INVESTIGATION OF THE MECHANISM OF COMPLEMENT ACTIVATION BY IMMUNOGLOBULIN G

Thesis by

Irving D. Sand

In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1982

(submitted 6 May 1982)

To my parents

Mrs. Ellen K. Sand and Mr. David Sand

ACKNOWLEDGMENTS

My greatest thanks go to Dr. John H. Richards whose insight and encouragement contributed tremendously to the progress of this research. I would like to thank David Strader for his assistance with the labeling project on UPC-10 IgG and the complement assays; and Dr. Richard Hardy for his assistance with the labeling project on 29B1 IgG. I am grateful to the Richards' research group for providing a stimulating and congenial atmosphere in which to work.

My thanks go to Dr. Michael Raftery and his group for their generosity in allowing me the use of equipment; especially, I thank Mr. John Racs who performed the amino acid analyses.

This work was supported by the National Institute of Health (Grant No. CA23028) and by an NIH training grant (PHS4GM07616A-3) and by graduate research assistantships (HL15162-09 and 6M16424-14).

DAI ABSTRACT

The synthesis of a tryptophan specific reagent, dimethyl (2-hydroxy-3-methyl-5-nitrobenzyl) sulfonium bromide, is described. The synthetic route incorporated ^{3}H and ^{14}C. The reagent was shown to specifically benzylate tryptophan which was either in solution as a free amino acid or in a protein. Physicochemical properties of the reagent which may be related to its specificity were examined.

Evidence is presented that a conformational change occurs in mouse IgG_{2a} when antigen binds and that this change is a necessary condition for the activation of the complement cascade. A $^{3}H/^{14}C$-labeled, tryptophan-specific reagent was used to monitor the reactivity of tryptophans in the Fc region of the immunoglobulin. This reactivity correlated with the ability of the antibody to fix complement. Thus binding monovalent and multivalent antigens caused decreases in the reactivity of Fc tryptophan residues by 15% and 22%, respectively. Hapten had no effect on the reactivity of these residues. After reduction and alkylation of disulfide bonds in the IgG to an extent which abolished the antibody's ability to fix complement, the reactivity of Fc tryptophan did not change when monovalent antigen was bound. The Fc tryptophan reactivity of similarly reduced and alkylated IgG increased by 25% when multivalent antigen was bound.

A combined classical-alternate pathway assay was developed which distinguishes effects on those components preceeding C3 in the complement cascade from effects on C3 and later components. When antigen-antibody aggregates were assayed by this method, fixation was observed in both the classical and alternate pathways. However, when oligomers of mouse IgG_{2a} produced by chemical crosslinking were assayed, no fixation of the components of the alternate pathway was observed. The time course of classical pathway fixation by these synthetic oligomers indicates that some of the components preceding C3 in the cascade were being enzymatically degraded.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

Chapter 3

Page

Chapter 4

LIST OF TABLES

ABBREVIATIONS

1.	Alternate buffer	0.01 M Hepes, 0.15 M NaCl, 0.007 M $MgCl_2 \cdot 6H_2O$, 0.02 M EGTA, pH 7.2 with 0.1% gelatin
2.	BSA	bovine serum albumin
3.	Classical buffer	0.01 M Hepes, 0.15 M NaCl, 0.00015 M $CaCl_2 \cdot 2H_2O$, 0.0005 M $MgCl_2 \cdot 6H_2O$, pH 7.4 with 0.1% gelatin
4.	CPL	circularly polarized luminescence
5.	DNP	2,4-dinitrophenyl
6.	DNP_{30}-BSA	multivalent antigen with an average of 30 DNP moieties per BSA molecule
7.	DNP_1-cytochrome C	monovalent antigen with one DNP group per cytochrome C molecule
8.	EDTA	ethylenediamine tetraacetic acid
9.	EGTA	ethyleneglycol-bis-(β-aminoethyl ether) N,N'-tetraacetic acid
10.	Fab	product of papain digestion of IgG which possesses the antigen binding site
11.	Fc	product of papain digestion of IgG analogous to the crystalizable fragment from rabbit IgG
12.	Fd	that portion of the Fab derived from the heavy chain
13.	Hepes	N-2-hydroxyethylpiperazine-N'-ethane-sulfonic acid

14.	HMNB	2-hydroxy-3-methyl-5-nitrobenzyl
15.	HMNB-DMSBr	dimethyl (2-hydroxy-3-methyl-5-nitrobenzyl) sulfonium bromide; modified Koshland's reagent
16.	HMNB-OH	2-hydroxy-3-methyl-5-nitrobenzyl alcohol
17.	HNB	2-hydroxy-5-nitrobenzyl
18.	HNBBr	2-hydroxy-5-nibrobenzyl bromide; Koshland's reagent
19.	HNB-DMSBr	dimethyl (2-hydroxy-5-nitrobenzyl) sulfonium bromide
20.	HNB-OH	2-hydroxy-5-nitrobenzyl alcohol
21.	IgG	immunoglobulin class G
22.	IgM	immunoglobulin class M
23.	NMR	nuclear magnetic resonance
24.	SDS	sodum dodecyl sulfate
25.	TMS	tetramethyl silane
26.	Tris	tris(hydroxymethyl)amino methane; tris base

ABSTRACTS

Chapter 1

Two mechanisms for the activation of complement by IgG are presented in this chapter. Previously reported evidence in support of each mechanism is reviewed and areas that are mechanistically uncertain are discussed.

Chapter 2

The synthesis of a tryptophan specific reagent, dimethyl (2-hydroxy-3-methyl-5-nitrobenzyl) sulfonium bromide, is described. The synthetic route incorporated tritium and ^{14}C thereby allowing the reagent to be used in a double radiolabeling paradigm. The reagent was shown to specifically benzylate tryptophan which was either in solution or in a protein. Physicochemical properties of the reagent which may give rise to this specificity were examined.

