INVESTIGATIONS OF THE EFFECT OF ANTIGEN BINDING UPON TRYPTOPHAN REACTIVITY IN IMMUNOGLOBULIN G

Thesis by

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Abbreviations

C_H2	second constant heavy chain domain unit in antibody structure
DNP	dinitrophenyl group
$(DNP)_1$-cytochrome c	cytochrome c carrier with one dinitrophenyl group covalently attached
$(DNP)_8$-cytochrome c	cytochrome c carrier with an *average* of eight dinitrophenyl groups per cytochrome c molecule
EAC1 cells	antibody coated erythrocytes with complement component C1 bound
EDTA	ethylenediamine tetra-acetic acid
Fab	product of papain digestion of IgG that possesses the antigen-binding site
Fc	product of papain digestion of IgG analogous to the crystallizable fragment from rabbit IgG
Fd	portion of the Fab derived from the heavy chain
HMNB-DMSBr	dimethyl(2-hydroxy-3-methyl-5-nitrobenzyl) sulfonium bromide; modified Koshland's reagent
IgG	immunoglobulin class G
IgM	immunoglobulin class M
SDS	sodium dodecyl sulfate
tris	tris (hydroxymethyl) amino methane; tris base

Abstract

A $^{3}H/^{14}C$-labelled reagent that reacts specifically with tryptophan residues, was used to monitor the reactivity of tryptophans in the Fc and Fab portions of IgG upon binding DNP-gly, $(DNP)_1$-cytochrome c, and $(DNP)_8$-cytochrome c. Binding of monovalent antigen caused a decrease in the reactivity of tryptophan residues in the Fc by 18%; binding of multivalent antigen caused decreases in the reactivity of tryptophan residues in Fc by 21% and Fab by 12%. Experiments to elucidate the interaction between IgG and complement component C4 were attempted.

Future studies include further characterization of $(DNP)_1$-cytochrome c and the complement fixation capability of IgM-ABPC22 with monovalent antigen. Also planned is an investigation of the interaction of C4 with IgM in soluble antibody-antigen complexes.

Introduction

As early as 1890 the destruction of foreign cells in immunized animals was found to require both antibodies provided through immunization and some heat labile portion of the serum. (Bordet, 1896, 1898) The unstable serum factor was subsequently called complement.

Antibodies may bind to the offending foreign substance, but they have no capacity to degrade it. Instead, the antibodies may effect the destruction of a foreign cell by initiating an interaction involving complement. Complement is a collective name for a complex set of proteins that, when activated in an ordered sequence, directly damages the cell and causes cytolysis (figure 1). Control proteins are included as well as proteins of the activation sequence. A cascade system with each step able to be inhibited offers the possibility of rapid activation coupled to a series of fail-safe controls. (Porter and Reid, 1978)

The classical pathway of complement activation is initiated by the association of immunoglobulins, IgG or IgM, to soluble antigens or antigens on cell surfaces. One of the proteins of the C1 complex, C1s, is triggered to become an active protease that cleaves and thereby activates C4 and C2. The activated complex of $\overline{C4b}$ and $\overline{C2a}$ constitutes C3 convertase, which subsequently cleaves C3. The resulting complex, $\overline{C4b2a3b}$, activates C5 again through cleavage. Self assembly of $\overline{C5b6789}$ on the cell surface then leads to lysis. (Eisen, 1980)

Further, an alternate pathway of complement activation, also called the properdin pathway exists; this pathway does not require antibody-antigen complexes. Instead, certain polysaccharides and other substances effect the cleavage of C3 via the activation of a set of serum proteins, including properdin, factor B, and factor D. Activated $\overline{C3b}$ thus formed then interacts with C5; the late steps of the pathway (C5-C9) are shared by the classical and alternate pathways

(figure 2). (Eisen, 1980)

The precise mechanism by which associations of antibody and antigen trigger the initiation of the classical cascade has long been of interest. The C1q subunit of C1 binds via one of its six antibody combining sites (figure 3) to IgG or IgM immunoglobulins of the appropriate subclass. (Reid and Porter, 1976) The interaction between C1q and antibody has been observed to involve the CH_2 domain of the Fc region of IgG, while binding of antigen takes place at the tips of the Fab portions of the molecule. (Kehoe and Fougereau, 1969, 1974; Colomb and Porter, 1975; Yasmeen et al., 1976; Isenman et al., 1977) Since the antigen binding site is spatially removed from the site at which C1q binds, one needs to consider how these two sites on the antibody molecule interact. There are two current models to explain the activation of complement by antibody-antigen complexes.

First, binding of antigen by the Fab portion may cause conformational changes in the Fc region; this model proposes that the C1q binding site in the Fc is exposed or formed by an allosteric change when antibody binds antigen. (Brown and Koshland, 1975, 1977) Circular polarization of fluorescence has detected spectral changes in IgG upon binding either monovalent or multivalent antigen. (Givol et al., 1974; Jaton et al., 1975; Schlessinger et al., 1975) These changes were interpreted as taking place in the Fc region since quite different patterns were observed when Fab units alone were allowed to interact with antigen. Also, the hinge portion of IgG shows a greater constriction upon antigen binding. (Colman et al., 1976; Deisenhofer et al., 1976; Huber et al., 1976) This may provide an avenue for transmission of information from Fab to Fc.

Second, the associative model proposes that antigen binding mainly causes clustering of the Fc regions of antibody molecules. Optimal complement activation is certainly accomplished by antibody-antigen complexes of more than

one antibody molecule. The clustering of Fc regions could facilitate multiple binding by the hexavalent C1q molecule. (Howard et al., 1978; Metzger, 1978)

Aggregation is important to complement activation; it is not clear whether it is sufficient alone for activation. Aggregation may be necessary, but not sufficient; for example, aggregation may be required to initiate or enhance conformational changes.

Possibly both allosteric changes initiated by antigen binding and aggregation of Fc regions are processes that are important in the initiation of the classical complement cascade. (Porter and Reid, 1979) For example, Brown and Koshland in 1975 reacted an anti-lac dye IgM with monovalent antigen, and observed complement activation. (Brown and Koshland, 1975) Each molecule of IgM contains five Fc regions; one could perceive IgM as an intramolecular aggregation of Fc regions. Still, without bound antigen, IgM does not activate complement.

Hoffman's model for C1-antibody-antigen interaction proposes that immunoglobulin is an allosteric protein acting on C1q as substrate, and that antigen is the allosteric regulator. (Hoffman, 1976) The antibody is in equilibrium between a tensed state and a relaxed state. The antibody's affinity for C1q is greater in the relaxed state. In the absence of antigen, the tense state predominates, but in the presence of antigen, the proportion of relaxed antibody to tense antibody is increased. The degree of enhancement of the relaxed state is related to the number of IgG-like units associated. In other words, the antibody molecules in an aggregate act cooperatively in their transition from the tense state to the relaxed state.

Part A

Studies on the Reactivity of Tryptophan Residues in IgG upon Binding Antigen

Introduction

Immunoglobulins are multichained proteins that are described by the formula $(HL)_{2n}$ where H represents the heavy chain (MW 50K) and L the light chain (MW 25K). For IgG, n=1 and for IgM, n=5 (figure 4). Figure 5 illustrates this chain structure for IgG and also the "domain" units which make up the chains for IgG. Each domain has roughly the same length, $\sim$ 110 amino acids, and each has a centrally placed disulfide bridge. (Hood et al., 1978) In addition, each domain exhibits the characteristic "immunoglobulin fold" (figure 6).

The allosteric model of complement activation asserts that a conformational change occurs in the Fc region upon antigen binding at the Fab. Much of the spectroscopic evidence for such a conformational change has been based upon the differences observed between spectra of IgG and spectra of the Fab portion alone. A method that could observe the Fc portion of the molecule directly would be helpful.

If a conformational change that initiates the complement cascade does occur in the Fc, one would expect that the binding site for Clq might be a prime site to look for such a change. As mentioned previously, Clq is believed to interact with IgG via the immunoglobulin's C_H2 domain. It has been suggested that a tryptophan residue in the C_H2 domain is likely to be involved in Clq binding, though perhaps not directly. Allan and Isliker modified two tryptophans in the Fc with 2-hydroxy-5-nitrobenzyl bromide and observed a dramatic drop in complement-fixing capacity of rabbit IgG aggregates. They suggested that it is the tryptophan at residue 277 which is concerned with the Clq binding site. (Allan and Isliker, 1974) Johnson and Thames have performed binding studies of Clq with synthetic analogues of the C_H2 region around

tryptophan 277 and their results suggest that C1q binding site is located in this vicinity. (Johnson and Thames, 1976) The same conclusion was drawn from work done with natural polypeptides that mimic C_H2. (Lee and Painter, 1980) Circularly polarized fluorescence has provided some evidence for a conformational change in the Fc region, and this technique actually reflects local environments of tryptophan residues. (Schlessinger, 1975) (In this technique the sample is irradiated at the absorption maxima of tryptophan, and primarily tryptophan's fluorescence is observed.)

In these experiments, a tryptophan specific reagent, dimethyl (2-hydroxy-3-methyl-5-nitrobenzyl) sulfonium bromide, was employed to monitor the reactivity of tryptophans in the immunoglobulin. This reagent, abbreviated HMNB-DMSBr, specifically benzylates tryptophan in solution as a free amino acid or in a protein. It is a modification of a Koshland's reagent, and was developed and characterized by Dan Sand in this laboratory. (Sand, 1982)

A double radiolabeling technique was employed to compare tryptophan reactivity in the presence and absence of antigen. Immunoglobulin with antigen bound was labelled with tritiated HMNB-DMSBr. Then this antibody was mixed with an equal amount of antibody labelled with ^{14}C-HMNB-DMSBr without antigen present.

The immunoglobulin under investigation was a hybridoma IgG_{2a} specific for dinitrophenyl haptens. Dan Sand's labelling experiments in the presence and absence of hapten and in the presence and absence of monovalent antigen were repeated, and the results were verified. In addition, a multivalent antigen was synthesized, and labelling experiments were undertaken to examine its effect on tryptophan reactivity in the Fc portion of IgG.

Experimental

HMNB-DMSBr

The synthesis and characterization of dimethyl (2-hydroxy-3-methyl-5-nitrobenzyl) sulfonium bromide was developed by Dan Sand in this laboratory and was described in his thesis. (Sand, 1982) The synthetic route can incorporate ^{3}H or ^{14}C, via introduction of tritiated $NaBH_4$ (New England Nuclear) or ^{14}C paraformaldehyde (Amersham Searle) at appropriate times in the synthesis (figure 7). Both tritiated and ^{14}C labelled reagent were prepared and purified by this method.

Isolation of 29B1 IgG_{2a}

The mouse hybridoma 29B1 produces an anti-dinitrophenyl IgG_{2a}. (Oi, 1979) Immunoglobulin from this tumor was harvested in SJL x BALBc mice. Ammonium sulfate precipitation was performed on ascites at 4°C. A saturated ammonium sulfate solution (pH 7.4) was added dropwise to the stirring ascites until the final solution was at 45% saturation. After 2 hrs. of stirring, precipitate was collected by centrifugation. The pellet was resuspended in a minimum of 50 mM tris buffer (pH 8.3), and this solution was applied to a Whatman DEAE-Sephacel column which had been equilibrated with the same buffer. All unbound protein was eluted with two bed volumes of buffer. A linear ionic gradient consisting of 0.5 M NaCl in the static chamber was used to elute the immunoglobulin. The first peak with an absorbance of 280 nm was pooled as the 29B1 IgG. The peak was concentrated by ultrafiltration at 4°C and dialyzed against 0.15 M NaCl, 0.02 M NaH_2PO_4 (pH 7.4) buffer. The protein was then chromatographed on LKB Ultragel AcA 34, and its purity determined by reducing SDS-polyacrylamide gel electrophoresis. If necessary, the immunoglobulin was rechromatographed on LKB Ultragel AcA 34 to improve purity.

