies which can provide enhanced stabilization through ionic forces, such as anti-phosphoryl choline myeloma proteins (Goetze and Richards, 1977), or charge-transfer interactions, such as anti-DNP myeloma proteins (Hardy and Richards, 1978). In addition, any favorable negative enthalpy terms for binding will be partially offset by negative entropy changes upon binding due to the large, flexible nature of the carbohydrate antigen. This is in contrast to some hapten-antibody systems in which a positive ΔS of binding is observed (Sehon, 1971), such an effect being attributed to the release of protein-bound water upon antigen binding. While keeping in mind that the association constants measured here of UPC-10 myeloma protein for a mixed population of branched and unbranched oligosaccharides are underestimates of the true association constants for the unbranched haptens, these values can be compared with those obtained for other anti-carbohydrate antibodies and their corresponding haptens. In all cases, the maximum association

constant observed between antibody and hapten is in the range 10^4-10^5 M^{-1} (this is true for anti-galactan (Jolley et al., 1973), anti-dextran (Sugii et al., 1979), and anti-inulin antibodies (Streefkirk and Glaudemans, 1977; Das et al., 1979)). In accordance with these findings with UPC-10 myeloma protein, there is no obvious relationship between affinity constants of intact anti-inulin antibodies and F_{ab} fragments for their corresponding oligosaccharides, although statistically significant differences can be measured (Streefkirk and Glaudemans, 1977). These observed differences might be attributable to protein conformational changes occurring at the binding site upon removal of the F_c fragment, although in the case of anti-carbohydrate antibodies this question has not been addressed.

APPENDIX

A relation between the binding of an unlabeled monovalent inhibitor and a labeled antigen of valency s to an antibody of valency n can be derived from the laws of mass action if the binding sites on the antibody molecule are independent. Thus, the total number of antibody binding sites is equal to the number of free sites plus the number of sites occupied by antigen plus the number of sites occupied by inhibitor. Using the subscripts "t" to indicate total, "f" to indicate free or unbound, and "b" to indicate bound,

$$n[Ab]_t = n[Ab]_f + s[Ag]_b + [I]_b \tag{1}$$

where [Ab], [Ag] and [I] represent the molar concentrations of antibody, labeled antigen, and unlabeled inhibitor, respectively. Dissociation constants for the binding of antigen and for inhibitor can be written as:

$$K_{Ag} = \frac{\text{(free Ab sites) (free Ag determinants)}}{\text{bound Ag determinants}} = \frac{n[Ab]_f\ s[Ag]_f}{s[Ag]_b} = \frac{n[Ab]_f\ [Ag]_f}{[Ag]_b} \tag{2}$$

$$K_I = \frac{\text{(free antibody sites) (free inhibitor)}}{\text{(bound inhibitor)}} = \frac{n[Ab]_f\ [I]_f}{[I]_b} \tag{3}$$

Equations 2 and 3 can be substituted into equation 1 to give:

$$n[Ab]_t = n[Ab]_f + \frac{n[Ab]_f\ s[Ab]_f}{K_{Ag}} + \frac{n[Ab]_f\ [I]_f}{K_I} \tag{4}$$

This equation can be rearranged:

$$[Ab]_f = \frac{[Ab]_t}{1 + \frac{s[Ag]_f}{K_L} + \frac{[I]_f}{K_I}} \tag{5}$$

This relation for the concentration of free antibody can be substituted into equation 2 to give an expression for bound antigen:

$$[Ag]_b = \frac{[Ag]_f \, n[Ab]_t}{K_L + s[Ag]_f + [I]_f \frac{K_L}{K_I}} \tag{6}$$

The degree of antibody saturation with antigen, r, is given by:

$$r = \frac{[Ag]_b}{[Ab]_t} \tag{7}$$

Substituting equation 6 into equation 7:

$$r = \frac{n[Ag]_f}{K_L + s[Ag]_f + [I]_f \frac{K_L}{K_I}} \tag{8}$$

Using the conventional notation of c for $[Ag]_f$, this expression can be rearranged to give:

$$c \left(\frac{1 - (\frac{s}{n})r}{n}\right) = \frac{K_L}{nK_I} [I]_f + \frac{K_L}{n} \tag{9}$$

Since $[I]_f$ cannot be measured directly, the approximation $[I]_f \sim [I]_{total}$ is valid if $[I]_b$ is low compared to K_I, a condition which is usually satisfied if $n[Ab]_t < K_I$ (Williams and Lefkowitz, 1978); if $n[Ab]_t \geq K_I$, the value of K_I which is measured will be an overestimate of the true K_I.

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Table 1

column fraction	Mn
91- 95	12,700
96-100	10,200
101-105	8,100
106-110	6,500
111-115	5,200
116-120	4,100
121-125	3,300
126-130	2,600
131-135	2,100
136-140	1,700

Table 2

BINDING PARAMETERS FOR UPC-10 MYELOMA PROTEIN

Temperature (°C)		37°	24°	4°
<u>whole antibody</u> (n = 2.0)				
antigen:	fraction 59-79	$K = 2.72 (\pm 0.05) \times 10^4\ M^{-1}$ $s = 1.85 (\pm 0.06)$	$K = 6.40 (\pm 0.03) \times 10^4\ M^{-1}$ $s = 1.75 (\pm 0.02)$	$K = 3.8 (\pm 0.4) \times 10^5\ M^{-1}$*
	fraction 59-79-OH	$K = 3.20 (\pm 0.03) \times 10^4\ M^{-1}$ $s = 1.56 (\pm 0.03)$		
	dodecamer			$K = 3.8 (\pm 0.4) \times 10^5\ M^{-1}$*
	heptamer	$K = 4800 (\pm 700)\ M^{-1}$		
	hexamer	$K = 3000 (\pm 600)\ M^{-1}$		
	pentamer	$K = 1600 (\pm 400)\ M^{-1}$		
	tetramer	$K = 290 (\pm 110)\ M^{-1}$		
<u>F_{ab} fragments</u>				
	fraction 59-79-OH	$K = 2.33 (\pm 0.04) \times 10^4\ M^{-1}$ $s = 1.72 (\pm 0.05)$		

*See discussion in text.
All binding constants are written as association constants.

Table 3

ASSOCIATION CONSTANTS OF UPC-10 MYELOMA PROTEIN FOR LEVAN FRACTION 59-79 AT 37°C

Competing Ligand	K_a
none	$2.72 (\pm 0.05) \times 10^4\ M^{-1}$
heptamer	$3.7 (\pm 1.3) \times 10^4\ M^{-1}$
hexamer	$3.3 (\pm 0.4) \times 10^4\ M^{-1}$
pentamer	$5.6 (\pm 1.7) \times 10^4\ M^{-1}$
tetramer	$4.1 (\pm 0.6) \times 10^4\ M^{-1}$

Table 4

NUMBER OF UNIQUE MOLECULAR STRUCTURES OF BRANCHED LEVAN OLIGOSACCHARIDES

Number of β2→1 branches	Number of glycosidic bonds							
	0	1	2	3	4	5	6	7
0	1	1	1	1	1	1	1	1
1	0	1	3	6	10	15	21	28
2	0	0	1	6	21	55	120	231
3	0	0	0	1	10	55	215	665
4	0	0	0	0	1	15	120	665
5	0	0	0	0	0	1	21	231
6	0	0	0	0	0	0	1	28
7	0	0	0	0	0	0	0	1

Table 5

EXPECTED PROPORTION OF BRANCHED FORMS IN OLIGOSACCHARIDE FRACTIONS

Number of β2→1 branches	Number of glycosidic bonds							
	0	1	2	3	4	5	6	7
0	1.0	0.82	0.58	0.38	0.23	0.13	0.07	0.04
1		0.18	0.38	0.50	0.51	0.44	0.33	0.23
2			0.03	0.11	0.23	0.35	0.41	0.41
3					0.02	0.08	0.16	0.26
4							0.02	0.06
5								
6								
7								

Table 6

COMPOSITION OF WELLS FOR EQUILIBRIUM DIALYSIS

A. Composition of Sample Wells

	chamber H		chamber P
well number	Ag (µl)	PBS/T (µl)	Ab (µl)
1	0	50	50
2	2	48	50
3	4	46	50
4	6	44	50
5	8	42	50
6	12	38	50
7	18	32	50
8	26	24	50
9	34	16	50
10	42	8	50
11	50	0	50

B. Composition of Control Wells

	chamber H		chamber P		
well number	Ag (µl)	PBS/T (µl)	Ab (µl)	Ag (µl)	PBS/T (µl)
1	50	0	0	0	50
2	50	0	0	0	50
3	50	0	0	0	50
4	50	0	0	0	50
5	0	50	25	25	0
6	0	50	25	25	0
7	25	25	25	0	25
8	25	25	25	0	25

Figure 1

This is a reference plot for the determination of ketohexose concentration using the orcinol assay with fructose as the standard.

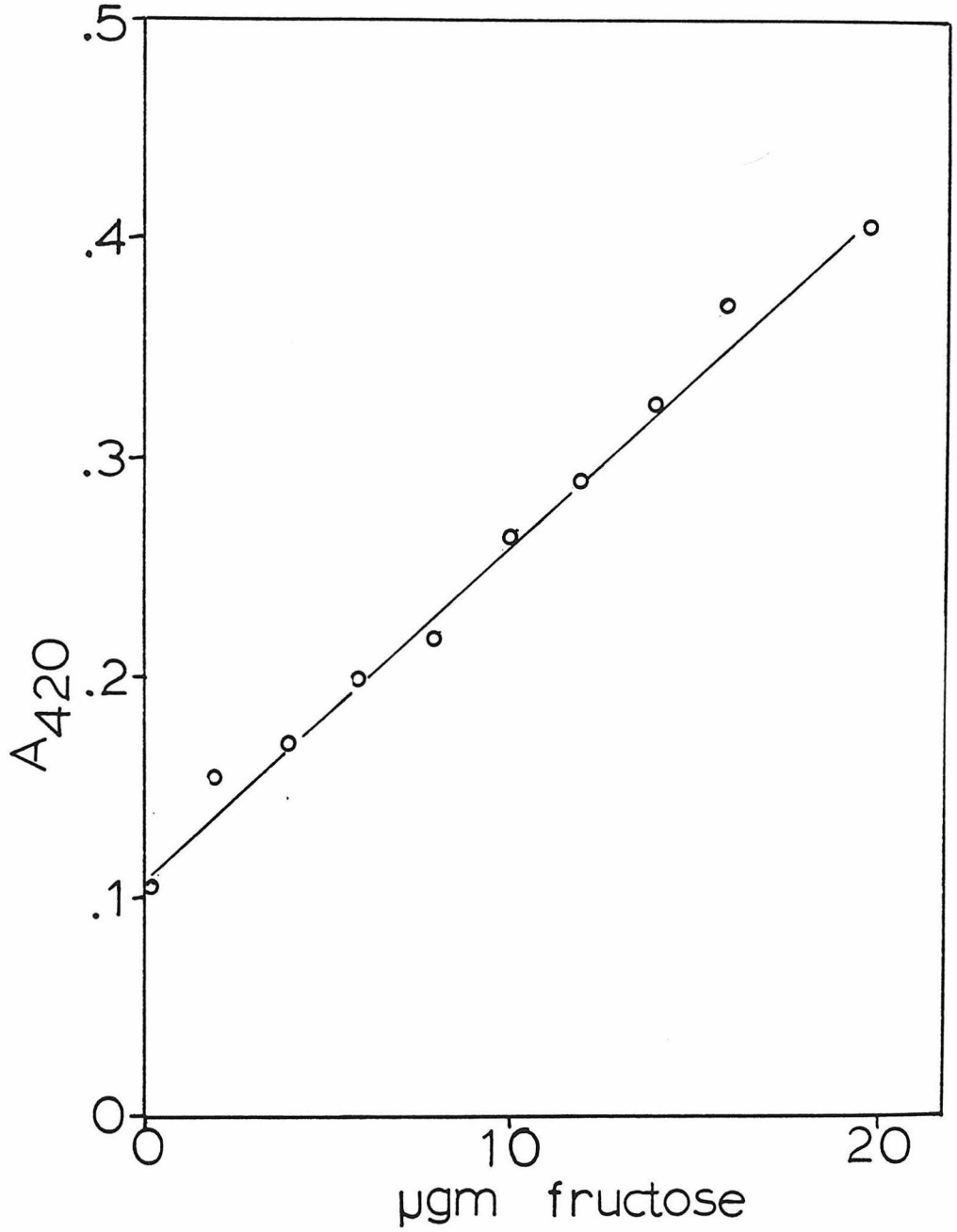

.5
.4
.3
.2
.1
0
A_{420}
0
10
20
µgm fructose

Figure 2

A column (5 cm diameter x 14 cm) of QAE-Sephadex (Q-50) was equilibrated with 0.05 M Tris, pH 7.6. UPC-10 ascites (100 mls) was filtered through glass wool, centrifuged at 8000 g for 30 min. and applied to the column which was then washed with 0.1 M Tris, pH 7.6 until the absorbance of the effluent at 280 nm had dropped below 0.09 OD units. A linear buffer gradient was begun from 0.1 M Tris, pH 7.6 to 0.1 M Tris, 0.5 M NaCl, pH 7.6 in a total volume of 1 l. The run was terminated at the completion of the gradient.

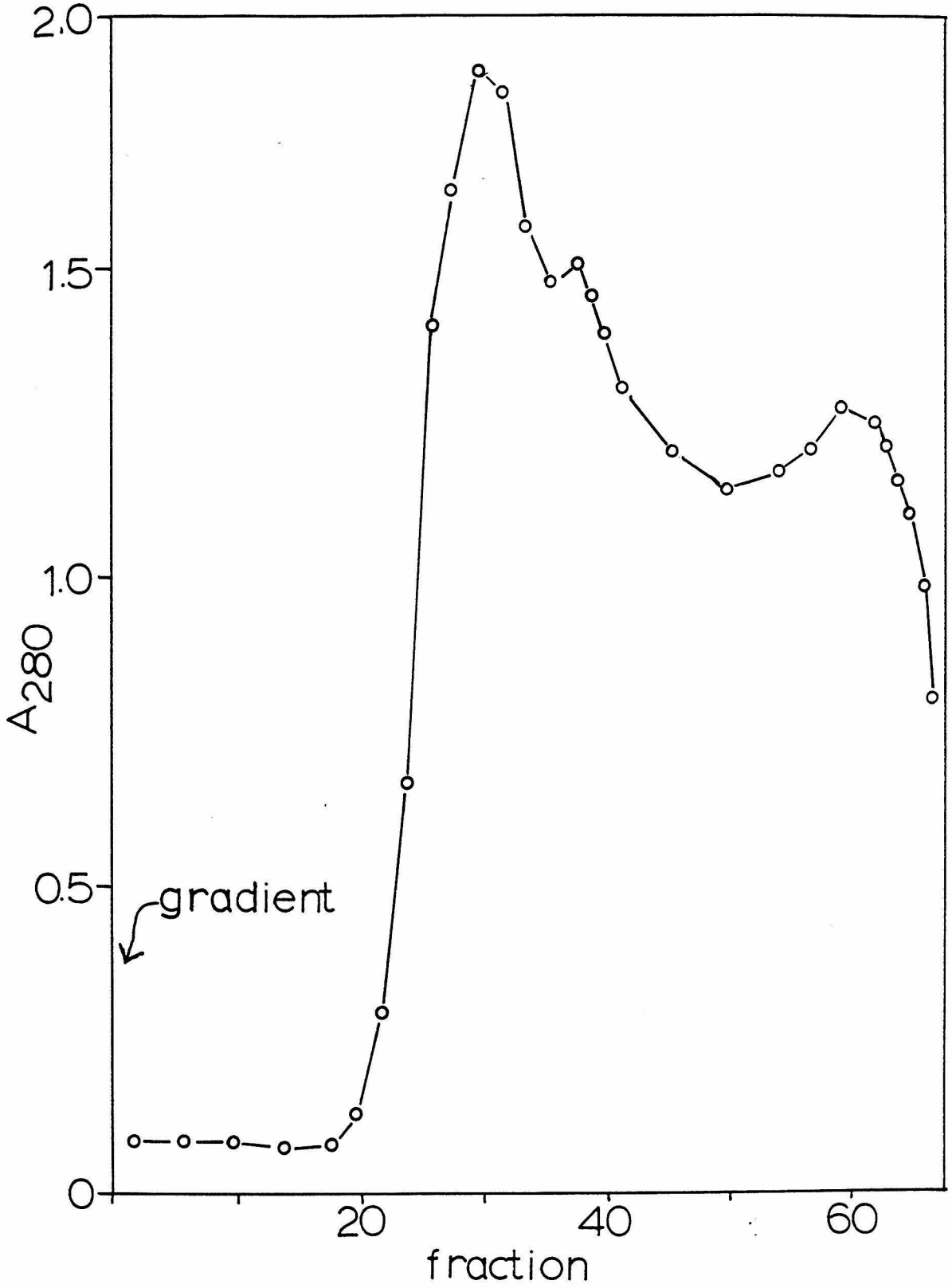

2.0
1.5
1.0
0.5
0
A280
gradient
20
40
60
fraction

Figure 3

The protein eluted from the QAE-Sephadex column shown in Fig. 2 was concentrated, dialyzed against running buffer and applied to a column (5 cm diameter x 9 cm) of DE-52 cellulose equilibrated with 0.02 M phosphate, pH 7.2 and washed with the same buffer. The indicated fractions were pooled.

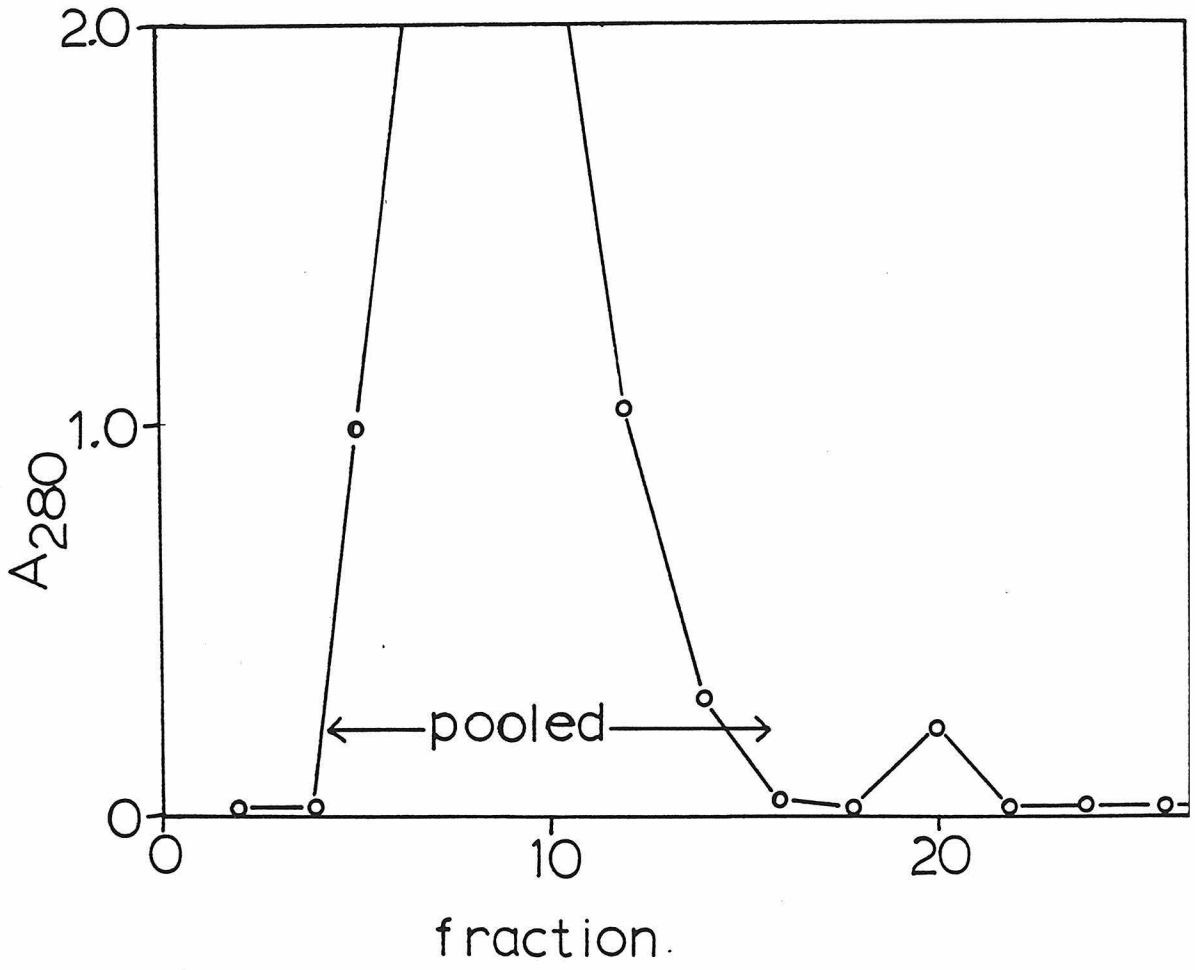

2.0
1.0
0
A_{280}
pooled
0
10
20
fraction.

Figure 4

The pooled fractions from the DE-cellulose column shown in Fig. 3 were concentrated, dialyzed against starting buffer and applied to a column (5 cm diameter x 30 cm) fo QAE-Sephadex (Q-50) equilibrated with 0.1 M Tris, pH 7.6 and washed with the same buffer. A linear buffer gradient was begun for 0.1 M Tris, pH 7.6 to 0.1 M Tris, 0.5 M NaCl, pH 7.6 in a total volume of 600 mls. The indicated fractions were pooled.

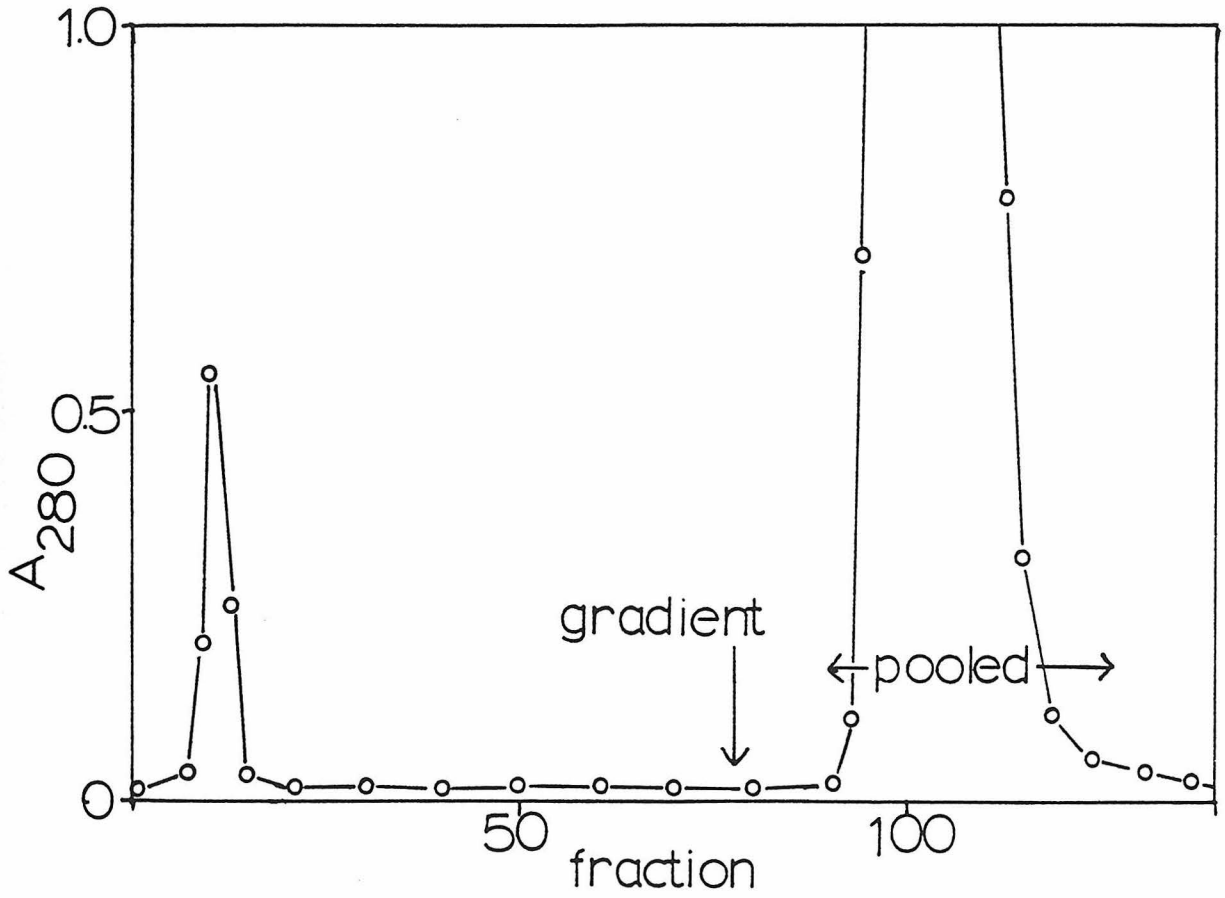

1.0
0.5
0
A_{280}
gradient
pooled
50
100
fraction

Figure 5

An analytical SDS electrophoresis slab gel with an exponential 8 → 18% acrylamide gradient was prepared and run at 20 mA. Samples were reduced before application. Sample #1 - standards; sample #2 - aliquot of pooled fractions from the QAE-Sephadex column shown in Fig. 4.

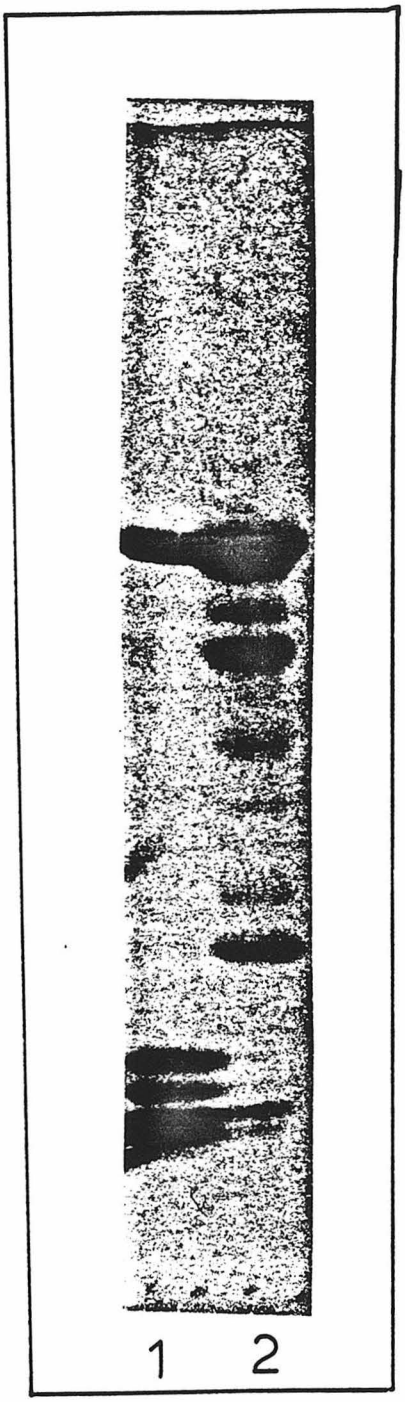

1
2

Figure 6

A) Pooled fractions from a Cibacron Blue-Sepharose column were concentrated, dialyzed against starting buffer and applied to a column (5 cm diameter x 20 cm) of QAE-Sephadex (Q-50) equilibrated and washed with 0.1 M Tris, pH 7.6. A linear buffer gradient was begun from 0.1 M Tris, pH 7.6 to 0.1 M Tris, 0.5 M NaCl, pH 7.6 in a total volume of 2 l. Open circles - absorbance at 280 nm. Closed circles - conductance. The inset shows an analytical SDS electrophoresis slab gel with an exponential 8 → 18% acrylamide gradient. Samples were reduced before application. Sample #1 - standards; sample #2 - aliquot of pooled fractions from above column.

B) Same column conditions as in A) except that the column length was 30 cm, buffer pH 7.45 and gradient volume 1600 ml (these differences would be expected to improve purity over that seen under conditions in A) above). Inset shows an SDS 7.5% acrylamide disk gel with aliquot of pooled fractions from above column, sample was reduced before application.

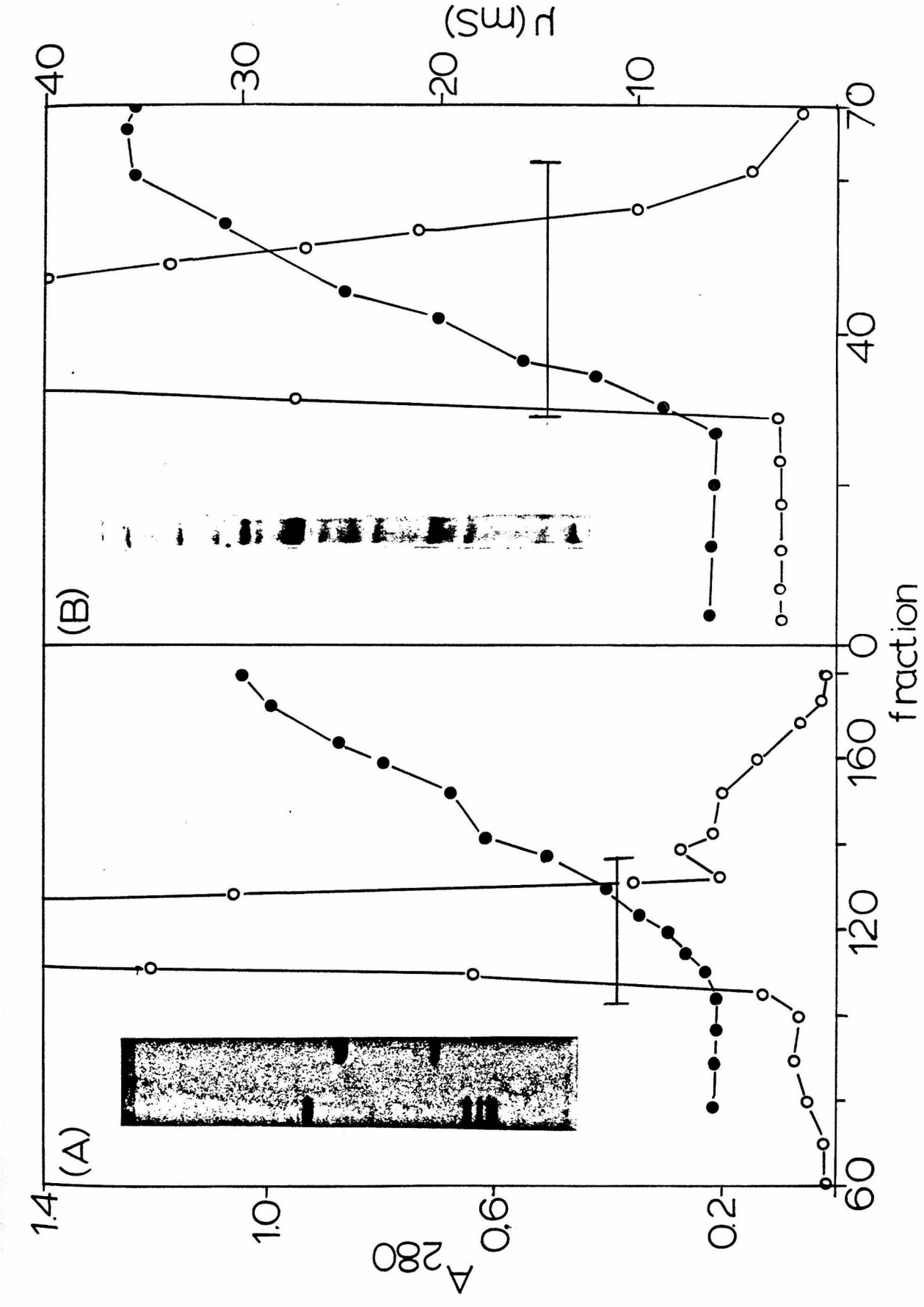

(A)
(B)
A_{280}
μ (mS)
fraction
1.4
1.0
0.6
0.2
40
30
20
10
60
120
160
0
40
70

Figure 7

Pooled fractions from the DE-52 cellulose column (described in text) were concentrated, dialyzed against running buffer and applied to a column (5 cm diameter x 30 cm) of CM-52 cellulose equilibrated and run with 0.05 M sodium acetate, pH 5.0. The indicated fractions were pooled. Inset shows SDS 7.5% acrylamide disk gels; samples were reduced before application. Sample #1 - aliquot of pooled fractions from DE-cellulose column described above. Sample #2 - aliquot of pooled fractions from CM-cellulose column shown in this figure.