Chapter 3

This chapter presents evidence that a conformational change occurs in mouse IgG_{2a} when antigen binds and that this change is a necessary condition for the activation of the complement cascade. A $^{3}H/^{14}C$-labeled reagent, which reacts specifically with tryptophan residues, was used to monitor the reactivity of tryptophans in the Fc region of the immunoglobulin. This reactivity correlated with the ability of the antibody to fix complement. Thus binding

monovalent and multivalent antigens caused decreases in the reactivity of Fc tryptophan residues by 15% and 22% respectively. Hapten had no effect on the reactivity of these residues. After reduction and alkylation of disulfide bonds in the IgG to an extent which abolished the antibody's ability to fix complement, the reactivity of Fc tryptophan residues did not change when monovalent antigen was bound. The Fc tryptophan reactivity of similarly reduced and alkylated IgG increased by 25% when multivalent antigen was bound.

Chapter 4

A combined classical pathway - alternate pathway complement fixation assay has been developed which distinguishes effects on those components preceding C3 in the cascade from effects on C3 and later components. When antigen-antibody aggregates are assayed by this method, fixation is observed in both the classical pathway and alternate pathway. However, when oligomers of mouse IgG_{2a}, produced by chemical crosslinking, are assayed, no fixation of the components of the alternate pathway is observed. The time course of classical pathway fixation by these synthetic oligomers indicates that some of the components preceding C3 in the cascade are being enzymatically degraded. Monovalent antigen did not alter the fixation of either pathway by these synthetic oligomers of IgG.

CHAPTER 1

Survey of Research on the Mechanism of Complement Activation

Complement is a system of proteins in serum which, when activated, performs various biological functions such as cell lysis, opsinization, vasodilation and chemotaxis. The classical pathway of complement activation begins with the association of immunoglobulins, either IgM or IgG, to cell surfaces or soluble antigens. C1 is then bound and activated by these immunoglobulin aggregates and the cascade is initiated by the proteolytic cleavage of C4 by $C\overline{1}$ and the subsequent formation of a $C\overline{14b2a}$ complex after the cleavage of C2. The active complex $C\overline{4b2a}$, termed C3 convertase, cleaves C3 forming a $C\overline{4b2a3b}$ complex which activates C5 also by a proteolytic cleavage. No further protease activity appears to be necessary for the completion of activation, but the self-assembly of $C\overline{5b6789}$ on the cell surface completes the cytolytic structure (Porter and Reid, 1979).

An alternative pathway to complement activation uses a different set of initiating proteins and enzymes, called the properdin system, to achieve the activation of C3 but from that point on the classical and alternate pathways are coincident. The alternate pathway achieves activation in the absence of immunoglobulin, C1, C2, and C4 (Gotze and Müller-Eberhard, 1976).

The present experimental evidence indicates that a minimum of two juxtaposed antibody molecules is required for complement activation either on the cell surface (Borsos and Rapp, 1965) or in solution (Cohen, 1968; and Wright *et al.*, 1980). The interaction of immunoglobulin aggregates with C1 is through C1q, a 410,000 dalton protein which appears to possess six sites with affinity for immunoglobulins (Reid and Porter, 1976). The binding site for C1q on IgG appears to be in the CH_2 domain (Yasmeen et al., 1976) but the amino acid residues forming the site have not been determined. Allan and Isliker (1974) have used chemical modification techniques to demonstrate the involvement of tryptophan 277 in complement activation; moreover, binding studies of synthetic (Johnson and Thames, 1976) and natural polypeptides (Lee and Painter, 1980) which duplicate the CH_2 region in the vicinity of tryptophan 277 indicate the location of the C1q binding site is in this region. However, others have proposed the basic region at the carboxyl end of the CH_2 domain as the C1q binding site (Burton et al., 1980). They base their proposed site on three factors: (1) availability for binding, (2) genetic conservation of amino acid residues between domains, and (3) the fact that polyionic molecules are capable of activating complement via C1.

One model for the interaction of immunoglobulin with C1 delineated by Hoffmann (1976a,b,c) considers C1q to be

the substrate of an allosteric protein, immunoglobulin, while antigen acts as an allosteric regulator. In this model the immunoglobulin is in equilibrium between a relaxed and a tensed state; the relaxed state must be achieved for complement activation to occur. In the Hoffmann model the protomer, the functional regulatory-catalytic unit, is the heavy-light chain structure. The ratio of molecules in the tensed state to molecules in the relaxed state in the absence of antigen or Clq is:

$$L_o = [T]_o/[R]_o > 1 \ . \qquad (1)$$

Each state, R and T, has a specific dissociation constant for both types of ligands. Initially treating every component as univalent for mathematical simplicity, Hoffmann defines these constants as:

$$K_{RA} = \frac{[R][A]}{[RA]} \qquad K_{TA} = \frac{[T][A]}{[TA]} \qquad (2)$$

and assumes

$$\frac{K_{RA}}{K_{TA}} < 1 \ . \qquad (3)$$

The affinity of immunoglobulins for Clq is also assumed to be greater in the R state than in the T state. In the presence of saturating amounts of antigenic ligand the ratio of the number of molecules in the T state to the number of molecules in the R state is given as:

$$L' = L_o\left(\frac{K_{RA}}{K_{TA}}\right)^N \qquad (4)$$

where N is the number of protomers acting concertedly. One assertion made by Hoffmann is that native IgG has two protomers but when aggregated by antigen the number of protomers is twice the number of IgG molecules in the aggregate. This requires that the IgG molecules in the aggregate act in concert allosterically; that is to say, they act cooperatively in their transition from the T state to the R state.