Synthesis of DNP-antigens

DNP-glycine

DNP-glycine was synthesized according to the procedure of Porter. (Porter, 1950)

Cytochrome C

Horse heart cytochrome c (Sigma Chemical Co.) was first purified via chromatography on Sephadex G-75, eluting with 0.05 M NaH_2PO_4 (pH 6.8) buffer.

$(DNP)_1$-cytochrome c

The synthesis of DNP_1-cychrome c was described by Dan Sand. (Sand, 1982) A nitro group was introduced into the protein at its only tryptophan residue via Koshland's reagent. The nitro group was reduced to an amine with $Na_2S_2O_4$. Dinitrofluorobenzene was then added at a pH of 6.3. The resulting modified protein was then purified by affinity chromatography on a column of ABPC-22 IgM coupled to Sepharose-4B. (Porathetal, 1973) This IgM is an anti-DNP protein. (Jarvis, 1981) The purified DNP_1-cytochrome C was desalted, lyophilized, and stored at -20°C.

$(DNP)_8$-cytochrome c

The synthesis of multivalent antigen, $(DNP)_8$-cytochrome c was accomplished by adding a 15-fold excess of 2,4-dinitrofluorobenzene (Aldrich Chemical Co.) to the purified cytochrome c at pH 10.1.

A solution of one part dinitrofluorobenzene per 50 parts water and 50 parts dioxane was prepared, and a volume of this solution which represented a 15-fold excess was added to a sample of cytochrome c in 0.5 M $NaHCO_3$ (pH 10.1). The reaction mixture was allowed to react for 24 hours with no stirring in the dark. Excess reagent was then removed by exhaustive dialysis against fresh 0.5 M $NaHCO_3$ buffer.

Labelling of IgG with HMNB-DMSBr

Immunoglobulin was labelled in the presence or absence of antigen. Solutions of cytochrome c carrier antigens were prepared by dissolving 1.85 mg DNP-glycine, 41.3 mg $(DNP)_1$-cytochrome c, 5.2 mg $(DNP)_8$-cytochrome c, 0.5 mg glycine, or 5.2 mg cytochrome c in 4 ml of 0.15 M NaCl, 0.02 M NaH_2PO_4, pH 6.8 buffer. One milliliter of a 5 mg/ml solution of 29B1 IgG2a was added. A six-fold excess of tritiated HMNB-DMSBr (4×10^{-4} M in 1×10^{-3} N HCl) was added dropwise at 0° with stirring. The solution was gently agitated for 24 hours at 4°C. A solution of IgG in the absence of antigen was labelled in the identical manner with ^{14}C-HMNB-DMSBr. Five milligrams of this ^{14}C labelled protein were added to each tritiated sample solution. The doubly labelled solution was immediately dialyzed against 0.10 M tris buffer, pH 8.0 to remove excess reagent. Thirty milligrams of completely unlabelled IgG was added to each sample at this point to minimize loss of radioactive label in subsequent steps. The solution was then made 0.03 M in $Na_2S_2O_4$ to remove DNP groups. One hour of reaction under nitrogen was followed by one half hour of oxygen bubbling through to remove excess $Na_2S_2O_4$. Dialysis against 0.15 M NaCl, 0.02 M NaH_2PO_4, pH 7.4 was followed by chromatography on Sephadex G-75. The fractions containing antibody free of hapten and antigen were collected and combined.

Papain Digestion

The immunoglobulin samples in 0.05 M sodium acetate, pH 5.5 were concentrated to 5 mg/ml by ultracentrifugation. The samples were made 0.001 M in cysteine and 0.002 M in EDTA, and 0.5 mg papain was added to each sample. Incubation for 45 minutes at 37°C was followed by addition of 3 mg p-chloromercuribenzoate to stop the reaction.

Separation of Fc and Fab Fragments

Each sample was dialyzed against 0.15 M NaCl, 0.02 M NaH_2PO_4, pH 7.4. The

Fc fragment was eluted with this same buffer on a DNP-sepharose column. The buffer was made 0.03 M in DNP-glycine and the Fab fragment was collected. The fractions containing Fc and Fab fragments were concentrated to one milliliter and their purity was checked by SDS-polyacrylamide gel electrophoresis.

Liquid Scintillation Counting

Each one milliliter sample was added to 10 ml Aquasol 2 scintillation counting fluid. Each sample was counted for fifty minutes on a Beckman LS-250 liquid scintillation counter programmed for a 3% counting error.

Results

Characterization of $(DNP)_8$-cytochrome c

Initially the absorbances at 280 nm and 365 nm were used to calculate the average ratio of DNP groups per protein molecule. This was the method used previously in this laboratory. The extinction coefficient of DNP at 365 nm is 16000, and the extinction coefficient of cytochrome c at 280 nm is 21200. (Mayer et al, 1970) Multiple modifications of cytochrome were performed in order to arrive at a procedure that produced several dinitrophenyl groups on a cytochrome c carrier. Cytochrome c is rich in lysine residues, containing 19 residues of lysine per molecule. (Margoliash et al, 1962) Thus modification of too many residues resulted in protein polymerization and precipitation.

Optimization of experimental procedure was attempted by varying both the excess of dinitrofluorobenzene and the pH at which the reaction was carried out. Under all conditions where a soluble product was obtained, the ratio of DNP groups to cytochrome c molecules did not exceed 1.7:1. This ratio was obtained using the absorbance at 280 nm for the protein and the absorbance at 365 nm for dinitrophenyl groups.

Ultraviolet and visible spectra revealed an absorbance of the dinitrophenyl moiety at about 260 nm, which distorted the protein absorbance at 280 nm. In addition, the main absorbance of cytochrome c at 400 nm interfered with the absorbance at 365 nm for DNP.

To correct for these difficulties, the visible absorbance of cytochrome c at 400 nm was used to estimate the protein concentration. Given A_{400}, the absorbance at 365 nm due to cytochrome c can be calculated and used to correct the observed value A_{365}. Thus the absorbance at 365 nm due solely to dinitrophenyl groups was obtained. With this procedure involving corrections for quantitating the extent of dinitrophenyl incorporation, the multivalent antigen

cytochrome c, with an *average* of 8 DNP groups per protein molecule, was prepared and characterized. In addition, the absorbance of cytochrome c at 528 nm confirmed the protein concentration that we had calculated.

Labelling of IgG with HMNB-DMSBr

Immunoglobulin 29B1 was labelled in the presence and absence of unmodified cytochrome, DNP-glycine, $(DNP)_1$-cytochrome c, and $(DNP)_8$-cytochrome c. The labelled antibody was then digested with papain, and the Fc and Fab fragments separated. The results of the double labelling experiments are given in Table 1. Each value given on the table is the average of duplicate experiments, and half of the difference between the duplicate values is given as the error. The concentration of dinitrophenyl moieties was sufficient to occupy greater than ninety percent of the antigen binding sites.

Discussion

Figure 8 illustrates the changes in tryptophan reactivity of IgG_{2a} 29B1 in the presence of hapten, monovalent antigen, and multivalent antigen. The presence of the hapten, DNP-glycine, only slightly increases tryptophan reactivity. There is a significant decrease in the reactivity of the Fc region's tryptophan residues upon binding either monovalent or multivalent antigen. These experiments confirm the trends observed for the monovalent antigen system that were observed by Dan Sand in this laboratory. In addition, a multivalent antigen utilizing the same protein carrier was examined for its effect on tryptophan reactivity. The presence of multivalent $(DNP)_8$-cytochrome c decreased tryptophan reactivity in both the Fab and Fc portions of the molecule.

The significant reduction in the reactivities of the tryptophan residues in the Fab portion of the molecule is noteworthy. It is possible to offer an explanation for this 12% reduction in reactivity in terms of the movement of the two Fab arms when both antigen-combining sites bind haptens on the same protein carrier.

These results strongly suggest that antigen binding causes a change in conformation or motional freedom of regions of the Fc portion such that tryptophan residues are less available to labelled HMNB-DMSBr. The monovalent $(DNP)_1$-cytochrome c is probably too small to interfere sterically with the availability of the antibody's tryptophan residues; also, aggregation is avoided in the monovalent antigen system.

These experiments support a mechanism of complement activation in which a change in the Fc occurs upon binding of antigen, and that change may represent a significant contribution to the triggering mechanism for the complement cascade.

Part B

Studies on the Nature of the Interaction between Complement Component C4 and Immunoglobulin G

Introduction

Complement component C4 is made up of three disulfide-linked peptide chains of molecular weight 93,000(α), 78,000(β), and 33,000(γ). (Schreiber and Muller-Eberhard, 1974; Nagasawa and Stroud, 1976) There are also non-covalent interactions between the chains, and 6 M guanidine or 1% sodium dodecyl sulfate is required to separate the chains after reduction. (Gigli et al, 1977) When hydrolysed by $\overline{C1s}$, C4 loses $\overline{C4a}$, a 8800 molecular weight piece from the amino terminus of its α chain. (Gorski et al, 1981) Active $\overline{C4b}$ remains and combines with activated $\overline{C2a}$ to form C3 convertase.

Only a small fraction (<10%) of activated $\overline{C4b}$ is found bound to antibody aggregates or antibody-coated cells; the rest becomes inactivated in the serum. (Goers and Porter, 1978; Muller-Eberhard et al, 1966) Component C4 labelled with ^{125}I was reacted with EAC1 cells, and only 5-10% of the C4 activated was observed bound to the cells. (Cooper and Muller-Eberhard, 1968) Heat-aggregated IgG binds C4, and this complex participates in the classical complement pathway (Ishizaka et al., 1961), at least through C3. (Sand, 1982) The radioimmunoassay results of Goers and Porter suggested that the $\overline{C4b}$ molecules that are effective in complement are those bound to the antibody. It was found that rabbit IgG on sheep erythrocytes could participate in a hemolytically active IgG/C4/C2 complex that was transferable from cell to cell. (Goers and Porter, 1978)

A reactive group, possibly an acyl group, may be generated when C4 is activated by $\overline{C1}$ and that this reactive group may react with IgG as well as with other adjacent structures. (Campbell, et al., 1980) The binding of $\overline{C4b}$ to IgG has been demonstrated to be a covalent interaction between the α'

chain of $\overline{C4b}$ and the heavy chain of the Fab portion of the immunoglobulin. (Campbell et al., 1980) Thus the IgG Fab arms provide binding sites for activated $\overline{C4b}$.

The nature of the binding site for activated C4 on antibody-antigen aggregates or antibody-coated red cells is far from clear. The question of whether the activated C4b moiety binds to the Fd at a specific site or whether the binding site is nonspecific, and simply reflects diffusion of the reactive C4b protein is addressed in this section.