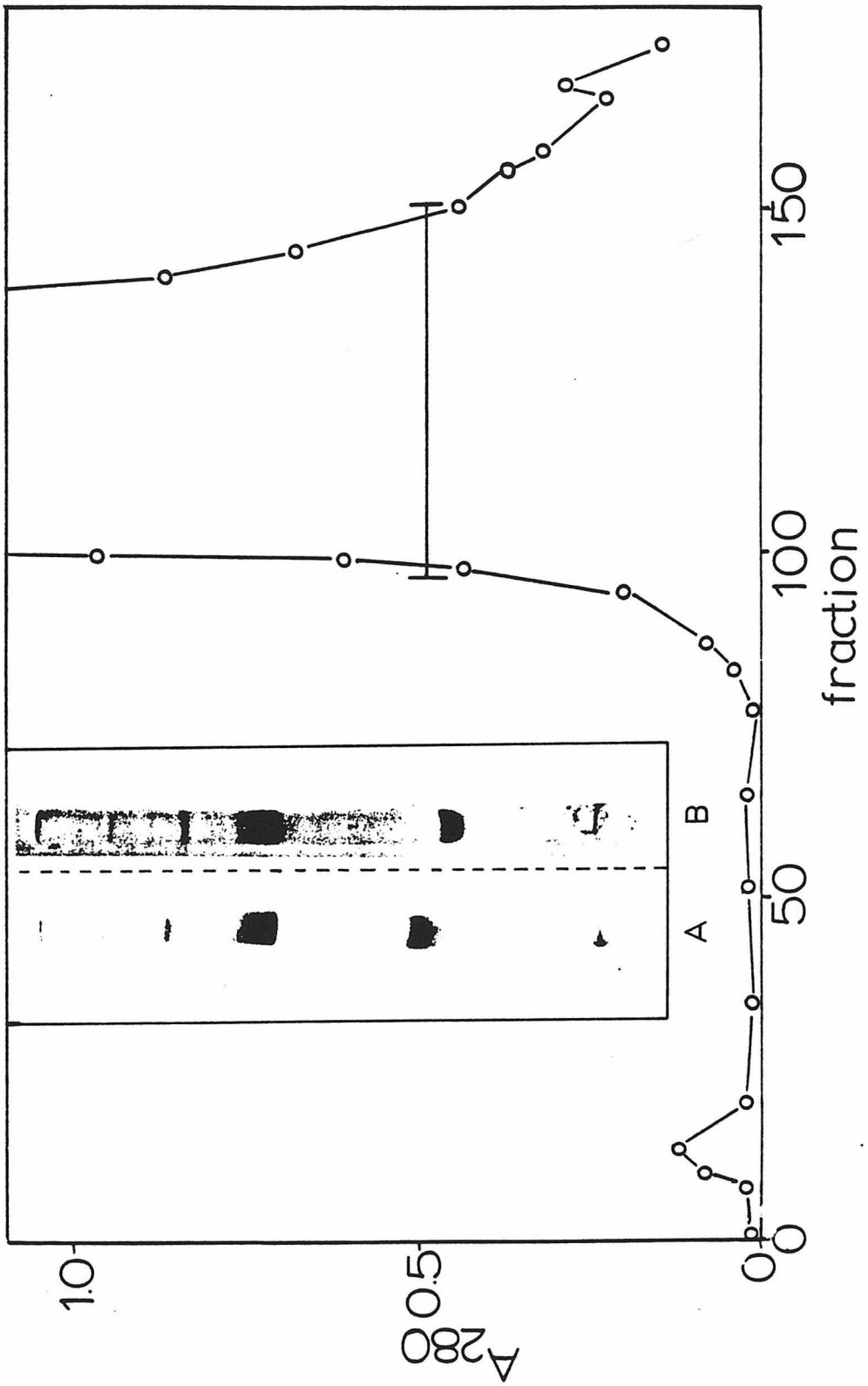

1.0
0.5
0
A_{280}
0
50
100
150
fraction
A
B

Figure 8

A quenched papain digestion mixture was dialyzed against running buffer and applied to a column (2.5 cm diameter x 23 cm) of CM-52 cellulose equilibrated with 0.01 M sodium acetate, pH 5.5 and run with 100 mls of the same buffer. A linear buffer gradient was begun from 0.01 M sodium acetate, pH 5.5 to 0.1 M sodium acetate, pH 5.5 in a total volume of 800 mls. The indicated fractions were pooled. Open circles - absorbance at 280 nm. Closed circles - conductance.

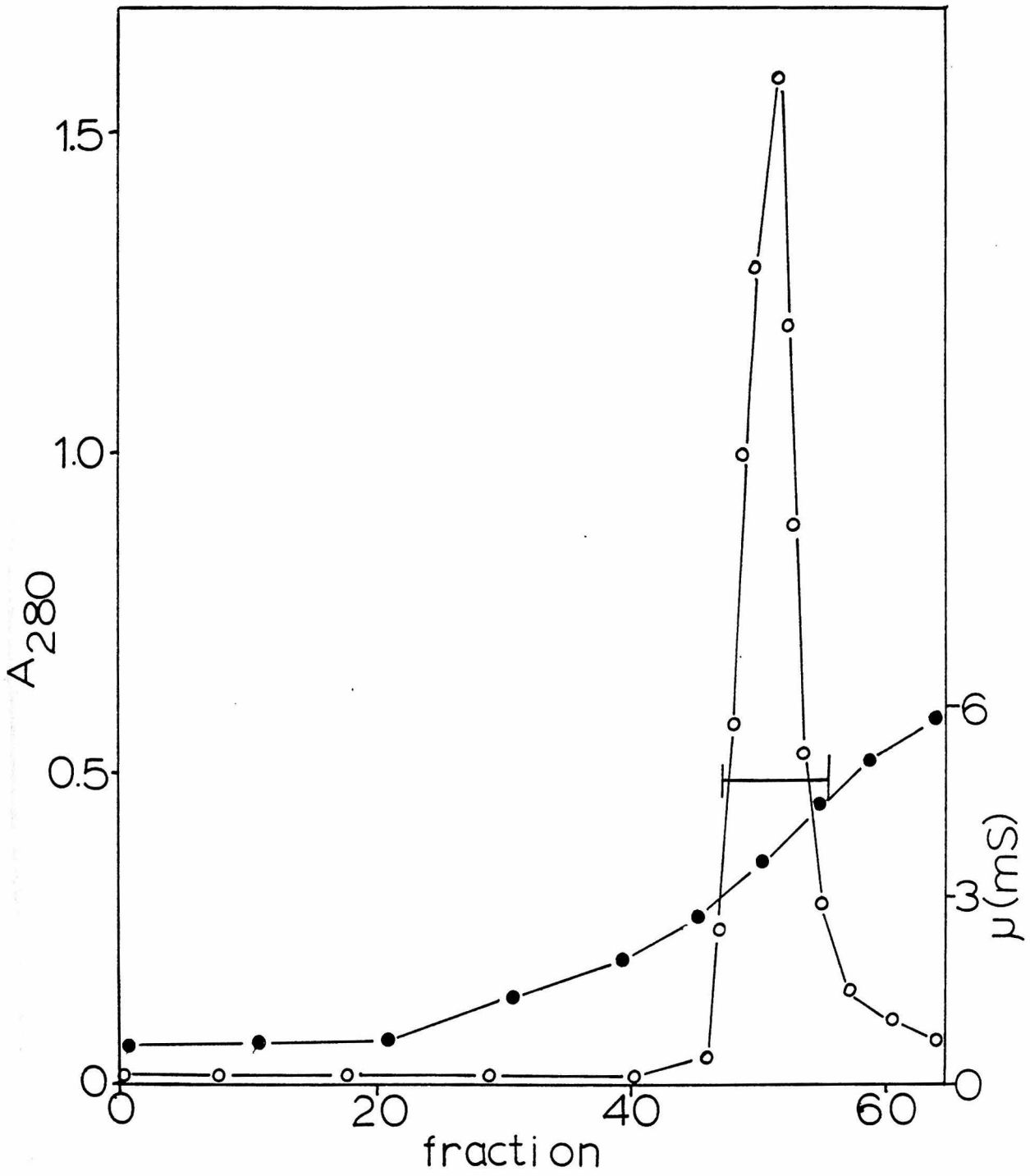

1.5
1.0
0.5
0
A_{280}
0
20
40
60
fraction
6
3
0
μ(mS)

Figure 9

An analytical SDS electrophoresis slab gel with an exponential 8 → 18% acrylamide gradient. Samples were reduced before application. Some samples were treated with whole Staphylococcus aureus before electrophoresis as follows. Pickled S. aureus (Calbiochem) in a 5% aqueous suspension were washed repeatedly in PBS and resuspended at a concentration of 25% (w/v). Aliquots of bacteria were added to samples and incubated 15 minutes at room temperature; the tubes were then centrifuged to pellet the bacteria and the supernatants were mixed with reducing sample buffer and processed as usual. Sample #1 - standards; sample #2 - same as sample #3 except that 7.5 μgm UPC-10 myeloma protein was treated with 50 μl of stock S. aureus solution; sample #3 - UPC-10 myeloma protein; sample #4 - papain digestion mixture after chromatography of CM-cellulose as shown in Fig. 8; sample #5 - same as sample #4 except that 7 μgm of digested protein was treated with 25 μl of stock S. aureus solution. Sample #3 shows that the antibody used for the digestion was well purified and contained no detectable albumin; heavy and light chains can be easily identified. Sample #2 indicates that the S. aureus treatment is effective in binding and removing UPC-10 myeloma protein from the sample. Sample #4 demonstrates that the digest consists predominantly of 2 closely spaced bands positioned at molecular weights close to that of the light chain, and a single band approximately 5000 daltons heavier. Since the doublet contains one band running at the light chain position and another marginally heavier, these probably correspond to the F_{ab} fragment, consisting of an intact light chain and a heavy chain fragment containing V_H, C_H1, and part of the hinge region. In addition, the bands comprising the doublet are

stronger in staining intensity than is the singlet, again suggesting that the doublet corresponds to the F_{ab} fragment and the singlet to the F_c fragment, in agreement wtih the expected molar ratio of $(F_{ab})/F_c = 2$. Sample #5 confirms this assignment as S. aureus cells are able to bind and remove from the samples the fragment corresponding to the singlet band, while not affecting the doublet. This identifies the singlet as being due to the F_c fragment, since protein A binds to the F_c portion of the immunoglobulin molecule (Goding, 1978).

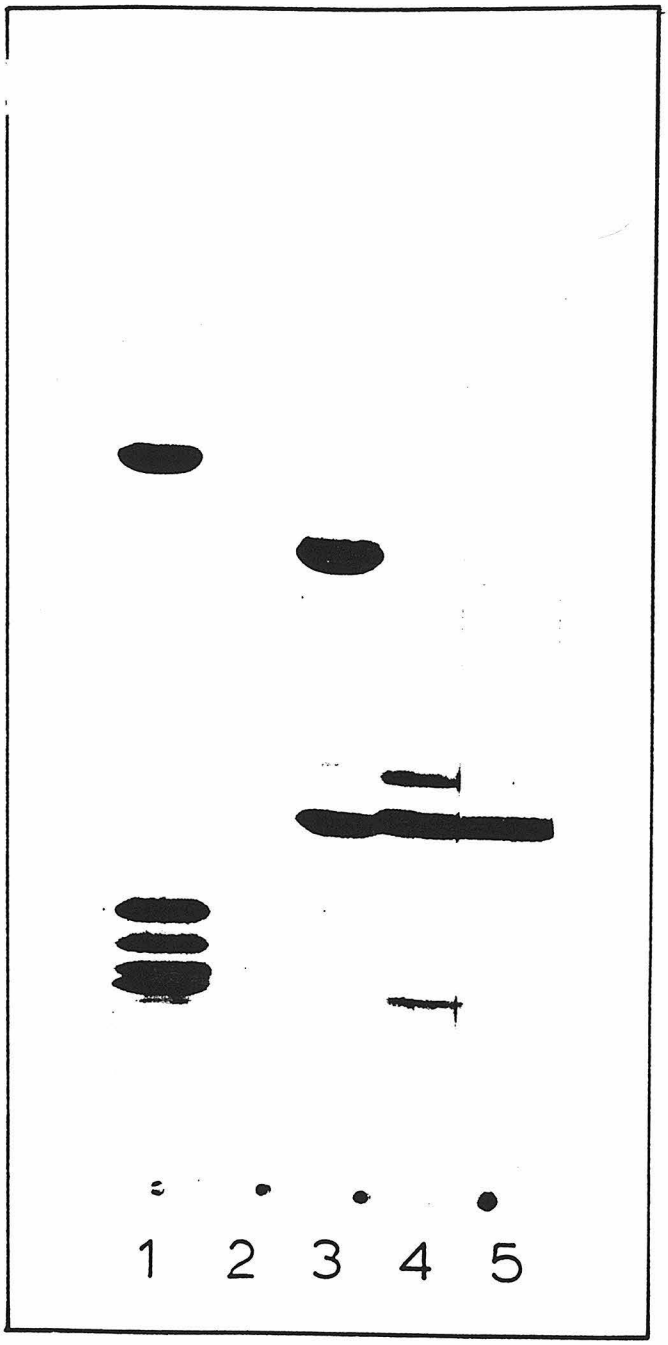

1 2 3 4 5

Figure 10

An Anphiline PAG isoelectric focusing gel was run in the pH range 3.5-9.5 as directed by the manufacturers (LKB). The sample (run in duplicate) consisted of pooled fractions of papain-digested UPC-10 myeloma protein after chromatography on CM-cellulose as shown in Fig. 8. Fixing, staining and destaining solutions were prepared as directed. The gel was fixed overnight and then cut in half to separate the duplicate samples. One half was stained with Coomassie brilliant blue and destained. The other half was washed for 48 hours at room temperature with frequent changes of PBS; the washing was considered complete when the pH of the wash solution was 7.4. The gel was then incubated for 48 hours at room temperature in 60 mls of 1% BSA (Sigma) in PBS to which were added 3.2 μg of ^{125}I-protein A (specific activity 4.4 μCi/μg protein). Following this treatment, the gel was washed with 1% BSA in PBS for 24 hours and then PBS alone for an additional 24 hours, dried and sealed in plastic. Autoradiography was performed using preflashed Kodak X-Omat AR film and an intensifying screen (Cronex Lightning-Plus, Dupont); the film was exposed for 8 hours at -70°C and developed.

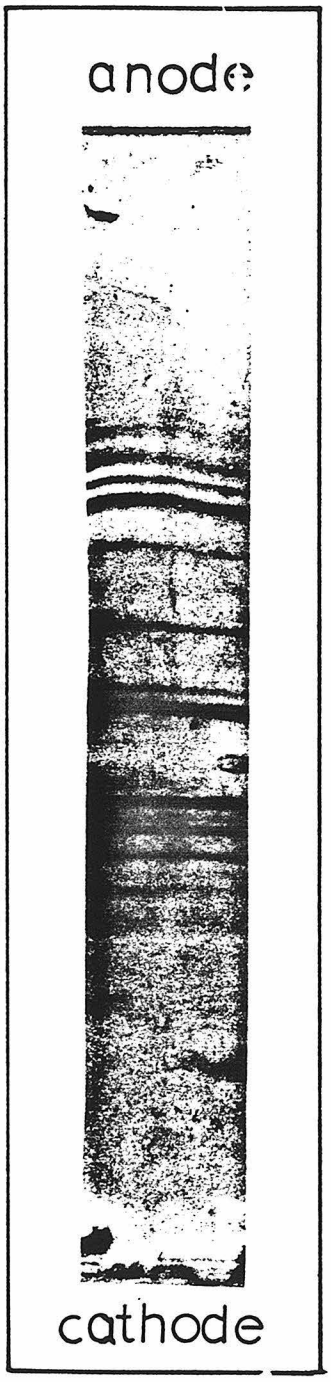

anode
cathode

Figure 11

A quenched papain digestion mixture was dialyzed against running buffer and applied to a column (2.5 cm diameter x 32 cm) of DE-52 cellulose equilibrated with 50 mM sodium phosphate, pH 6.6 and washed with the same buffer. A linear buffer gradient was begun from 50 mM sodium phosphate, pH 6.6 to 50 mM sodium phosphate, 2 M sodium chloride, pH 6.6 in a total volume of 500 mls. The indicated fractions were pooled. Open circles - absorbance at 280 nm. Closed circles - conductance.

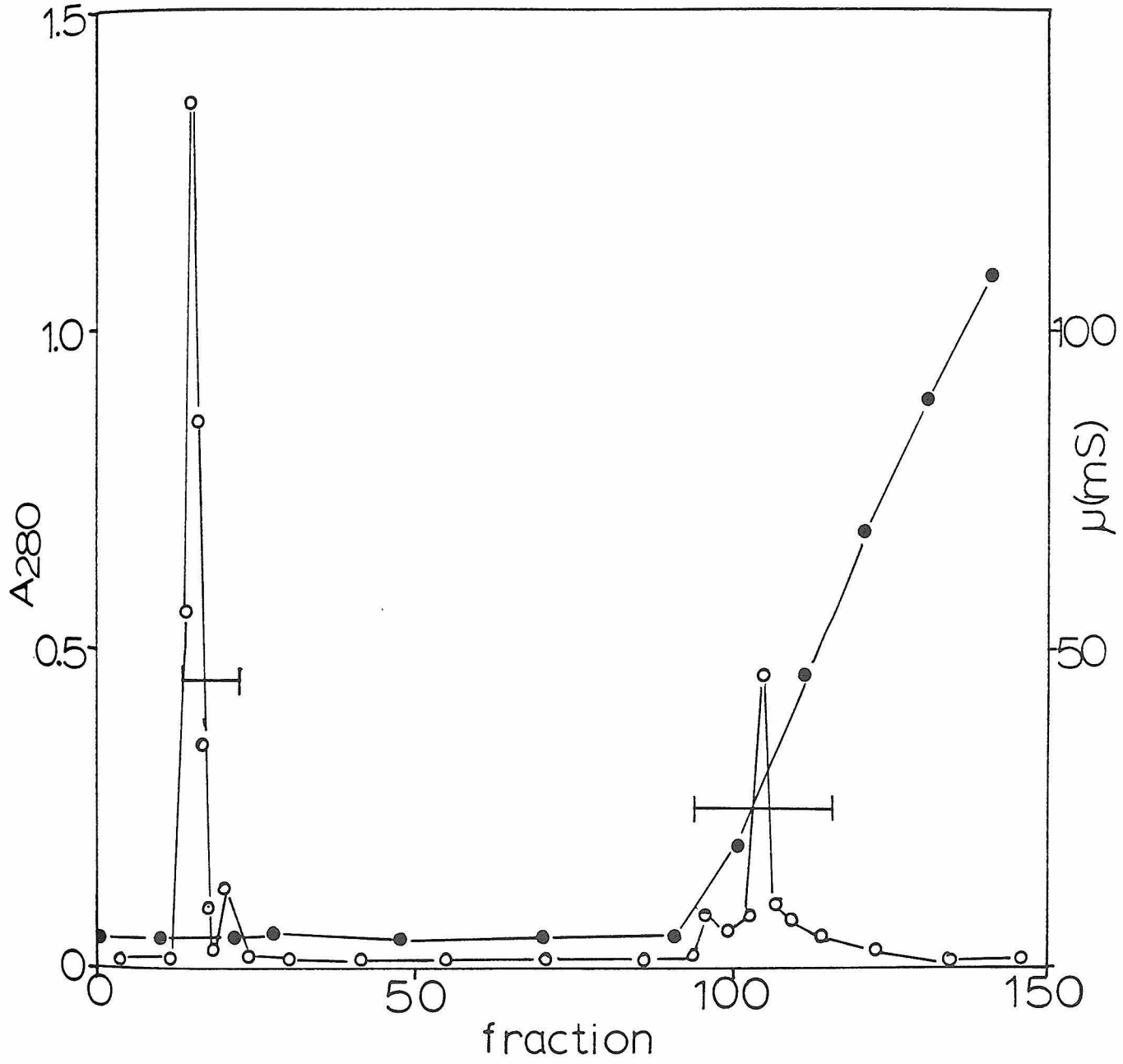

1.5
1.0
0.5
0
A280
0
50
100
150
fraction
100
50
μ(mS)

Figure 12

Analytical ultracentrifugation of UPC-10 myeloma protein (3.8×10^{-6} M in PBS) was performed at 22°C and 52,000 rpm. The double-sector cell was scanned at 12 minute intervals at 280 nm and the absorbance data were converted to the derivative plots of the successive scans shown as described in the text.

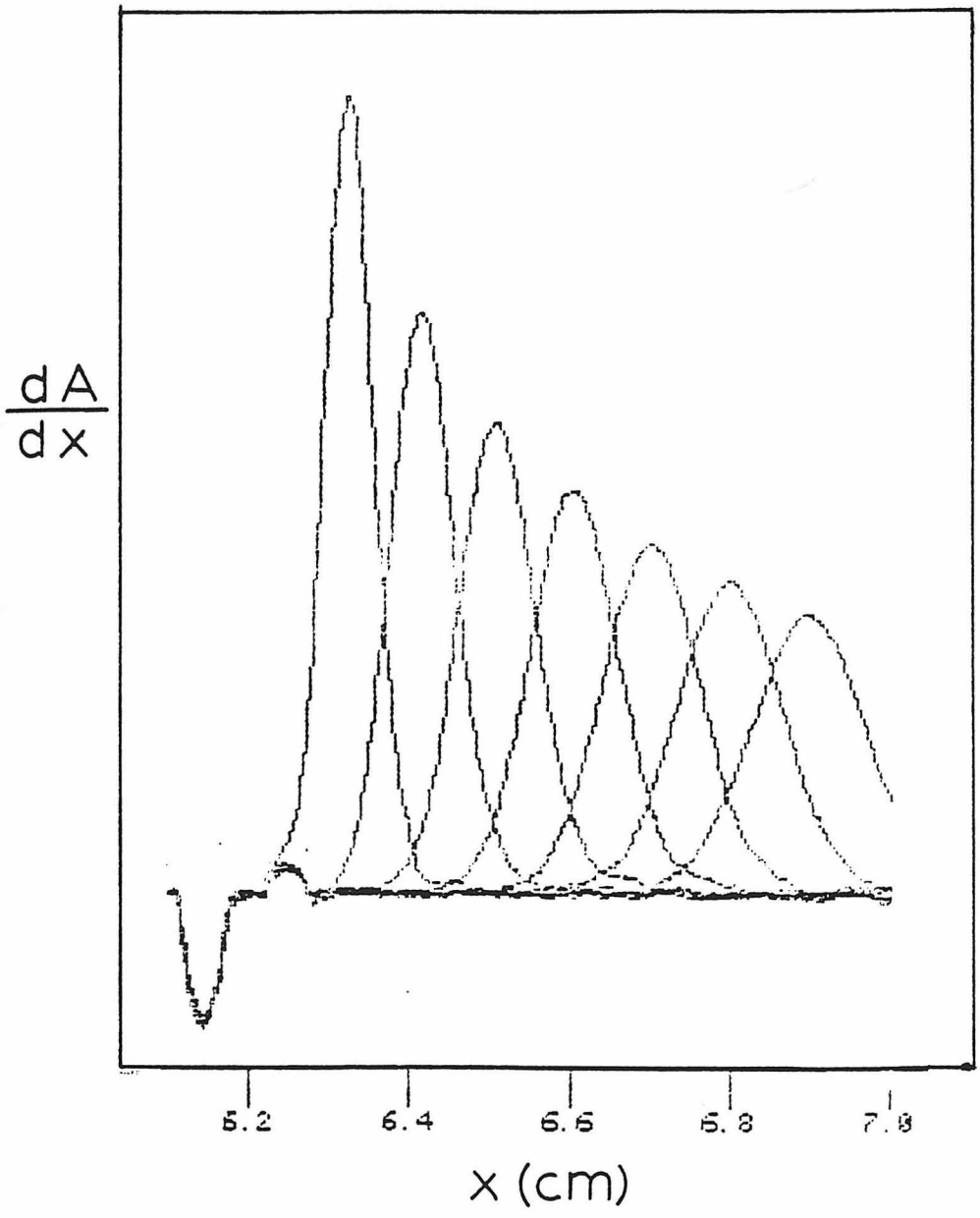

dA
dx
5.2
6.4
6.6
6.8
7.0
X (cm)

Figure 13

Chromatography of the fractions from the column shown in Fig. 14 was performed on Whatman 3 MM paper in 10:4:3 ethyl acetate/pyridine/water with ascending development. From left to right, samples are fructose, fraction 59-79, 80-90, 92-97, 99-105, 107-113, 115-124, 127-140 and fructose twice. Thus, these samples are successive peaks from the Biogel column. Sugars were visualized using $AgNO_3$ with brief heating. From this pattern the following identifications were made:

fraction	127-140	fructose
	115-124	dimer
	107-113	trimer
	99-105	tetramer
	92- 97	pentamer

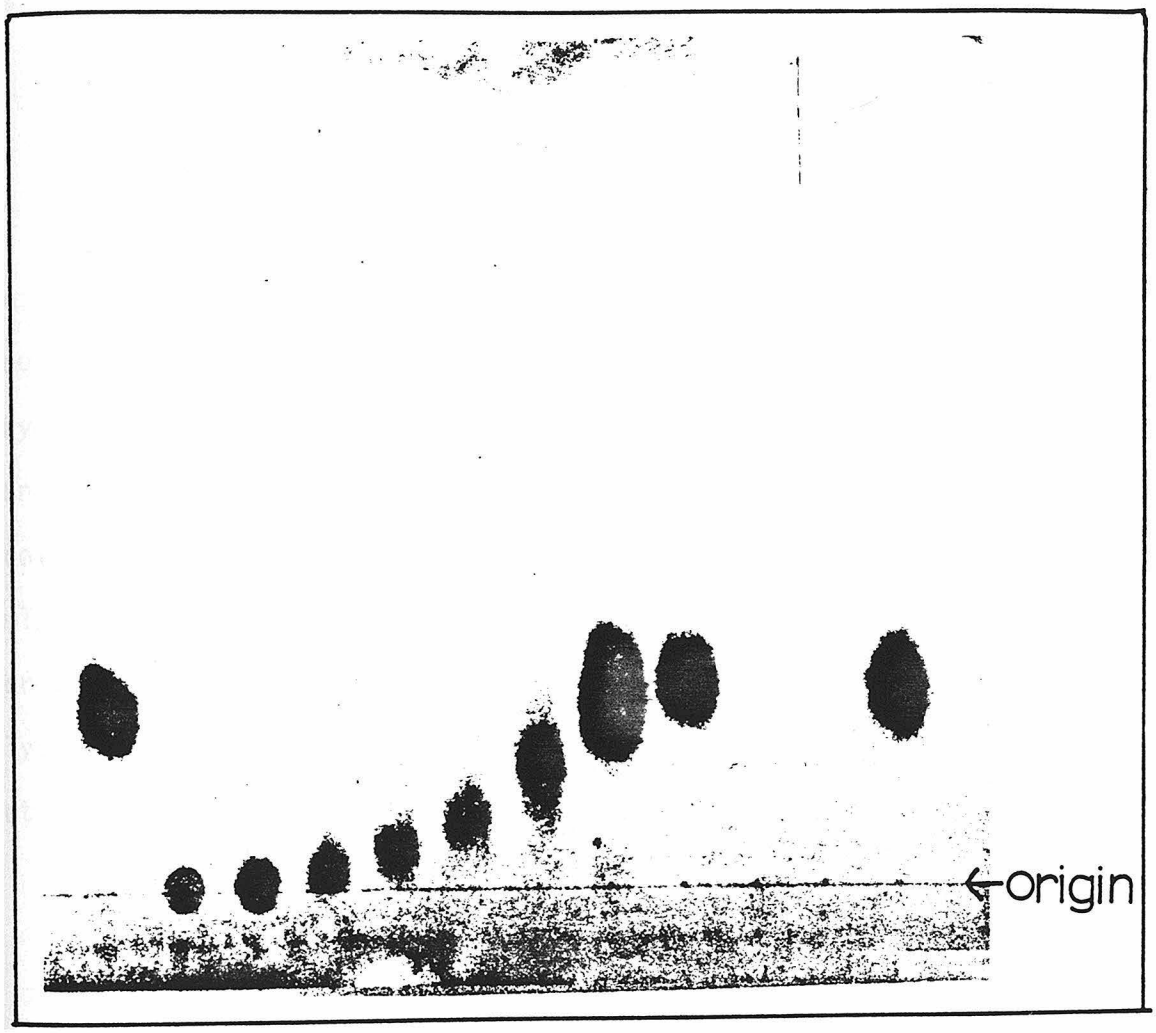

origin

Figure 14

A column (2.5 cm diameter x 170 cm) of Biogel P-2, -400 mesh was equilibrated with distilled water. Lyophilized, crude bacterial levan hydrolysate (250 mgs) was dissolved in water, centrifuged to remove undissolved material, and applied directly to the column bed. The column was then washed with water at a flow rate of approximately 0.7 ml/min.; 12 minute fractions were collected and assayed using the orcinol method. The indicated fractions were pooled and lyophilized--the numbered peaks correspond to monomer, dimer, trimer, etc. as indicated in Fig. 13.