The initial motivation for the development of this model came from the observation of cooperativity in the binding of C_1^- to dinitrophenylated erythrocytes covered with antibody (Thompson and Hoffmann, 1974a,b). Although some objection has been made to the experimental procedure (Metzger, 1978), this cooperativity is a direct indication of the allosteric nature of the protein.

Experimental evidence frequently cited in support of the allosteric model is the activation of complement by IgM which had bound monovalent antigen (Brown and Koshland, 1975). IgM specifically against p-(p-dimethylaminobenzeneazo)-phenyl-β-lactoside (Lac dye) was used to activate complement but monovalent antigen caused the anti-Lac IgM to activate complement without observable aggregation of IgM. Furthermore this complement fixation could be inhibited by the hapten. The authors interpreted these results as reflecting

a conformational change in the Fc region of IgM which alters the functional properties of the immunoglobulin. The authors interpreted the smaller binding constant for monovalent antigen as compared to hapten in terms of the thermodynamic coupling of binding antigen and changing conformation. In this scheme, some of the free energy evolved when antigen is bound is coupled to the conformational change of the immunoglobulin which presumably has a positive free energy term. The summation of the two steps leads to a less negative free energy for the overall reaction and, therefore, a smaller binding constant. Metzger (1978) points out that this need not be the case because the bulk of the monovalent antigen may simply give it less affinity for the IgM than the hapten.

The results of Brown and Koshland are readily interpretable in terms of the model of Hoffmann. The discrimination between hapten and monovalent antigen on the basis of complement activation may be related to the difference in the association constants in the T and R states for the particular ligand. As seen in equation 4 the only difference in L' between saturating hapten and saturating monovalent antigen will come from the quantity $(^{K}RA/K_{TA})$, because the number of associated protomers, N, and the state of the equilibrium in the absence of ligand, L_o, will be unchanged. The agreement of the complement fixation results with the allosteric model only requires that $^{K}RA/K_{TA}$ for the monovalent antigen be significantly less than $^{K}RA/K_{TA}$

for the hapten. If one assumes the values used by Hoffmann (1976a), then this decrease must be on the order of 50%.

Several techniques have been used to observe conformational changes in the Fc portion of IgG upon binding antigen. Schlessinger *et al.* (1975) used circularly polarized fluorescence to monitor conformational changes in IgG. The systems under study included: (1) a hapten-like tetra-D-alanine with anti-poly-D-alanine IgG, (2) the multivalent antigen RNase with anti-RNase IgG, and (3) the monovalent hapten, lysozyme "loop" peptide, with anti-"loop" IgG. Both the monovalent and multivalent antigens caused changes in the circularly polarized fluorescence spectra of their respective IgG when bound, but tetra-D-alanine did not. These spectral changes were interpreted as reflecting conformational changes in the immunoglobulin. The changes observed when Fab bound antigen did not fully account for the changes observed in the entire immunoglobulin so that some of the spectroscopic difference between changes in the spectrum of whole immunoglobulin and the spectrum of Fab were attributed by the authors to changes in the Fc region. This work assumed that spectroscopic alterations arising from the enzymatic digestion used to produce Fab would be negligible. When the disulfide bonds of the IgG were reduced and alkylated the spectroscopic changes in IgG resulting from antigen being bound

resembled those changes in Fab. The reduction of disulfide bonds had been previously shown to reduce complement activation significantly (Schur and Christian, 1964; Press, 1975) so that the elimination of spectroscopic changes by a process which reduces complement activation was used to demonstrate a link between the spectroscopic observation and effector function.

Henney and Ishizaka (1968) found the appearance of new antigenic determinants in the Fc region of IgG in antigen-antibody aggregates which were not apparent in free IgG. These uncovered determinants showed cross-reactivity with the Fc region of IgG aggregates produced by heat or ethanolic denaturation. The appearance of new antigenic determinants was attributed to a conformational change in the Fc region and the cross-reactivity indicated to the authors that similar conformational changes occur in the process of denaturation, thus enabling antigen-free aggregates of IgG to fix complement. However it can be argued that antigen and Fc in close proximity, which may be the case for antigen-antibody aggregates, act together to form a new antigenic determinant.

Additional evidence for a conformational change occurring in the Fc region of IgG upon antigen binding is the appearance of previously unexposed Protein A binding sites on rabbit IgG when antigen is present (Mota *et al.*, 1981). Protein A from *Staphylococcus aureus* (SpA) binds

in the region where the CH_2 domain attaches to the CH_3 domain (Deisenhofer *et al.*, 1978). Mota and his coworkers found that rabbit, anti-ferritin IgG formed the stable complex $(IgG_2SpA_1)_2$; this complex could bind a small fragment of Protein A, fSpA, but was not retained on an affinity column consisting of Protein A covalently linked to sepharose. However, when the $(IgG_2\text{-}SpA_1)_2$ complexes were reacted with an excess of ferritin to give $(IgG_2\text{-}SpA_1)_2\cdot$(ferritin), the complexes were retained on the affinity column. The authors argue that this retention is the result of exposure of a binding site for Protein A upon binding antigen which had previously been buried. If the binding of Clq were analogous, then binding antigen might expose sites for Cl which had previously been buried.

Aggregates of IgG produced by denaturation (Ishizaka and Ishizaka, 1960) and chemical crosslinking (Ishizaka *et al.*, 1967; Wright *et al.*, 1980) appear to fix complement by the classical pathway. Augener *et al.* (1971) have shown $C\overline{1}$ binding by aggregates of Fc from IgG to be nearly as efficient as aggregates of whole IgG. This ability to fix complement by IgG aggregates produced by such diverse means has given rise to the aggregation theory of complement activation which, in its simplest form, states that antigen serves to aggregate IgG and aggregation increases the avidity of Clq for IgG by overcoming the entropy involved in sequentially binding free molecules from solution. A

more refined model has been proposed by Metzger (1974) which assumes multiple Clq binding sites on IgG and suggests that the cooperativity observed by Thompson and Hoffmann (1974a,b) results from these sites being stabilized by the initial binding of Clq to the antigen-antibody aggregate and eventually being bound by other Clq's giving rise to an activating aggregate comprised of multiple Cl's.