The early components (C1 and C4) of the classical pathway were combined and allowed to react with aggregates of IgG_{2a} 29B1 in order to effect activation. We hoped that binding of $\overline{C4b}$ to the Fd portion of the immunoglobulin would occur and that subsequent enzyme digests of the antibody molecule with $\overline{C4b}$ attached would elucidate whether $\overline{C4b}$ was bound discretely or randomly to the Fab.

Experimental

29B1 IgG_{2a}

The IgG_{2a} hybridoma 29B1 was used to produce antibody, and the antibody was isolated and purified as described previously.

Complement Component C1

Component C1 was obtained from fresh human serum. (Gigli, Porter, and Sim, 1976) The euglobin precipitate of serum which contained C1 also contained a high concentration of proteolylytic enzymes. The difficulty of isolating component C1 lay in its easy conversion to activated $\overline{C1}$ by these proteases. In this particular preparation, repeated additions of the protease inhibitor, phenylmethylsulfonyl chloride, during the isolation steps greatly enhanced the proportion of C1 isolated in its unactivated form.

The isolation included addition of the serum to an excess of 5 mM aqueous $CaCl_2$, after which the euglobin precipitate was collected by centrifugation. The precipitate was resuspended in 0.04M sodium acetate buffer and run down a column of Sepharose 6B.

In addition human C1 in functionally pure form was obtained from Cordis Laboratories in order to compare its behavior to that of the protein isolated.

Complement Component C4

Human C4 was obtained from Cordis Laboratories in functionally pure form.

Preparation of Antibody Aggregates

IgG_{2a} 29B1 was used to make antibody aggregates, by the method described by Ziccardi. (Ziccardi, 1981) This procedure involved incubation of a 10 mg/ml sample at 63°C for 20 minutes followed by centrifugation.

Cyanogen Bromide Cleavage

The antibody after reduction with dithiothreitol was dissolved in 98-100% formic acid and then diluted with water. (Steers et al, 1965) Quantitative

cleavage of the heavy chain at methionine residues was achieved by adding solid CNBr and allowing the mixture to stand at 5°C for 24 hours. (Givol and Porter, 1965)

Chemical Crosslinking of IgG

Immunoglobulin G from hybridoma 29B1 was crosslinked by a modification of "Method B" described by Wright. (Wright et al, 1980) The antibody molecules were polymerized in the presence of the bifunctional cross-linking reagent dithiobis (succinimidylpropionate) in the absence of antigen. The reagent was added dropwise at 0°C to the IgG in sodium borate buffer (pH 9.0). The mixture was stirred at 4°C for exactly two hours, followed by immediate chromatography on Sephadex G-25. Peak fractions corresponding to IgG dimer were combined and further purified on Ultragel AcA 34 column.

SDS-PAGE

SDS-polyacrylamide slab gel electrophoresis was used to gauge the purity of proteins and to separate components of mixtures. The gels were stained generally with Coomassie Blue, or with silver when appropriate. (Merril, 1981)

Activation of Complement Components

The activation of C1 and C4 under physiological conditions has been described. (Ziccardi, 1981) The early classical complement pathway was reconstituted from the purified components at concentrations corresponding to their normal serum concentrations: 135 mg/ml C1 and 409 mg/ml C4. The purified component mixture was in tris-buffered saline (pH 7.5) that was 1.5mM in Ca^{++} and 0.6mM in Mg^{++}.

Aggregated 29B1 or dimers of IgG 29B1 were added to the complement mixture to effect activation.

Results

Attempts were made to characterize the cyanogen bromide digest of IgG_{2a} 29B1 by SDS-PAGE. The difficulty lay in the range of molecular size of the fragments to be separated. SDS/polyacrylamide gels of various acrylamide concentrations (12% to 16%) failed to effect satisfactory separation.

The early classical complement pathway was reconstituted with C1 and C4 as described in the experimental section. Addition of IgG_{2a} 29B1 aggregates should have caused activation of C1 and C4, and thus resulted in binding of C4 to the aggregates. In addition, the activation of complement components C1 and C4 by chemically crosslinked dimers of 29B1 was attempted.

In all cases, no results have been obtained to date since a satisfactory resolution of the CNBr fragments of IgG has not been achieved.

Discussion

Complement components C1 and C4 of the classical cascade were reassembled from purified components in physiological solution. Either heat-aggregated IgG or chemically cross-linked dimers of IgG were used to effect activation.

Attempts to characterize and separate peptides resulting from the cyanogen bromide digest of IgG have been unsuccessful to date. Cyanogen bromide was chosen because it has been demonstrated to produce six fragments from the heavy chain. (Givol and Porter, 1965) If C4 attachment could be demonstrated on one of the six peptides, via comparison of SDS-PAGE of IgG/$\overline{C4b}$ digests with SDS-PAGE of IgG digests, further digests of that particular peptide with other enzymes might determine whether $\overline{C4b}$ binds to a specific site on the IgG molecule. If binding were specific, one would expect to be able to detect $\overline{C4b}$ only on one specific peptide resulting from any one enzyme digestion of the immunoglobulin. (Incomplete enzyme digestion might distort the results; further digestion could alleviate the problem.) Note that this would not *prove specific* binding, but it was intended as a preliminary experiment.

The peptide produced by CNBr cleavage which includes the N-terminus of the heavy chain has been named C-1 (not to be confused with a complement component), and it includes the Fd portion of the molecule. (Givol and Porter, 1965) Since the site of the C4b attachment to the immunoglobulin has been shown to occur on the Fd portion, one would expect to observe C4b attached to C-1 after cyanogen bromide digestion. Attempts to observe satisfactory separation on SDS-PAGE of the fragments have failed thus far. HPLC may make separation of the fragments achievable. Fragments can be separated on a Sephadex G-100 column. However, separation on a column requires a large scaling up of the experiment and requires prohibitive quantities of C4. Isolation of C4 from large quantities of outdated human serum may make this possible.

Future Research

Experiments testing the ability of $(DNP)_8$-cytochrome c to activate complement are called for. The combined classical-alternate pathway assay (Sand, 1982) will be used to test for activation of the classical pathway and check for diminution of components.

Further characterization of $(DNP)_1$-cytochrome is necessary to absolutely confirm that it is indeed monovalent. The modification of this protein involves reaction of its single tryptophan residue with Koshland's reagent, after which the nitro group is reduced to an amine with $Na_2S_2O_4$. This amine is modified by dinitroflourobenzene at pH 6.3. A control experiment will be carried out under identical conditions, but omitting reduction with $Na_2S_2O_4$ and using tritiated dinitrofluorobenzene. Lack of incorporation of radioactive label into the protein would indicate that the reaction conditions are such that dinitrofluorobenzene reacts only at the modified tryptophan site.

Studies of the interaction between the IgG_{2a} 29B1 and $(DNP)_1$-cytochrome c have been carried out (Sand, 1982), and confirmation of these results have been described in this report. The interaction of this monovalent antigen with immunoglobin of class M is also of interest. An IgM antibody secreted by the myeloma ABPC22 is available in this laboratory. This tumor provides a homogeneous population of immunoglobulin M which binds dansyl- and DNP- derivatized proteins. The investigations of Richard Hardy in this laboratory indicate that the monovalent antigens $(DNP)_1$-LBTI or $(DNP)_1$-BSA can trigger ABPC22 to activate complement. (Hardy, 1981) This result is in agreement with the experiments of Brown and Koshland (1975) in which a heterogeneous population of IgM raised in rabbits against lac dye could activate complement upon binding monovalent antigen. The observation that IgM with monovalent antigen can initiate complement activation is consistant with a mechanism of complement activation in which antigen induces a conformational change in the Fc portion of the antibody.

The ability of complexes of IgM ABPC22 and monovalent antigen $(DNP)_1$-cytochrome c to activate the classical complement cascade will be investigated.

Cytochrome c is viewed as a better protein carrier than bovine serum albumin and lima bean trypsin inhibitor for several reasons. $(DNP)_1$-cytochrome c has been characterized to be likely monovalent, and future investigation will improve this characterization. The procedure for modifying BSA leaves some doubt as to its monofunctionality, and spectrophotometric analysis assures only that the average ratio of DNP groups per BSA molecule is one. Lima bean trypsin inhibitor was modified at its only tryosine residue, but the monomeric form of this protein undergoes a concentration-dependent dimerization (Krahn and Stevens, 1971) which would result in a divalent antigen. In addition, cytochrome c is a very compact molecule with a low molecular weight of 12384 daltons. Its small size decreases the possibility of blocking sites on the Fc region of the immunoglobulin.

The classical complement activation capability of IgM ABPC22 with $(DNP)_1$-cytochrome c will be observed employing a standard microcomplement ^{51}Cr release assay (Bengali et al, 1980), and the method of Riches and Stanworth (1980) will be used to gauge any possible capacity of the system to activate the alternate complement pathway.

A related facet to the classical complement activation problem involving C4 is particularly intriguing.

Much evidence exists that $\overline{C4b}$ does bind to immunoglobulin G and that this IgG-bound $\overline{C4b}$ can participate in the complement cascade. (Ishizaka, 1961; Willoughby and Mayer, 1965; Goers and Porter, 1978; Campbell, 1980) Evidence has suggested that hemolytic activity is due primarily to $\overline{C4b}$ bound to the IgG molecule and not to $\overline{C4b}$ bound to the cell surface. Recently, Circolo and Borsos have found that cell bound IgM does not bind detectable amounts of $\overline{C4b}$. The hemolytically active C4b can be shown to be bound to the cell surface. (Circolo and Borsos, 1982)

In order to elucidate the interaction between immunoglobulin and C4, the

location of C4b in soluble complexes of IgM ABPC22, $\overline{C4b}$ and $(DNP)_1$-cytochrome c will be probed. Previous studies of IgM and C4 have utilized antigens on cell surfaces; the investigation of the interaction between IgM and C4 in soluble complexes may reveal an interaction that is quite different. In this system, in which the antigen $(DNP)_1$-cytochrome c is small and monovalent, will $\overline{C4b}$ be bound to the IgM molecule as indicated by studies of IgG, or will no $\overline{C4b}$ be bound to the IgM as the results of Circolo and Borsos (1982) suggest for cellular antigens? These experiments will utilize C4 labelled with ^{125}I by the method of Reboul (1979) and Campbell (1980). It may be that the roles of the IgG molecule and the IgM molecule in the classical complement cascade are quite different. Much research done on the mechanism of complement activation has assumed IgM to be a functional pentamer of IgG; such may not be the case. Indeed, many functions of IgM and IgG are quite different in the immune system. IgM is produced first in the primary immune response, while IgG is produced later and is the main antibody produced in a secondary immune response. Class M antibody usually has a lower affinity for antigen per site, but an overall high avidity for antigen due to its multiplicity of binding sites. Class G antibody also crosses the placenta to enter the fetal circulation, but IgM does not. It is possible that IgM behaves in a manner fundamentally different than IgG in the activation of the complement cascade.