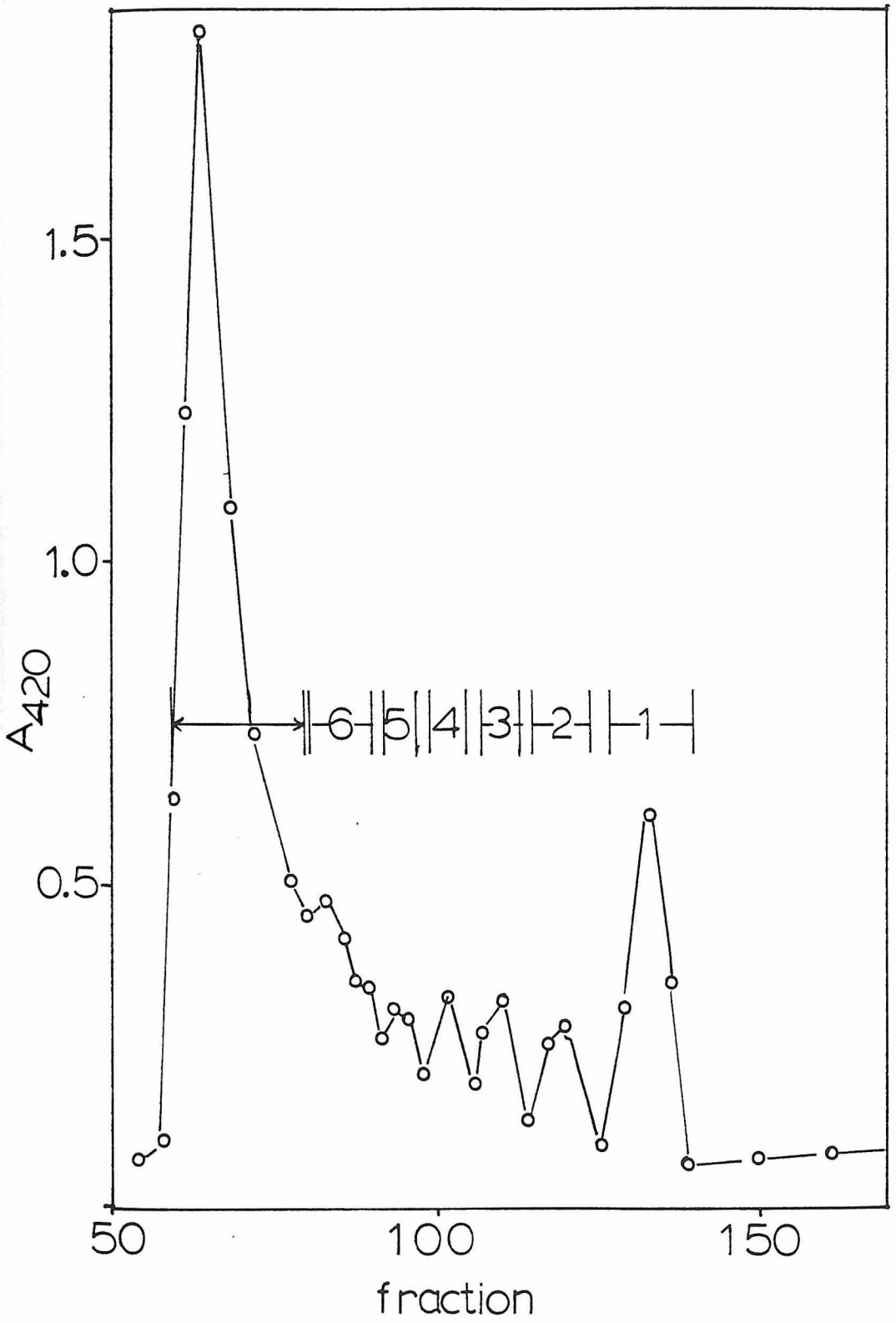

1.5
1.0
0.5
A_{420}
6
5
4
3
2
1
50
100
150
fraction

Figure 15

A plot of elution volume (V_e) minus void volumn (V_o) versus logarithm molecular weight was prepared for the Biogel P-2 column peaks shown in Fig. 14 and identified in Fig. 13.

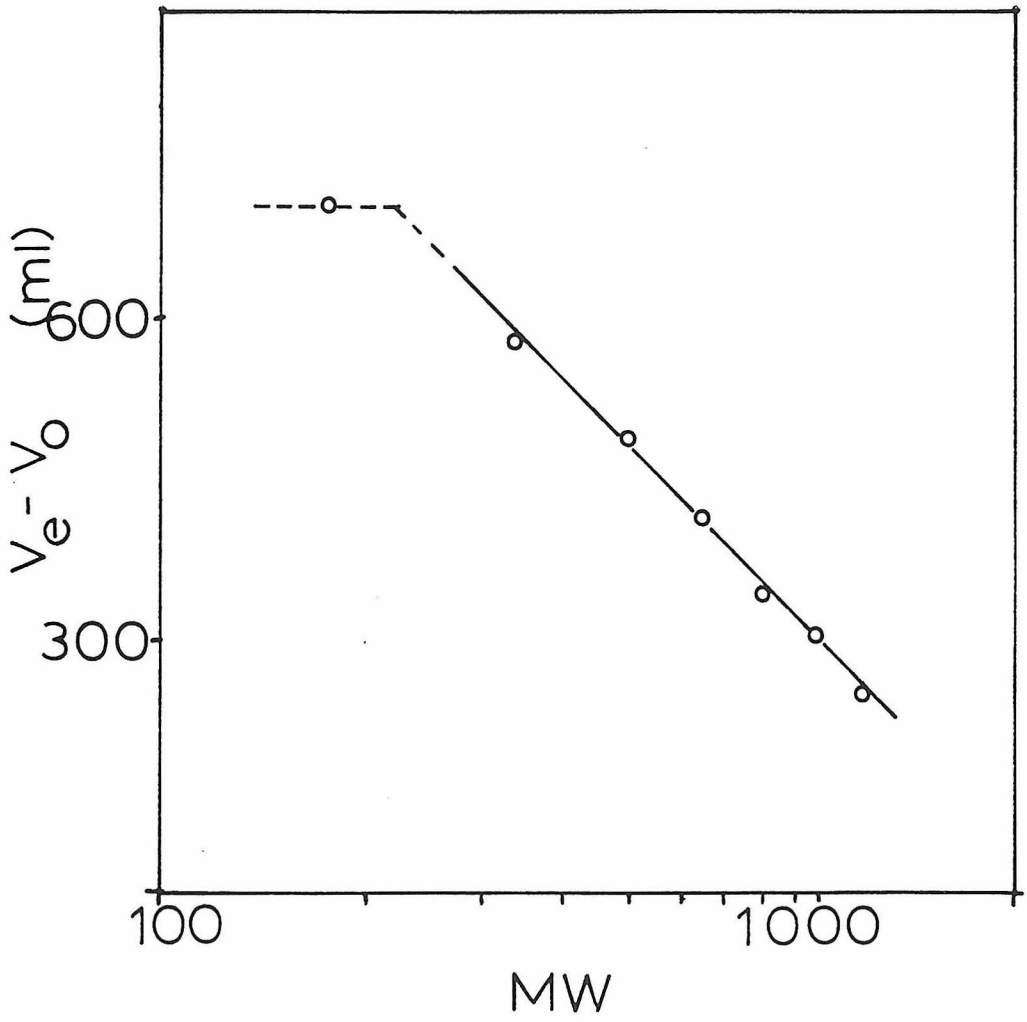
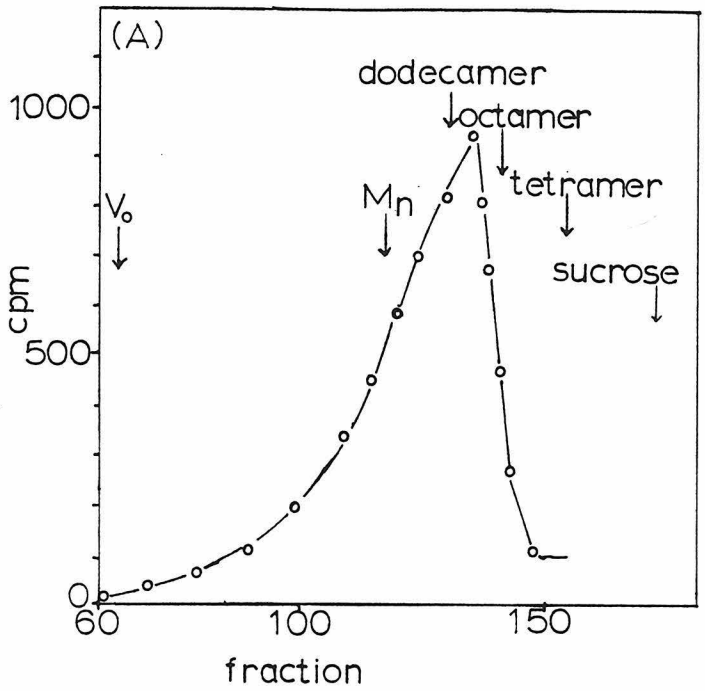

$V_e - V_o$ (ml)
600
300
100
1000
MW

Figure 16

A) A column (2.5 cm diameter x 170 cm) of G-50 Sephadex superfine was equilibrated and washed in distilled water and calibrated with ^{14}C-sucrose, ^{3}H-tetramer, octamer and dodecamer. The molecular weight distribution of fraction 59-79 (Fig. 14) was then determined using this calibrated column.

B) A plot of (elution volume - void volume) versus logarithm molecular weight was prepared for the calibrated column described above. This relation was used to calculate the molecular weight of the eluted material as a function of elution volume and allowed the determination of M_n from the elution profile of fraction 59-79.


(A)
dodecamer
octamer
tetramer
sucrose
Mn
Vo
1000
500
0
cpm
60
100
150
fraction

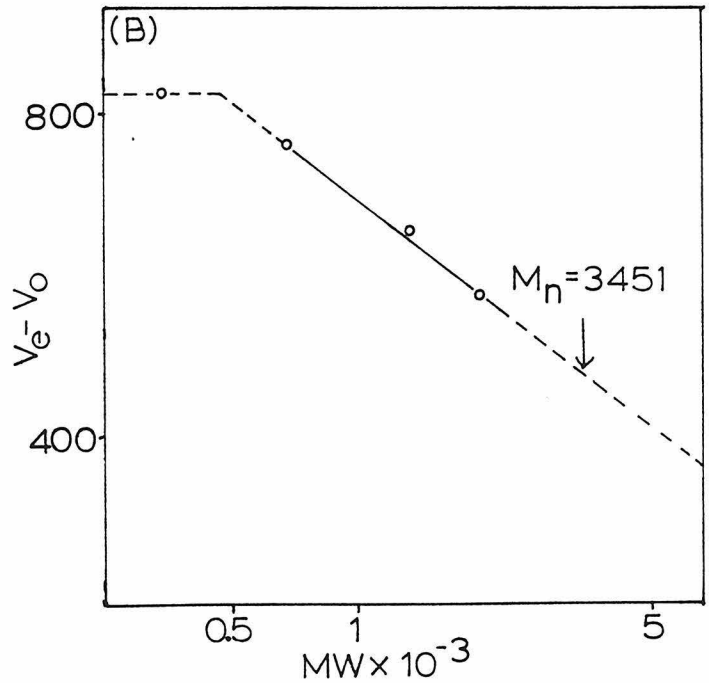

(B)
800
400
Ve-Vo
Mn=3451
0.5
1
5
MW x 10-3

Figure 17

The calibrated column described in Fig. 16 was loaded with 16 mg of fraction 59-79 and a trace amount of fraction 59-79-O^{3}H dissolved in 0.5 ml of H_2O; the column was run in distilled water, 100 λ aliquots were taken for counting, and the arbitrarily determined fractions were pooled as indicated.

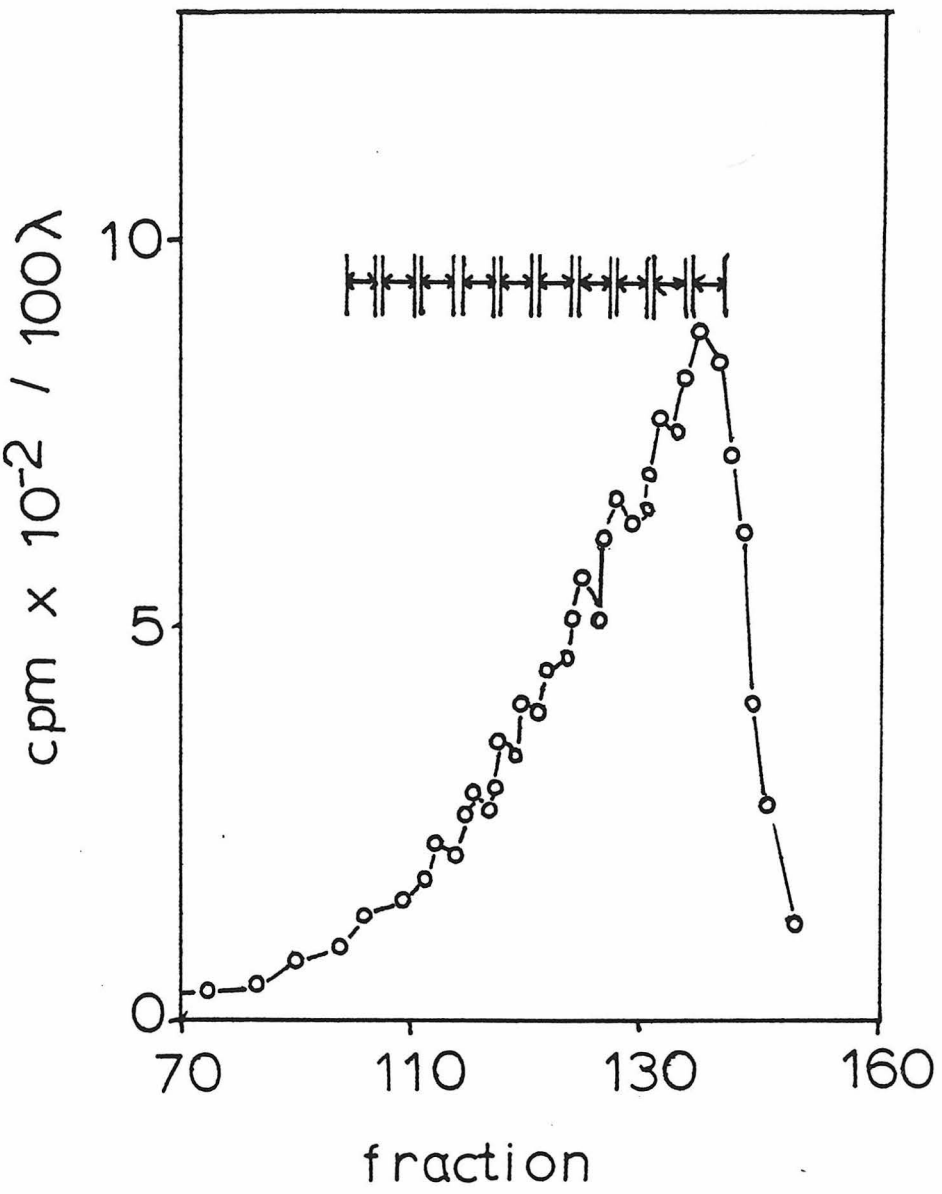

cpm x 10^{-2} / 100λ
10
5
0
70
110
130
160
fraction

Figure 18

A representative Scatchard plot of bacterial levan fraction 59-79 binding to whole UPC-10 myeloma protein (9.9×10^{-5} M) in PBS at 37°C.

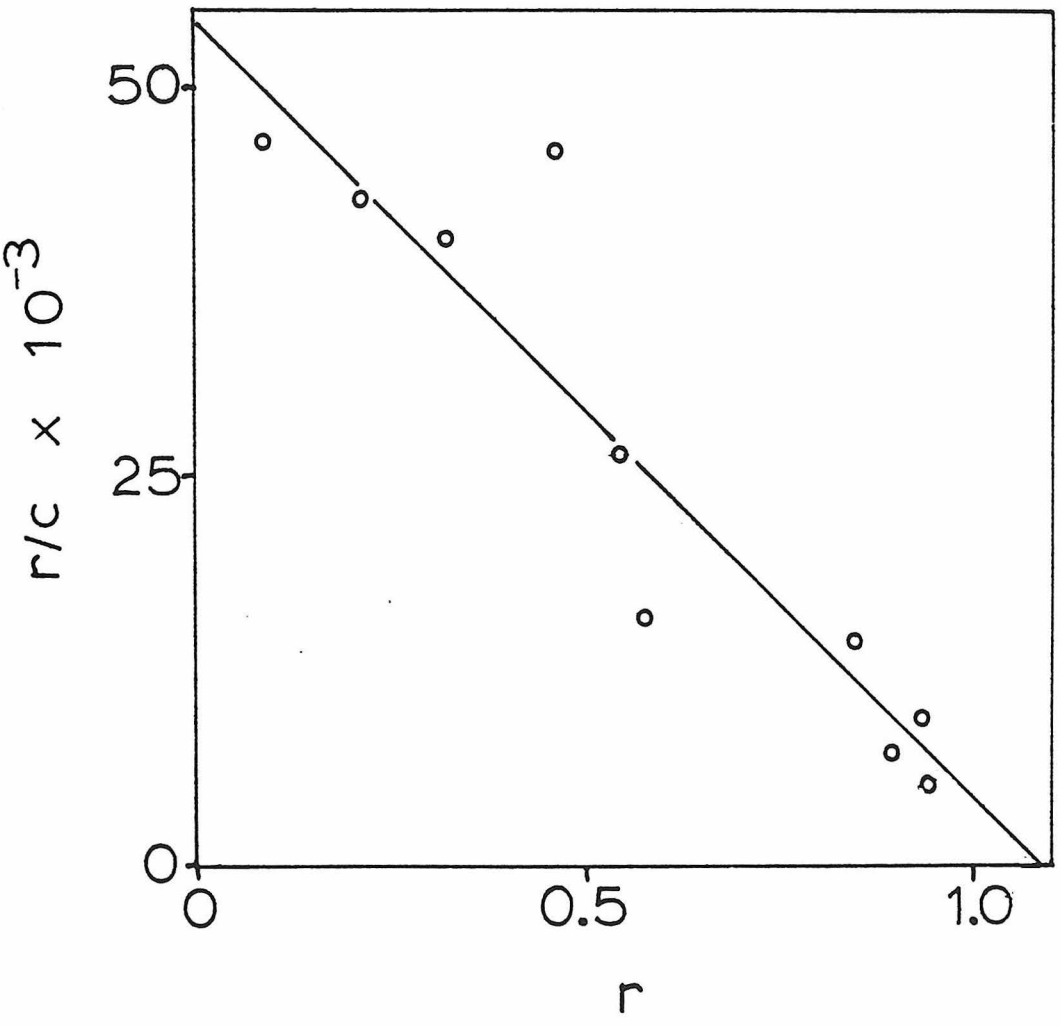

50
25
0
r/c x 10^{-3}
0
0.5
1.0
r

Figure 19

A plot of ΔG of binding bacterial levan fraction 59-79 to whole UPC-10 myeloma protein versus temperature was prepared and used to calculate thermyodynamic binding parameters.

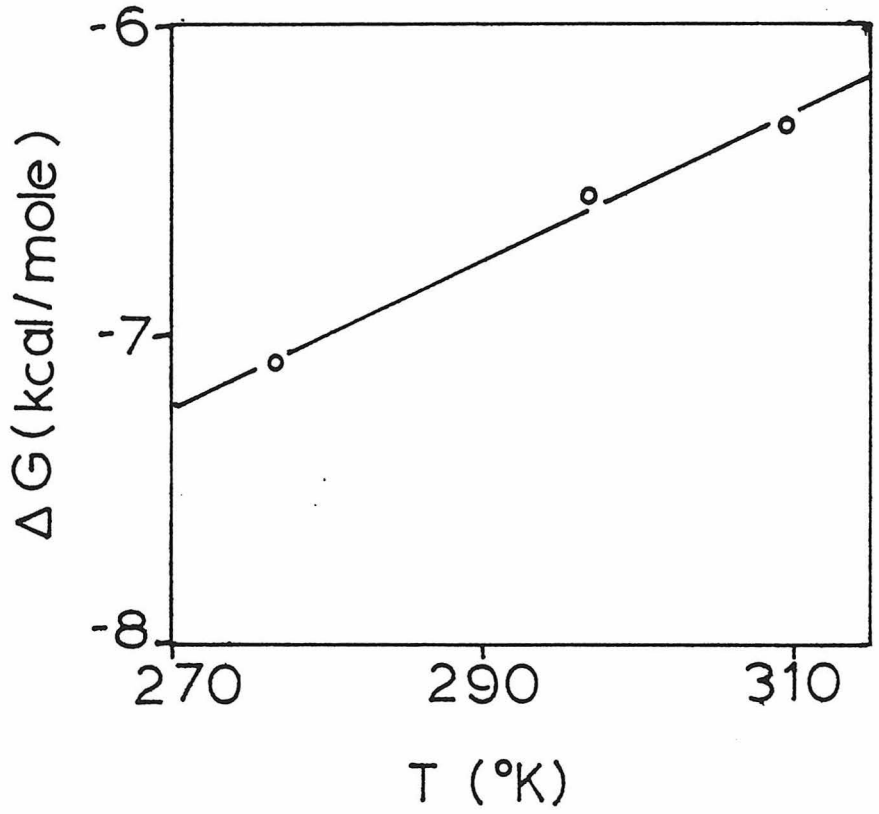

-6
-7
-8
ΔG (kcal/mole)
270
290
310
T (°K)

Figure 20

A Sips plot of bacterial levan fraction 59-79 binding to whole UPC-10 myeloma protein (9.9×10^{-5} M) at 37°C was prepared and used to calculate the index of heterogeneity, a.

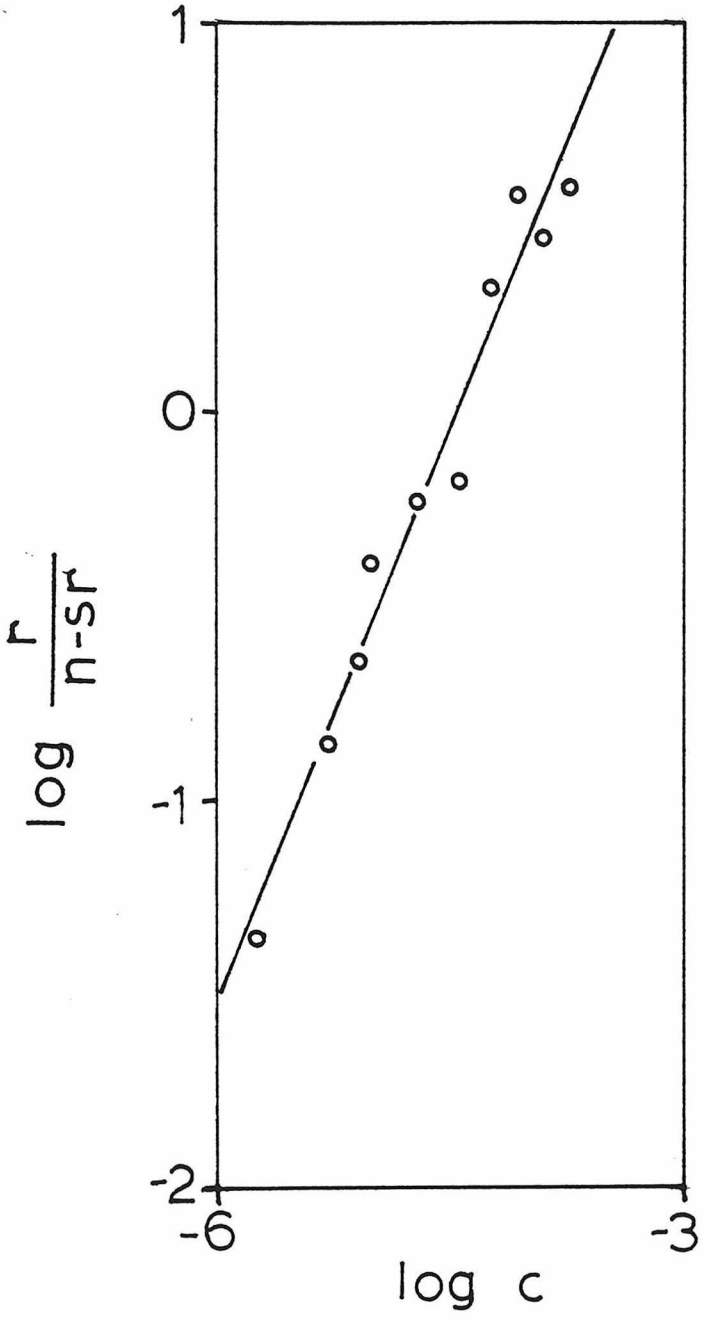
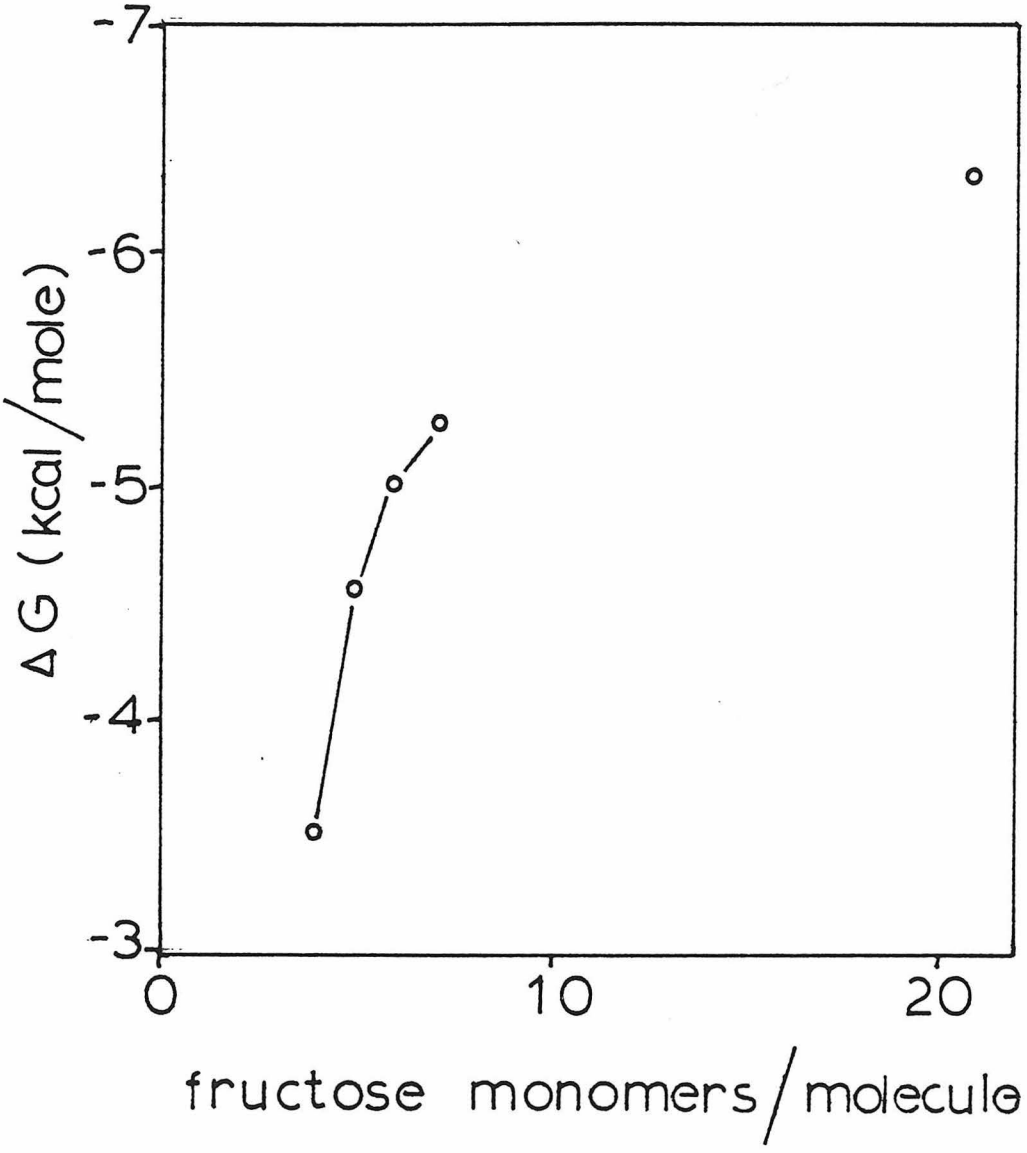

1
0
-1
-2
-6
-3
log r/(n-sr)
log c

Figure 21

A plot was prepared of ΔG of binding bacterial levan oligosaccharide preparations to whole UPC-10 myeloma protein (9.9×10^{-5} M) at 37°C versus oligosaccharide size.


-7
-6
-5
-4
-3
ΔG (kcal/mole)
0
10
20
fructose monomers/molecule

Chapter III: Characterization of Complement Fixation by UPC-10 Myeloma Protein

INTRODUCTION

Relationship of Antibody Structure to Complement Fixation

While all antibody molecules have the ability to bind antigen, some antibody classes have additional secondary activities, including complement fixation, binding of antibodies to lymphocytes, macrophages, and mast cells, and segregation of specific antibody classes by seromucosal and placental barriers (Winklehake, 1978). Since different antibody classes have correspondingly different secondary functions, the immune system is not only able to produce antibodies to react with a foreign antigen, but can also vary the nature of secondary functions which will be possible. A subset of these secondary activities, most notably mast cell triggering and complement activation, are possible only in the presence of antigen and in this work will be referred to as effector functions. In this way, the antibody molecule can serve to link an antigen-specific recognition event to an antigen-nonspecific effector activity.

In addition to primary sequence variations which provide the basis for the differing effector functions belonging to different antibody classes and subclasses (Nisonoff et al., 1975), other structural features of the antibody molecule are important in relation to its ability to express effector functions. Foremost among these features are inter-chain disulfide bonds, which in IgG have been shown by several studies to be critical structural elements that must be intact for antibody-mediated complement fixation to occur (Schur and Christian, 1964). Binding studies between human IgG1 and C1 have provided evidence that an intact interheavy chain disulfide bond is a necessary condition

for C1 binding since antibody molecules possessed the same ability to activate complement after reduction of a number of other disulfide bonds, as long as the interheavy chain disulfide bond was preserved (Isenman et al., 1975). A mechanism for such functional dependence upon the interheavy chain disulfide bond has been provided by electron microscopic observations of reduced rabbit IgG molecules, which have shown that the loss of the single interheavy chain disulfide bond results in massive alterations of the protein structure through the unfolding of the C_H2 region and the subsequent extension of the F_{ab} arms upon interaction with a bivalent hapten (Seegan et al., 1979). Such an extension of the F_{ab} segments has been supported by studies on the ability acquired upon reduction of specific IgG molecules to bridge the gap between erythrocytes in solution while the unreduced IgG molecules were unable to bind to the surfaces of two adjacent erythrocytes which were kept at a minimum distance by mutual charge-charge repulsion (Romans et al., 1977). Furthermore, unfolding of the antibody molecule upon cleavage of the interheavy chain disulfide bond explains the enhanced susceptibility of such reduced molecules to enzymatic degradation (Nisonoff et al., 1975). Nevertheless, isolated F_c fragments retain the ability to bind C1 whether or not the interheavy chain disulfide bond is reduced, suggesting that the loss of C1 binding ability of the reduced IgG molecule is due to increased flexibility of the F_{ab} arms, rather than the actual dismantling of the C1 binding site through unfolding of the tertiary structure of the molecule (Isenman et al., 1975). Thus, the expression of the ability to activate complement of the intact IgG molecule appears to depend upon the integrity of the interheavy chain disulfide bond.