The analysis of fluorescence depolarization and X-ray crystal data (McCammon and Karplus, 1977) indicates a great deal of flexibility of the Fab arms of IgG with respect to the Fc region. The interpretation of X-ray data from IgG_{Dob} (Silverton *et al.*, 1977) finds little interaction between CH_1 and CH_2 regions on the same heavy chain. These two aspects of IgG structure, flexibility and transdomain isolation, support the aggregation theory by denying a physical mechansim by which changes in occupation of the binding site are transmitted to the Fc region. However, other researchers (Huber *et al.*, 1976) have speculated that such flexibility between CH_1 and CH_2 diminishes by the constriction of the hinge region when antigen is bound, thereby providing an avenue for transmission of information about the occupation of the binding site by antigen.

While studies using circularly polarized luminescence have found spectral changes attributed to antigen binding,

other techniques have found no such dependence. These include NMR studies of gadolinium III interacting with rabbit IgG (Dower et al., 1975) and ESR studies of spin labels attached to the carbohydrate of CH_2 (Willan *et al.*, 1977). The latter experiment seems particularly relevant to complement fixation because the oligosaccharide found in the CH_2 domain of IgG seems to be involved in the activation of C1.

The dependence of chemical and enzymatic reactivity of IgG on the presence of bound antigen has also been examined (Wright *et al.*, 1978). Knowing the integrity of disulfide bonds in IgG was requisite for complement activation, these authors followed the kinetics of reduction of disulfide bonds between the heavy chains in the presence and absence of hapten and multivalent antigen. In neither case was the rate of reduction significantly altered by the presence of ligand. Similarly the rate of proteolytic cleavage of IgG by papain to give Fab and Fc fragments did not change when either hapten or multivalent antigen was bound. These negative results were interpreted as evidence against any significant conformational change occurring on the carboxyl side of the CH_1 domain. One objection to this interpretation of the proteolytic observations is that alterations in the rate of proteolysis will most likely arise from effects on the rate limiting step of the process. If the rate limiting step for proteolysis is independent of the conformation of the IgG then the overall rate will probably not reflect

conformational changes in IgG. Furthermore recent work on the effect of reduction of IgG on its ability to activate complement implicated intrachain disulfide bonds as being crucial for the activation of complement instead of those disulfide bonds between heavy chains (Johnson and Hoffmann, 1981). Thus the kinetics of the reduction of inter-heavy chain disulfide bonds may not be directly relevant to complement activation.

While the aggregation theory has an appealing simplicity, it cannot account for all the experimental observations concerning the cooperativity of $\overline{C_1}$ binding and the activation of complement by (Lac_1-RNase·IgM). At the same time conformational changes attributed to antigen binding have not been unambiguously assigned to the Fc region of IgG. Furthermore, the evidence for activation of complement by antibody aggregates in the absence of any antigen appears to obviate the need for any binding of an antigenic ligand. The work presented in this thesis will focus on the observation of conformational changes resulting from antigen binding, the assignment of these changes to the Fc region, and the differences existing between complement activation by IgG-antigen aggregates and complement activation by aggregates produced by chemically crosslinking IgG.

REFERENCES

Allan, R. and Isliker, H., *Immunochem.* (1974) *11*, 243.

Augener, W., Grey, H.M., Cooper, N.R., and Müller-Eberhard, H.J., *Immunochem.* (1971) *8*, 1011.

Borsos, T. and Rapp, H.J., *J. Immunol.* (1965) *95*, 559.

Brown, J.C. and Koshland, M.E., *Proc. Natl. Acad. Sci. USA* (1975) *72*, 5111.

Burton, D.R., Boyd, J., Brampton, A.D., Easterbrook-Smith, S.B., Emanuel, E.J., Novatny, J., Rademacher, T.W., van Schravendijk, M.R., Sternberg, M.J.D., and Dwek, R.A., *Nature* (1980) *288*, 338.

Cohen, S., *J. Immunol.* (1968) *100*, 407.

Deisenhofer, J., Jones, T.A., Huber, R., Sjodahl, J., and Sjoquist, J., *Hoppe Seyler's Z. Physiol. Chem.* (1978) *359*, 975.

Dower, S.K., Dwek, R.A., McLaughlin, A.C., Mole, L.E., Press, E.M., and Sunderland, C.A., *Biochem. J.* (1975) *149*, 73.

Gotze, O. and Müller-Eberhard, H.J., *Adv. Immunol.* (1976) *24*, 1.

Henney, C.S. and Ishizaka, K., *J. Immunol.* (1968) *100*, 718.

Hoffmann, L.G., *Immunochem.* (1976a) *13*, 725.

Hoffmann, L.G., *Immunochem.* (1976b) *13*, 731.

Hoffmann, L.G., *Immunochem.* (1976c) *13*, 737.

Huber, R., Deisenhofer, J., Colman, P.M., Matsushima, J., and Palm, W. *Nature* (1976) *264*, 415.

Ishizaka, K. and Ishizaka, T.J., *J. Immunol.* (1960) *85*, 163.

Ishizaka, T., Ishizaka, K., Salmon, S., and Fudenberg, H., *J. Immunol.* (1967) *99*, 82.

Johnson, B.A. and Hoffmann, L.G., *Mol. Immunol.* (1981) *18*, 181.

Johnson, B.J. and Thames, K.E., *J. Immunol.* (1976) *117*, 1491.

Lee, J.P. and Painter, R.H., *Mol. Immunol.* (1980) *17*, 1155.

McCammon, J.A. and Karplus, M., *Nature* (1977) *268*, 765.