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Table 1: $^{3}H:^{14}C$ Ratios obtained from labelling studies

	Fc	Fab
Controls:		
29B1 + unmodified cytc	0.99 ± 0.02	1.00 ± 0.07
29B1 + glycine	0.97 ± 0.09	0.98 ± 0.05
Antibody with hapten:		
29B1 + DNP-glycine	0.99 ± 0.07	1.02 ± 0.07
Antibody with monovalent antigen:		
29B1 + $(DNP)_1$-cytc	0.81 ± 0.03	0.95 ± 0.05
Antibody with multivalent antigen:		
29B1 + $(DNP)_8$-cytc	0.78 ± 0.07	0.88 ± 0.06

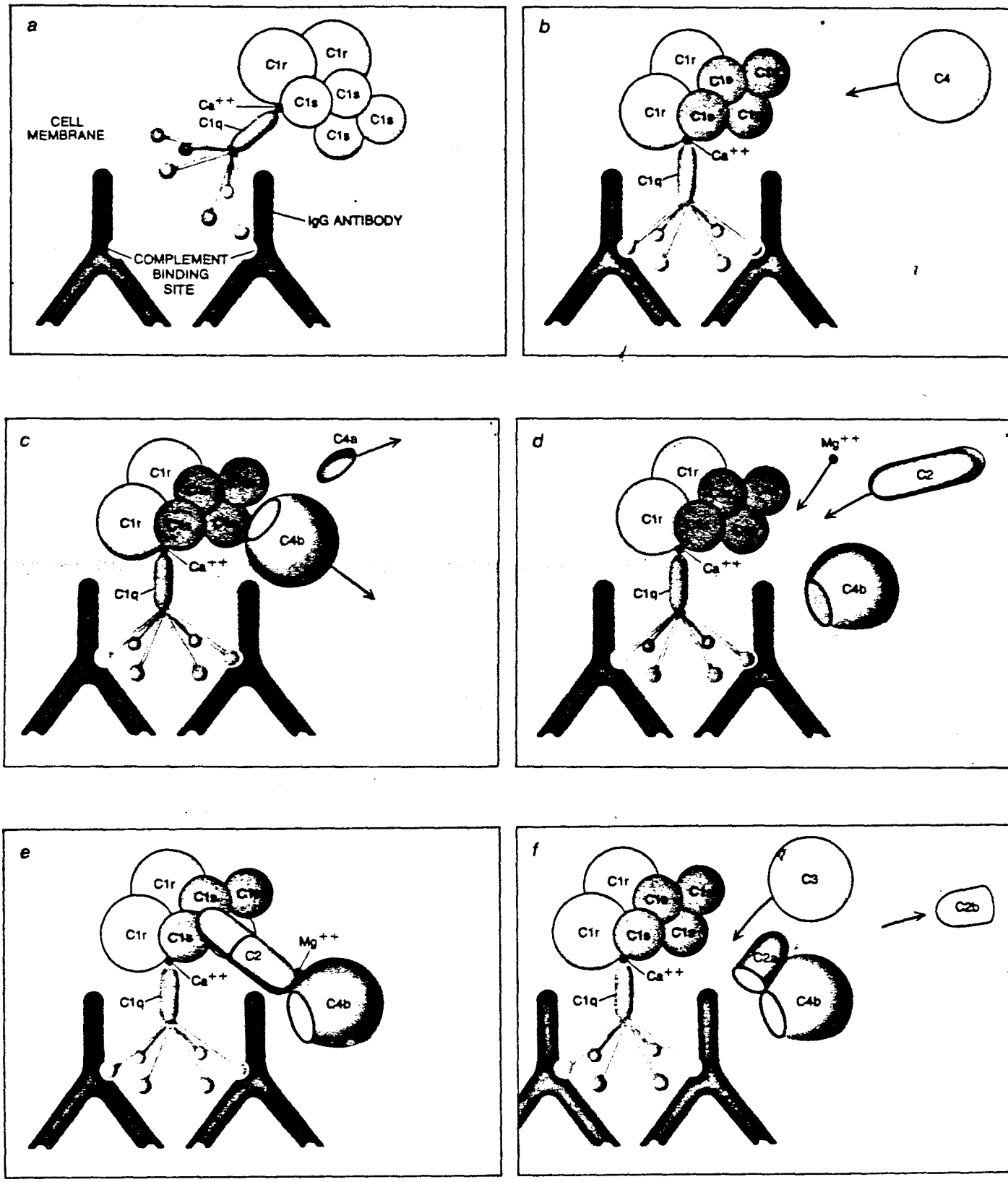


Figure 1. Classical Pathway of Complement Activation

a. Complement factor C1, consisting of subunits C1q, C1s and C1r, binds to the antibody.

b.c. The $\overline{C1}$ complex becomes enzymatically active and cleaves C4.

d. $\overline{C4b}$ binding to the cell surface is illustrated, although it is also found covalently bound to the Fd portion of IgG.

e.f. C2 is cleaved by the $\overline{C1s}$ subunit and combines with $\overline{C4b}$ to form C3

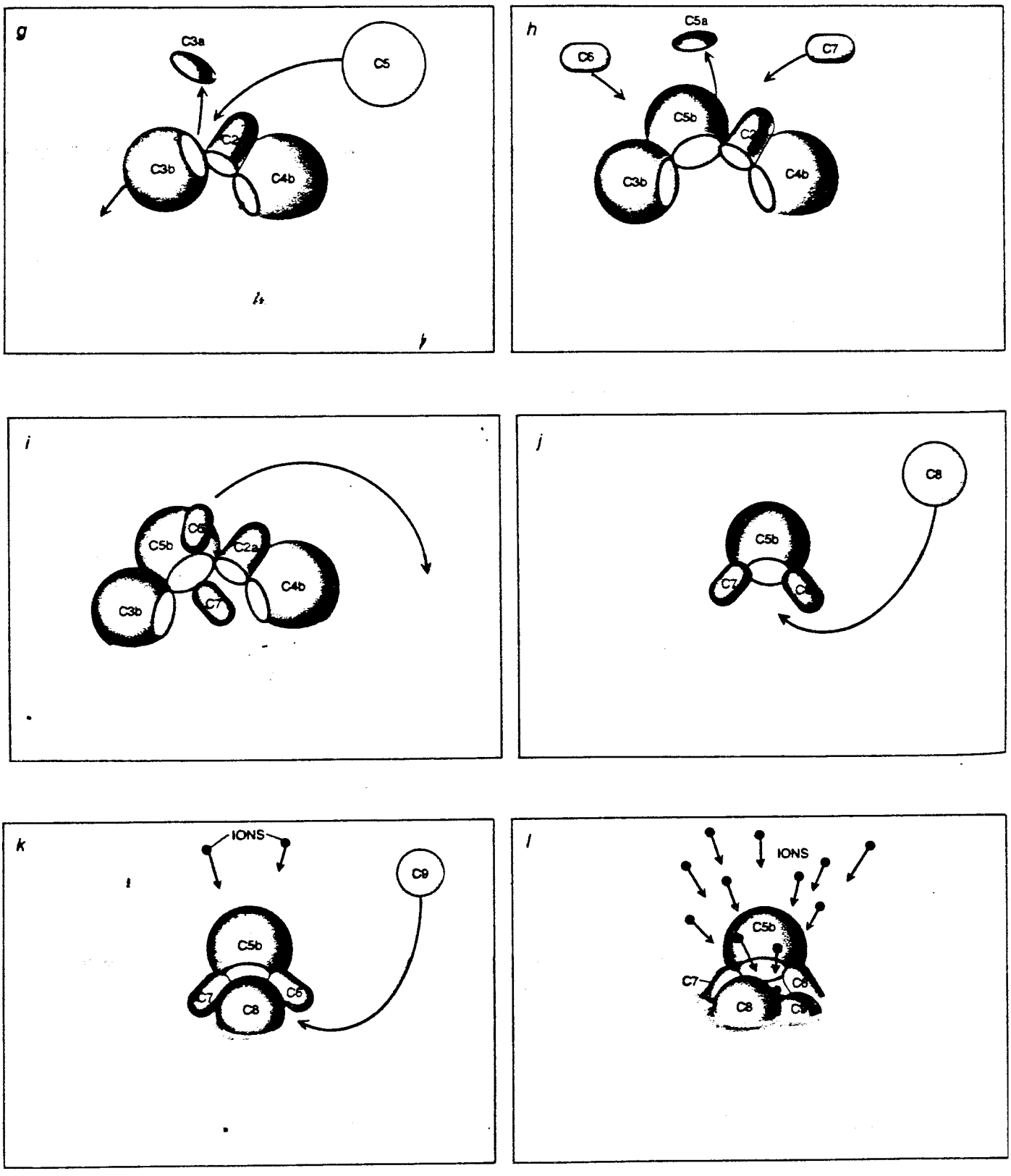


Figure 1 (cont.)

g. C3 convertase splits C3 into $\overline{C3a}$ and $\overline{C3b}$

h. The complex $\overline{C4b}$-$\overline{C2a}$-$\overline{C3b}$ cleaves C5 in the last enzymatic step of the cascade.

i.j.k.l. The remaining components assemble themselves in such a way that a small hole is formed in the membrane.

From M. Mayer, "The Complement System," Sci. Am. 229, 54 (1973).

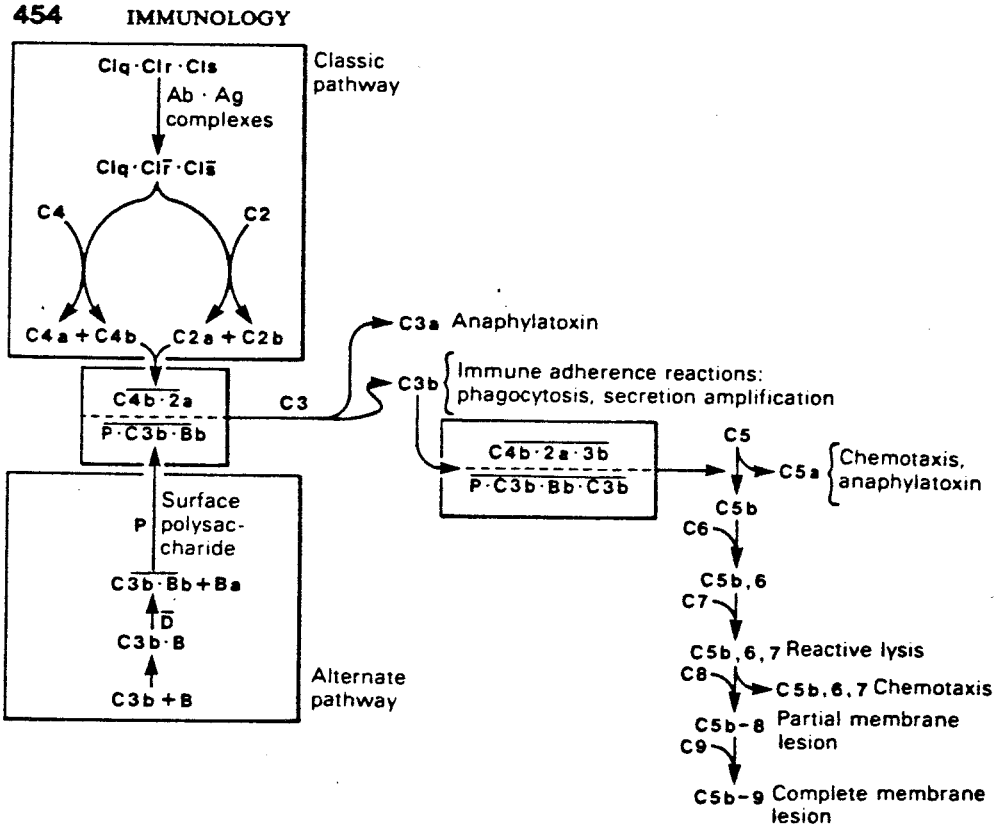


Figure 2: Reaction sequence of the complement system, depicting both the Classic and Alternate Pathways