The roles of other antibody structures, such as Ig-linked carbohydrate, in the expression of effector functions are largely unclear. Although the Ig-linked carbohydrate as a whole does not appear to participate in a general way in the expression of effector functions, individual portions of the carbohydrate may have functional significance in specific cases, such as the importance of Ig-linked sialic acid to the binding of rabbit IgG to fetal yolk sac membranes (Tsay and Schlamowitz, 1975). Conflicting evidence has been obtained concerning the role of Ig-linked carbohydrate in complement fixation. In one study, glycosidase digestion of rabbit anti-pneumococcal antibody abolished its ability to fix complement, whereas the same treatment left the complement activity of anti-staphylococcal, anti-E. coli and anti-streptococcal IgG unaffected (Williams et al., 1973). Another study has indicated that extensive treatment of rabbit IgG with neuraminidase, β-galactosidase, β-N-acetylglucosaminidase, and endo-β-N-acetylglucosaminidase D resulted in approximately an 80% loss of complement fixing ability, suggesting that either the carbohydrate interacts with complement components directly or serves to stabilize regions of the IgG molecule which bind to C1q (Koide et al., 1977). The latter is highly likely, since the major oligosaccharide in rabbit IgG is linked to the heavy chain in the C_H2 domain (Nisonoff et al., 1975), a site which has been implicated in complement activation. The failure of deglycosylated IgG to activate complement has been specifically linked to its inability to bind C1q (Winklehake et al., 1980).

Localization and Characterization of Effector Sites

Largely through the use of proteolytic enzymes, it has been possible to precisely dissect the IgG molecule into its individual domains. Thus, fragments corresponding to the V_H-V_L domain (F_v) (Inbar et al., 1972; Hochman et al., 1973), V_HC_H1-V_LC_L domains (F_{ab}) (Porter, 1959), $(C_H2C_H3)_2$ domains (F_c) (Porter, 1959), C_H2 domain (Ellerson et al., 1972; 1976), $(C_H3)_2$ domain (Ellerson et al., 1976; Ovary et al., 1976) and $(V_LC_L$-$V_HC_H1C_H2)_2$ domains (F_{acb}) (Ovary et al., 1976; Arlaud et al., 1976; Colomb and Porter, 1975) have been prepared and studied in relation to their ability to carry out various effector functions. Functional assays on such fragments have shown that complement fixation is mediated through a site contained in the C_H2 domain (Yasmeen et al., 1976; Ellerson et al., 1972; Ovary et al., 1976; Arlaud et al., 1976; Colomb and Porter, 1975), although one study suggested that the C_H3 domain stabilized an IgG-C1q complex (Arlaud et al., 1976). (The C_H3 domain has been shown to be involved more directly in the cellular binding of IgG (Yasmeen et al., 1976; Foster et al., 1980)).

Further dissection of the C_H2 domain has been carried out to localize the C1q binding site to a specific region(s) of the primary sequence. A cyanogen bromide fragment from MOPC 173 (mouse IgG2a) corresponding to positions 253-314 of the heavy chain has been shown to contain complement fixing ability (Kehoe et al., 1974; Kehoe and Fougereau, 1969). Synthetic peptide analogs have been prepared to mimic portions of the C1q binding site, based on the sequence of human IgG1 from residues 274-281 (Boackle et al., 1979; Edelman et al., 1969); this study suggested that Trp 277 was the most essential amino acid in this

sequence for both C1 binding and C2 conversion, while the presence of Tyr 278 and Lys 274 enhanced these activities but were less important (Boackle et al., 1979). The importance of Lys 274 to these functional aspects helps explain the reduced complement fixing ability of human IgG2 and IgG4, both of which have Glx in position 274, as opposed to the high ability of human IgG1 (which has the lysine at position 274) to fix complement (Boackle et al., 1979).

Additional evidence concerning the structure of the C1q binding site has been obtained by labeling studies aimed at different positions in the molecule. The specific labeling of tryptophan residues in non-specific rabbit IgG by 2-hydroxy-5-nitrobenzyl bromide (NBB) has suggested that at least one tryptophan in the F_c fragment near the hinge region plays an important role in the activation of complement, although the exact position of the critical residue could not be established (Allan and Isliker, 1974a). A comparison of C1q binding versus assays for hemolytic activity has shown that, although the ability of NBB-modified antibody aggregates to fix whole complement in a hemolytic assay is drastically reduced, such aggregates have an affinity for free C1q which is similar to that seen for unlabeled antibody, and that C1q bound to modified antibody is not inactivated in any way, but is still able to initiate the classical pathway when erythrocytes coated with unmodified antibody are added (Allan and Isliker, 1974b). These observations suggested that the tryptophan residues which are susceptible to NBB-labeling are not directly involved in C1q binding, but rather are necessary for the maintenance of the conformation of a C1q-binding site, and that alteration of such a site does not affect the affinity of antibody-C1q binding but rather destroys the ability of the

antibody to induce a conformational change in the C1q molecule necessary for further steps in the complement cascade (Allan and Isliker, 1974b).

A labeling study concerning the modification of carboxyl groups in the F_c fragment and the resulting effect of complement fixation has been carried out using non-immune human IgG (Vivano-Martinez et al., 1980). Acidic groups were chosen for reaction as there are a number of aspartic and glutamic acid residues near Trp 277 in the primary sequence of human IgG (Edelman et al., 1969). Modification of 4 out of 7 acidic residues by condensation with glycine ethyl ester using ethyl-dimethyl-aminopropyl-carbodiimide abolished the ability of isolated F_c fragments to fix complement. No significant alterations in the conformation of the fragment were seen as assessed by circular dichroism measurements, although subtle differences were attributed to modification-induced structural changes within the local vicinity of the C1q binding site. No attempt was made in this study to determine which acidic residues in the fragment were labeled under these conditions. This observation that a decrease in the number of acidic groups on the antibody molecule eliminates its ability to fix complement is indirectly supported by experiments studying the inhibitory effect of pyridoxal-5-phosphate on the interaction between C1q and insoluble immune complexes of ovalbumin and rabbit anti-ovalbumin (Allan et al., 1979). Molecules of C1q were treated with pyridoxal-5-phosphate, which formed Schiff bases with sensitive lysine residues; after stabilization by borohydride reduction, these modified C1q molecules had lost their ability to bind to immune complexes. In this way, these labeling studies suggest the importance of acidic groups on the antibody molecule

and basic groups on the C1q molecule which may interact with each other to provide energy for the binding to occur.

Additional labeling experiments have been carried out on other residues amenable to chemical modification, such as tyrosines and arginines, although the involvement of arginines in complement fixation cannot be expected based on either primary or tertiary sequence data. Modification of human IgG with tetranitromethane to yield 7.3 moles of nitrotyrosine per mole of IgG abolished 72% of the complement fixing activity of the protein when absorbed onto latex particles (Johnson and Thames, 1976), suggesting that the tyrosine residue important to complement fixation is Tyr 278, as it is adjacent to Trp 277. In like manner, treatment of human Ig with the 2,3-butanedione reagent (Grossberg and Pressman, 1968) modified 16 residues of arginine per IgG molecule and abolished 77% of the anticomplement activity as measured by the latex adsorption technique (Johnson and Thomas, 1976). Although these experiments help to define the nature of the interactions between IgG and C1q molecules, it is impossible to understand the precise molecular details of such an interaction without knowing which specific residues are being labeled and how such labeling alters the tertiary structure of a C1q binding site on the IgG molecule.

Burton et al. (1980) have suggested a location on IgG for the C1q binding site based upon the crystallographic structure of IgG and the effect of specific chemical modifications of the IgG molecule upon its ability to activate complement. Suggesting that the primary interactions between IgG and C1q molecules are ionic in nature, these investigators have proposed a C1q binding site on the IgG molecule consisting of the last two (C-terminal) β-strands of the C2 domain.

These stretches of polypeptide chain are accessible to solvent as determined from crystal structures and contain amino acid residues that, if chemically modified to eliminate any ionic change, decrease the binding interactions between IgG and C1q molecules.

No definitive estimation of the number of binding sites on C1q for antibody molecules has been obtained. Due to the striking hexameric structure of the C1q molecule, however, it has often been assumed that C1q possesses six binding sites, one at the end of each globulin head (Porter, 1979). This attractive possibility is contradicted by ultracentrifugal analysis on complexes formed between human IgG and C1q, which suggests that at least 10 antibody molecules are bound per C1q molecule (Schumaker et al., 1976). Based on this evidence, these authors postulated a 12-site model of C1q, although they could not rule out the possibilty that each C1q molecule could bind 18 antibody molecules.

Quantitative measurements of the interactions between human C1q and IgG and IgM have been obtained by measuring the ability of these classes of antibody molecules to inhibit the binding of labeled C1q to solid-phase IgM molecules (Sledge and Bing, 1973). IgG was found to have a binding constant $K_{assoc.} = 9 \times 10^3\ M^{-1}$, and IgM bound with an affinity of $K_{assoc.} = 1.6 \times 10^5\ M^{-1}$.

It is important to recognize that the interactions between C1 and IgG may fall into two classes: those which result in binding of IgG to C1q, and those which modify the native structure of the C1 molecule (with subunits q, r, and s) in order to produce activated C1. Using IgG fragments, it has been observed that isolated C_H2 domains bound C1 as well as did native IgG, although the fragment retained only 3% of the

activity of intact IgG in a whole complement-fixing assay (Yasmeen et al., 1976). This was also shown by the NBB modification of tryptophan residues which resulted in IgG molecules unable to activate complement but that retained 80% of the binding capacity of native IgG for C1q (Allan and Isliker, 1974b). These observations suggest the duality of IgG-C1q interactions and are supported by the fact that C1r and C1s can not be converted to their active forms in the absence of C1q (Ziccardi and Cooper, 1976).

Possible Mechanisms of Complement Activation

During the past decade a great many experiments have been carried out to determine the molecular mechanism whereby antibody molecules activate effector functions upon combination with antigen. Two major models have emerged from this work concerning the nature of this mechanism. An allosteric model has suggested that antigen binding causes a conformational change in the antibody molecule in a region distant from the antigen-binding sites and is thereby able to either enhance the affinity of an effector molecule (C1q, F_c receptor, etc.) for the antibody or generate effector activity in otherwise inactive antibody-effector molecule complexes. This model has also been modified to include restrictions of inter-domain flexibility caused by antigen binding (distortion model). Alternatively, an associative model postulates that effector functions are triggered by two or more antibody molecules which have been clustered together due to the multivalent nature of the antigen. Thus, binding of effector molecules to immunoglobulin is enhanced by the immobilization of the antibody onto the antigenic surface. Although these two models represent extreme

possibilities, most studies on the effect of antigens on antibody structure have formulated experimental results in terms of one of these two models (for a recent review, see Metzger, 1978).

As many different systems have been employed in the investigation of antibody structure and effector functions, it is important to analyze the possible variations between them in light of the effect such variations might have on the generalization of experimental conclusions. The nature of the antibodies employed probably provides the greatest source of variability between these studies. Thus, immunoglobulins have been investigated most commonly from humans, mice, and rabbits, although many other species have been studied that belong to each class and subclass that is known for the various species. Finally, antibody preparations, while specific for a given antigen, are often heterogeneous with respect to the immunoglobulin molecules present, so that conclusions that might be drawn from a homogeneous antibody study may not be applicable to a similar study in which antibody preparations containing 10 or more different proteins were used. Unfortunately, without a detailed knowledge of antibody structure and its interaction with antigen, it is often impossible to draw conclusions concerning the general validity of a particular system under study.

An Allosteric Model

Intrinsic spectroscopic properties

The majority of observations which have supported an allosteric model have been spectroscopic in nature. While utilizing many different experimental approaches to the question of antibody conformation, all of these studies are similar in their utilization of either a natural or an

artificially induced feature of the molecule to serve as a probe of the antibody molecule's conformation.

Since the involvement of Trp side chains has been implicated in C1q binding both by model peptide studies (Boackle et al., 1979) and by Trp modification studies (Allan and Isliker, 1974a), the exposure of aromatic side chains to solvent has been studied by solvent perturbation UV difference spectroscopy using 20% ethylene glycol (Isenman et al., 1977). Using fragments corresponding to the isolated domains, these studies suggested the exposure of 1 out of 2 tryptophans in the monomeric C_H2 fragment, whereas in the dimeric C_H3 fragment only 0.4 out of 2 tryptophans was exposed; the exposure of an essential tryptophan in the C_H2 domain correlates well with the ability of C_H2 to interact with C1q, while C_H3 does not allow such binding. In addition, removal of a C-terminal peptide from the intact C_H3 domain not only resulted in greater solvent exposure of tryptophan residues (0.9 out of 2) but also endowed the fragment with the ability to bind C1q. These results indicated that potential C1q binding sites may be present at several positions of the antibody molecule, but that tertiary folding limits the availability to C1q of such sites, except for the C1q binding site in the C_H2 domain (Isenman et al., 1977). This line of reasoning could conceivably be carried further by postulating that, just as tertiary folding may inhibit the expression of C1q binding sites in the C_H3 domain, the same folding might modulate the availability of the C1q binding site in the C_H2 domain as well.

Several studies have examined the effect of hapten binding on the circular dichroism of an antibody molecule with conflicting results. Using heterogeneous rabbit anti-DNP antibodies, it has been observed

that the binding of ε-DNP-lysine to F_{ab} fragments caused no change in the CD spectrum; it was also noted that the CD spectrum of intact antibody was the sum of the separate contributions of F_{ab} and F_c portions (Cathou et al., 1968). Alternatively, two independent studies on monoclonal rabbit IgG with activity against Type III pneumococcal capsular polysaccharide have suggested that CD changes due to hapten binding are possible. An early investigation showed that the binding of a hexasaccharide produced changes in the circular dichroism of the antibody molecule for each of three antibodies which were studied, although the three different antibodies did not exhibit identical hapten-induced effects (Holowka et al., 1972). A later study using a different set of three monoclonal anti-(Type III pneumococcal polysaccharide) antibodies found that all three of these proteins showed similar CD spectral alterations upon interaction with a hexasaccharide antigen, and that these effects could be ascribed to conformational changes in the F_{ab} regions of the molecule (Jaton et al., 1974). Taken together, these experiments suggest that ligand-induced alteration of the circular dichorism spectrum of an antibody can indeed be observed and ascribed to conformational changes of the antibody molecule, although no results were obtained that implicated such conformational alterations occurred in the F_c portion of the molecule.

Although changes in the intrinsic fluorescence of specific antibody molecules upon addition of hapten are often used in the determination of binding constants (Jolley and Glaudemans, 1974), such changes are by no means universal or similar in character (Jolley et al., 1973). With respect to the fluorescence of an antibody of the IgG class, a study of the fluorescence of a rabbit monoclonal anti-(Type III pneumococcal

polysaccharide) IgG showed an 8 nm blue shift of the emission maximum with significant quenching upon binding a hexasaccharide antigen (Jaton et al., 1975a). Like the hapten-induced changes in the circular dichroism spectra of this antibody, the fluorescence changes could be attributed to the F_{ab} portions, and apparently did not involve the F_c portion to a measurable degree.

In contrast to the uninformative nature of fluorescence spectra in the study of antibody conformation and its relation to the expression of effector functions, the circular polarization of fluorescence (CPL) emitted by tryptophan residues in the antibody molecule has provided the strongest evidence for conformational changes which may or may not be involved in complement activation. CPL spectra of biomolecular systems have been shown to reflect almost exclusively the contributions of tryptophanyl residues; the observation of an altered CPL spectrum is thus considered to be evidence for a variation in the asymetry of the excited state of one or all of the tryptophans in a protein, just as alterations in circular dichroic spectra are taken as evidence of changes in the asymetry of the ground state of appropriate chromophores in the protein (Richardson and Riehl, 1977).

In a study using monoclonal rabbit antibody with activity against Type III pneumococcal polysaccharide (Jaton et al., 1975b) it was found that the CPL spectrum of the antibody was altered upon binding the carbohydrate antigen, and that oligosaccharides of increasing size produced changes in the CPL spectrum of increasing magnitude. When the same oligosaccharide haptens were allowed to combine with the F_{ab} fragments, the CPL changes observed were not as large as were seen with the intact antibody, thus suggesting a conformational change in the

antibody molecule which could be attributed to the F_c portion. In addition, reduction and alkylation of the interchain disulfide bonds eliminated all of the spectral changes associated with the F_c portion. This study stressed that observation that small, haptenic determinants, such as the tetrasaccharide, were able to induce both F_{ab} and F_c associated conformational changes to some degree (Jaton et al., 1975b).

Other investigators, using heterogeneous antibody preparations, have examined the CPL spectra of antibodies directed against either RNAse, poly-alanine, phosphoryl choline, or the loop region of lysozyme upon combination with their corresponding antigens (Givol et al., 1974; Schlessinger et al., 1975). With all antibody preparations except anti-phosphoryl choline, antigen-induced CPL spectral changes were observed for both intact antibodies and F_{ab} fragments; such changes were observed at high antigen concentration, suggesting that the CPL effects were due to antigen binding and not aggregation. In these three systems, there were marked differences upon binding antigen between the spectra observed for the intact antibody and for F_{ab} fragments, suggesting that antigen binding resulted in a conformational change, as monitored by CPL, that partly occurred in the F_c portion of the molecule. It was noted by the authors, however, that an alternative explanation for the dissimilarity of the spectra of the antibody and F_{ab} fragment might be that the structural constraints imposed upon the F_{ab} arms by the F_c portion of the molecule could alter the structure of the F_{ab} arms themselves, and thus account for the observed CPL differences. Reduction of the interheavy chain disulfide bond abolished the CPL changes attributed to the F_c regions. Upon combination with phosphorylcholine or tetra-alanine, the corresponding antibodies did not

exhibit any F_c conformational changes, suggesting that large antigenic determinants were required to induce such structural alterations, in contrast to the anti-Type III pneumococcal polysaccharide study which indicated that the tetrasaccharide was capable of causing conformational changes in the F_c portion of the antibody molecule (Jaton et al., 1975).

A more detailed study of anti-"loop" antibodies with "loop" (residues 64-80 of lysozyme), a dimeric derivative of "loop", and poly-"loop" has suggested that two different types of antigen-induced CPL changes can be found for this heterogeneous antibody preparation (Pecht et al., 1977). The CPL spectra obtained with free antibody, antibody and "loop", and antibody and bis-"loop" were different from each other, while F_{ab} fragments, either free or complexed with either one of the two ligands ("loop" or bis-"loop"), all exhibited the same CPL spectrum. Again, reduced antibody showed the same CPL spectra when complexed with antigen as that seen with the F_{ab} fragments. This work suggested that there may be more than one type of antigen-induced conformational change which can be monitored by CPL, although it is possible that this observation was a result of the heterogeneity of the antibody preparation.

In addition, assays of the abilities of the various antigens to cause antibody-mediated complement fixation showed that monovalent antigens, such as "loop" and intact lysozyme, failed to cause fixation to occur, while bis-"loop"-antibody complexes observed in the ultracentrifuge showed predominantly (> 95%) antibody monomers and were able to activate complement, encouraging these authors to conclude that divalent antigen caused a briding between F_{ab} arms that was evidenced

by CPL-monitored conformational changes and the appearance of effector function activity, thus lending support to a mechanism of the expression of effector functions through the antigen-induced distortion of antibody structure (Pecht et al., 1977). However, the possibility that the observed complement fixation by bis-"loop"-antibody complexes was due to a small amount of antibody aggregates (dimers and larger) was not ruled out.

Biophysical properties of antigen-antibody complexes

Although several studies have attempted to define the solution conformation of unliganded IgG by physical techniques (Chapter 1), little attempt has been made to investigate the effect that complexation with ligands has on the solution structure of antibody molecules. The focus of these biophysical studies has been to observe both tertiary and segmental structural changes.

One early study concluded on the basis of electron micrographs of liganded and unliganded heterogeneous IgG that when the antibody molecules were crosslinked by antigen the angle between the F_{ab} arms increased, and these authors postulated that when the IgG molecule "clicks open" it reveals a site that is able to interact with complement (Feinstein and Rowe, 1965). Such a hypothesis has been refuted by ultracentrifugation studies on anti-lactoside IgG and two corresponding univalent antigen fragments that indicate that the sedimentation coefficient of the antibody molecule increases slightly upon complexation with hapten, suggesting that rather than "clicking open", the antibody molecule has been induced to assume a more compact shape upon binding ligand (Warner and Schumaker, 1970).

Evidence for a hapten-induced change in the volume of an antibody molecule has also been obtained by small-angle X-ray scattering measurements of heterogeneous rabbit antibodies directed against poly-D-alanine. While the addition of tetra-D-alanine to F_{ab} or $(F_{ab})_2$ fragments did not produce a change in protein volume or radius of gyration (Pilz et al., 1975), this hapten did produce significant decreases in both parameters when intact anti-poly-D-alanine antibody was used, supporting the ultracentrifugation evidence that the antibody molecule may assume a more compact shape upon binding haptens (Pilz et al., 1973).

The technique of thermal perturbation difference spectroscopy has been applied to IgG and its fragments to observe conformational transitions (Troitsky et al., 1973); in this method, UV spectra are taken as a difference between the sample protein at a given temperature and an identical sample at a control temperature. The observation of a conformational transition occurring between 27-38°C, which was attributed to an alteration in the asymetric environment of one or more tyrosine residues, was found to be present for a number of different antibody preparations, suggesting that this temperature effect may be a general one for IgG molecules. This structural transition was localized to the F_{ab} portion of the molecule (Zav'yalov et al., 1977a). Using heterogeneous IgG specific for a given antigen, addition of a dansyl hapten to anti-dansyl antibody stabilized the high-temperature state of the protein (Zav'yalov et al., 1975), while addition of DNP-lysine to anti-DNP antibody stabilized the low-temperature form (Zav'yalov et al., 1977b). This thermal transition is not exhibited by all antibodies,

however, as the myeloma protein (IgA) MOPC 315 did not show this effect (Zav'yalov et al., 1977b).

Measurements of the polarization of fluorescence designed to determine the rotational relaxation time of anti-dansyl-IgG when complexed to dansyl haptens have indicated that the antibody becomes more rigid when hapten is bound (Tumerman et al., 1972). Although these studies suggest that changes in the segmental arrangement of flexibility of the antibody molecule might occur as a result of hapten binding, they do not allow a correlation between antibody structure and the expression of effector functions.

Temperature-jump experiments examining the binding of various sized oligosaccharides to homogeneous rabbit anti-(Type III pneumococcal polysaccharide) IgG found a marked dependence of association rate constant on chain length, and a length-independent dissociation rate constant, as opposed to the expected results of a dissociation rate which reflected the stability of the complexes and an association rate which was nearly diffusion controlled (Maeda et al., 1977). A mechanism was proposed which included a rapid preequilibration step between antibody and hapten followed by a slow isomerization between two conformations of the hapten-antibody complex; preliminary studies of F_{ab} fragments, however, suggested that this isomerization took place in the F_{ab} region and did not involve other portions of the molecule. This observation was paralleled by studies on the anti-galactan myeloma proteins (IgA) XRPC-24 and J-539 with corresponding oligogalactan haptens in which the kinetics of binding were followed by temperature-jump fluorescence spectroscopy (Vuk-Pavlovic et al., 1978). Both F_{ab} fragments and intact antibody exhibited a fast hapten binding

step followed by a slower protein isomerization, suggesting that hapten binding to these proteins causes a structural rearrangement in the binding pocket which provides more favorable hapten-protein interactions. An additional anti-galactan myeloma protein (IgA) T-601 has been studied by this technique, as well as heterologous recombinants of heavy and light chains from these three myeloma proteins, all of which show the two-step binding kinetics, indicating that such a binding mechanism may be of a general nature and not restricted to a few isolated proteins (Zidovetzki et al., 1980), although similar efforts on anti-DNP myeloma proteins (IgA) have shown that MOPC 460 exhibits a slow protein isomerization upon hapten binding (Lancet and Pecht, 1976) while MOPC 315 does not (Haselkorn et al., 1974). However, these studies do not provide any evidence for hapten-induced conformational changes outside the F_{ab} fragment.

Extrinsic spectroscopic labels

Even though many labeling reagents have been used to modify antibody molecules in an effort to elucidate structural details, this approach has been largely ignored with respect to antigen-induced conformational changes. Although studies have been carried out which employed both NMR and EPR spectroscopy to observe the behavior of artificially introduced labels, these experiments have not been uniformly successful in demonstrating conformational changes.

Variable results obtained through the use of EPR have been most likely a result of differences in the specific residues which were spin-labeled. Due to the attachment of carbohydrate to rabbit IgG at a location (Asn 297) in the vicinity of the presumed C1q binding site,

antibody molecules specific for the DNP group were spin-labeled through oxidation of the IgG carbohydrate using periodate, followed by treatment with 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl free radical and reduction of the resulting imine to give a product that was predominantly labeled at the terminal carbohydrate residue of neuraminic acid (Willan et al., 1977). Observation of the electron spin resonance signal from such modified antibody showed that the spin label was fairly mobile, both in the free IgG molecule and in IgG molecules that were crosslinked with multisubstituted DNP-bovine gamma globulin, suggesting that no antigen-induced conformational change occurred, or more likely that the label was unable to detect such a change even if it did occur. With the same carbohydrate-labeling technique, another study obtained similar results using a less well-defined system (Nezlin et al., 1978).

Through the use of other labeling methods, however, conformational changes have been detected. Labeling of antibody preparations with an iminoxyl radical under conditions where surface residues, predominantly lysines, were tagged resulted in conjugates in which the spin-label appeared to be completely mobile (Kaivarainen and Nezlin, 1976); with the same label, these conditions could be altered to provide labeling of histidine residues, in which case the spectra showed that the label was present in two immobilized environments with different correlation times (Kaivarainen and Nezlin, 1976). Upon addition of antigen, spin-labeled F_{ab} prepared by this technique showed an increase in the amount and degree of immobilization of the more immobile component. The observations were true for both anti-human hemoglobin and anti-bovine IgG antibodies. These results were taken as evidence of

conformational changes occurring upon antigen binding which produced an alteration of the environment of the spin label.

Through the use of ion-binding to both native and modified antibody, NMR studies have provided evidence concerning antibody conformation. One study took advantage of the affinity of rabbit IgG for Gd(III) ions to observe the effect of antigen upon antibody conformation through observation of solvent-water proton relaxation rates (Dower et al., 1975). Using homogeneous rabbit anti-(Type III pneumococcal polysaccharide) antibody, it was shown that addition of a 28-unit oligosaccharide to the antibody resulted in an apparent alteration of the binding site for Gd(III) in the F_c region, but that this change appeared to occur as a function of antibody crosslinking, and was not related merely to the occupancy of antigen-binding sites (Willan et al., 1977).

Another labeling study employed rabbit anti-poly-alanine IgG which had been modified by the insertion of a mercuric ion into the interheavy chain disulfide bridge (Vuk-Pavlovic et al., 1979). This specific introduction of mercuric ions and the resultant increase in disulfide bridge length of 3 Å were found not to cause significant structural alterations in the antibody molecule as assessed by CPL spectra. The environment of the mercuric ions was monitored by observing the line width of ^{35}Cl ions in solution; binding of ^{35}Cl to weak coordination sites on the mercuric ion causes line-broadening of the ^{35}Cl signal resulting from the induced asymmetry of the electric field gradient at the ^{35}Cl nucleus which provides another relaxation mechanism in addition to those present for ^{35}Cl in solution (Stengle and Baldeschwieler, 1966). Thus, ^{35}Cl in solution with native antibody

exhibited a sharper NMR signal than ^{35}Cl in the presence of mercuriated antibody, due to the additional relaxation possible by coordination with mercuric ion. Addition of divalent antigen to the mercuriated antibody resulted in a sharpening of the ^{35}Cl NMR signal, and this effect was taken as evidence of a decrease in the ability of antibody-bound mercuric ions to coordinate to chloride, presumably caused by an alteration of protein structure. This change could not be accounted for simply as aggregation of antibody caused by addition of divalent hapten, since n increase in the size of molecular complexes would tend to broaden, rather than sharpen, the ^{35}Cl signal through an increase in the rotational correlation time (Vuk-Pavlovic et al., 1979).

Other methods

In addition to binding antigen or effector molecules such as C1q, some classes of IgG from various species are able to bind protein A from Staphylococcus aureus in the F_c region of the antibody (Kronvall et al., 1970). While chicken IgG does not normally bind protein A, immune complexes formed between a preparation of chicken Ig and the corresponding antigen, nicotinic acetylcholine receptor, did bind protein A, although only a subpopulation of the IgG preparation developed this activity (Barkas and Watson, 1979). As this antigen-induced protein A binding occurred even under conditions of large antigen excess, these authors suggested that the induction of protein A reactivity was a result of a conformational change for which antigen binding was responsible.

One method used to monitor antigen-induced conformational changes in the antibody molecule has been to observe the development of new

antigenic determinants on the antibody molecule itself. Soluble immune complexes formed from rabbit antibodies with activity against either BSA (Henney et al., 1965) or ferritin (Henney and Stanworth, 1966) have the ability to elicit auto-antibodies in rabbits possessing identical allotypes, suggesting that combination with antigen caused a conformational change in the antibody molecule and revealed an antigenic determinant which is normally hidden and not recognized by the rabbit as "self". A further investigation of such antigenic determinants specific for antigen-antibody complexes showed that these determinants were located in the F_c region and were similar to, but not identical with, antigenic determinants found only on artificially aggregated IgG, supporting the concept that the expression of effector functions by both antigen-antibody complexes and aggregated IgG may depend on structural alterations in the F_c region of the molecule (Henney and Ishizaka, 1968).

In summary, a large body of evidence exists that circumstantially implicates conformational changes alone as needed for the initiation of complement fixation activity. Three requirements must be met to confirm the validity of an allosteric mechanism:

1) a site must be clearly defined on the antibody molecule that interacts with C1q

2) one must be able to show hapten-induced conformational changes at such a C1q site in an intact antibody molecule

3) one must be able to correlate any observed conformational changes with the expression of effector activity.

These requirements have not been met for a single antibody-antigen system and therefore the studies described above cannot be used as conclusive proof of an allosteric model.

An Aggregational Model

Much of the support for an aggregational mechanism for the expression of effector functions comes largely as a result of lack of evidence for a conformational mechanism, in part due to the difficulty of studying components which are in ill-defined physical states (such as denatured or aggregated antibody). Although some systems have been utilized which could be well-characterized, an aggregational hypothesis has received only a small fraction of the effort spent in looking for conformational changes in antibody molecules.

Early observations of complement fixation by multimeric IgG complexes

Very early in the efforts to study complement fixation by antibody-antigen complexes it was realized that fixation curves mimicked precipitation curves obtained under the same conditions (Osler et al., 1948). Although the action of complement was at that time only poorly understood, these results suggested that the aggregation of antibody molecules might play an important role in this process.

The role of aggregation of IgG in complement activation was further studied by the artificial generation of antibody aggregates by treatment with a wide variety of denaturing or crosslinking agents, including heat, urea/mercaptoethanol, bis-diazobenzidine (Ishizaka and Ishizaka, 1960), and organic solvents (Waldesbuhl et al., 1970). These studies

emphasized the finding that aggregated IgG prepared by a wide variety of methods was active in complement fixation.