Metzger, H., *Adv. Immunol.* (1974) *18*, 169.

Metzger, H., *Contemp. Top. in Mol. Immunol.* (1978) *7*, 119.

Mota, G., Moraru, I., Sjoquist, J., and Ghetie, V., *Mol. Immunol.* (1981) *18*, 373.

Porter, R.R. and Reid, K.B.M., *Adv. Protein Chem.* (1979) *33*, 1.

Press, E.M., *Bochem. J.* (1975) *149*, 285.

Reid, K.B.M. and Porter, R.R., *Biochem. J.* (1976) *155*, 19.

Schlessinger, J., Steinberg, I.Z., Givol, D., Hochman, J., and Pect, I., *Proc. Natl. Acad. Sci. USA* (1975) *72*, 2775.

Schur, R.H. and Christian, G.D., *J. Exp. Med.* (1964) *120*, 531.

Silverton, E.W., Mavia, M.A., and Davies, D.R., *Proc. Natl. Acad. Sci. USA* (1977) *74*, 5140.

Thompson, J.J. and Hoffmann, L.G., *Immunochem.* (1974a) *11*, 431.

Thompson, J.J. and Hoffmann, L.G., *Immunochem.* (1974b) *11*, 537.

Willan, K.J., Golding, B., Givol, D., and Dwek, R.A., *FEBS Lett.* (1977) *80*, 133.

Winkelhake, J.L. and Kasper, D.L., *J. Immunol.* (1972) *109*, 824.

Wright, J.K., Engel, J., and Jaton, J-C., *Eur. J. Immunol.* (1978) *8*, 309.

Wright, J.K., Tschopp, J., Jaton, J-C., and Engel, J., *Biochem. J.* (1980) *187*, 775.

Yasmeen, D., Ellerson, J.R., Dorrington, K.J., and Painter, R.H., *J. Immunol.* (1976) *116*, 518.

CHAPTER 2

The Synthesis and Reactivity of a Modified Koshland's Reagent

INTRODUCTION

Horton and Koshland (1965) developed a chemical reagent, 2-hydroxy-5-nitrobenzyl bromide, which showed great selectivity among naturally occurring amino acids for tryptophan. This reagent has subsequently been used to examine the role of tryptophan in such proteins as pepsin (Dopheide and Jones, 1968), chymotrypsin (Oza and Martin, 1967), and immunoglobulin G (Allan and Isliker, 1974). Because of the unique spectral properties of the hydroxy-nitrobenzyl group in the visible region at high pH, it has been used as a facile means of quantifying tryptophan in proteins (Barman and Koshland, 1967).

The sulfonium salt of this benzyl bromide was found to retain the specificity for tryptophan which the original Koshland's reagent possessed and additionally had a number of useful qualities not found in the earlier reagent (Horton and Tucker, 1970). The dimethyl (2-hydroxy-5-nitrobenzyl) sulfonium bromide was more soluble and more stable in aqueous solutions than its corresponding benzyl bromide. This allowed for the preparation of reagents in aqueous solutions and decreased the molar excess of reagent needed to derivatize the same number of tryptophan residues.

Another analog of Koshland's reagent was the 2-methoxy-5-nitrobenzyl bromide, but it was found that this reagent lost much of its specificity for tryptophan; which led to the conclusion that a hydroxyl group ortho to the bromomethyl group was necessary for the reagent's specificity (Horton *et al.*, 1965).

Some of the reaction products of Koshland's reagent and the sulfonium salt of this reagent with tryptophan ethyl esters have been isolated and characterized (Loudon and Koshland, 1970). There appeared to be at least two monosubstituted and two disubstituted derivatives with the possibility of acid catalyzed rearrangement of the mono-substituted forms (Figure 1). This study indicated that the selectivity of Koshland's reagent arises from two properties: (1) the hydrophobic nature of the reagent and (2) the ability of nitrobenzene compounds to form charge-transfer complexes with tryptophan.

This chapter details the synthesis, purification and characterization of a modified Koshland's reagent, dimethyl (2-hydroxy-3-methyl-5-nitrobenzyl) sulfonium bromide. This reagent was developed to incorporate ^{3}H and ^{14}C in a tryptophan-specific reagent. The incorporation of radio-isotopes allows for the detection of reagent at lower levels and with greater accuracy than spectroscopy because of the difficulties in quantitating the extent of reaction caused by perturbations in the spectra of the derivatized protein

arising from benzylation of the tryptophan residues (Horton and Koshland, 1967). The use of two distinguishable radioisotopes makes double radiolabeling techniques possible (Kaplan, 1971).

MATERIALS AND METHODS

Dimethyl (2-hydroxy-3-methyl-5-nitrobenzyl) sulfonium bromide

The synthetic route to dimethyl (2-hydroxy-3-methyl-5-nitrobenzyl) sulfonium bromide (abbreviated HMNB-DMSBr) is diagrammed in Figure 2. Salicylaldehyde was reduced to 2-hydroxybenzyl alcohol using the procedure of Chaikin and Brown (1949). It was in this reduction that ^{3}H was introduced. Tritiated $NaBH_4$ (New England Nuclear, 100 mCi at 282 mCi/mmol) was dissolved in 0.2 ml of H_2O/methanol (1:1, v:v) made 0.1 N in KOH. A solution of salicylaldehyde in the same solvent system was made up by dissolving 2 grams of salicylaldehyde in 2 ml of methanol and adding this dropwise to 6 ml of 0.1 N KOH in 1:1 H_2O/methanol. The tritiated $NaBH_4$ solution was placed in a 250 ml round bottom flask and the salicylaldehyde in 0.1 N KOH was added dropwise with stirring. After 15 minutes a solution of nonradioactive $NaBH_4$ (0.15 gm in 12 ml of 0.1 N KOH H_2O/methanol) was added dropwise and the reaction stirred at ambient temperature for 24 hours producing 2-hydroxybenzyl alcohol.