(P=properdin, B=factor B, D=factor D)
(Eisen, 1980)

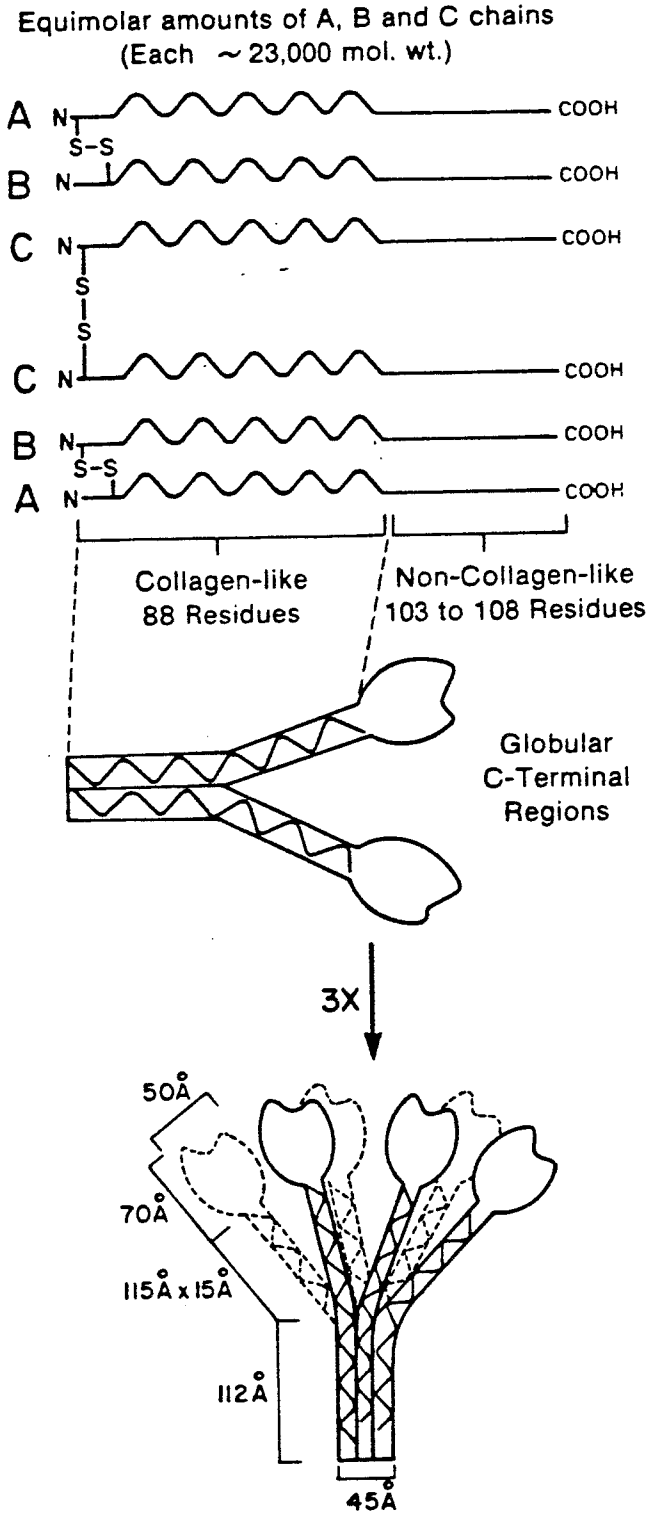


Figure 3: Peptide chain structure of C1q
Dimensions are from electron microscope studies.
(Porter, R.R. and Reid, K.B.B. Nature 1978, 275, 699)

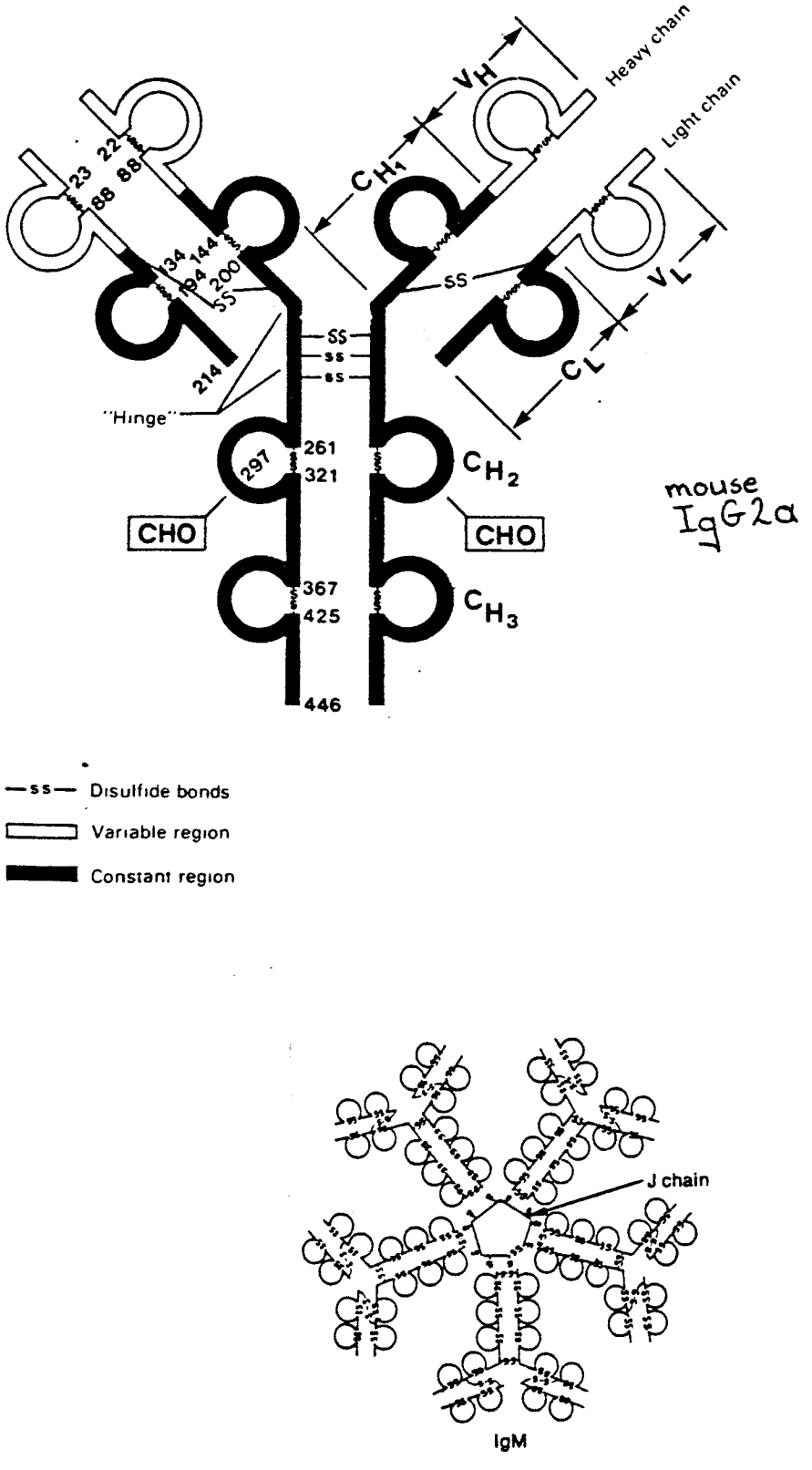


Figure 4: Schematic models of immunoglobulin G molecule and immunoglobulin M molecule (Eisen, 1980)

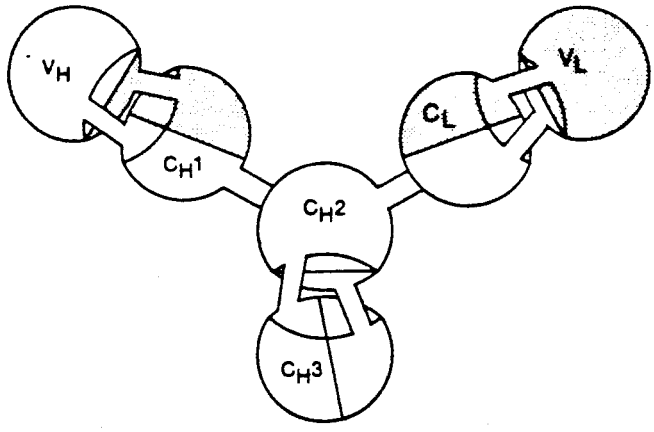


Figure 5: Domain structure of IgG molecule
(Poljak, R. Nature 1972, 235, 137)

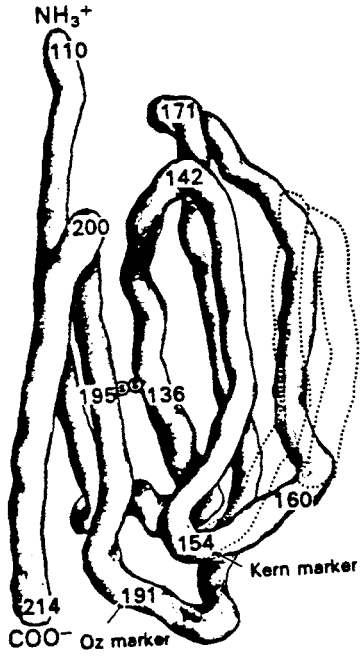


Figure 6: Diagram of the basic immunoglobulin fold.
The domain shown here is C_L which starts at residue 110 and extends to 214, the carboxyl terminus. The C_H1 domain has a very similar structure. The V_L and V_H domains contain an additional loop of polypeptide (dasked lines).
(Poljak, R. Proc. Natl. Acad. Sci. 1973, 70, 3306)