Likewise, it became apparent that the nature of the antigen could control the aggregation of antibody molecules in an immune response. Experiments using monovalent and bivalent DNP-haptens with anti-DNP antibody showed that the bivalent hapten could crosslink antibody molecules and induce complement fixation, whereas the monovalent hapten could not (Ishizaka et al., 1964).

Several experiments using antibody with activity against sheep erythrocytes indicated that complexes containing more than one IgG molecule were required to activate complement. By estimating the number of antigenic determinants on the cell surface for various preparations of hemolytic IgG and counting (by direct observation) the number of membrane lesions produced on the cell surface after treatment with IgG and complement, it was suggested that two molecules of IgG were required to form a site on the membrane which could then be attacked by complement (Humphrey and Dourmashkin, 1965).

A quantitative complement transfer assay was developed which allowed the measurement of the number of IgG molecules required to produce one hemolytic lesion on a membrane surface (Borsos and Rapp, 1965a). In this assay, molecules of C1 were first activated on the surface of antibody-bearing sheep erythrocytes; the resulting C1 was then quantitatively transferred to sheep erythrocytes coated with C4, at which point the remaining complement components were added and the degree of cell lysis was measured. This technique allowed the direct quantification of the number of C1 molecules activated based upon the "one-hit" theory of immune hemolysis (Mayer, 1961). With this assay, it

was possible to determine the dependence of the number of C1 molecules produced as a function of antibody concentration (Borsos and Rapp, 1965b); the resulting dose-response curves indicated that two IgG molecules were required to form a site on the cell capable of activating C1.

Further evidence for a model requiring two IgG molecules for complement activation has come from a study utilizing a chemical modification of IgG which virtually abolishes complement fixing ability but leaves antigen-binding properties unaffected (Cohen and Becker, 1968). Native and treated antibody were mixed in varying proportions and added to antigen at equivalence to provide immune complexes which were tested for ability to fix complement (Cohen, 1968). It was reasoned that if only one IgG molecule bound to antigen was required for complement fixation, then the ability of the mixed immune complexes to fix complement would be directly proportional to the amount of native IgG present in the complexes; however, if more than one IgG were required in proximity to one another to fix complement, then the amount of complement fixation resulting from the mixed immune complexes would always be less than expected on the basis of the amount of native antibody present in those complexes. The results which were obtained from such an analysis showed that two adjacent IgG molecules were required for complement fixation to ccur.

Lack of correlation between antibody spectroscopy and biologic activity

Although the CPL studies on rabbit anti-(Type III pneumococcal polysaccharide) discussed earlier provided strong evidence for conformational changes of the antibody which occurred upon binding small

haptenic determinants such as the hexasaccharide and were maximal for oligosaccharides consisting of 16 sugar residues or larger (Jaton et al., 1975), such conformational changes did not correlate with the appearance of effector function activity. Indeed, the 16-unit oligosaccharide was found to be incapable of triggering antibody-mediated complement fixation, and such activity did not appear until the oligosaccharide consisted of 21 or more sugar residues (Jaton et al., 1976). Further examination by ultracentrifugation of the antibody-antigen complexes formed with various-sized oligosaccharides indicated that clusters of antibodies that were active in complement fixation had to consist of at least 4 antibody molecules held together through binding interactions with the antigen, while complexes consisting of one, two, or three antibody molecules were unable to fix complement (Jaton et al., 1976); this is in contrast with the earlier studies mentioned above which suggested that antibody dimers have the ability to activate complement.

Therefore, this study suggested that while CPL may monitor conformational alterations occurring in the antibody molecule upon antigen binding, such changes are insufficient for complement fixation to occur.

In a more detailed study, complement activation was measured through the use of C1-binding and C4-consumption assays, again with homogeneous rabbit anti-(Type III pneumococcal polysaccharide) IgG and bivalent (16 sugar residues) and trivalent (26 sugar residues) antigens (Medgyesi et al., 1979). Using these assays, which were found to be more sensitive than the whole complement consumption assay previously used, Jaton et al. (1976) found that complexes of antibody with either

the bivalent or trivalent antigens were capable of binding C1 and consuming C4. Fractionation by gel chromatography of antibody-antigen complexes allowed the use of complexes containing a given number of antibody molecules (monomers, dimers, etc. up to hexamers) in the C4-consumption assay; unfortunately, no data were presented which would allow an estimation of the stability of such complexes during the time required for the isolation and assay. With the bivalent antigen, complexes consisting of 2 or more antibody molecules were found to be active, while trivalent antigen provided activity even with monomeric complexes, which were proposed to be closed cyclic structures. Although most complexes with trivalent antigen were more active in consuming C4 than the corresponding complexes with the bivalent antigen, hexameric complexes with bivalent antigen were significantly more active than hexameric complexes with trivalent antigen (92% vs. 65%); this observation led the authors to suggest that in addition to the number of antibody molecules clustered togehter in a complex by antigen, complement activation may depend upon the spatial arrangements possible in such complexes.

An indirect confirmation of the inadequacy of the observed conformational changes to initiate effector function activity has come from the observation that the binding constant of Staphlococcus protein A to the F_c portion of the antibody molecule was independent of antigen binding for four homogeneous anti-(Type III pneumococcal polysaccharide) IgG preparations (Wright et al., 1978). If a large scale conformational change is necessary in the F_c portion to expose a C1q binding site, it was argued that such a structural alteration might be expected to affect the binding of protein A in some way. This issue

has also been addressed by studying the ability of rabbit immune complexes to compete with monomeric IgG for protein A binding sites (Sandor and Langone, 1981); an increase in affinity for protein A correlated with the aggregation of antibody molecules and was not related to the occupancy of antigen-binding sites. These findings are in contrast to the experiments using chicken IgG (Kronvall et al., 1970) discussed earlier.

Defined IgG complexes

The use of bivalent haptens has allowed the preparation of relatively stable IgG complexes which could be separated and assayed for complement fixing ability (Hyslop et al., 1970). Although IgG complexes differing in size were poorly resolved, it could be observed that dimers and probably trimers did not fix whole complement, while larger complexes were active. Addition of monovalent haptens to solutions of large, active complexes abolished the activity of the preparation to fix complement, confirming the reversible nature of these complexes and indicating that the assemblage of IgG into a multimeric form was a requirement for the development of the ability to activate complement.

To simplify the complexity of working with poorly defined non-covalent aggregates of antibody and antigen, it was recognized that bifunctional crosslinking reagents could be employed to prepare a series of antibody complexes. Ishizaka and co-workers took this approach by crosslinking non-immune rabbit IgG with bis-diazotized benzidine and then fractionating the complexes by gel filtration (Ishizaka et al., 1965). Assays of complement fixing ability indicated that the smallest aggregate which would still activate complement had a sedimentation

coefficient of 9.6S (dimer). Unfortunately, this activity could not be unambiguously assigned to the dimer as contamination from larger aggregates could not be completely ruled out.

The search for well-defined aggregates was extended through the use of bivalent affinity labeling reagents, with which it has been possible to prepare covalently bound artificial antibody complexes (Segal and Hurwitz, 1979) and to assay such complexes for complement fixation ability (Segal et al., 1979). Using heterogeneous rabbit anti-DNP antibody crosslinked with one of three bivalent reagents, little or no complement fixing activity could be detected from any oligomer in solution. When these same complexes were absorbed onto the surface of trinitrophenylated erythrocytes, complement fixation activity was detected which increased in parallel with oligomer size. A lower threshold of oligomer size with respect to complement fixation could not be found in the cell surface assay, suggesting that a "unit signal", or minimum number of IgG molecules needed in a cluster for activity did not exist for this system.

This work has been substantiated by a similar study of chemically crosslinked IgG with activity against Type II pneumococcal polysaccharide (Tschopp et al., 1980a). The ability of antibody complexes alone to activate C1 as measured by C1s cleavage was found to be dependent upon complex size, with monomer showing the lowest (although measurable) activity, indicating that no "unit signal" greater than monomer exists for this system. In addition, the concentration of a given IgG complex at which 50% of the C1s present was activated was approximately the same concentration as that needed for 50% saturation of C1q under the same conditions, suggesting that activation of C1s may

merely reflect the ability of IgG complexes to bind to C1q. The activation of C1s was not influenced by the presence of a specific hapten, a monovalent nonasaccharide, in clear conflict with the requirements of an allosteric model. These functional studies were supplemented by electron microscopic observations which showed IgG dimers bound to the globulin heads of the C1q molecule, while equilibrium sedimentation measurements afforded a value of 10^6 M^{-1} for the association constant of binding of dimers to C1q (Tschopp et al., 1980b).

Using non-immune rabbit IgG which had been chemically crosslinked (Wright et al., 1980a), the investigators performed whole complement fixation assays which showed that dimers, trimers, and tetramers of antibody alone could trigger the complement cascade, with potency increasing in that order (Wright et al., 1980b). Elimination of the crosslinks by reductive agents abolished the fixation activity of the preparation.

Special cases of complement fixation by monovalent antibody

DNP-poly lysine

While complement fixation by IgG has been found to occur predominantly only in the presence of multivalent antigens, it has been observed with monovalent antigens under special conditions. During an investigation of complement fixation by anti-DNP IgG and bivalent haptens, it was discovered that the antibody when complexed with 1-ε-DNP-lysine$_{20}$ was a potent activator of complement (Goers et al., 1975). The requirements for native antibody (rather than F_{ab} or F_c fragments, or antibody in which the interheavy chain disulfide bond had

been reduced and alkylated) and the specific DNP-hapten suggested that activation was proceeding via the classical pathway, and this mechanism was supported by measuring the depletion of individual complement components. In addition, the poly-lysine chain was required for activity, and succinylation to remove the multiple positive charges on the chain abolished the complement fixing activity. Further examination of this system revealed that the poly-lysine chain on the hapten was able to interact with C1r and C1s such that C1s became activated, and that this interaction could only occur if hapten-antibody complexes were bound to C1q (Goers et al., 1977).

Although this monovalent system does not allow general observations to be made concerning other IgG systems, it has made possible a more precise dissection of the events leading to activation of C1 than is afforded by typical antibody-antigen systems. C1q binding assays provided the observation that reduced and alkylated IgG bound as well to C1q as did native antibody, in spite of the fact that reduction of the interheavy chain disulfide completely abolished the complement fixing ability expected upon the addition of the DNP-poly-lysine hapten (Goers et al., 1977). This finding reemphasized the duality of C1-IgG interactions by providing a direct indication that two interactions must exist, one of which is reduction-insensitive and serves to bind C1q to the IgG molecule, and the other of which is reduction-sensitive and serves to cause the activation of C1.

IgM

An important distinction should be made between complement fixation by IgM and IgG. Since IgM is a pentameric molecule, it is inherently a complex of C1q binding sites, and thus might be expected to activate

complement in a monomeric fashion. Using heterogeneous IgM with specificity for the determinant phenyl-β-lactoside, Brown and Koshland (1975) found that these IgM preparations fixed complement in the presence of a monovalent protein-hapten conjugate, although the hapten alone was unable to elicit this response; the dependence of complement fixation depended directly upon the occupancy of binding sites by antigen, while high levels of antigen did not inhibit the fixation, suggesting that the mechanism of the expression of effector functions in this case did not involve aggregation of antibody molecules by a multivalent antigenic contaminant. Although no direct spectroscopic or physical evidence was obtained concerning the conformation of the IgM molecules, it was assumed that a conformational change was involved in this process. Additional studies with a number of different hapten-carrier conjugates clarified the observation that the simple binding of antigen to sites on the antibody molecule was sufficient to cause the presumed conformational changes in the F_c portion of the IgM molecule (Chiang and Koshland, 1979a). The role of the carrier was also examined, and although speculative, a model was proposed suggesting that non-specific interactions between the carrier and antibody caused alterations in the tertiary structure of unstable hinge regions in distant portions of the antibody molecule, thereby creating a new complement fixing activity (Chiang and Koshland, 1979b). Direct evidence concerning the conformation of IgM as a function of antigen binding was obtained by measuring the accessibility of the J chain of IgM antibody to anti-J chain antibodies; using IgM antibodies specific for either azophenyl-β-D-lactoside or azobenzenearsonate haptens, these investigators observed enhanced J chain exposure when the IgM molecules

were complexed with mono-substituted or multi-substituted hapten-carrier conjugates (Brown and Koshland, 1977). Since the J chain is attached to the terminal C_H4 domain of the μ chain and is normally folded within the F_c structure, this enhancement of J chain accessibility was taken as evidence of a conformational change as a result of antigen binding.

Since these studies indicate that 1) IgM is able to fix complement even at high levels of antigen, under conditions at which all antigen-binding sites are occupied and aggregation of IgM molecules is therefore prevented, and b) an apparent correlation exists between occupancy of antigen-binding sites and expression of complement fixation activity, it seems fairly clear that conformational changes alone may be both necessary and sufficient in IgM molecules for the expression of effector functions. However, other investigators have suggested using equine IgM that multiple attachment between the antigen and IgM molecule is necessary for complement activation to occur (Siegel and Cathou, 1980). Unfortunately, more precise details of the mechanism of effector function triggering can not be obtained for IgM without using homogeneous antibody preparations.

Studies on IgG complexes, produced either through interactions with multivalent antigen or the use of bifunctional chemical crosslinking agents, have clearly shown that in some cases the antibody complexes in the absence of any antigen have the ability to mimic complement fixation activities. The inability of other IgG complexes to trigger complement fixation functions might be due either to differences in the antibody-antigen system, the method of antibody complex preparation, or the specific assays employed to quantitate complement fixation. Although a definitive picture cannot yet be drawn based upon the

evidence so far obtained, it seems clear that aggregational processes must play a necessary, if not sufficient, role in complement fixation by IgG molecules.

Quantitative Complement Assays

Since one of the in vivo functions of the complement cascade is to lyse foreign cells, the quantitative measurement of complement activity in a biologic fluid has traditionally utilized erythrocytes coated with hemolytic antibodies as an indicator system. Such an approach was particularly valuable since an erythrocyte when lysed releases hemoglobin which is easily measured by spectrophotometric techniques. Standard procedures have been described in which the total reaction volume is on the order of 10 ml, requiring the use of large amounts of complement and cells (Kabat and Mayer, 1961). Although a method has been designed to scale down the assay (Wasserman and Levine, 1961), this technique still requires a total reaction volume of 0.7 ml and a cell concentration of 5×10^{7}/ml. The number of cells used in each single determination dictates the range of complement activity which can be accurately measured; this is due to the fact that the hemolytic response to the amount of complement present is sigmoidal, and thus complement activity can only be measured accurately between 10% and 90% hemolysis.

The von Krogh relationship (von Krogh, 1916) has been commonly used to describe this sigmoidal response:

$$x = K \left(\frac{y}{1-y}\right)^{1/n}$$

where x is the amount of complement added, expressed in mls of diluted complement containing fluid, usually serum. The variable y represents the fraction of cells lysed as determined by the quantification of

released hemoglobin. These measured quantities are related by the constant K, which is the volume of fluid required to hold one CH_{50} unit of complement. One CH_{50} unit is that amount of complement required for lysis of 50% of the cells present under the assay conditions of concentration, cell sensitivity, time and temperature. The exponent 1/n is a reflection of the steepness of the response curve and depends upon a number of factors, including cell concentration (Kabat and Mayer, 1961); this dependence arises from the ability of a single complement activating focus (C1) to generate multiple C4b,2a complexes which are then able to either absorb directly onto the initiating cell membrane or to diffuse to an immediately adjacent cell (Chapter 1).

Logarithmic transformation of the von Krogh relationship allows the determination of K after titration of a source of complement against a fixed number of cells under standardized conditions. The large number of variables involved in this assay, such as the rapidity of heating and cooling samples, and the degree of cell sensitization (age of cells, activity of hemolysin preparation), make it impossible to meaningfully compare quantitative complement activity results from different laboratories.

The quantitative measurement of complement activity is required in order to analyze the ability of immune complexes to activate, or fix complement in standard preparations via the classical pathway. A two-stage procedure is commonly employed in this analysis (Kabat and Mayer, 1961). A known amount of complement is first incubated with immune complexes to allow complement activation to occur; once activated in the absence of cells these complement components rapidly lose

activity. Sensitized cells are then added to the reaction mixture in order to measure the amount of complement activity remaining after the first incubation. Again, the experimental results depend highly upon the exact procedures used, so that the results of complement activation by antigen-antibody complexes can be meaningful only if highly ritualized protocols are followed.

Just as the number of sensitized cells used for an indicator system determines the range of complement activity which can be accurately measured, so too does the activity of complement present determine the amounts of antibody and antigen which are required in order to observe any complement fixation. Unfortunately, both the "macro" and "micro" assays described by Wasserman and Levine (1961) would have required prohibitively large amounts of levan and levan fragments for a functional analysis of the UPC-10 myeloma protein system. A restriction in the availability of antigen therefore required an improvement in the sensitivity of the indicator system which would allow the entire assay to be sealed down in terms of cells, and therefore antigen, needed.

MATERIALS AND METHODS

General

UPC-10 myeloma protein, levan from Aerobacter levanicum and perennial ryegrass, and oligosaccharide fractions derived from bacterial levan were isolated and characterized as described in Chapter 2.

Guinea pig serum was prepared from blood obtained from animals of the DHC/BA strain (Caltech) by heart puncture. The tubes of blood were allowed to clot at room temperature for 30 minutes and the clots were retracted during a 2-hour incubation at 4°C. The serum was separated from the clot by centrifugation at 2000 rpm in a GLC-2 centrifuge (Sorvall) at 4°C for 5 minutes and the serum supernatant was recentrifuged to remove all traces of cells. The serum was then immediately frozen and stored at -70°C.

Buffers

The buffers used for complement fixation are designated as follows: GVB^{2+}- 5.5 mM sodium barbitol, 0.142 M NaCl, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1 g/l gelatin, pH 7.4; EDTA-GVB- 5.5 mM sodium barbitol, 0.142 M NaCl, 1 g/l gelatin, 0.01 M EDTA, pH 7.4. All solutions used in complement assays were prepared with double glass distilled water.

Phosphate-buffered saline consisted of 20 mM sodium phosphate, 0.15 M sodium chloride, pH 7.4.

Preparation of Sensitized Erythrocytes (EA)

Washed sheep erythrocytes were obtained from Colorado Serum Supply Co. and were used within three weeks of receipt. One volume of cells

(10^9 cells/ml) was washed three times with EDTA-GVB and mixed with an equal volume of hemolysin (Cordis Laboratories) diluted 1/200 in EDTA-GVB; this cell suspension was incubated with occasional shaking at 37°C for 30 minutes, then at 0°C for an additional 30 minutes. The activated erythrocytes (EA) were washed once with EDTA-GVB and twice with GVB^{2+} and were stored in GVB^{2+} at 4°C for a maximum of three weeks. In order to calculate cell concentrations, an aliquot of a cell suspension was diluted into distilled water and the absorbance due to hemoglobin measured; the absorbance of a 1 mM solution of hemoglobin was taken to be 13.8 OD units at 541 nm (Antonini and Brunon, 1971) and a value of 10^{-11} grams was used for the amount of hemoglobin contained in the average sheep erythrocyte (Payne et al., 1976).

Macro Assay

Titration of whole complement activity in guinea pig serum

Freshly thawed guinea pig serum was diluted 300 fold into GVB^{2+} and stored at 0°C until used. All samples were prepared in duplicate according to Table 1 and were incubated at 37° for 30 minutes with shaking every 10 minutes. Ice-cold GVB^{2+} (0.5 ml) was then added to tubes 1-7 and all tubes were centrifuged for 5 minutes at 2000 rpm in a GLC-2 centrifuge (Sorvall) at 4°C. The supernatant absorbances were measured at 541 nm.

Absorbance values for duplicate tubes were averaged and used to calculate the percentage lysis from the following formula:

where A = absorbance of any sample tube (1-6)

A_7 = absorbance of control tube 7

A_8 = absorbance of control tube 8

The number of complement units per ml of diluted serum needed to cause 50% lysis was graphically determined by plotting the volume of diluted serum added vs. percent lysis for each tube on probability times log paper (Figure 1).

Whole complement fixation assay

All tubes were prepared in duplicate. All dilutions were made into freshly prepared GVB^{2+}; UPC-10 myeloma protein was in PBS. Sample tubes were prepared with 50 μgms antibody, 2 CH_{50} units of guinea pig complement, antigen, and enough GVB^{2+} to bring the total volume to 0.9 ml. These tubes were incubated at 37° for 1 hour with gentle shaking, and then were rapidly cooled in an ice-water bath. An aliquot (0.6 ml) of a suspension of EA in GVB^{2+} (2.5×10^8 cells/ml) was added to each tube, after which the tubes were incubated at 37° for an additional 30 minutes. Ice-cold GVB^{2+} (0.5 ml) was then added to each tube and the tubes were centrifuged at 2000 rpm in a GLC-2 centrifuge (Sorvall) at 4°C for 5 minutes. The absorbances of the supernatants were measured at 541 nm.

In addition to the sample tubes with antibody, four controls were run: 1) for each set of tubes with antibody and antigen, a corresponding set with the same amount of antigen without antibody; 2) a pair of tubes with antibody, complement, and buffer alone (antibody control); 3) a pair of tubes with complement and buffer alone (buffer control); and 4) a pair of tubes with buffer alone (cell control).

Absorbance values were averaged for each pair of tubes and used to calculate the precentage of complement fixation using the following formula:

$$\% \text{ fixation} = 100 - \frac{A-x}{y-x} \times 100$$

where A = absorbance of sample at 541 nm

x = minimal lysis = absorbance of cell control

y = absorbance of antibody or buffer control, for tubes with antibody and antigen or antigen alone, respectively.

Micro Assay

For the micro assay, all samples were prepared in the wells of a microtiter plate instead of tubes. Flexible 96-well plates were obtained from Cooke Laboratory (Alexandria, VA); these were superior to standard hard plates as they could be conveniently floated in a water bath to carry out the desired incubations.

Two different incubation protocols for the micro assay were followed as indicated (Kabat and Mayer, 1961). One was identical to the macro assay in that the sample mixture was incubated at 37° for one hour, after which cells were added and incubated at 37° for an additional 30 minutes. A second procedure replaced the initial one hour with an 18 hour incubation at 4°C. In either case, the corresponding incubation protocol was used for the complement titration as well, in order to take account of the thermal and temporal lability of whole complement activity.

Preparation of ^{51}Cr-EA

Sensitized erythrocytes were loaded with $Na_2{}^{51}CrO_4$ by a method based on that of MacDonald et al. (1975). Cells were washed with GVB^{2+} and resuspended at a concentration of 10^8 cells/ml. An equal volume of $Na_2{}^{51}CrO_4$ (1 Ci/ml) in physiologic saline (Amersham) was added and incubated 2 hours at 37°. The cells were then washed three times with GVB^{2+} and resuspended at a concentration of 5×10^8 cells/ml. Cells were loaded with the radioisotope immediately prior to each experiment.

Titration of whole complement activity in guinea pig serum

This procedure was identical to the titration used for the macro assay except that the total volume in each sample was reduced to 100 µl. Increasing amounts of freshly thawed diluted guinea pig serum were diluted to 100 µl with GVB^{2+} and allowed to incubate as described above. Identical aliquots of 10 µl of a ^{51}Cr-EA suspension were added to each well and incubated at 37° for 30 minutes. The cells were pelleted by centrifugation and 10 µl aliquots in duplicate were removed from each supernatant for counting. The radioactivity data were analyzed in a manner similar to that described for the absorbances in the macro titration.

Whole complement fixation assay

Mixtures of antibody, antigen and 2 units of complement were diluted to a total volume of 100 µl with GVB^{2+}. Incubations were carried out at 37° for one hour or at 4° for 18 hours, after which time the plates were chilled in an ice-water bath and an aliquot (10 µl) of a

suspension of ^{51}Cr-EA (5 x 10^8 cells/ml) was added to each well. The cells were incubated at 37° for 30 minutes, chilled to 0°C, and pelleted by centrifugation. Aliquots (10 µl) were removed for determination of radioactivity on a gamma counter (Beckman). The radioactivity data were analyzed as described for the absorbance data in the macro assay.

Sedimentation velocity analysis

Ultracentrifugation experiments were performed in the laboratory of Dr. V. Schumaker, UCLA using instrumentation which has been described elsewhere (Schumaker et al., 1980). Briefly, a Beckman Model E analytical ultracentrifuge equipped with double-sector cells, xenon light source, mirror optical system, and photoelectric scanner was used. Output from the scanner was fed directly to a PDP 11/45 computer, reading approximately 40 data points per mm of cell length. These data were plotted as optical density vs. distance and then converted to a derivative plot using a Fourier transform smoothing procedure to reduce noise.

RESULTS

Complement Fixation Assay

Both macro and micro assay procedures have been used in the following experiments. The macro assay relies upon the spectrophotometric determination of released hemoglobin from sensitized erythrocyte as an indicator system, while the micro assay employs sensitized erythrocyte preloaded with $^{51}CrO_4$, thereby allowing the accurate measurement of the degree of lysis of fewer cells. This is in accord with the original description of red cell labelling by radioactive chromium (Gray and Sterling, 1950) and recognizes the first use of such a technique in a complement assay (Weinrach et al., 1958). Indeed, many recent complement methods have been published using this approach (Joiner et al., 1979; Bengali et al., 1980).

The difference between the macro and micro assays can be seen in their volumes (0.9 mls vs. 0.1 mls) and in the number of sensitized cells used (1.5×10^8 vs. 5×10^6), respectively. Representative plots of both macro and micro complement titrations are shown in Fig. 2. Application of the von Krogh relationship to these data provides values of K, or specific complement activities, for each serum. In addition, values of 1/n can be calculated to be 0.19 and 0.13 for the macro and micro assays, respectively. The shift to a smaller value of 1/n upon going to the micro assay implies a steeper sigmoid response curve, and therefore a more sensitive measure of complement fixation (Wasserman and Levine, 1961).

The choice of assay used for any particular experiment rested largely upon the availability of antigen. Due to the microliter volumes

required for the micro assay, this procedure inherently had more fluctuations in the resultant data, so that the macro assay was performed when possible. Two different protocols were used for the first incubation step of the micro assay. The mixture of antibody, antigen, and complement was either incubated at 37°C for 1 hour or 4°C for 18 hours. The 4°C incubation was used with poorly-binding antigens in order to enhance antigen-antibody interactions; in addition, a low-temperature assay has been shown to provide more efficient complement fixation (Kabat and Mayer, 1961).

Complement Fixation by UPC-10 Myeloma Protein and Ryegrass Levan

The dependence of complement fixation by UPC-10 myeloma protein upon the concentration of ryegrass levan as measured using the macro assay is shown in Fig. 3. Antibody concentration in the intial incubation step was 3.7×10^{-7} M. It can be seen that the 50% lysis point for antibody-mediated (classical) activation occurs at an antigen concentration of 0.25 mg, or 5.5×10^{-5} M, assuming a molecular weight for the grass levan of 2000 daltons (Bell and Palmer, 1949). The ability of the ryegrass levan to activate complement in the absence of antibody is the result of the alternate pathway; the presence of UPC-10 myeloma protein provides a 2-3 fold reduction in the amount of carbohydrate needed to activate the same amount of complement. Due to the limited amounts of grass levan available, it was not possible to extend the assay into higher antigen/antibody ratios.

The failure of the curve corresponding to antigen in the absence of antibody to plateau at 0% fixation for decreasing amounts of added antigen is due to the finite amount of time required to quench each

reaction mixture after the second incubation, thereby providing the complement in those tubes at low antigen concentrations more time in which to lyse the sensitized erythrocytes than in the control tubes which were quenched first. This problem was corrected in later experiments by cooling all sample mixtures simultaneously to 0°C following the second (cell) incubation.

Complement Fixation by UPC-10 Myeloma Protein and Bacterial Levan

The combined results from several macro complement assays using 3.7 x 10^{-7} M antibody and levan isolated from Aerobacter levanicum are shown in Fig. 4. Fig. 5 shows the corresponding micro complement assays using a 4°C initial incubation step with 6.7 x 10^{-7} M antibody. The curve exhibits classical activation and indicates that the amount of bacterial levan required for 50% fixation in the macro assay was only 2 nanograms, or 1.1 x 10^{-12} M using a molecular weight for the bacterial levan of 1.75 x 10^{7} daltons (Arigad and Feingold, 1957). The striking observation seen with this antigen in both assays is that the ability of antibody to activate the classical pathway was lost when large amounts of antigen were added.

The dependence of the initiation of complement fixation using whole bacterial levan upon antibody concentration was examined using micro assays with an initial 37° incubation and is shown in Fig. 6. Over this range of bacterial levan concentrations, there is no observed dependence of complement fixation upon antibody concentration; under these conditions, the formation of complexes able to activate complement depends solely upon the concentration of antigen, and this observation

may reflect the strong affinity of UPC-10 myeloma protein for this antigen.

Complement Fixation by UPC-10 Myeloma Protein and Levan Fractions

The relative abilities of levan fractions (isolated by G-50 Sephadex chromatography as described in Chapter 2) to fix complement in combination with UPC-10 myeloma protein were measured using micro complement assays in order to investigate the effect of antigen size upon the formation of antigen-antibody complexes with complement fixation activity. Complement fixation curves were obtained using 6.7×10^{-7} M antibody in the sample wells; antibody and antigen were allowed to incubate with complement at 4°C in the first step of the assay in order to enhance the binding affinity of antibody for antigen. The von Krogh relation was fitted to the experimental curves and was used to determine the amounts of antigen required to elicit 50% complement fixation, as shown in Table 2.

The series of sized antigen fractions can be clearly seen to vary in their ability to activate the classical pathway of complement in combination with UPC-10 myeloma protein. Smaller molecular weight levan fractions are less able to assemble antibody molecules into active complexes, and therefore require antigen concentrations (on a weight basis) higher than those needed for the high molecular weight levan fractions in order to achieve 50% complement fixation.

The dependence of complement fixation using levan fractions 59-79 and 91-95 upon antibody concentration was explored using micro complement assays with 37°C and 4°C initial incubation steps, respectively, and is shown in Figures 7 and 8. It is evident that the

antibody concentration markedly affects the ability of both antigens to assemble active complexes. This is in contrast to the results obtained with the whole bacterial levan, for which no antibody concentration dependence of complement fixation was found.

In addition to the levan fractions consisting of a range of polysaccharide molecular weights, smaller oligosaccharides isolated using sequential chromatography on Biogel P-2 and Sephadex G-50 as described in Chapter 2 were tested in the micro complement assay with a 37° initial incubation step. None of these oligosaccharides containing either 15, 16 or 19 sugar residues were found to fix a measurable amount of complement as tested up to an antigen concentration of 3×10^{-4} M in the presence of 6.7×10^{-7} antibody, conditions under which fraction 59-79 was found to fix complement strongly (Fig. 7).