The alcohol was further reduced to o-cresol with Raney nickel catalyst (Papa *et al.*, 1942). A 10% solution

of NaOH in H_2O (50 ml) was added to the 2-hydroxybenzyl alcohol in the same 250 ml round bottom flask used in the borohydride reduction. The solution was heated to 90°C and 6 gm of Raney nickel catalyst was added in small portions with stirring over a period of 2.5 hours. Octyl alcohol was occasionally added to diminish foaming of the reaction mixture when the catalyst was added. The mixture was kept at 90°C for one hour after all the catalyst had been added and then it was allowed to cool to room temperature. The reaction mixture was then filtered and added dropwise to 50 ml of 6 N $HCl_{(aq)}$. The acidified solution was extracted twice with 30 ml of diethyl ether and the o-cresol was recovered after removal of the ether by rotary evaporation. Water (90 ml) was added to the o-cresol in a 250 ml round bottom flask and the solution cooled in an ice-water bath. The o-cresol was nitrated para to the hydroxy group (Clemence and Raiziss, 1934) by dissolving 1.65 gm of $NaNO_2$ in the aqueous o-cresol solution and adding 10 ml of a 5% $H_2SO_{4(aq)}$ solution with vigorous stirring while keeping the reaction mixture cold. The resulting 2-methyl-4-nitrosophenol was oxidized with dilute $HNO_{3(aq)}$ and the crude 2-methyl-4-nitrophenol was recrystallized from hot H_2O, filtered, and dissolved in 7 ml of 30% $HBr_{(aq)}$.

Bromomethylation was executed following a published procedure (Buehler et al., 1967) using 0.5 gm of paraformaldehyde and 100 µl of concentrated H_2SO_4. The solution of 2-methyl-4-nitrophenol with paraformaldehyde and H_2SO_4 in

30% $HBr_{(aq)}$ was stirred for three hours. The solid product was collected by filtration, dissolved in dimethylsulfide, and, after 12 hours, the crude dimethyl (2-hydroxy-3-methyl-5-nitrobenzyl) sulfonium bromide was collected by filtration. The product was recrystallized by dissolving the solid in a minimal amount of methanol at 45°C, cooling this solution to room temperature, adding diethyl ether until an initial precipitate formed and then cooling the mixture in an ice-water bath.

The synthesis of ^{14}C HMNB-DMSBr began with the bromomethylation of 2-methyl-4-nitrophenol using 2 mCi of ^{14}C paraformaldehyde (Amersham. Searle Corp., 250 mCi/gm) which was diluted with 94 mg of non-radioactive paraformaldehyde.

Reaction of HMNB-DMSBr with Free Amino Acids

The specificity of HMNB-DMSBr for tryptophan in solution of free amino acids was ascertained in the following manner. A solution of natural amino acids was made up in a 0.05 M NaH_2PO_4 buffer at pH 6.8. All the amino acids were 1×10^{-3} M. HMNB-DMSBr was added as a solid and in three trials, the final HMNB-DMSBr concentrations were 9.4×10^{-4} M, 4.9×10^{-3} M, and 9.7×10^{-3} M. After the reactions were stirred at 4°C for 24 hours, 50 µl aliquots were analyzed on a Beckman 140B amino acid analyzer.

Reaction of HMNB-DMSBr with Reduced and Alkylated Chicken Lysozyme

Chicken lysozyme (Grade I, Sigma Chemical Co.) was dissolved to 1 mg/ml in 0.10 M NaCl, 0.05 M tris buffer pH 8.0. A fivefold molar excess of dithiothreitol was added to the protein solution. After stirring at ambient temperature for two hours, the reduced protein was alkylated by adding a tenfold molar excess of iodoacetamide and gently agitating this reaction solution for 24 hours while protected from light. The reduced and alkylated chicken lysozyme was then dialyzed against 0.10 M NaCl, 0.05 M NaH_2PO_4, pH 6.8 and concentrated to 1 mg/ml by ultrafiltration. A tenfold molar excess of HMNB-DMSBr was added to the protein as a solid and the solution stirred for 24 hours at room temperature. The protein was then exhaustively dialyzed against 0.10 M tris buffer pH 8.0 at 4°C to remove the benzyl alcohol produced by hydrolysis and then desalted by dialysis against H_2O. Aliquots of 50 µl of 2.0 M mercaptoethane sulfonic acid (Pierce Chemical Co.) were added to the protein and the hydrolysis was performed under nitrogen at 110°C for twelve hours. The acid was partially neutralized by the addition of 50 µl of 2 M $NaOH_{(aq)}$ and aliquots were analyzed as before.

Reaction of HMNB-DMSBr with Rabbit IgG

Antibodies were raised in rabbits against pneumococci III bacteria to high titer and restricted heterogeneity using the innocculation regimen of Kimball *et al.* (1971). The IgG was isolated from serum by dialyzing 200 ml of serum against 0.1 M tris buffer pH 9.0 at 4°C and applying to a sephadex QAE-50 column (5 x 30 cm) equilibrated with the tris buffer. All unbound protein was eluted with this starting buffer and the IgG was eluted with a linear ionic gradient formed by placing 0.3 M NaCl in 0.1 M tris pH 9.0 in the static chamber and having 0.1 M tris pH 9.0 in the mixing chamber. The IgG was the first peak eluted. After concentrating the IgG peak by ultrafiltration, it was applied to a cibacron blue column (Pharmacia Fine Chemicals, Upsala, Sweden) and eluted with this saline-phosphate buffer.