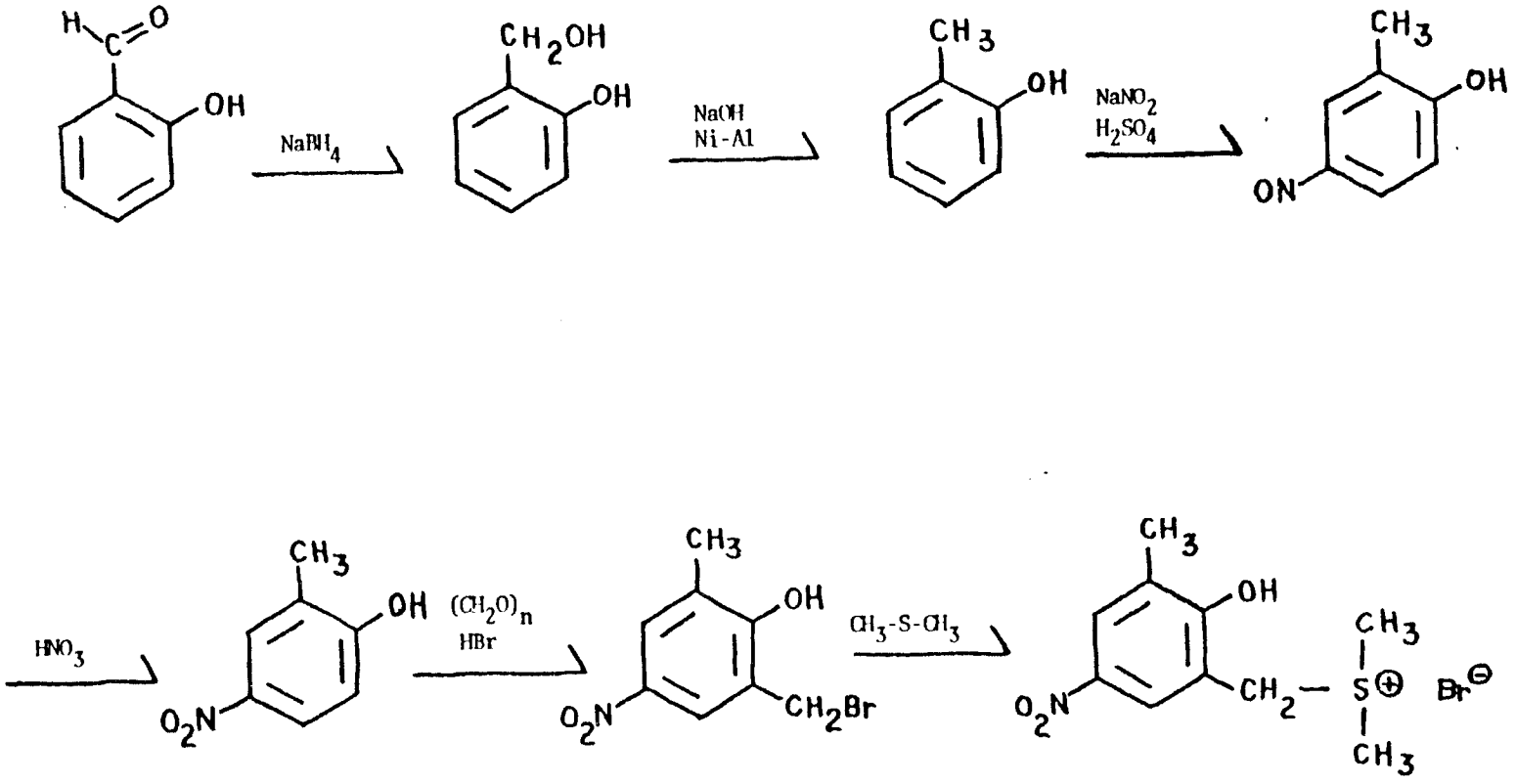
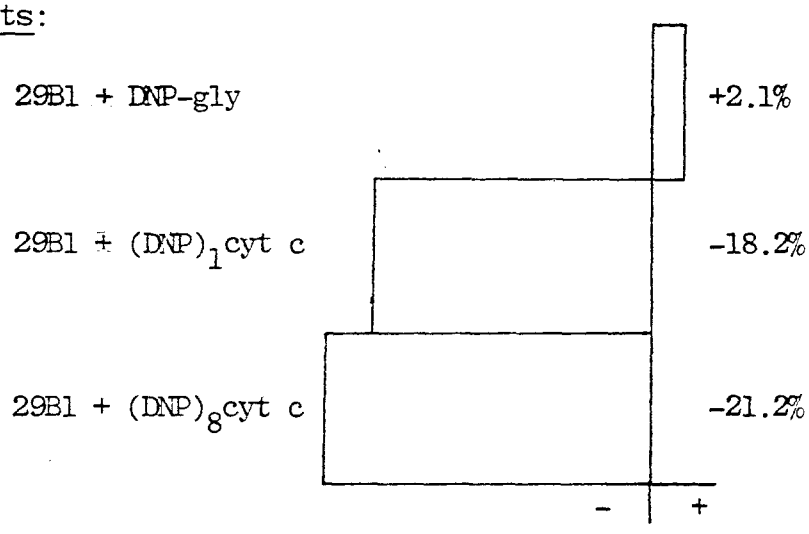


Figure 7. Synthetic route from salicylaldehyde to dimethyl (2-hydroxy-3-methyl-5-nitrobenzyl) sulfonium bromide
Tritium was incorporated in the initial reduction with $NaBH_4$. The incorporation of ^{14}C ocurred in the bromomethylation of 2-methyl-4-nitrophenol with paraformaldehyde and HBr(aq). (Sand,1982)

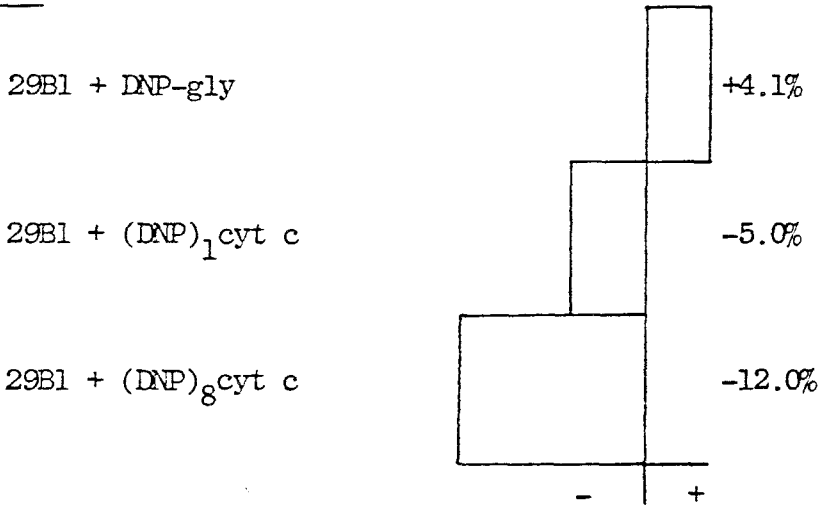
Figure 8.

Summary of changes in tryptophan reactivities of IgG in the presence of hapten, monovalent atigen, and multivalent antigen
The values expressed are percentage change of incorporation of label in the presence of ligand compared to incorporation of label in absence of ligand.

Fc fragments:



Fab fragments:



PROPOSITIONS

Proposition Abstracts

Proposition I

Cooperativity among metal atoms in a cluster has been used to explain the rate enhancement of carbonyl dissociation with progressive phosphine substitution.

$$Ir_4(CO)_{12} \xrightarrow{1} Ir_4(CO)_{11}(PPh_3) \xrightarrow{1320} Ir_4(CO)_{10}(PPh_3)_2 \xrightarrow{41800} Ir_4(CO)_9(PPh_3)_3$$

Another explanation for this enhancement is the great steric bulk of the triphenyl phosphine ligand. The investigation of CO exchange processes in complexes of the type $Ir_4(CO)_{11}PX_3$ is proposed in order to assess the relative contributions of electronic cooperativity and spatial consideration. Phosphines could be employed that have roughly equivalent size, but very different π-accepting characteristics.

Proposition II

Upon activation, $\overline{C4b}$ exhibits a metastable binding site for the Fd portion of IgG in immune aggregates. Evidence suggests that C4 contains an internal thioester, which upon cleavage of C4 by C1s is exposed and activated. The activate acyl of the thioester may react with nucleophiles on the Fd. Experiments are proposed to investigate the effect of antigen-binding and C1-binding on the availability/reactivity of nucleophilic groups in the Fd portion of the immmunoglobulin. Double labelling experiments with a small, activated ester are suggested.

Proposition III

The study of the globin gene family of vertebrates has contributed much to knowledge of evolution, development, and gene clusters. The isolation and sequencing of a hemoglobin gene from the annelid worm, Glycera dibranchiata is proposed because comparison to vertebrate globin, vertebrate nyoglobin, and leghemoglobin *may* help explain the evolution of iron heme-bearing pigments that function in oxygen storage.

Proposition I

Transition-metal cluster compounds are of interest to chemists largely because of their potential applications in catalysis. The special properties that adjacent metals give the molecule or the catalytically active fragments that the molecule releases may explain the reactivity of clusters as homogeneous catalysts. (Muetterties, 1976; Smith and Basset, 1977; Pittman and Ryan, 1978) Clusters are also being utilized to model substrate bonding and the reactions of substrates on metal surfaces. (Muetterties, 1975; Muetterties, 1979; Muetterties et al, 1979)

Because it has been suggested that the tetranuclear phosphine-substituted carbonyls of iridium may serve as catalysts in the hydroformylation of olefins (Drakesmith and Whyman, 1973), this class of compounds is of particular interest.

The tetrairidium dodecacarbonyl molecule is a tetrahedral cluster of metal atoms with carbonyl groups attached linearly to the apices of the iridium polyhedron. The four iridium atoms are held together solely by metal-metal bonds (figure 1,A). (Churchhill and Hutchinson, 1978) Substitution of phosphine groups for carbonyls results in a change in the skeletal structure of the complex: three of the carbonyl groups occupy bridging positions. The diphosphine-substituted cluster, $Ir_4(CO)_{10}(PPh_3)_2$, sports one basal axial phosphine and one basal equatorial phosphine (figure 1,B). The phosphines in $Ir_4(CO)_9(PPh_3)_3$ occupy basal axial, basal equatorial, and basal equatorial positions (figure 1,C). (Albano et al, 1967) These stereochemistries persist in solution for the analogous $Ir_4(CO)_{10}(PPh_2Me)_2$ and $Ir_4(CO)_9(PPh_2Me)_3$ as revealed by ^{13}C-NMR studies. (Stuntz and Shapley, 1977) Carbonyl scrambling has been demonstrated by NMR in the phosphine-substituted clusters, indicating facile terminal-bridging exchange. (Cattermole et al, 1974)

Norton reported a cooperative effect in the reactions of the triphenyl phosphine-substituted iridium carbonyls, suggesting the importance of interactions among metal atoms in clusters. The kinetic parameters for progressive carbonyl substitution in the cluster revealed a substantial rate enhancement for carbonyl dissociation with progressive phosphine substitution. (Karel and Norton, 1974)

$$Ir_4(CO)_{12} \xrightarrow{1} Ir_4(CO)_{11}(PPh_3) \xrightarrow{1320} Ir_4(CO)_{10}(PPh_3)_2 \xrightarrow{41800} Ir_4(CO)_9(PPh_3)_3$$

These results are often interpreted as being supportive evidence that electronic interactions can be transmitted from neighboring metal atoms to the active catalytic center in a cluster. (Pittman and Ryan, 1978)

In contrast, in the analogous cobalt system, no such enormous rate enhancement was noted. (Darensbourg and Incorvia, 1979, 1980).

$$Co_4(CO)_{12} \xrightarrow{1} Co_4(CO)_{11}[P(OMe)_3] \xrightarrow{0.62} Co_4(CO)_{10}[P(OMe)_3]_2 \xrightarrow{1.46} Co_4(CO)_9[P(OMe)_3]_3$$

In this case the ligand replacing CO is the small, good π-acceptor $P(OMe)_3$. One possible explanation for the much greater enhancement in the iridium system is the greater steric bulk of the triphenyl phosphine ligand as compared to the small trimethyl phosphite group. Sizeable steric acceleration is well-documented in mononuclear carbonyl phosphine and phosphite derivatives. (Darensbourg and Graves, 1979) The study of CO dissociation in the complexes $Ir_4(CO)_9[PR_3]_3$, R=Me,nBu, Et, i-Pr, Ph has demonstrated that carbonyls are more

easily lost when the phosphine is larger. (Darensbourg and Baldwin-Zuschke, 1982)

An interesting question follows: In clusters of the type $Ir_4(CO)_{12-n}(PX_3)_n$, X=OR, R, is the ease with which a carbonyl dissociates related to a electronic cooperativity among the metal atoms, and/or b) spatial confrontations between ligands dictated by the cluster's framework? Thus, it would be helpful to know to what extent the steric qualities or the electronic qualities of the ligand affect the cluster.