Sedimentation Velocity Analyses of Antigen-antibody Complexes

Sedimentation velocity analyses were performed for the complexes formed between UPC-10 myeloma protein and all of the bacterial levan fractions which had been studied in complement fixation assays. The derivative plots from a representative experiment conducted at 60,000 rpm in a titanium ANF rotor at 12.9-14.7°C using an antibody concentration of 3.3×10^{-6} M and variuos concentrations of levan fraction 91-95 are shown in Figure 9. Comparing these profiles to that obtained for pure UPC-10 myeloma protein (Chapter 2), it can be seen that the antigen produced a marked disturbance in the sedimentation characteristics of the antibody through its ability to complex with multiple antibody molecules. It can also be seen that the degree of alteration from the Gaussian curve expected for a single,

non-associating migrating species (Schachman, 1959) is increased with increasing antigen concentration, further supporting the role of antigen as a cross-linking agent. However, it was not possible to obtain clearly separated molecular complexes for most levan fractions with antibody even at high speed (60,000 rpm) and low temperatures (4°C), most likely due to the relatively weak association between UPC-10 myeloma protein and the levan fragments (Chapter 2). Such a situation allows a continually reassociating pool of antibody and antigen to exist. For this reason, two criteria were used in the evaluation of complex formation for those levan fractions which did not produce clearly defined complexes in combination with antibody. In addition to the distortion of the Gaussian curve expected for a single migrating species as discussed above, the sedimentation coefficient was measured from the derivative plot for the major peak. This value could then be compared to that expected for UPC-10 myeloma protein alone ($S_{20,W}$ = 6.52, Chapter 2); increases in the sedimentation coefficient above that expected for pure antibody would reflect the contribution of complex formation, even in the absence of any apparent isolated complexes. Representative derivative plots of UPC-10 myeloma protein alone and in the presence of levan fraction 59-79 are shown in Fig. 12 (Chapter 2) and NN. It can be calculated that the sedimentation coefficient for the major derivative peak obtained as a result of antibody migration had indeed increased from $S_{20,W}$ = 6.52 S to 7.90 S upon addition of antigen.

The results of similar analyses performed for all levan fractions are shown in Table 3. Arranged in order of decreasing molecular weights, it can be seen that complex formation as assessed by peak shape

distortion is strongly dependent upon molecular weight, so that the small oligosaccharides studied (containing 10-19 sugar residues/molecule) produced little or no change in the derivative peak profile. Consideration of the sedimentation coefficient of the major species suggests that it is a much poorer determinant of complex formation, largely because significant amounts of faster-migrating complexes may form without affecting the sedimentation of monomeric IgG molecules.

The apparent paradox in the abilities of 15-mer and 19-mer to form aggregates comparable to those exhibited by fractions 111-115 and 116-120 containing higher molecular weight molecules is most likely due to the fact that these oligosaccharides of defined size were tested at molar concentrations greater than ten times those used for the larger fractions; this was done in an effort to observe whether any complex formation could be detected with a given fraction. It is noteworthy that decamer was unable to form aggregates even at the highest concentration of any which were examined.

In order to examine an antibody-antigen mixture under conditions at which 50% complement fixation had been observed to occur, a solution was prepared containing 0.1 mg/ml UPC-10 myeloma protein and 120 ng/ml levan fraction 59-79 (Table 2); samples were spun at 60,000 rpm and scanned at 230 nm. The derivative plot, shown in Fig. 11, exhibited only minor alterations from that expected from a monomer antibody preparation, in that the leading edge of the migrating protein boundary had developed some irregularity in shape and the sedimentation coefficient for the observed peak had increased to $S_{20,W}$ = 6.90 S. These suggestions of complex formation are much more subtle than the results obtained with

larger ratios of fraction 59-79 to antibody, and it is important to note that antigen concentrations listed in Table 3 are in excess of those needed for 50% complement fixation for those fractions with such activity.

DISCUSSION

In order to be able to investigate the ability of an antibody preparation in combination with its corresponding antigen to activate the classical complement pathway, it is imperative to use quantitative complement assays which are accurate and reproducible. Due to the biological variability inherent in crude sera or batches of erythrocytes, it is critically important to maintain controls upon each step of the procedure, as shown by the fact that all sera were assayed separately for complement activity and cells were used within a brief three-week period following sensitization. Added to these complications is the fact that many of the reactions, including the fixation of complement by immune complexes and the lysis of sensitized erythrocytes by complement, do not proceed rapidly to completion but instead follow a course which is strongly temperature and concentration dependent (Kabat and Mayer, 1961), making it very difficult to compare in a quantitative fashion experimental results from two different laboratories.

The micro complement assays which have been described in these studies, although largely based upon standard methods (Wasserman and Levine, 1961), have been modified in order to provide extreme sensitivity of biologic function while requiring a minimum amount of the limited antigen. This goal was accomplished through the combination of radioisotopic loading of erythrocytes with ^{51}Cr to provide a sensitive measure of lysis, a scaling down of the amounts of reagents--complement, antibody, antigen, and sensitized cells--required for the assay, and a facilitation of the use of such small quantities through the use of microtiter plates and microliter sampling techniques. These modifications made it possible to improve the sensitivity of the assay

as reflected in a smaller value of 1/n in the von Krogh analysis (Wasserman and Levin, 1961).

By use of the quantitative complement assay the abilities of the whole levans isolated from perennial ryegrass and Aerobacter levanicum to initiate classical complement fixation in combination with UPC-10 myeloma protein were studied. Both levans were seen to be active in this regard, although bacterial levan was more potent by a factor of 5×10^7 on a molar basis. Such a dissimilarity could be explained by either differences in binding affinity to the antigen or differences in the number of antigenic determinants per levan molecule. Although specific binding constants cannot be obtained between UPC-10 myeloma protein and these antigens, it can be argued that since the grass antigen has an average molecular weight of 5000 daltons (Bell and Palmer, 1949) and therefore contains approximately 30 β2→6 linked fructofuramose rings, each levan molecule must contain at least one full antigenic determinant, based upon the observations that carbohydrate-binding antibodies have antigen-binding sites which are complementary to 3-6 sugar residues (Chapter 1). Thus, the observed relative inability of the grass antigen to initiate the classical complement pathway in combination with antibody cannot be due to a lack of antigen binding by the antibody, but rather must be explained by the much greater propensity of the bacterial antigen to contain multiple antigenic determinants, due to its higher molecular weight.

The superior potency of the bacterial antigen to trigger the complement fixation activity of UPC-10 myeloma protein allowed for approximately a 10^6 fold range of antigen concentrations to be tested in the fixation assay. Thus, five zones of antigen concentrations could

be defined; the first three zones--insufficient antigen to produce any fixation, sufficient antigen to produce partial fixation, and sufficient antigen to produce maximal (100%) fixation--were demonstrated for both the bacterial and grass antigens. However, the ability to examine relatively high concentrations of bacterial levan in the fixation assay made it possible to observe a zone in which increasing amounts of antigen partially inhibited complement fixation, followed by a zone in which complement fixation was completely inhibited (Fig. 5).

It is important to consider whether the colligative effect of adding increasing amounts of carbohydrate to the assay system had either an inhibitory effect upon the complement components or a lytic effect upon the cells, both of which could account for the decrease in complement fixation at high concentrations of bacterial antigen. This region of inhibition at high antigen concentrations (high-zone inhibition) occurs around a 6×10^{-9} M (100 μg) concentration of bacterial levan. In contrast, classical complement fixation is first seen with the grass levan at a concentration of 2×10^{-5}-2×10^{-4} M. Any non-specific interaction of the bacterial antigen with cells or complement to produce the high-zone inhibition would also be expected to exist for the grass antigen as well, due to their structural similarity. Thus, the fact that classical complement activation with the grass levan takes place at carbohydrate concentrations at least 2000-fold higher than those of concern with the bacterial levan strongly suggests that non-specific factors are not responsible for the high zone inhibition seen in the bacterial levan assay.

Another possible cause for the high-zone inhibition seen with the bacterial levan would be the potential increase in solution viscosity

caused by the progressive addition of a high molecular weight carbohydrate to the sample, thereby possibly producing an increasing tendency for the erythrocytes to lyse during mixing or pipetting steps. However, since the buffer used for all samples (GVB^{2+}) contains 1 gram of gelatin per liter, the addition of as much as 100 μg of bacterial levan (to give 0.1 g levan/l) cannot provide even a significant change in the viscosity of this solution.

With the elimination of non-specific causes for the high-zone inhibition of classical complement activation observed with the bacterial levan, this effect must be due to the specific interactions of antibody and antigen that lead to the development of this effector function. Such interactions can be analyzed in light of the two models which have been formulated for the expression of effector functions--the conformational and aggregational mechanisms of classical complement activation. A purely conformational model would require that the expression of the effector function would reflect solely the degree of antigen binding site occupancy so that a single antibody molecule bound to two separate antigen molecules would exhibit maximal ability to fix antigen. Since such species are favored at high antigen/antibody ratios, the expected curve for percent fixation versus antigen concentration would show the maximal development of fixation at some antigen concentration, above which the degree of fixation would remain maximal. There is no explanation in a conformational model for the presence of the observed high-zone inhibition, as outlined by Metzgar (1978). Alternatively, an aggregational model predicts just such a behavior. If multimeric antibody complexes are required in order for complement fixation to be initiated, such complexes will only be present

within an intermediate range of antigen/antibody ratios. With less antigen, most of the antigen-binding sites on the antibody will be vacant so that complexation will not occur. Correspondingly, higher antigen/antibody ratios will produce a situation in which each antibody molecule is favored to bind two separate antigen molecules, again preventing the formation of complexes containing multiple antibody molecules. Such considerations provide the explanation for the familiar precipitin curve seen with antibody and multivalent antigen (Kabat, 1968) which depends upon the formation of large, insoluble immune complexes. In this way, the observed complement fixation ability of the UPC-10 myeloma protein seen as a function of the bacterial levan concentration provides strong evidence for an aggregational mechanism.

In addition to the strict conformational model, a modified hypothesis has been advanced which postulates that distortion of the antibody molecule through interaction with antigen, possibly by altering the angle between the F_{ab} arms, in some way facilitates the binding between the antibody and C1q (Metzgar, 1978). Such a distortion model has received support from a study in which monomeric antibody molecules, each presumed to exist as a closed cyclic structure in combination with a single antigen molecule, were found to produce C4 consumption (Medgyesi et al., 1979). Since the formation of such complexes would be progressively inhibited by an increase in antigen/antibody ratio above a certain point, it is not possible to rule out such a mechanism from studies on the UPC-10 myeloma protein. In order to do so, the affinity of the antibody for antigen would have to be great enough to allow sufficient time for isolation and functional assessment of such monomeric complexes.

One fascinating incidental finding of these studies is that the solely β2→6 linked grass levan is a potent activator of the alternate complement pathway, while the bacterial levan, containing β2→1 branches, is totally devoid of such activity. Although current understanding of the initiation steps of the alternate pathway is incomplete, proposed mechanisms have focused upon the role of C3b and its interactions with target surfaces; it has been suggested that C3b is generated spontaneously and deposited randomly upon cell surfaces, with the discrimination as to whether alternate pathway will proceed being determined by the capacity of the C3b molecule to interact appropriately with cell surface molecules, probably carbohydrates (for a recent review see Muller-Eberhard and Schreiber, 1980). Such a mechanism does not attempt to explain the distinctive abilities of various carbohydrates to activate the alternate pathway. Through specific molecular alterations, some important structural features of streptococcal cell walls (Greenblatt et al., 1978) and glucaon (Inai et al., 1976) in relation to their ability to trigger the alternate complement pathway have been eludicated, but these have not provided a generalized mechanism for such activation.

Through the use of bacterial levan fractions which had been sized according to molecular weight by gel chromatography, it was possible to investigate the effect of antigen size upon its ability to assemble antibody molecules into complement-activating complexes. Above a certain size (greater than approximately 10,000 daltons), antigen molecular weight does not appear to affect the complement-fixing ability of the immune complexes, although antigens smaller than 10,000 daltons are markedly less potent in this regard. This agrees well with the

findings of Medgyesi et al., 1979) with a rabbit anti-pneumococcal Type III polysaccharide antibody, in that larger, multivalent antigens were more effective in complement fixation than were bivalent antigens. Unfortunately, it was not possible with either the rabbit antibody or UPC-10 myeloma protein to measure these antigen binding constants directly, so that the question of whether the impaired complement fixation abilities of smaller bacterial levan fractions were due to poorer binding or to decreased multivalency cannot be answered.

The observed dependence of complement fixation using these levan fragments upon antibody concentration is in contrast to the lack of dependence using the whole bacterial antigen. This dependence suggests a weaker avidity of the antibody for the fragments, so that antigen-antibody binding, and therefore complex formation, is enhanced by increasing either the antigen or antibody concentrations. More importantly, the results shown in Figure 8 clearly demonstrate that both the appearance and disappearance of complement fixation with increasing antigen concentration depend in a specific way upon the concentrations of antibody and antigen. This reemphasizes the fact that the high-zone inhibition of complement fixation, a feature required by an aggregational hypothesis, is a specific property of the interactions between antibody and antigen and is not an artifact created by non-specific colligative effects due to the addition of antigen to the sample solutions.

The failure of the branched bacterial oligosaccharides containing 15 or 19 sugar residues to produce any detectable complement fixation in combination with antibody is in contrast to the observed ability of the unbranched oligosaccharide of the pneumococcal Type III series to

activate complement in combination with the corresponding antibody. Assuming an upper limit on the size of an antigen binding site to be complementary to six sugar rings (Cisar et al., 1975) an unbranched oligosaccharide approximately 20 residues in length would be expected to be functionally bivalent. However, the proportion of bacterial levan oligosaccharides containing only β2→6 linkages (no β2→1 branches) drops rapidly with increasing chain length (Chapter 2). Since UPC-10 myeloma protein binds only to unbranched segments of β2→6 linked fructose (Cisar et al., 1974), it is not surprising that an undetectable portion of these bacterial oligosaccharides would contain two separate antigenic determinants.

The direct observation of complex formation between bacterial levan fragments and UPC-10 myeloma protein was made possible through the use of analytical ultracentrifugation. Although specific molecular complexes could not be separated under the most favorable conditions of maximal speed and minimal temperature, most likely due to the relatively weak association between UPC-10 myeloma protein and levan oligosaccharides (Chapter 2), it is striking that all of the levan fragments which showed measurable amounts of complement-fixing ability in combination with antibody (fractions 91-95 through 106-110; Table 2) were also most active in the formation of immune complexes as assessed by the ultracentrifugal analysis. The functional assay of complement fixation as an indicator for complex formation is so extremely sensitive that it can detect antigen-antibody complexes at a concentration that is so small as to be undetectable in the ultracentrifuge; for this reason it was not possible to correlate the appearance of complement fixation with the appearance of immune complexes as seen in the ultracentrifuge.

That such a correlation does exist, however, is shown in Fig. 11, in which antibody and antigen concentrations were adjusted to correspond to 50% complement fixation in the micro assay; the increase of antibody sedimentation coefficient to $S_{20,W}$ = 6.90 S from 6.52 S suggests the role of antigen as an aggregating agent.

SUMMARY

The studies described in this thesis represent the first use of a myeloma protein of the IgG class to investigate antigen-induced effector functions. Through the application of the homogeneous antibody secreted by the UPC-10 plasmacytoma, it has been possible to explore the nature of the antigen-antibody interactions which enable the antibody to fix complement. One of the limitations of this system was imposed by the difficulty encountered in the preparation and purification of the various levan antigens; this restriction was resolved by the development of a sensitive complement assay which could be performed with nanogram quantities of antigen.

The availability of both ryegrass and bacterial levan made possible a comparison of the relative abilities of these two polysaccharide preparations to initiate classical complement activation in association with the UPC-10 myeloma protein with respect to antigen molecular weight and the presence of branching in the levan chain. The importance of antigen size was more closely evaluated through the investigation of the ability of bacterial levan fragments of well-defined chain length to initiate complement fixation, as well as the analytical ultracentrifugation analysis of the resulting immune complexes. From these studies a number of clear conclusions may be drawn.

The studies of complement fixation clearly demonstrate that the ability of immune complexes to fix complement exists only in a specific range of antibody:antigen ratios, with these ranges being determined by several factors, including antibody concentration, antigen size, and the affinity of the antibody for the antigen determinant. It has been possible to show here that the observed behavior of these immune

complexes in complement fixation is not consistent with a mechanism of complement activation which does not require the aggregation of IgG molecules as a necessary, if not sufficient, step. This requirement for aggregation has been underscored by results from ultracentrifugation experiments which suggest that only those antigens which can be demonstrated to form aggregates with antibody molecules have the capacity to induce classical complement fixation. Thus, the use of a homogeneous myeloma protein with a well-defined series of antigen preparations has led to an increased understanding of the mechanism of complement activation by IgG.

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Table 1

COMPOSITION OF SAMPLE WELLS FOR MACRO COMPLEMENT TITRATION

Tube Number	1	2	3	4	5	6	7	8
UBS	0.8	0.7	0.6	0.5	0.4	0.3	0.9	---
Guinea pig serum (1/300)	0.1	0.2	0.3	0.4	0.5	0.6	---	---
EA (2.5×10^8 cells/ml)	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
H_2O	---	---	---	---	---	---	---	1.4

Tube #7 is 0% lysis control.
Tube #8 is 100% lysis control.
All volumes in milliliters.

Table 2

RESULTS OF MICRO COMPLEMENT FIXATION ASSAY USING UPC-10 MYELOMA PROTEIN AND BACTERIAL LEVAN OLIGOSACCHARIDES

levan fraction (Ag)	approximate molecular weight	nanograms Ag required for 50% complement fixation*
59 - 79	1000 - 12700	12
91 - 95	12700	10
96 - 100	10200	8
101 - 105	8100	35†
106 - 110	6500	212†

*Using 10 μg Ab/well.
†Estimated from best fit of von Krogh relation.

Table 3

ULTRACENTRIFUGATION OF UPC-10 MYELOMA PROTEIN IN COMBINATION WITH LEVAN OLIGOSACCHARIDE PREPARATIONS

Fraction	distortion of peak shape	sedimentation coefficient of major species
59-79 (1.1×10^{-5} M)	+++	7.9 S
91-95 (7.8×10^{-7} M)	+++	6.59 S
96-100 (9.8×10^{-7} M)	+++	6.39 S
101-105 (1.2×10^{-6} M)	+++	6.86 S
106-110 (1.5×10^{-6} M)	+++	6.70 S
111-115 (1.9×10^{-6} M)	+	6.52 S
116-120 (2.4×10^{-6} M)	+	6.86 S
19-mer (3.2×10^{-5} M)	++	7.9 S
15-mer (4.0×10^{-5} M)	+	7.3 S
Decamer (5.5×10^{-5} M)	-	6.64 S

Antibody concentration in the range 3.3×10^{-6} M - 3.8×10^{-6} M.

Centrifuge runs maintained between 22-25°C.

Figure 1

A macro whole complement titration was performed as described in the text. The percentage lysis observed with the addition of various amounts of 300-fold diluted guinea pig serum was plotted as shown on probability x logarithm paper; this plot was used to calculate the amount of diluted serum necessary to produce 50% cell lysis, and thus the number of CH_{50} units in whole serum (811 CH_{50}/ml in this experiment).

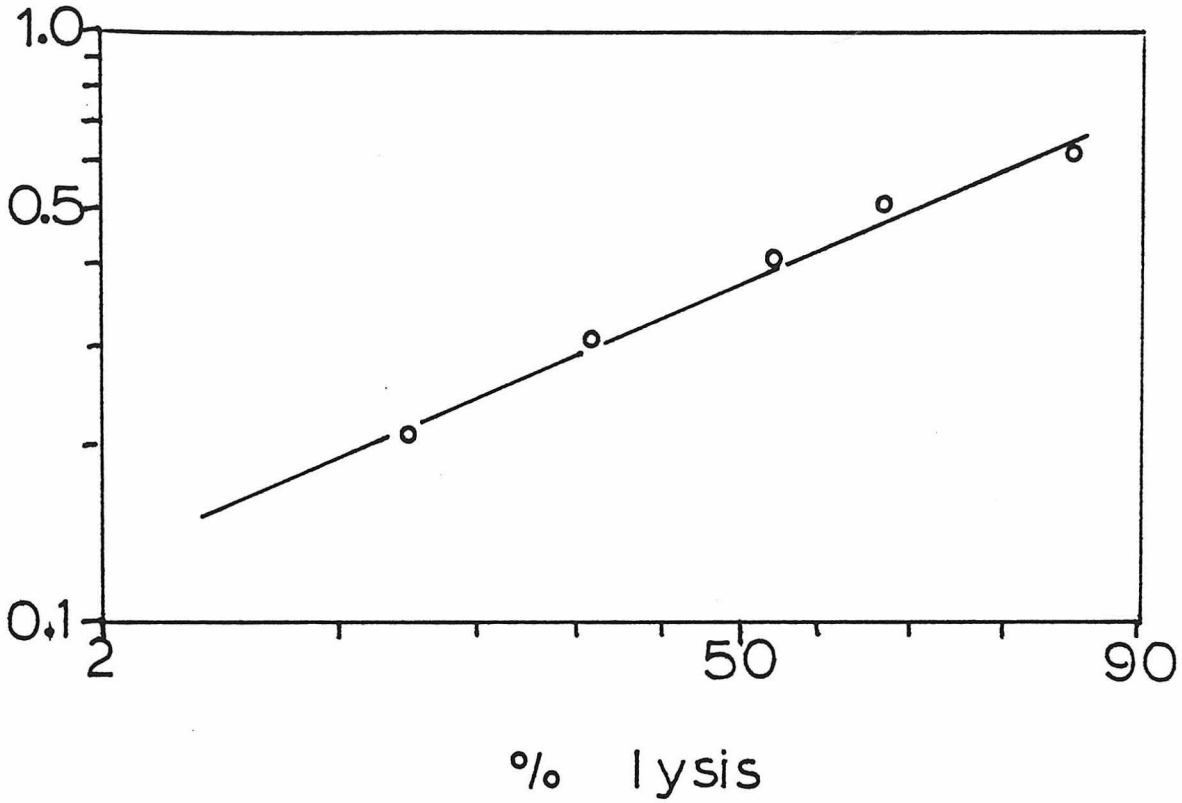

1.0
0.5
0.1
2
50
90
% lysis

Figure 2

A) A representative macro whole complement titration is shown as percentage lysis versus volume of 300-fold diluted serum added; 938 CH_{50}/ml serum was measured for this preparation. Closed circles - experimental data. Open circles - best fit to experimental data using von Krogh relation to determine values for K and 1/n (see text).
B) Similar to A), a micro whole complement assay using a one hour 37°C incubation is shown; 10,000 CH_{50}/ml serum was measured for this preparation.

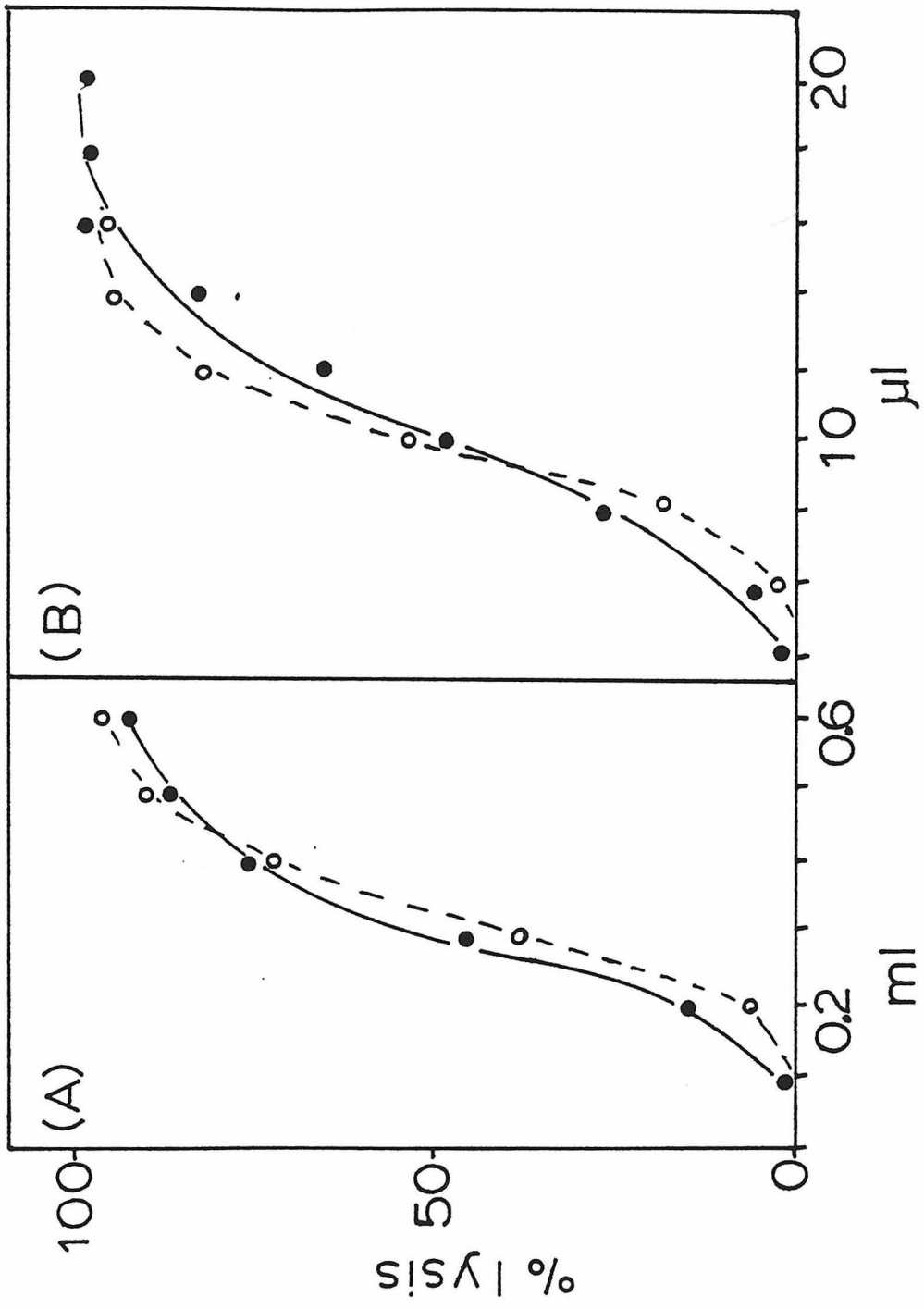

% lysis
100
50
0
(A)
(B)
0.2
0.6
ml
10
20
µl

Figure 3

A macro whole complement assay was performed using UPC-10 myeloma protein (55 μg/ml) with perennial ryegrass levan as the antigen. Closed circles - antigen and antibody present. Open circles - antigen without antibody present.

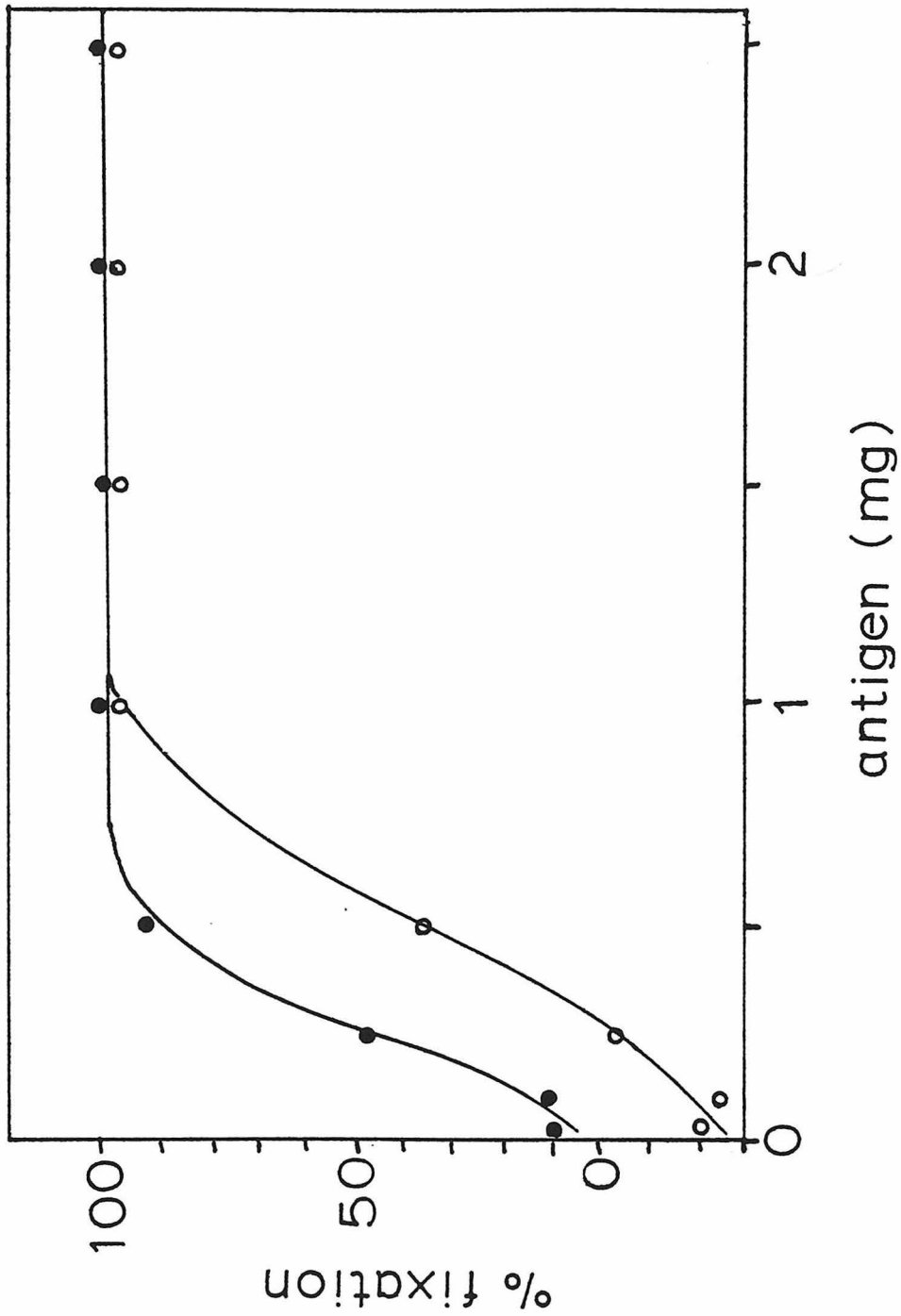

% fixation
100
50
0
0
1
2
antigen (mg)

Figure 4

The aggregate results of several macro whole complement assays using UPC-10 myeloma protein (3.7×10^{-7} M) and bacterial levan as the antigen are shown. The initial incubations of antibody, antigen and complement were performed for one hour at 37°C. Closed circles - antibody and antigen present. Open circles - antigen without antibody present.