Either HNB-DMSBr or HMNB-DSMBr was added as a solid to a 1 mg/ml solution of rabbit IgG in 0.1 M NaCl, 0.05 M NaH_2PO_4 pH 6.8, chilled in an ice-water bath until a fivefold molar excess of Koshland's reagent was achieved. The reaction solution was gently agitated for 12 hours at 4°C then exhaustively dialyzed against 0.05 M tris buffer pH 8.0. The protein concentration was assayed by the absorbance at 280 nm using $E^{1\%}_{1cm} = 13.5$ for rabbit IgG (Stevenson and Dorrington, 1970), $\varepsilon = 18000\ M^{-1}\ cm^{-1}$ for the 2-hydroxy-5-nitrobenzyl moiety at pH > 10 (Horton

and Koshland, 1967) and $\varepsilon = 17500\ M^{-1}\ cm^{-1}$ for the 2-hydroxy-3-methyl-5-nitrobenzyl moiety at pH > 10.

The digestion of derivatized IgG with pepsin and the separation of the peptic fragments were performed according to the procedure of Allan and Isliker (1974).

Partition of HNB-OH and HMNB-OH between Diethyl Ether and Phosphate Buffer

A basic solution (1 ml) of ^{14}C-HMNB-DMSBr (0.0287 M) and HNB-DMSBr (0.0287 M) in 0.1 N NaOH was heated to 40°C for 24 hours and then diluted with 25 ml of 0.5 M NaH_2PO_4 pH 6.8. Diethyl ether (25 ml) was added to the phosphate buffer solution in a 250 ml separatory funnel and this was agitated for 30 minutes at room temperature. The aqueous phase was collected; the ether phase was dried with $MgSO_{4\,(anh)}$, the ether evaporated by gently heating under vacuum, and the residue was redissolved in 3 ml of the phosphate buffer.

Liquid scintillation counting of samples from the aqueous and ether phases was performed on a Beckman LS-250 liquid scintillation counter programmed for a 3% counting error. The samples were prepared by adding 10 ml of Aquasol 2 liquid scintillation fluid (New England Nuclear Inc.) to 1 ml of the phosphate buffered sample.

Difference Spectra of HNB-OH and HMNB-OH with Tryptophan

Difference spectra were obtained following the procedure of Loudon and Koshland (1970). The reagents

were dissolved in 0.1 M $NaHCO_2$, pH 3.2 to a final concentration of 3.0×10^{-4} M for 2-hydroxy-3-methyl-5-nitrobenzyl alcohol or 2.7×10^{-4} M for 2-hydroxy-5-nitrobenzyl alcohol. Then varying amounts of tryptophan were added to achieve a concentration range from 10 mM to 75 mM. The measurements of the change in absorbance were made at 380 nm at room temperature. A second series of measurements was made in 0.1 M NaH_2PO_4, pH 6.8 using the same benzyl alcohol and tryptophan concentrations.

RESULTS

Synthesis of HMNB-DMSBr

The NMR spectrum of 2-hydroxy-3-methyl-5-nitrobenzyl bromide, obtained from the bromomethylation of 2-methyl-4-nitrophenol as described and prior to the addition of dimethyl sulfide, is shown in Figure 3. The spectrum was taken with a Varian EM-390 spectrometer using d_6-acetone as the solvent and TMS as the zero standard.

The crystals of dimethyl (2-hydroxy-3-methyl-5-nitrobenzyl) sulfonium bromide appeared as short, cream-colored needles. The product decomposed at 156°C with the evolution of dimethyl sulfide. The calculated and observed values for the elemental analysis were respectively:
C 38.97; H 4.58; N 4.54; O 15,57; S 10.40; Br 25.93, and
C 38.97; H 4.59; N 4.44; O 15.38; S 9.50; Br 25.78.
The tritiated, modified Koshland's reagent had a specific

activity of 18 μCi/mmol and the ^{14}C modified Koshland's reagent was 13 μCi/mmol.

Reactivity of HMNB-DMSBr with Free Amino Acids

Table 1 lists the results of studies on the reactivities of various amino acids toward HMNB-DMSBr. The peak areas of the chromatograms and those areas normalized to the area of the alanine peak are given. At concentrations of reagent 5 to 10 times that of tryptophan, all of this amino acid was derivatized; the reagent did not attack any other amino acid with comparable vigor. The reactivity of dimethyl (2-hydroxy-5-nitrobenzyl) sulfonium bromide toward cysteine has been previously demonstrated (Horton and Tucker, 1970) and similar reactivity was found for the modified Koshland's reagent with cysteine.

Reactivity of HMNB-DMSBr with Reduced and Alkylated Chicken Lysozyme

When native chicken lysozyme is reacted with an excess of HMNB-DMSBr, the protein is highly derivatized and forms a precipitate. To avoid this problem the chicken lysozyme was completely reduced and alkylated prior to derivatization as described in Materials and Methods.

After exhaustive dialysis the reduced and alkylated chicken lysozyme had incorporated 1.06 2-hydroxy-3-methyl-5-nitrobenzyl moieties per molecule of protein. This was calculated using $E^{1\%}_{1cm}$ = 24.7 at 280 nm for chicken lysozyme

(Davies *et al.*, 1969). Table II gives the amino acid analysis for reduced and alkylated chicken lysozyme before and after derivatization with HMNB-DMSBr. The amino acid analysis shows 2.5 out of 3.3 or 76% of the recoverable tryptophan underivatized compared to 4.9 out of 6 or 82% of the tryptophan being unreacted as measured spectrophotometrically for the whole, reduced and alkylated protein. It is also noteworthy that amino acids shown to be reactive with less selective benzyl halides, in particular, methionine, serine, and tyrosine (Horton and Koshland, 1967) were not measurably derivatized by this procedure.