It would be interesting to investigate CO dissociation in clusters using ligands of varying electronic properties. Such studies could help discern whether any cooperation does take place among metal atoms in the cluster. Mono-phosphine substituted clusters of the type $Ir_4(CO)_{11}L$ would be most useful since the steric characteristics of L will be minimized.

The first four ligands of interest would be perhaps PF_3, $P(OCH_2CH_2Cl)_3$, $P(OMe)_3$, and $P(OEt)_3$. These ligands have very different π-accepting characteristics, as judged by the infrared carbonyl stretching frequencies of $Ni(CO)_3L$ where L=PF_3, $P(OCH_2CH_2Cl)_3$, $P(OMe)_3$, $P(OEt)_3$. ("Tolman parameter," Tolman, 1970) The π-accepting capability ordering is:

$$PF_3 > P(OCH_2CH_2Cl)_3 > P(OMe)_3 > P(OEt)_3$$

These four phosphine ligands also have roughly equivalent size as indicated by their respective ligand cone angles: 104°, 110°, 107°, and 109°. (Tolman, 1977)

Given ligands of equivalent size but different electronic properties, the rates of dissociation of CO from $Ir(CO)_{11}L$, L=PF_3, $P(OCH_2CH_2Cl)_3$, $P(OMe)_3$, $P(OEt)_3$ would be very enlightening. The rate of carbonyl dissociation could

measured by observing the rate of exchange of carbonyls in the complex with a ^{13}CO atmosphere. Carbon-13 enrichment could be detected via carbonyl infrared stretching frequencies. The absorption region for the bridging carbonyls (1820-1700 cm^{-1}) is an especially sensitive indicator of such enrichment; absorption peaks shift to lower frequencies as ^{13}CO is incorporated.

If the metal atoms are cooperating, then a better π-acceptor on one metal center should decrease the rate of exchange of carbonyls. One would see the fastest rate for $Ir_4(CO)_{11}[P(OEt)_3]$ and the slowest rate for $Ir_4(CO)_{11}[PF_3]$. Subsequent studies could investigate these same ligands in complexes of the type $Ir_4(CO)_{10}L_2$ in an effort to demonstrate the *relative* contributions of electronic cooperativity and spatial consideration.

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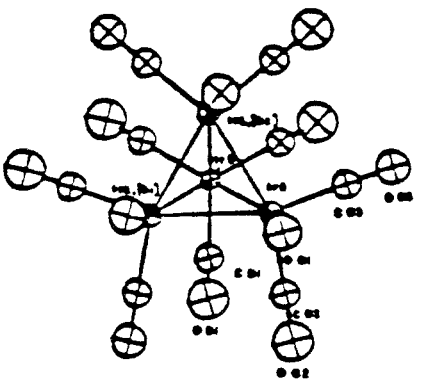
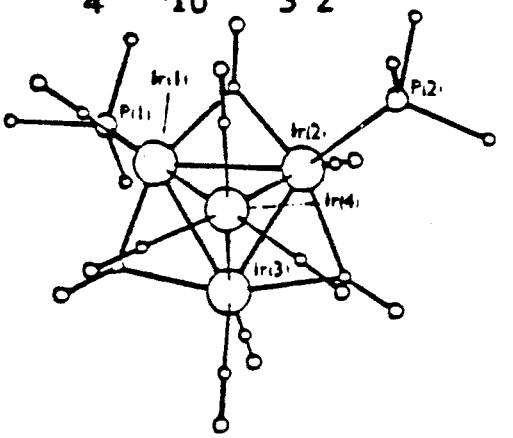
FIGURE 1

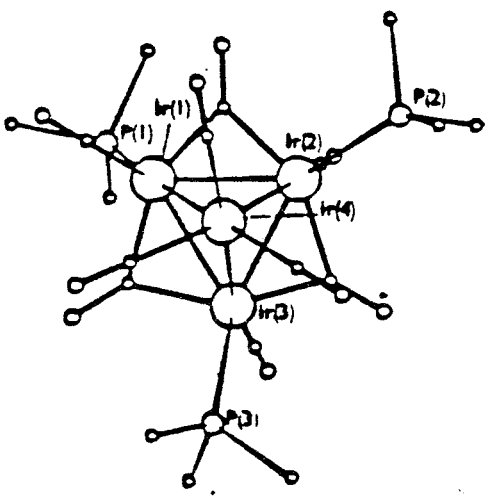
A. $Ir_4(CO)_{12}$



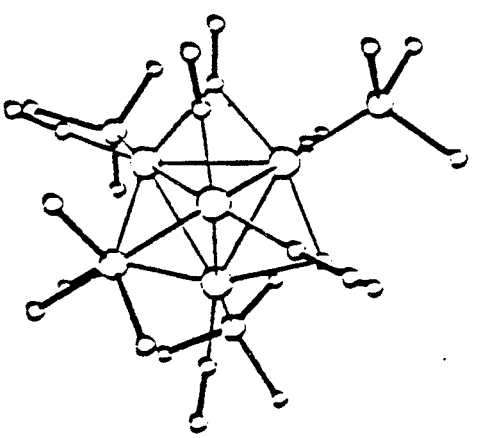
B. $Ir_4(CO)_{10}(PPh_3)_2$



C. $Ir_4(CO)_9(PPh_3)_3$



D. $Ir_4(CO)_8(PMe_3)_4$



Proposition II

The generation of a conformational change in the Fc region upon antigen binding in the Fab region has long been considered a model for activation of the classical complement cascade. Since $\overline{C3b}$ and $\overline{C4b}$ seem to bind only to the Fd portion of IgG in antibody-antigen aggregates (Gadd and Reid, 1981; Campbell, et al., 1980), it seems worthwhile to investigate the possibility that a conformational change in the Fab region upon antigen binding is a necessary condition for $\overline{C3b}$ or $\overline{C4b}$ to bind and to take part in the rest of the classical complement cascade.

There is good evidence that unactivated complement components C3 and C4 each contain an internal thioester linkage. Upon cleavage of C4 into $\overline{C4a}$ and $\overline{C4b}$ by the active proteinase $\overline{C1s}$, a transient reactive group is generated elsewhere on $\overline{C4b}$'s α-chain that allows $\overline{C4b}$ to bind covalently to the Fd portion of IgG (Campbell, et al., 1980) or to the surface of erythrocytes. (Law, et al., 1980) The same phenomenon is observed for $\overline{C3b}$ upon activation by C3 convertase ($\overline{C4b2a}$). (Gadd and Reid, 1981; Law and Levine, 1977; Law, et al., 1979) This reactive group exposed upon activation reacts rapidly with water; thus $\overline{C4b}$ and $\overline{C3b}$ are rapidly inactivated unless binding to antibody or cell surface occurs.

The interaction between $\overline{C3b}$ and antibody or cell surface is through an ester or amide bond (Gadd and Reid, 1981; Law and Levine, 1977) in which the acyl group is contributed by a glutamic acid residue from the α-chain of $\overline{C3b}$. (Law, et al., 1979a; Law, et al., 1979b) The glutamyl residue and cysteinyl residue that make up this thioester in C3 and C4 are separated by two amino acids in the primary sequence. The thioester site is contained in a sequence of eight amino acids that are almost completely conserved among C3, C4, and α_2-macroglobulin. (Thomas, et al., 1982; Campbell, et al., 1981; Swenson and

Howard, 1980)

The reactivities of the thioester linkage in the unactivated components C3 and C4 toward nucleophiles are much less than the reactivities of the components upon activation. These observations have been explained by a thioester "buried" within the protein; upon cleavage of C4 by $\overline{C1s}$ or C3 by C3 convertase, the thioester is exposed and provides a very reactive acyl group. (Tack, et al., 1980; Harrison, et al., 1981) This acyl group can then react with nucleophiles on antibodies or cell surfaces, or it can be inactivated through reaction with water.

As mentioned earlier in this report, there is evidence that $\overline{C4b}$ binds to the Fd portion of antibody in antibody-antigen aggregates and that it is this $\overline{C4b}$ bound to antibody which is hemolytically active. (Campbell, et al., 1980) Binding of $\overline{C4b}$ elsewhere on the antibody molecule was *not* detected. Component $\overline{C3b}$ is also covalently bound to the Fd in antibody-antigen aggregates. (Gadd and Reid, 1981) If activation of C3 or C4 generates a reactive thioester subject to nucleophilic acyl substitution, why does reaction only occur in the Fd portion of the immunoglobulin molecule? A few speculations come to mind immediately. First, perhaps C1 with its long collagen-like extensions actually cleaves C4 at a place nearer the Fab arms; since the thioester is hydrolysed rapidly, C4b either reacts with Fab (or cell surface) which is near at hand or is inactivated by water. This does not easily explain why binding of C4b to the light chain of the Fab region is not detected. Second, perhaps C1's presence at the Fc region blocks certain nucleophilic groups of the Fc from reacting with the nascent thioester of $\overline{C3b}$ or $\overline{C4b}$. Third, perhaps binding of antigen increases the reactivity/availability of nucleophilic groups in the Fd portion of IgG.

With these questions in mind, it is appropriate to investigate the availability of nucleophilic groups in the IgG molecule. A reactive ester such as pentachlorophenyl acetate might be used to label IgG. The reaction conditions (pH, reagent excess, etc.) could be varied in order to discover conditions under which the more reactive/available nucleophilic groups react with the ester. Once such conditions are established, a double-labelling experiment could be carried out in the presence and absence of antigen. For example, tritiated pentachlorophenyl acetate is allowed to react with antibody with antigen bound. Then this ^{3}H-labelled IgG is mixed with IgG which has been labelled with ^{14}C-pentachlorophenyl acetate in the absence of antigen. Comparison of ^{3}H:^{14}C ratios obtained should indicate whether the binding of antigen affects nucleophilic groups on the IgG molecule. Digestion with papain and affinity chromatography can separate Fc and Fab regions so that the ^{3}H:^{14}C ratios for the two fragments can be determined. One should realize that the physical presence of antigen may very well affect the availability of nucleophiles at the antigen-binding site.

Another phase of the project would be to perform the same experiments under conditions where C1 is also bound to the antibody. The results may reveal whether the presence of C1 on the Fc might be blocking or otherwise affecting nucleophilic groups of the IgG molecule.