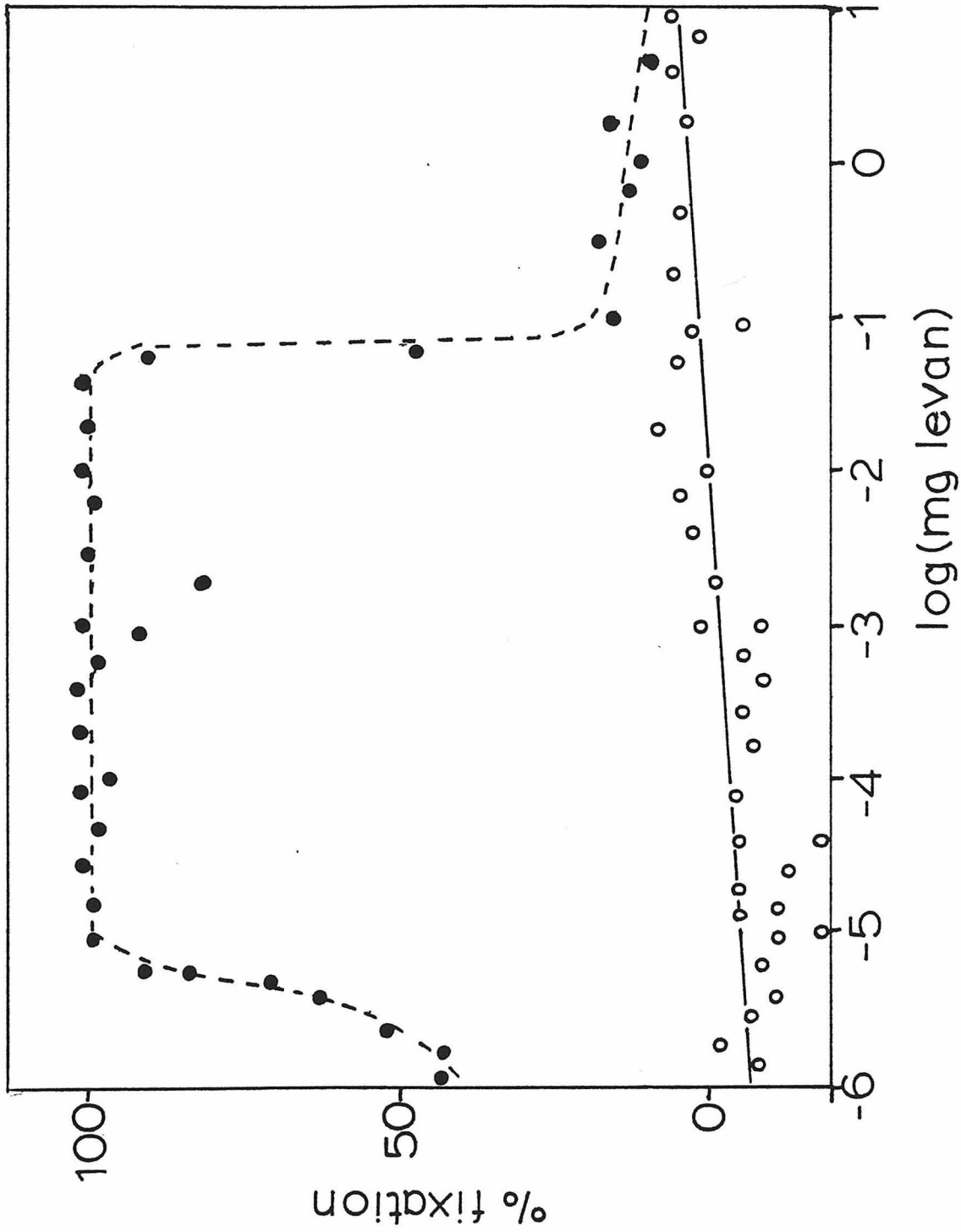

% fixation
100
50
0
1
0
-1
-2
-3
-4
-5
-6
log(mg levan)

Figure 5

The aggregate results of several micro whole complement assays using UPC-10 myeloma protein (6.7×10^{-7} M) and bacterial levan as the antigen are shown. The initial incubations of antibody, antigen and complement were performed for 18 hours at 4°C. Open circles - antibody and antigen present. Closed circles - antigen without antibody present.

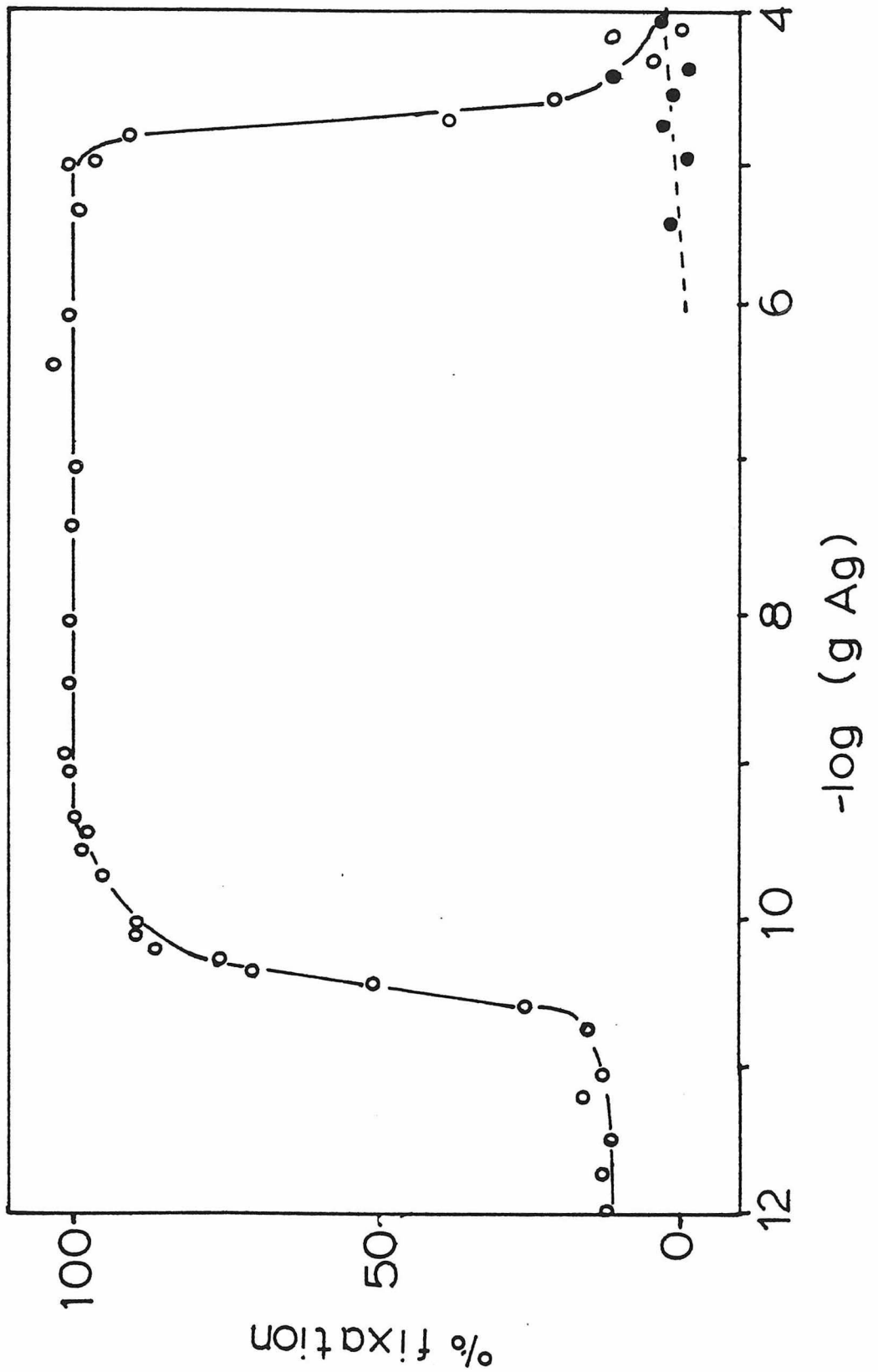

% fixation
100
50
0
12
10
8
6
4
-log (g Ag)

Figure 6

The aggregate results of several micro whole complement assays using UPC-10 myeloma protein and bacterial levan as the antigen are shown. The initial incubations of antibody, antigen and complement were performed for one hour at 37°C. Crosses (connected by solid line) - antibody (3.3×10^{-6} M) and antigen present. Triangles (connected by interrupted line) - antibody (3.3×10^{-7} M) and antigen present. Closed circles (connected by dotted line) - antibody (1.3×10^{-7} M) and antigen present. Open circles (connected by solid line) - antigen without antibody present.

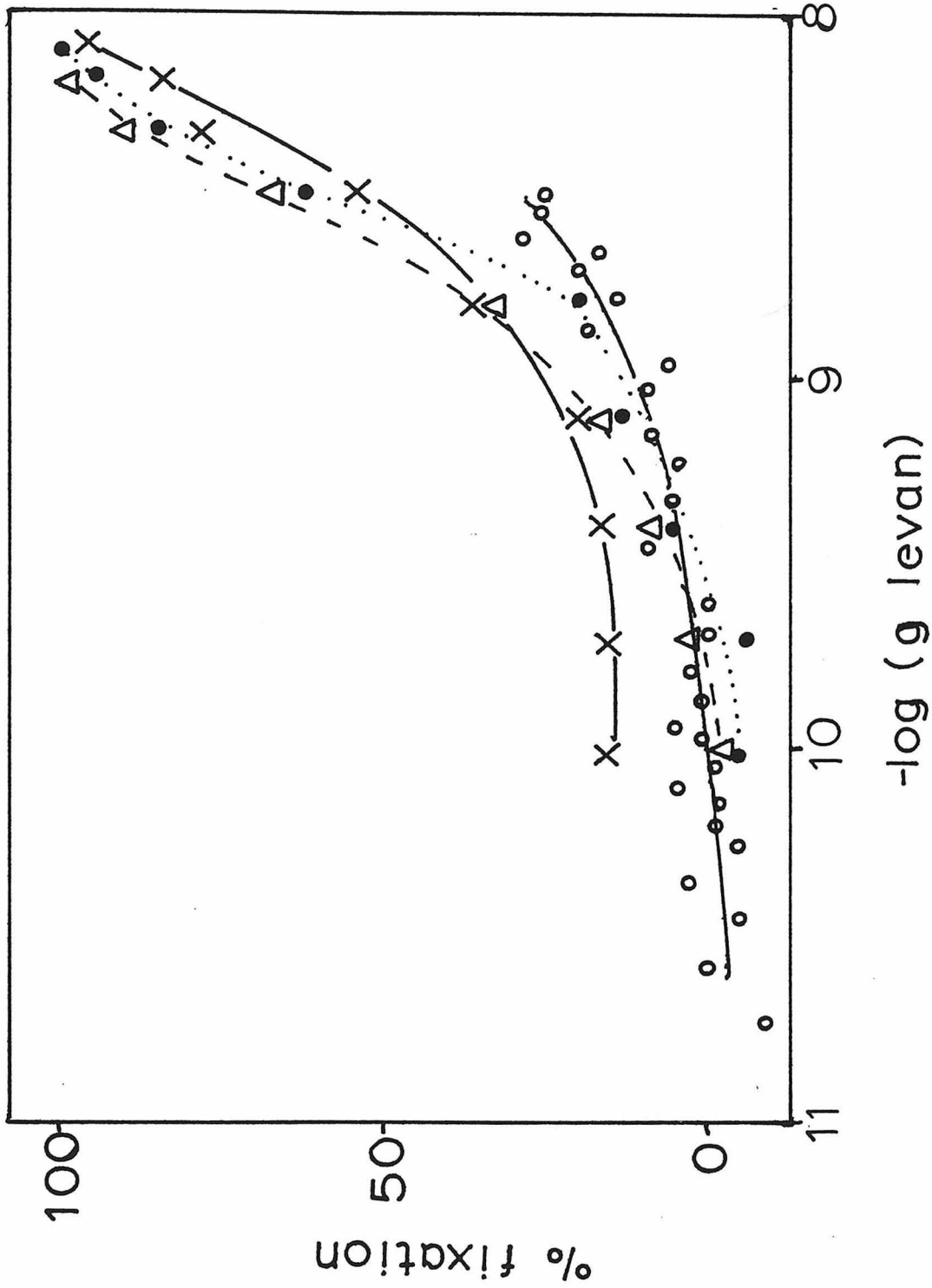

% fixation
100
50
0
8
9
10
11
-log (g levan)

Figure 7

The aggregate results of several micro whole complement assays using UPC-10 myeloma protein and levan fraction 59-79 as the antigen are shown. The initial incubations of antibody, antigen and complement were performed for one hour at 37°C. Closed circles (connected by solid line) - antibody (3.3×10^{-6} M) and antigen present. Open circles (connected by interrupted line) - antibody (6.7×10^{-7} M) and antigen present. Crosses (connected by dotted line) - antibody (3.3×10^{-7} M) and antigen present. Triangles (connected by solid line) - antibody (1.3×10^{-7} M) and antigen present.

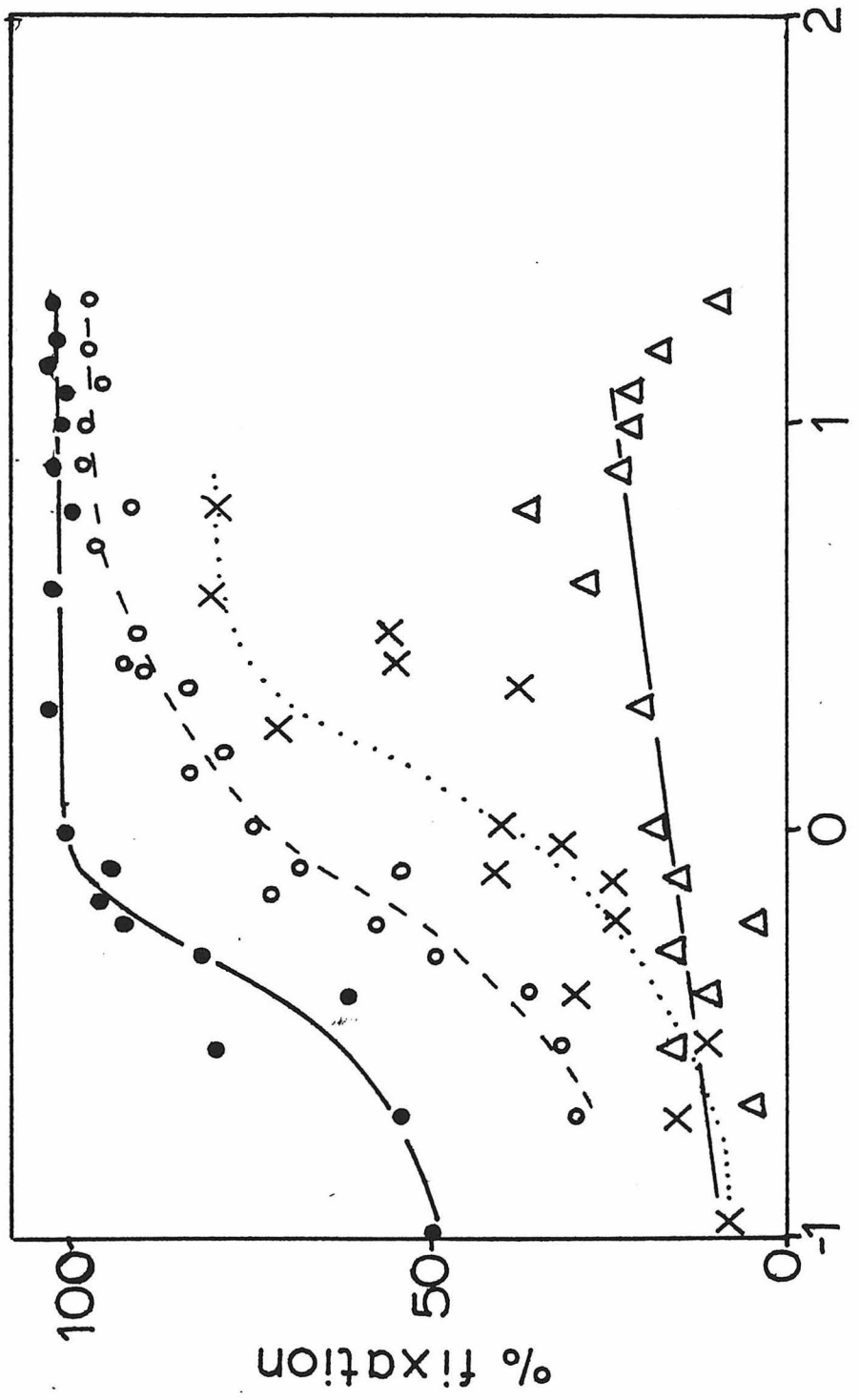

% fixation
100
50
0
-1
0
1
2
log µg Ag

Figure 8

The aggregate results of several micro whole complement assays using UPC-10 myeloma protein and levan fraction 91-95 as the antigen are shown. The initial incubations of antibody, antigen and complement were performed for 18 hours at 4°C. Open circles (connected by solid line) - antibody (6.7×10^{-7} M) and antigen present. Crosses (connected by dotted line) - antibody (3.3×10^{-7} M) and antigen present. Triangles (connected by interrupted line) - antibody (1.3×10^{-7} M) and antigen present.

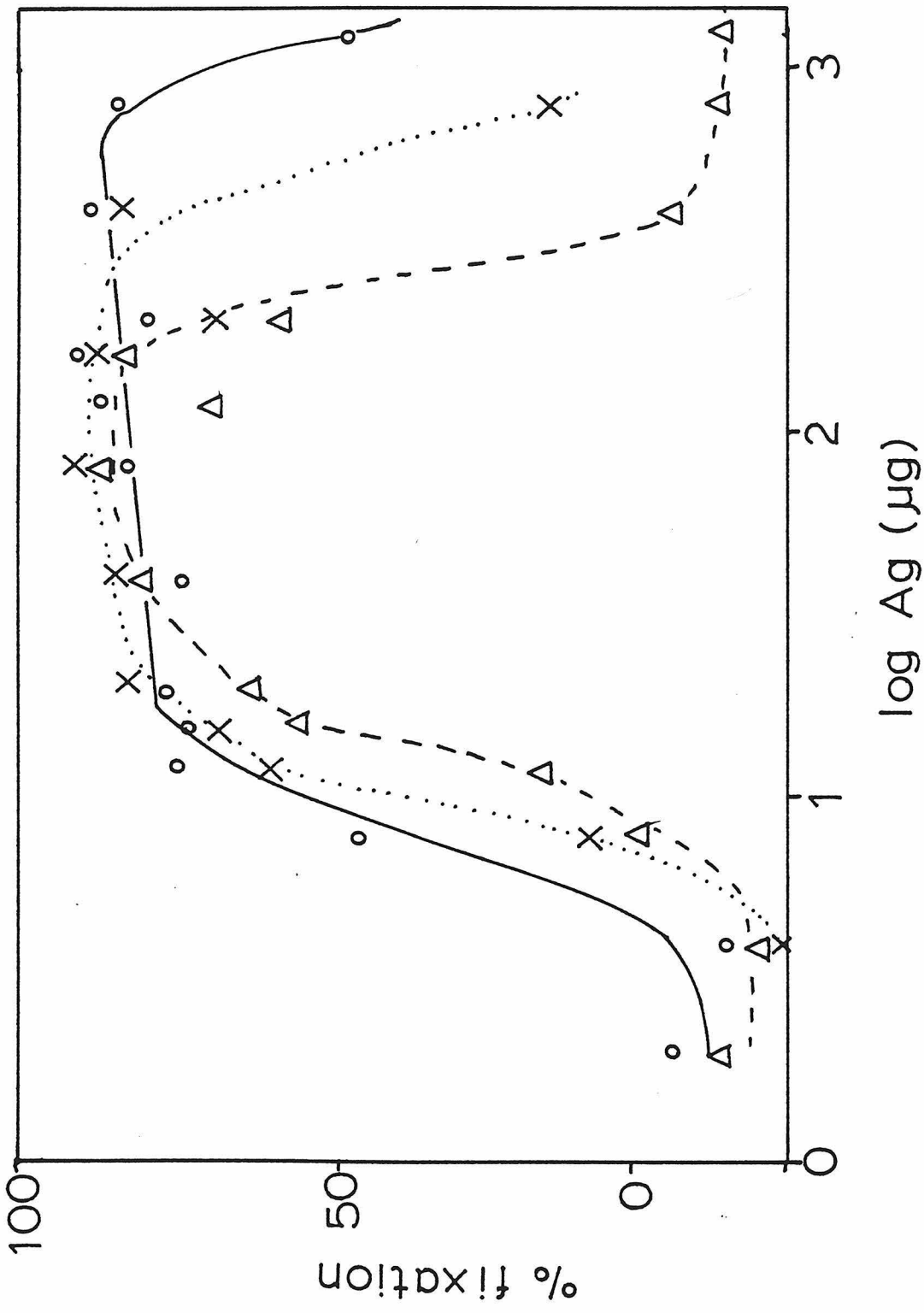

% fixation
100
50
0
0
1
2
3
log Ag (µg)

Figure 9

Analytical ultracentrifugation of UPC-10 myeloma protein (3.3×10^{-6} M in PBS) was performed at 12.9-14.8°C and 60,000 rpm in the presence of levan fraction 91-95. The double-sector cell was scanned at 12 minute intervals at 280 nm and the absorbance data were converted to the derivative plots of the successive scans shown as described in the text. A - antibody and levan fragment 91-95 (7.9×10^{-7} M). B - antibody and levan fragment 91-95 (1.6×10^{-6} M). C - antibody and levan fragment 91-95 (3.1×10^{-6} M).

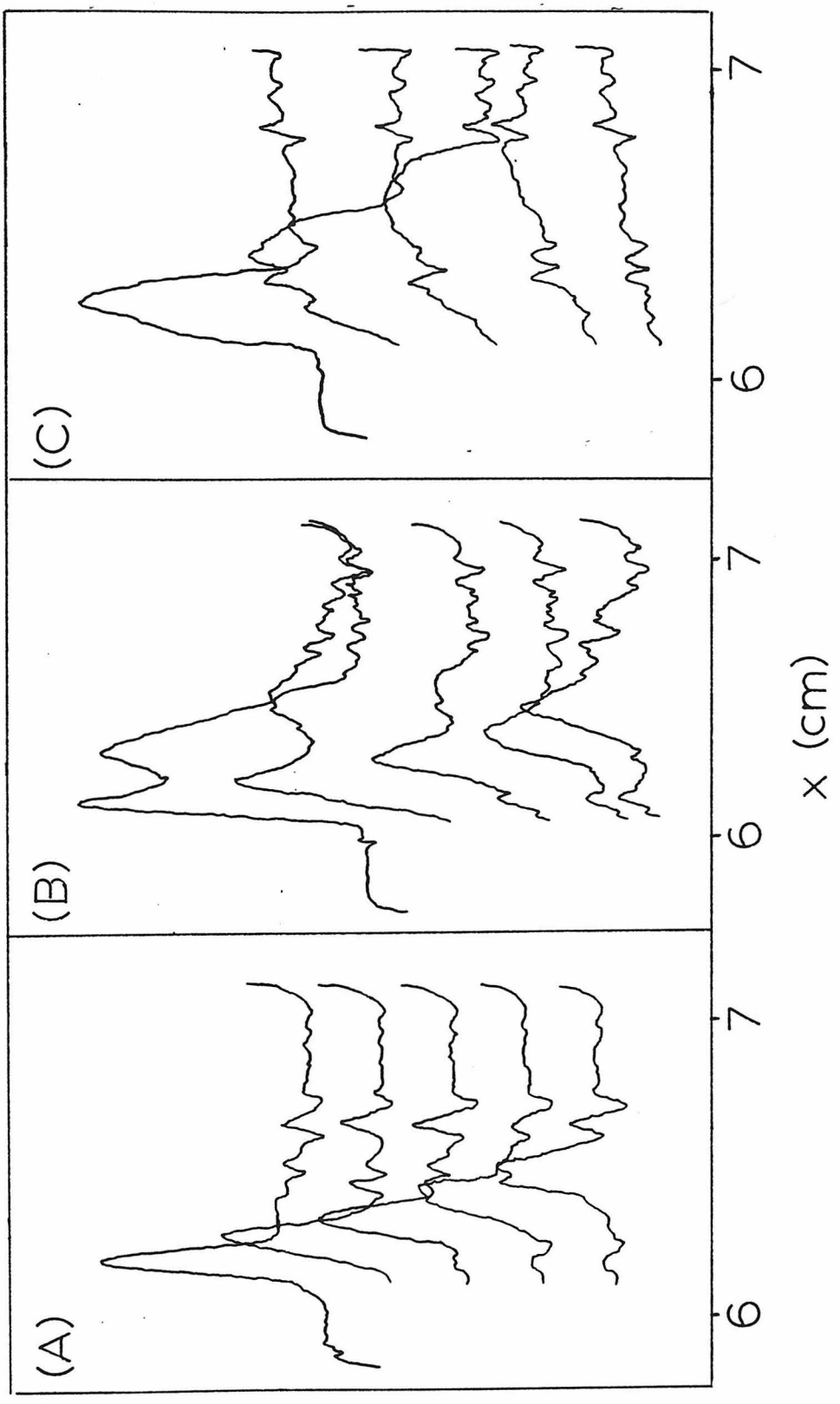

(A)
(B)
(C)
6
7
6
7
6
7
x (cm)

Figure 10

Analytical ultracentrifugation of UPC-10 myeloma protein (3.65×10^{-6} M in PBS) was performed at 22°C and 52,000 rpm in the presence of levan fraction 59-79 (1.9×10^{-5} M). The double-sector cell was scanned at 12 minute intervals at 280 nm and the absorbance data was converted to the derivative plots of the successive scans shown as described in the text. (Note: There is a 20 minute interval between the third and fourth scans shown).

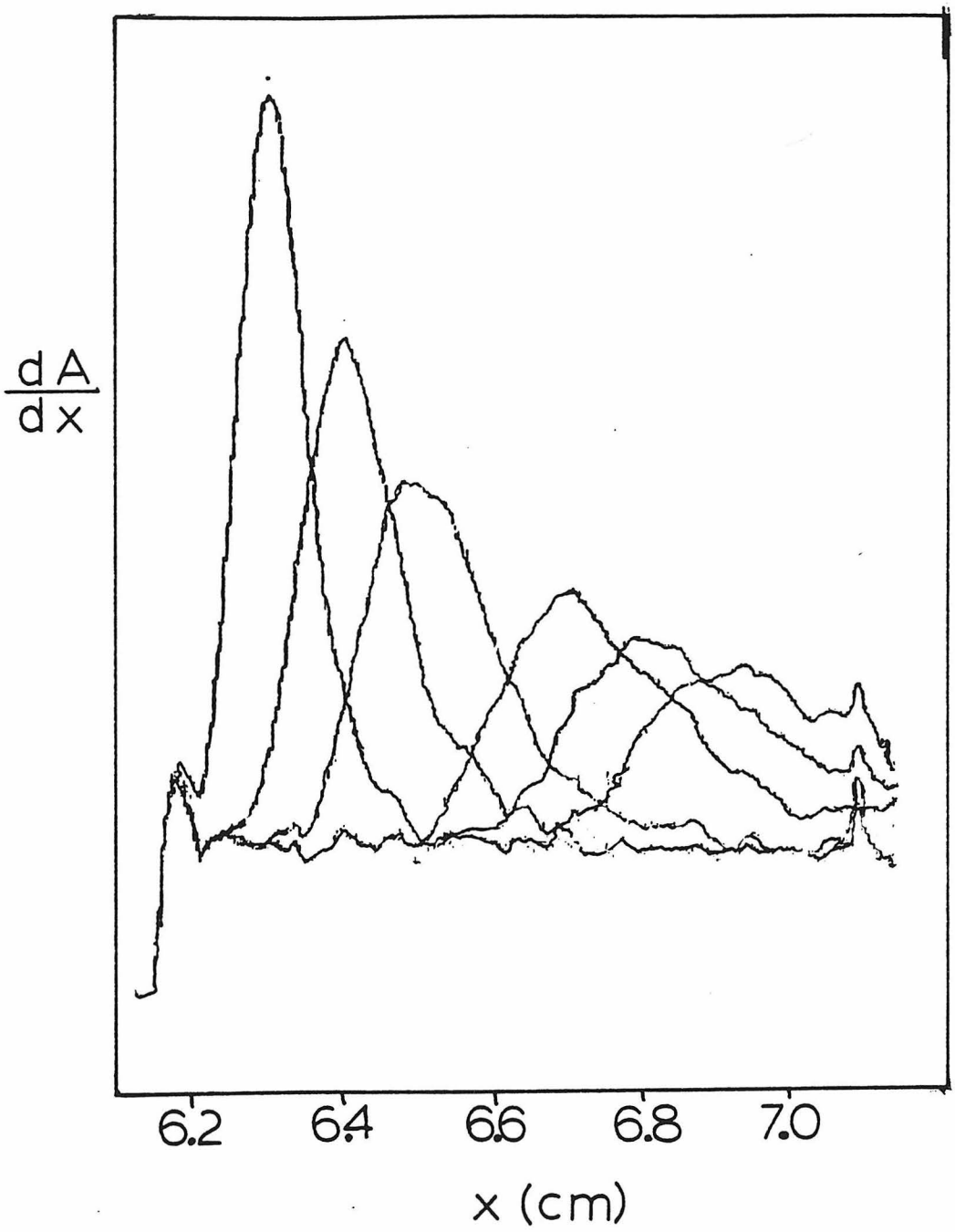
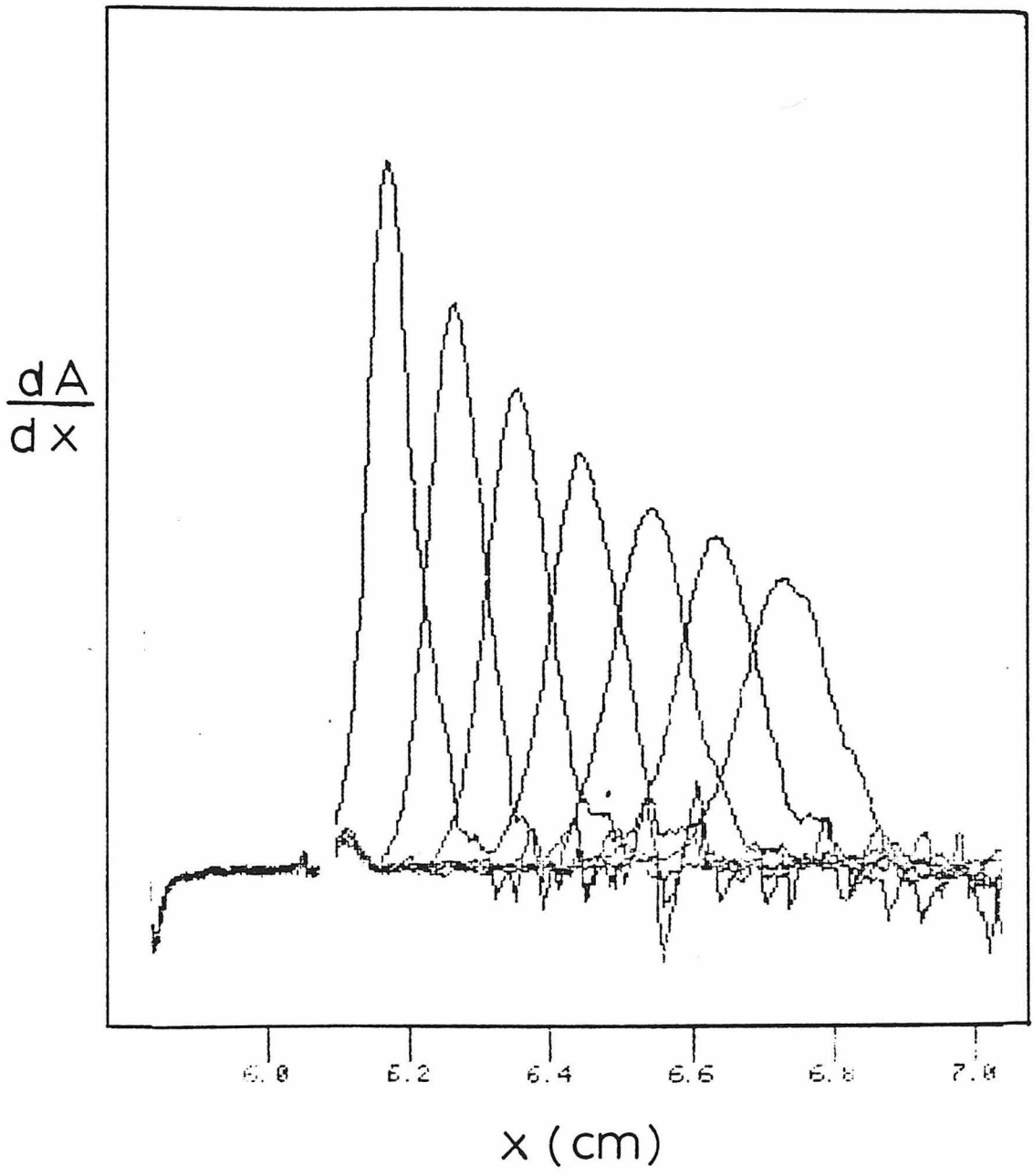

dA/dx
6.2
6.4
6.6
6.8
7.0
x (cm)

Figure 11

Analytical ultracentrifugation of UPC-10 myeloma protein (6.7×10^{-7} M in PBS) was performed at 9.8 - 11.1°C and 60,000 rpm in the presence of levan fraction 59-79 (3.5×10^{-8} M) (these concentrations correspond to those which produced 50% complement fixation in the micro whole complement assay). The double-sector cell was scanned at 12 minute intervals at 230 nm and the absorbance data were converted to the derivative plots of the successive scans shown as described in the text.


dA
dx
6.0
6.2
6.4
6.6
6.8
7.0
x (cm)

PROPOSITIONS

PROPOSITION I

Immune complexes form in serum when antibody molecules combine specifically with their corresponding antigen. Large amounts of these complexes are frequently produced as a result of an excessive antibody response to either foreign or self antigens, such as is found in autoimmune diseases. When high concentrations of immune complexes are present in the circulation, they are deposited along vascular and glomerular basement membranes, producing inflammatory changes and tissue damage (Gilliland and Mannik, 1980). Although the rapid clearance of these antibody aggregates is usually accomplished by the reticuloendothelial system, impaired clearance of model immune complexes has been found to be present in two autoimmune diseases--primary biliary cirrhosis (Jaffe et al., 1978) and systemic lupus erythematosis (Frank et al., 1979).