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Proposition III

More is probably known of the evolution of the mammalian globin gene family than any other system. Vertebrate hemoglobin is composed of two chains each of α-like and β-like globin polypeptides. The initial interest in these genes was chiefly their developmental aspects: each type of chain is encoded by a gene under differential, but coordinate control. (Proudfoot et al., 1980; Efstratiadis et al., 1980)

Investigation of the structure of globin genes revealed a 3 exon/2 intron structure that was characteristic of every functional globin gene studied. Further, every gene contained two introns which interrupted the protein-coding sequence at *homologous* positions. Intron positions and lengths for globin genes of mouse, man, rabbit, chicken, sheep, goat, and frog are given in Table 1. The homology in sequence and similarity in structure of these genes has led to the suggestion that the two introns were present before the duplication that gave rise to separate α- and β-globin genes, an event estimated to have occurred 500 million years ago. (Dayhoff et al., 1972)

The nitrogen-fixing nodules of legumes contain a myoglobin-like protein termed leghemoglobin. (Baulcombe and Verma, 1978) The amino acid sequence homology that leghemoglobin genes share with vertebrate globin genes suggests that leghemoglobins and globins have a common evolutionary origin. (Hunt et al., 1978) The leghemoglobin genes contain three introns, the first and third of which are in homologous positions to the globin gene introns. (Hyldig-Nielsen et al., 1982) The middle "extra" intron has provoked considerable attention.

Mitika Go used C^{α}-C^{α} distance maps to demonstrate four structural units in the globin polypeptide. Two of these peptide units correspond to the first

and second exon in the globin chains. The remaining two peptide units are coded by the middle exon. If that "extra" intron in leghemoglobin were present in globin, each of the four structural units in the protein would correspond to a separate exon. (Gō, 1981)

Given these intriguing facts on globin and leghemoglobin gene structure, examination of the gene coding for myoglobin should prove interesting. Comparisons between the *protein* structure and amino acid sequence suggest that the myoglobin and globin genes originated from a common phylogenetic ancestor about 600-800 million years ago, before the divergence of α- and β-globin genes. (Hunt et al., 1978; Czelusniak et al., 1982) Would the myoglobin gene contain three introns at positions homologous to the intron positions in the leghemoglobin gene?

Very recently (Blanchetot, et al., February 24, 1983), the myoglobin gene from grey seal has been isolated and characterized. The gene contains two introns at positions precisely homologous to globin introns; however, the introns of length 4800 base pairs and 3400 base pairs are unusually long. These lengths are totally unlike the globin gene intron lengths (Table 1), and the complete myoglobin gene was determined to be 9,200 base pairs long. (Blanchetot, et al., 1983)

The "extra" intron in the leghemoglobin genes and these surprisingly long introns in the myoglobin genes should focus more attention on the study of the hemoglobin-like proteins of invertebrates. The nomenclature is difficult here. Such proteins in invertebrates are called hemoglobins in the literature; it may or may not be correct to do so. In any case, in the following paragraphs hemoglobin-like proteins in invertebrates will be referred to as hemoglobin.

The distribution in invertebrates of the red fluid variously called cruorine,

erythrocruorin, hemoglobin, or myoglobin, depending on when it was described and where it is located, has been the object of many studies. (Terwilliger, 1980) In the 1800's, E. Ray Lankester described the apparent irregular occurence of this "red pigment" among the invertebrates. (Lankester, 1872) Does this intermittent distribution represent the descent from an ancestral hemoglobin-like protein or the repeated development of oxygen-binding heme proteins as different organisms respond to similar environmental pressures? Svedberg suggested that all hemoglobin structures were based on an oxygen-binding polypeptide unit with molecular weight 14-17,000 daltons. (Svedberg, 1933) Thus far, the most primitive phylum for which the presence of hemoglobin has been demonstrated is Phylum Protozoa. Two species of Paramecium, P. caudatum (Sato and Tamiya, 1937) and P. tetraurelia (Smith, et al., 1962) have so far been shown to contain hemoglobin. Paramecium hemoglobin is a monoheme polypeptide of molecular weight 13,400 daltons (Smith, et al., 1962), similar to myoglobin's molecular weight. It exhibits in general spectroscopic and chemical reactivity typical of vertebrate globin and myoglobin. (Smith et al., 1962) The structure of this hemoglobin has not been studied; so its relation to the vertebrate globins cannot be assessed.

The complete amino acid sequence (147 residues) has been determined for the monomeric hemoglobin of the common bloodworm, Glycera dibranchiata. (Imamura et al., 1972) Amino acids at 23% of the sites are identical to those in sperm whale myoglobin, and 20% of the positions are identical to human β-globin. Although these degrees of correspondence seem low, the overall conformation of the annelid hemoglobin chain is remarkably similar to the arrangement found in vertebrate globins. Crystallographic studies reveal seven helical segments which are disposed in the familiar "myoglobin fold". (figure 1) (Padlan and Love, 1974) Notably the D helix is absent and the distal

histidine in vertebrate globins is occupied by leucine. (Padlan and Love, 1974) The protein has a molecular weight of 15,590 daltons. (Imamura et al., 1972)

Since the sequence of this invertebrate hemoglobin gene may prove invaluable to the understanding of globin evolution, it is proposed that the gene be isolated and sequenced. The protein is not of prohibitively low concentration in the organism; it should be possible to include the gene in a DNA library made from mRNA. (Grunstein and Hogness, 1975; Benton and Davis, 1977) The cDNA library could be screened with a ^{32}P-labelled copy of a vertebrate globin or myoglobin gene. If the degree of homology is not sufficient for the probe to pick out the hemoglobin gene, an alternate method could be attempted. Since the amino acid sequence for the G. dibranchiata hemoglobin is known (Imamura, et al., 1972), a mixture of synthetic oligonucleotides that could code for a portion of the peptide chain could be used as probes (as in Singer-Sam et al., 1983). A problem with this technique is that there will be a range of melting temperatures for a mixture of probes. This might make it difficult to distinguish a low melting, but well-matched probe from a probe that contains more mismatches but that melts at a higher temperature, due to its base composition.

Once the cDNA clone is isolated, it can be used to screen a genomic library constructed from either restriction enzyme fragments or sheared DNA. (Maniatis et al., 1978) The genomic recombinant molecules can be isolated, prepared and cloned (Lacy et al., 1979), and the appropriate fragment could be isolated and sequenced. (Sanger et al., 1980)

Knowledge of the sequence of the G. dibranchiata hemoglobin gene may lead to better understanding of the evolution of hemoglobin-like proteins. Possible sequence homologies between this gene and the vertebrate globin and myoglobin genes would be important, as would similarities in intron/exon organization, position, and length.

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Table 1: Intron Lengths and Positions for Globin-like Genes

	gene	IVS1 length (bp)	IVS2 length (bp)	IVS1 amino acid position	IVS2 amino acid position	reference
uman α-globin	α2	117	140	31,32	99,100	1
ouse α-globin	α	122	135	31,32	99,100	2
oat α-globin	α	108	103			3
rog α-globin (X. laevis)	α'	171	338	31,32	99,100	4,13
uman β-globin	β	130	850	30,31	104,105	5
ouse β-globin	β^{maj}	116	653	30,31	104,105	6,2
abbit β-globin	β1	126	573	30,31	104,105	7
oat β-globin	β^{A}	128	906	29,30	103,104	8,3
rog β-globin (X. laevis)	β^{1}	192	841	29,30	103,104	4,13
rey seal myoglobin	Mb	∿4800	∿3400	31,32	105,106	9

		IVS1 length (bp)	IVS2 length (bp)	IVS3 length (bp)	IVS1 amino acid position	IVS2 amino acid position	IVS3 amino acid position	reference
oybean leghaemoglobin	Lba	119	233	680	31,32	68,69	103,104	10
	Lbc_1	169	234	285	31,32	68,69	103,104	10
	Lbc_2	119	190	197	31,32	68,69	103,104	11
	Lbc_3	119	99	229	31,32	68,69	103,104	12

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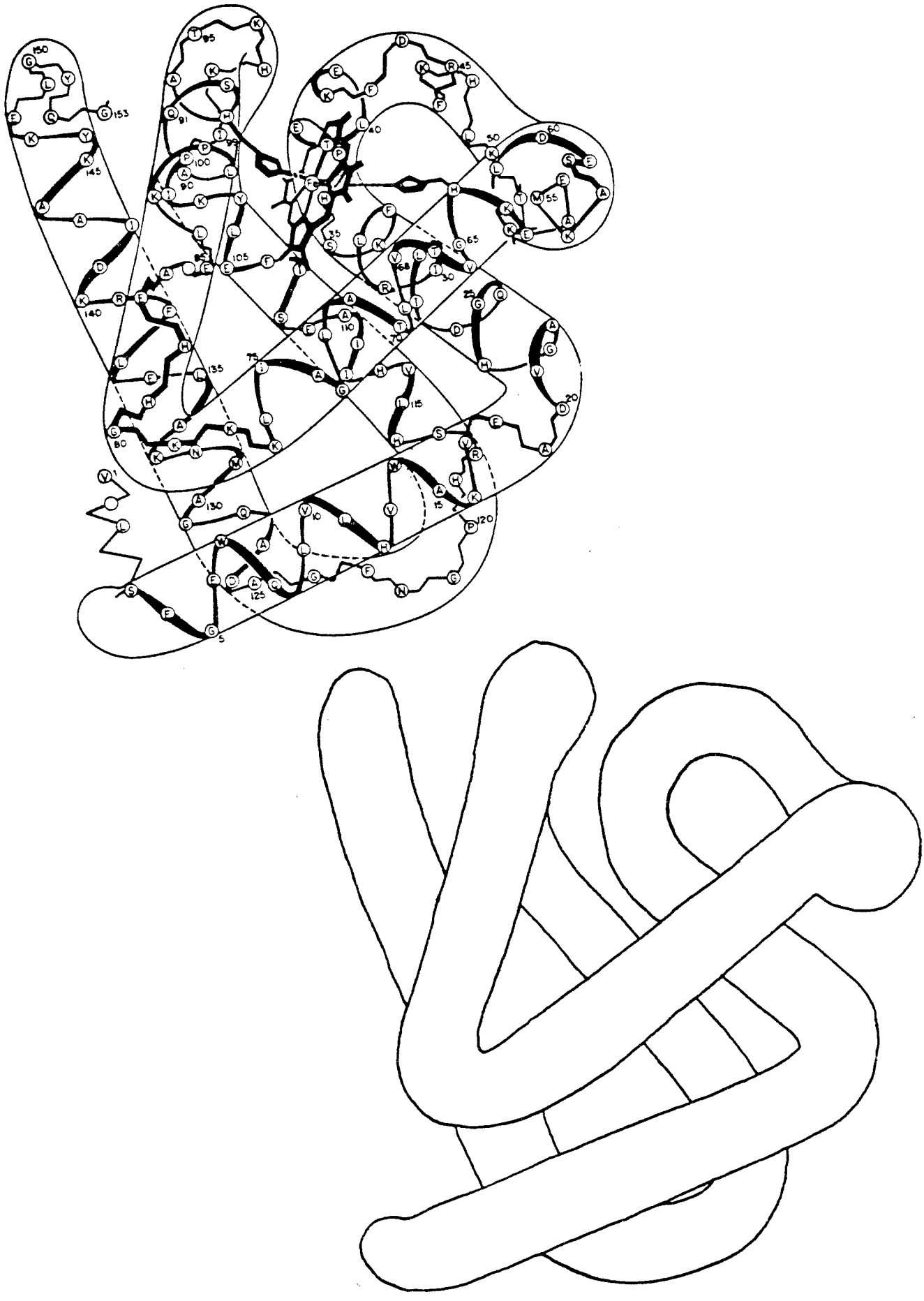

Figure 1. The "Myoglobin Fold"
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