Immune complexes are cleared from the circulation as a result of cellular interactions, predominantly involving mononuclear phagocytes (McKeever and Spicer, 1980) and hepatocytes (Frommel and Rachman, 1979). These interactions are mediated through cell-surface receptors, including an F_c-receptor which binds IgG molecules and a C3b receptor which recognizes an activated form of the third complement component absorbed onto immune complexes. Recently, it has been shown that the clearance of model immune complexes containing IgG molecules in rats depends upon the exposure of terminal galactose residues which are present on the carbohydrate chain of IgG molecules (Kornfeld et al., 1971; Thornburg et al., 1980).*

*The galactose receptor may in fact be the F_c-receptor, since the carbohydrate chain is attached to the C_H2 domain in the F_c portion of the antibody molecule (Nisonoff et al., 1975), and the C_H2 domain has been implicated in the binding of the IgG molecule to the F_c-receptor (Diamond et al., 1979). Such a possibility could be supported experimentally if cells bearing F_c-receptors on their surfaces failed to bind deglycosylated IgG molecules.

It has been suggested that the antibody molecule undergoes a conformational change upon binding antigen and thereby exposes the carbohydrate chain to binding by the galactose receptor on hepatocytes (Thornburg et al., 1980). Such a conformational mechanism is based upon crystallographic studies which show the carbohydrate chains of human IgG to be sequestered between two folds of polypeptide chain in the C_H2 domain (Diesenhofer et al., 1976). The postulated antigen-induced conformational change also explains why IgG molecules in the absence of bound antigen have a much longer circulating half-life (21 days; Wells, 1978) than do IgG molecules assembled into immune complexes (4 min.; Thornburg et al., 1980). However, this mechanism fails to explain the observation that immune complexes formed in antigen excess have much longer circulating half-lifes than do those formed in antibody excess (Weigle, 1961); such an observation suggests a role for antibody multivalency in the cellular binding of immune complexes--an aggregational model.

The studies which have utilized model immune complexes in order to observe the clearance of such complexes have been hampered by the fact that these antibody preparations have been heterogeneous (Thornburg et al., 1980; Hopf et al., 1976; Finbloom et al., 1980; Finbloom and Plotz, 1979a,b), thereby preventing a quantitative analysis of the effect of antigen binding upon the clearance of antibody molecules. It is possible to avoid such complications through the use of a homogeneous IgG myeloma protein preparation, such as that obtainable from the UPC-10 plasmacytoma (Lieberman et al., 1975). Using this myeloma protein and its corresponding levan antigen (Cisar et al., 1974), one should be able

to perform both *in vivo* and *in vitro* experiments to explore the mechanism of immune complex clearance.

In vitro experiments utilizing isolated hepatocytes (Thornburg et al., 1980) could be performed to determine the association constants between UPC-10 myeloma protein and the cell-surface galactose receptor, both in the absence of the levan antigen and in the presence of a saturating concentration of a monovalent levan oligosaccharide. A clear distinction between a conformational and aggregational mechanism of clearance could thus be obtained, since a conformational model would require an increase in association constant for the antibody molecule binding to the cell receptor upon filling one or two antigen-binding sites, while an aggregational model would predict the association constant for binding between the antibody molecule and cell to be unaltered by the vacancy or occupancy of the antigen-binding site on the IgG molecule.

In vivo experiments using UPC-10 myeloma protein allow the possibility to examine the clearance mechanism under physiologic conditions. Previously, such studies have often used preformed immune complexes which would begin to dissociate when diluted into the blood of an experimental animal (Finbloom et al., 1980; Thornburg et al., 1980); such a problem has been avoided through the stabilization of immune complexes by covalent crosslinks (Segal and Titus, 1978; Finbloom and Plotz, 1979a,b), although the question as to whether chemical modification might alter the structure of the antibody molecule and thereby its cell-binding characteristics has not been addressed. These problems could be eliminated by introducing the desired antigen into the animal's blood prior to the addition of antibody; if rapid mixing is

obtained, the immune complexes would form *in vivo* and thus accurately reflect the concentrations of antigen and antibody present in the circulation. Bacterial levan is an ideal antigenic substance for such a study since it does not activate the alternate pathway of complement and thereby produce anaphylactic shock in the animal; also, it does not contain any galactose residues and therefore cannot interact directly with the cellular galactose receptor. The effects of immune complex formation and the resulting classical complement activation could be avoided by pretreatment with parenteral antihistamines or by depletion of C3 caused by cobra venom factor (Bockow and Mannik, 1981). This approach would allow the determination of the clearance rate for UPC-10 myeloma protein as a function of the blood antigen concentrations. A conformational model suggests that the half-life of circulating UPC-10 myeloma protein would be minimal under conditions of antigen excess, while an aggregational model would predict that the clearance rate for this IgG antibody would be similar under conditions of antibody or antigen excess. In this way, both *in vivo* and *in vitro* experiments utilizing a homogeneous myeloma IgG could be performed to analyze the clearance of immune complexes from the circulation.

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PROPOSITION II

An inflammatory reaction is a response of vascular tissue to injury and is associated with many changes in the surrounding blood vessels as a process of defense and repair occurs. Central to these actions is an increase in vascular permeability in the area of inflammation; large molecular weight plasma proteins, including immunoglobulin and complement, which are normally confined to the vascular space, become able to diffuse freely into the interstitial space. Many chemical and biologic mediators have been identified which can produce an increase in vascular permeability, including histamine, serotonin, kinins, fibinopeptides, prostaglandins, various products of polymorphonuclear (PMN) leukocytes and lymphocytes, and the complement peptides C3a and C5a (see Robbins and Cotran, 1979).

Although many different mediators have been identified that produce an increase in vascular permeability, the mechanisms by which this is accomplished remain largely unknown. Physiologic experiments have suggested that "pores" exist across the endothelial surface to allow the transport of hydrophilic compounds into the interstitial space, although it has not been possible to anatomically identify such structures. Two current opposing views hold that these pores are represented either by a system of pinocytic vesicles which carry plasma protein across the endothelial cell, or by intercellular junctions (Robbins and Cotran, 1979).

C3a and C5a are protein fragments produced as a result of complement activation. Both molecules share the abilities to induce smooth muscle contraction, increase vascular permeability, and stimulate the release of cellular products from mast cells, basophils and PMN; in addition, C5a possesses chemotactic activity for PMN (for a recent

review, see Hugli, 1978). Initial studies which examined the effect of C3a on the microvasculature of skin established that it was capable of eliciting a vascular response characterized on a microscopic level as a widening of intercellular endothelial junctions and disruption of the basement membrane (Lepow et al., 1970). In addition, mast cells in this area were found to be degranulated, and pretreatment with antihistamine compounds partially abolished the response. These observations led to the conclusion that the increase in vascular premeability observed upon C3a injection into skin was mediated through the release of histamine from mast cells. At the same time, this conclusion failed to establish the importance of C3a-induced histamine release from mast cells in the production of enhanced vascular permeability as a general mechanism, since antihistamine compounds are of questionable significance in many clinical inflammatory reactions (Wedmore and Williams, 1981).

Recently, further studies have suggested that C5a may produce an increase in vascular permeability that is histamine-independent (Williams and Jose, 1981). It was found that PMN play a central role in this process by an unknown mechanism (Wedmore and Williams, 1981), leading to the proposal that C5a may induce the influx of PMN at the damaged site and then in some way activate these cells to produce the observed increase in vascular permeability. The possibility that C3a may also participate in a histamine-independent mechanism has not been addressed, although there are several compelling reasons to suspect that such a mechanism may exist. First, pretreatment with antihistamine compounds only partially abolishes the vascular permeability changes induced by C3a (Lepow et al., 1970). Second, although C3a and C5a do not share extensive primary sequence homology, these proteins do appear to share

many structural characteristics, including an extensive disulfide bonding pattern and a C-terminal arginine residue which is critically important to biologic activity (Hugli, 1978). Third, although C3a lacks chemotactic activity for PMN, it has been observed that C3a at 10^{-7} M concentrations can stimulate the release of cellular enzymes through interactions with a membrane receptor (Chenoweth and Hugli, 1978). These reasons support an reexamination of the mechanism of vascular permeability alteration by C3a.

In order to assess the relative participation of mast cells and PMN in this C3a-induced process, it is necessary to be able to eliminate each cell or its effects from the assay system. Previous studies have demonstrated the effectiveneess of nitrogen mustard in the removal of PMN from the circulation (Wedmore and Williams, 1981). The effects of mast cell degranulation may be eliminated either by pretreatment with H_1-antagonists or through the prior induction of degranulation by compound 48/80 (Johnson and Moran, 1969).

Thus, the role of C3a in the development of increased vascular permeability may be examined in the series of four experiments outlined below:

1) The baseline activity of C3a in the vascular permeability assay may be determined with both mast cells and PMN present in the assay tissue.

2) The involvement of mast cells may be evaluated by pretreatment of the tissue with 48/80 and H_1-antagonists, thereby eliminating histamine released from mast cells as a potential mediator.

3) The involvement of PMN may be examined by pretreatment with nitrogen mustard to remove circulating PMN.

4) The experiments described in 2) and 3) may be combined to evaluate the possible activation of C3a in the vascular permeability assay that are mediated by neither mast cells nor PMN. A possible mechanism would require C3a (or C5a) to interact directly with endothelial cells to induce widening of the intercellular junctions or stimulation of the vesicle transport process.

Thus, these experiments should help to define the possible role of C3a in the inflammatory process.

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PROPOSITION III

For many years it has been recognized that protein phosphorylation-dephosphorylation serves as a cardinal mechanism for the control of intracellular events by external physiologic factors. While glycogen metabolism was the first system for which phosphorylation was observed to function in this regulatory role, the same principles are currently known to govern over 35 enzyme systems catalyzing various cellular processes (for a recent review, see Cohen, 1982). Although the vast majority of such phosphorylations take place on serine and threonine residues (99.97%), a new class of phosphorylations has recently been described which take place on tyrosine residues, accounting for the remaining 0.03% of the cellular protein phosphorylations (Sefton et al., 1980). At present, the two systems known to initiate tyrosine phosphorylation are the epidermal growth factor (EGF) receptor and the transforming gene products of certain RNA tumor viruses.

EGF is a peptide hormone that binds to a specific membrane receptor and induces the proliferation of cells of epidermal and epithelial origin (for a recent review, see Carpenter and Cohen, 1979). Using the human epidermal carcinoma cell line A431 which has a high concentration of EGF receptors on its surface, Carpenter et al. (1979) showed that an enhanced rate of phosphorylation of various cellular proteins occurred as a direct consequence of EGF binding; further analysis revealed that this EGF-stimulated phosphorylation occurred on tyrosine residues (Ushiro and Cohen, 1980). Affinity labelling experiments have suggested that the kinase activity stimulated by EGF resides in the receptor molecule itself (Buhrow et al., 1982), thereby implying that the receptor spans the membrane, exposing the EGF-binding site in the

extracytoplasmic face of the membrane and the protein kinase site on the cytoplasmic face.

Likewise, tyrosine phosphorylation activity has been associated with certain RNA tumor viruses, including Rous sarcoma virus (RSV). The product of the src gene of RSV which is crucial for transformation, $pp60^{src}$, as well as its cellular counterpart, $pp60^{sarc}$, have been shown to be tyrosine kinases (for a recent review, see Hunter, 1980). Although the possible effects of tyrosine phosphorylation by $pp60^{src}$ are unknown, it has been shown to act in vivo upon several (at least 7) unknown proteins. One of these proteins, the so-called 36K protein, has been shown to undergo tyrosine phosphorylation via both $pp60^{src}$ and the EGF-receptor kinase, suggesting a similarity in action in these two enzyme systems (Cooper and Hunter, 1981). Intriguingly, the EGF receptor and the viral transforming protein from RSV, avian sarcoma viruses Y73 and PRC II, and Synder-Theilin feline sarcoma virus, all possess sites of tyrosine phosphorylation (Patschinsky et al., 1982). This raises the question of how these kinases are themselves phosphorylated. In the case of $pp60^{src}$, it has been suggested that this kinase may phosphorylate itself by either an intra- or intermolecular mechanism (Collett et al., 1980), while other investigators using different purification methods have been unable to reproduce these results (Levinson et al., 1980). An analysis of the phosphorylation sites in these kinases has revealed a striking similarity in their primary sequences; the tyrosine subject to phosphorylation is uniformly located in close proximity to a glutamic acid residue and 7 residues to the C-terminal side of a basic amino acid (Patschinsky et al., 1982). This repetition of structure strongly

suggests either that these kinases are able to phosphorylate each other or that they are all subject to phosphorylation (and possibly functional activation or modification) by another tyrosine kinase with the required specificity.

Using this sequence data, Pike et al. (1982), have prepared a synthetic peptide similar to the sequence surrounding the tyrosine phosphorylation site in $pp60^{src}$ (Arg-Arg-Leu-Ile-Glu-Asp-Asn-Glu-Tyr-Thr-Ala-Arg-Gly) and have shown that this peptide can be phosphorylated by the EGF-receptor-kinase in vitro, suggesting that the phosphorylation of these transformation gene products is accomplished by the EGF receptor in vivo. However, the cellular significance of such a mechanism is not known at present. Thus, the current understanding of tyrosine phosphorylation cannot answer how $pp60^{src}$ and the EGF receptor become themselves phosphorylated and cannot rule out the presence of one or more unknown tyrosine kinases in these cells.

For this reason, it should be informative to look for other tyrosine kinases in A431 which, in addition to the EGF receptor, have a specificity corresponding to the phosphorylation site in $pp60^{src}$. One approach to such a search would involve the conversion of the corresponding synthetic peptide into an affinity label. Although commonly used to study membrane receptors (for a review, see Hanstein, 1979), photoaffinity labels have not been used extensively for peptide modification; one advantage that photoaffinity labels possess is that reaction conditions and times can be carefully chosen in order to maximize the extent and specificity of the labelling reaction, in contrast to conventional affinity labels in which the chemically active group has the potential to react with the peptide itself from the moment

that such a group is introduced. Specific photoaffinity labels have been designed with peptide modification in mind (Galardy et al., 1974) and have been used in the modification of the insulin molecule (Yeung et al., 1980). For this study of kinase labelling, a suitable peptide modification could be accomplished through the introduction at the N-terminus of a p-azidobenzoate group as the N-hydroxysuccinimide ester (Gallardy et al., 1974). Such a peptide could be used as outlined below to answer the following questions:

a) "How many cellular tyrosine kinases can be identified with a specificity corresponding to $pp60^{src}$?" Extracts of A431 cells could be incubated with radiolabelled, photoaffinity-labelled peptide and photolyzed, with the identification of labelled tyrosine kinases accomplished by electrophoretic methods. In addition to the EGF-receptor, one would expect to identify any other tyrosine kinases capable of phosphorylating $pp60^{src}$. Such an approach allows the identification of the kinases responsible for tyrosine phosphorylation, in contrast to other methods, including the use of antibodies specific for phosphotyrosine haptens (Ross et al., 1981), which recognize only the products of the phosphorylation step. Alternatively, enhanced specificity might be achieved through the use of $[\gamma^{32}P]ATP$ in the incubation mixtures; by using ^{32}P as a tracer, one might limit the labelled proteins to those which both interacted with and phosphorylated the target peptide.

b) "What is the primary sequence specificity of the transformation gene products?" If $pp60^{src}$, for example, carries out tyrosine phosphorylations on sequences similar to those found on itself and the EGF-receptor, then it should be labelled through the use of the modified

peptide. However, a lack of $pp60^{src}$ labelling would suggest that it is incapable of autophosphorylation and must rely on other tyrosine kinases for this reaction.

c) "What is the role of tyrosine phosphorylation in the action of EGF and in the transforamtion event by RNA tumor viruses?" If the modified peptide specifically binds and covalently couples with a tyrosine kinase, it has the potential of eliminating that molecule's enzymatic activity; in this way, either the EGF-receptor or $pp60^{src}$ could be inactivated in studies designed to assess the effect of such inactivation on subsequent cellular function.

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PROPOSITION IV

Myasthenia gravis (MG) is an autoimmune disorder which is characterized by the production of antibodies capable of binding specifically with the nicotinic acetylcholine receptor (AcChR) on the postsynaptic membrane of the neuromuscular junction. The first experimental evidence of such an etiology was obtained when rabbits which had been immunized with acetylcholine receptor from electric eels later developed symptoms identical to those seen in myasthenia gravis (Patrick and Lindstrom, 1973). Through the study of this experimental autoimmune myasthenia gravis (EAMG), it has been possible to examine the role of the immune system in human MG (for a recent review, see Lindstrom, 1979).

The course of EAMG may be divided into an acute and chronic phase. Approximately 8-11 days following the initial immunization with AcChR, an acute phase of EAMG is observed if and only if Bordatella pertussis is included as an immunizing adjuvant (Lennon et al., 1975); this acute phase of EAMG has no counterpart in human MG (for a clinical review, see Drachman, 1978). This stage of the disease is characterized by three related events. Anti-AcChR antibodies are first detected in the serum at this time at a low concentration (Lindstrom et al., 1976a). Coincident with the development of muscular weakness, focal membrane lysis can be seen at the tips of postjunctional membrane folds, presumably due to the interaction between bound antibody and complement (Sahashi et al., 1978). This is rapidly followed by a massive phagocytic invasion of the neuromuscular junction (Engel et al., 1976a), producing functional denervation of most of the affected endplates (Lambert et al., 1976). This phagocytic invasion can be blocked by prior depletion of C3, the third component of complement (Lennon et al.,

1978). The combined effects of focal lysis and phagocytic invasion produce a simplified structure of the postjunctional membrane in which the complex folding normally seen in an area subjacent to the nerve terminal is lost, resulting in a smoothed surface (Engel et al., 1976b). Since the acetylcholine receptor is localized at the tips of these postjunctional folds (Fertuck and Salpeter, 1974), the degenerative effect of the loss of these folds may solely reflect the loss of receptor molecules (Engel et al., 1976b).

In contrast to the acute phase of EAMG, the chronic phase closely resembles human MG (Lindstrom, 1979). It appears approximately 30 days following the initial immunization and is associated with an elevated serum concentration of anti-AcChR antibodies (Lindstrom et al., 1976b). The focal lysis of postjunctional folds is continued throughout the chronic phase, suggesting that the maintenance of the simplified membrane structure is a dynamic, potentially reversible, process (Engel et al., 1976b). It has been proposed that the membrane changes seen in human MG are due solely to the effects of this complement-mediated focal lysis, and are not initiated by phagocytic invasion, which is only seen in EAMG (Lindstrom, 1979).

Anti-AcChR antibodies play a dual role in the pathogenesis of EAMG--through the direct effect of binding to the acetylcholine receptor and as a result of triggering the complement system. The importance of antibodies to MG was strongly doubted initially, as there is poor correlation between anti-AcChR antibody titers and disease severity in a population of MG patients (Lindstrom et al., 1976c); through the use of plasmapheresis, however, disease severity in an individual patient has been shown to correlate closely with the concentration of anti-AcChR

antibody (Newsome-Davis et al., 1978). The acetylcholine receptor is composed of 4 polypeptide subunits (Raftery et al., 1974); the fact that immunization with any of the individual subunits of AcChR is able to induce EAMG (Lindstrom et al., 1978) suggests that there are multiple myasthenogenic determinants on the receptor molecule (Lindstrom, 1979). The responsibility of anti-AcChR antibody in the generation of EAMG has also been shown through passive transfer experiments; rats which have been injected with anti-AcChR antibodies develop symptoms of acute EAMG (Engel et al., 1979). It has also been shown that EAMG is produced solely as a response to immunization with AcChR, and is not due to minor components in the receptor preparation (Claudio and Raftery, 1980).

Electrophysiologic studies have shown that the binding of anti-AcChR antibodies to rat muscle receptor results in a 15% decrease in the mean conductance of an activated receptor and a 23% decrease in the mean open time (Heinemann et al., 1977). Since a large safety margin exists for neuromuscular transmission in the rat, it has been estimated that greater than 67% of muscle AcChR must be bound with antibody before muscular weakness or electromyographic changes may be observed (Lennon et al., 1978).

The binding of bivalent antibody also results in crosslinking of the membrane receptor, and this has been shown to lead to an increased rate of receptor degradation (Drachman et al., 1978). This increased degradation is a specific result of receptor aggregation in the membrane (Lindstrom and Einarson, 1979) and is inhibited by low temperature and metabolic poisons, such as DNP and NaF (Heinemann et al., 1977). In the absence of a compensatory increase in AcChR synthesis, this antibody-induced increase in degradation may be responsible for the

majority of the lost AcChR which is observed (Lindstrom and Einarson, 1979).

The exact nature of the destructive effect caused by interactions between antibody and complement in the course of MG is not clear at present. As previously discussed, C3 is required for the phagocytic invasion seen in acute and passive EAMG (Lennon et al., 1978). In addition, the decreases in miniature end-plate potentials (MEPP) and numbers of acetylcholine receptors in murine muscle seen after the passive transfer of anti-AcChR antibody from human MG patients have been found to depend upon the presence of C3 (Toyka et al., 1977). Although C5 was found not to be involved in the observed decreases in MEPP upon antibody binding to the receptor, the effect of C5 in the loss of acetylcholine receptor from the surface of the muscle cell was not studied (Toyka et al., 1977). However, the terminal complement component, C9, has been localized on the postsynaptic membrane and on disintegrating junctional folds, and this clearly implicates the membrane attack complex of complement in the pathology of MG (Sahashi et al., 1979).

It is important to note that the destructive changes seen as a result of this autoimmune disease are at least partially reversible; this is shown by the improvement in symptoms after plasmapheresis (Dau et al., 1979). In addition, it has been observed that pregnant patients with MG may show a remission of symptoms, and it has been suggested that this phenomenon may be due to the immunosuppressive action of α-fetoprotein upon antibody function (Abramsky and Brenner, 1979).

The central role of anti-AcChR antibodies in the pathology of MG allows the possibility that the course of the disease might be changed

through a suppression of the effects caused by the binding of antibody to the receptor molecule. Such a suppression could be achieved by the introduction of F_{ab} fragments prepared from anti-AcChR antibody. These fragments would be able to effectively block the binding of intact, bivalent antibody to the receptor molecules, and would be therefore expected to eliminate the increased rate of receptor degradation (Lindstrom and Einarson, 1979). In addition, F_{ab} fragments would be unable to activate the classical pathway of complement (Porter, 1979) and could therefore block the complement-mediated focal lysis of the postjunctional membrane folds and the C3-associated decreases in miniature end-plate potentials. Since the simplification of the architecture of the postjunctional membrane seen in acute EAMG is at least partially reversible (Engel et al., 1979), it is possible that blockage of intact antibody binding to the receptor through competition with F_{ab} fragments might lead to improved postjunctional membrane structure. Although F_{ab} fragments are most likely able to produce the same inhibition of receptor function (decreased mean open time and mean conductance of activated receptors) as in intact antibody, it is possible that functional improvement of neuromuscular transmission might be gained in spite of this inhibition as the number of receptors increases and the postjunctional membrane recovers its native structure. One study has indeed shown that binding of F_{ab} fragments prepared from anti-AcChR antibody to isolated receptors decreases the total amount of α-bungarotoxin which is specifically bound by the acetylcholine receptor by only 15% (Mihovilovic and Martinez-Carrion, 1979).

The question as to whether F_{ab} blockade of anti-AcChR antibody binding is able to produce a clinical improvement in neuromuscular transmission can be addressed directly by studying the effects of such a blockade in rats with chronic EAMG (Lennon et al., 1975). It should be possible to easily purify IgG from rats with chronic EAMG and prepare F_{ab} fragments by treatment with papain (Porter, 1959). It would be important to analyze the effect of F_{ab} blockade on a number of functional parameters, including amplitude of miniature end-plate potentials, number of receptors present at the junction, and electrophysiologic properties. If it were found that such an F_{ab} blockade produced an improvement in neuromuscular transmission, it could be extended to a trial in human MG patients without any significant alteration in experimental procedures.

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PROPOSITION V

Conjugation is a bacterial process whereby DNA is transferred following cell-to-cell contact. The genetic apparatus required for conjugation is encoded in a variety of conjugative plasmids, the best studied of which is the sex factor F. These plasmids may, in addition, encode genes not related to the conjugation process, thereby allowing the efficient transfer of useful phentotypes between bacterial cells (for a recent review see Willetts and Skurray, 1980).

F consists of approximately 100 kilobases of closed circular double-stranded DNA (Willetts and Skurray, 1980) and contains several insertion sequences (Davidson et al., 1975); these sequences allow F to exist as an autonomous DNA segment, to integrate into the bacterial chromosome to form an Hfr strain, or to excise with an adjacent chromosomal segment to form a F' plasmid. Approximately one-third of the F molecule is devoted to the conjugation process.

Nineteen plasmid genes have been identified to participate in conjugation; these tra (for transfer) genes may be divided into 4 basic groups (Willets and Skurray, 1980). Twelve genes are required for pilus formation, two for stabilization of the mating pairs, and six for the metabolism of conjugal DNA. The fourth group consists of a single gene, tra J, which is required for the expression of the other transfer genes (Finnegan and Willetts, 1973) as well as those involved in surface exclusion (Achtman et al., 1980). These genes are arranged so that tra J and its promoter precede the other transfer genes, with transcription proceeding in the same direction and the entire block of transfer genes, designated tra Y→Z, serving as a single operon (Willetts and Skurray, 1980).

Conjugation is not mediated solely by F plasmid genes, however. Four separate chromosomal mutations--sfr A, sfr B (Beutin and Achtman, 1979), cpx A and cpx B (McEwen and Silverman, 1980a,b)--have been described which affect the expression of the tra Y→Z operon. Recent work has shown that cpxA2 cpxB1 mutants are unable to participate in DNA donor activity as a result of their inability to produce tra operon mRNA, and this deficiency has been associated with an absence of the Tra J protein (Sambucetti et al., 1982). Furthermore, these mutants can be shown to produce tra J mRNA, although the gene product does not accumulate in its cellular location in the bacterial outer membrane (Kennedy et al., 1977; Sambucetti et al., 1982). As the cpx mutations have been shown to alter the protein composition of both the inner and outer bacterial membranes (McEwen and Silverman, 1982), it has been suggested that these mutations affect the translocation of envelope proteins, such as Tra J, to the outer membrane where they are required for cellular functioning (Sambucetti et al., 1982). However, it has not been possible to explain how Tra J may exert a regulatory role on the expression of the tra Y→Z operon from its location in the outer membrane.

Indeed, Tra J might be envisioned to function either as a structural or enzymatic component in the outer membrane to control conjugation. As a structural element it could interact with other membrane components, such as Tra D which is associated with both the inner and outer membranes (Achtman et al., 1979), in order to exert a regulatory effect upon cytoplasmic events. Intriguingly, Tra J is immunologically related to and may be a precursor of F pilin (Manning and Achtman, 1979); such a mechanism would allow the cell to produce the

machinery for conjugation only if the necessary structural elements, such as pilin, were present in the required location. Alternatively, Tra J might serve as an enzymatic component, the activity of which could be modulated by events at the outer membrane; the genetic regulatory role of Tra J would again have to be mediated through other cellular components, a result either of the modification of these components or their response to soluble factors elaborated as a function of Tra J activity.

To determine whether Tra J mediates its genetic regulatory activity in a structural or enzymatic way, it should be possible to examine whether the positive effect of Tra J in the outer membrane on the expression of the tra Y→Z operon may be transferred from Tra J-containing cells to cpx mutants. Quite simply, the bacteriophage λ has been prepared so that it contains an intact tra J sequence and its promoter (Ippen-Ihler, 1978); this vector could be used to infect UV-irradiated F^- cells to produce Tra J in the outer membrane. Extracts of these infected cells prepared by sonication and centrifugation could then be used to treat cpxA2 cpxB1 mutants which lack only Tra J in the outer membrane, looking for an aquisition of DNA donor activity by the mutant cells. If Tra J serves as a structural element in the outer membrane or as an enzymatic factor which alters other membrane proteins, these transfer experiments would not reconstitute the expression of tra Y→Z in the cpx mutants. On the other hand, if Tra J contains an enzymatic activity which is mediated by soluble factors in the periplasmic space, the transfer of these factors to cpx mutants would be expected to induce the expression of the transfer genes, provided that appropriate permeability of the outer

membrane to such soluble factors could be established, as through EDTA treatment to reduce Mg^{+} concentrations (Wheat, 1980). In this way, transfer experiments between F^{-} cells infected with the λp(tra J) transducing bacteriophage and cpx mutant cells should help to define the genetic regulatory role of Tra J in the bacterial outer membrane.

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