Reactivity of HMNB-DMSBR with Rabbit IgG

The molar ratio of covalently linked 2-hydroxy-3-methyl-5-nitrobenzyl moieties to IgG molecules was 0.41 with a standard deviation of 0.02, when labeled as described above, as compared to 0.60 for the (2-hydroxy-5-nitrobenzyl): IgG ratio under identical conditions. Unlike the tryptophan reactivity found in rabbit IgG benzylated with 2-hydroxy-5-nitrobenzyl bromide (Allan and Isliker, 1974), tryptophan residues in both the Fab and Fc regions were derivatized. Figure 4 is the chromatograph of the peptic digest of labeled rabbit IgG on sephadex G-75, and the absorbances at 410 nm and 280 nm are shown. The distribution of label between Fab and Fc is approximately the same for HMNB-DMSBr and HNB-DMSBr.

Partition of HMNB-OH and HNB-OH between an Organic Phase and an Aqueous Phase

After evaporating the ether, the mixture of 2-hydroxy-3-methyl-5-nitrobenzyl alcohol and 2-hydroxy-5-nitrobenzyl alcohol from the organic phase has redissolved in 0.5 M NaH_2PO_4. One millilitre of this solution had 16627 cpm and, at pH > 10, an absorbance of 26.12 at 410 nm. One millilitre of the aqueous phase after the diethyl ether extraction had 233.2 cpm and, at pH > 10, an absorbance of 0.370.

Association of HMNB-OH with Free Tryptophan in Solution

Figure 5 shows the plot of -(ln Δ absorbance) versus -(ln[trp]) for the benzyl alcohols of Koshland's reagent and modified Koshland's reagent at pH 3.2. Linear regression of these data gives a slope of 1.063 for 2-hydroxy-5-nitrobenzyl alcohol and 1.024 for 2-hydroxy-3-methyl-5-nitrobenzyl alcohol with a correlation coefficient of 0.99. No evidence of complexation was observed at pH 6.8 when difference spectra were taken from 340 nm to 600 nm.

DISCUSSION

The modified Koshland's reagent, dimethyl (2-hydroxy-3-methyl-5-nitrobenzyl) sulfonium bromide, appears to have the same selectivity for tryptophan that was observed for

the dimethyl sulfonium salt of the original reagent (Horton and Tucker, 1970). The similarity in reactivity is consistent with the similarity in hydrophobicity and electronic properties observed between 2-hydroxy-5-nitrobenzyl alcohol and 2-hydroxy-3-methyl-5-nitrobenzyl alcohol in the partition experiments and visible spectra. Values for the cpm per absorbance unit in one millitre of sample were 638 for the organic phase and 630 for the aqueous phase. These results indicate that there is no significant difference in the partition coefficients of the alcohols in this system. The close agreement of the slopes for the plots of -ln(Δ absorbance) versus -ln [trp] shows that the interaction of the alcohols with tryptophan in formate buffer is approximately the same. Loudon and Koshland (1970) have asserted that these two characteristics, hydrophobicity and charge-transfer interaction, lead to the specificity of Koshland's reagent for tryptophan. The absence of observable complexation at pH 6.8, the pH at which labeling with the modified Koshland's reagent is performed, may be due to the ionization of the phenol at this pH. The nitrophenolic anion, stabilized as shown in Figure 6 by the benzyl alcohol, would have less affinity for electron donation from tryptophan than the uncharged nitrophenol. The increased absorbance at 410 nm for the two benzyl alcohols at this pH indicated significant ionization which was not present at pH 3.2.

The quinone methide species has been postulated to explain the reactivity of HNB-DMSBr (Loudon and Koshland, 1970) and similar resonance structures can be drawn for HMNB-DMSBr as shown in Figure 7. If the slight decrease in the reactivity of HMNB-DMSBr with rabbit IgG as compared to HNB-DMSBr with rabbit IgG cannot be attributed to differences in electronic or hydrophobic properties between the two reagents; then perhaps there is a small steric factor which arises from the methyl group ortho to the benzylating methylene.

Despite its somewhat decreased reactivity, the modified Koshland's reagent, dimethyl (2-hydroxy-3-methyl-5-nitrobenzyl) sulfonium bromide is a selective reagent for tryptophan and can readily be made radioactive with ^{3}H and ^{14}C.

REFERENCES

Allan, R. and Isliker, H., *Immunochem.* (1974) *11*, 175.

Barman, T.E. and Koshland, D.E., Jr., *J. Biol. Chem.* (1967) *242*, 5771.

Buehler, C.A., Kirchner, F.K., and Deebel, G.F., *Org. Syn. Coll. Vol.* (1967) *3*, 468.

Canfield, R., *J. Biol. Chem.* (1963) *238*, 2698.

Chaikin, S.W. and Brown, W.G., *J. Am. Chem. Soc.* (1949) *71*, 122.

Clemence, L.W. and Raiziss, G.W., *J. Am. Pharm. Assoc.* (1934) *23*, 536.

Davies, R.C., Neuberger, A., and Wilson, B.M., *Biochim. Biophys. Acta* (1969) *178*, 294.

Dopheide, T.A.A. and Jones, W.M., *J. Biol. Chem.* (1968) *243*, 3906.

Horton, H.R., Kelly, H., and Koshland, D.E., Jr., *J. Biol. Chem.* (1965) *240*, 722.

Horton, H.R. and Koshland, D.E., Jr., *J. Am. Chem. Soc.* (1965) *87*, 1126.

Horton, H.R. and Koshland, D.E., Jr., "Methods in Enzymology", (1967) XI, 556.

Horton, H.R. and Tucker, W.P., *J. Biol. Chem.* (1970) *245*, 3397.

Kaplan, H., *Biochem. J.* (1971) *124*, 289.

Kimball, J.W., Pappenheimer, A.M., and Jaton, J.-C., *J. Immunol.* (1971) *106*, 1177.

Loudon, G.M. and Koshland, D.E., Jr., *J. Biol. Chem.* (1970) *245*, 2247.

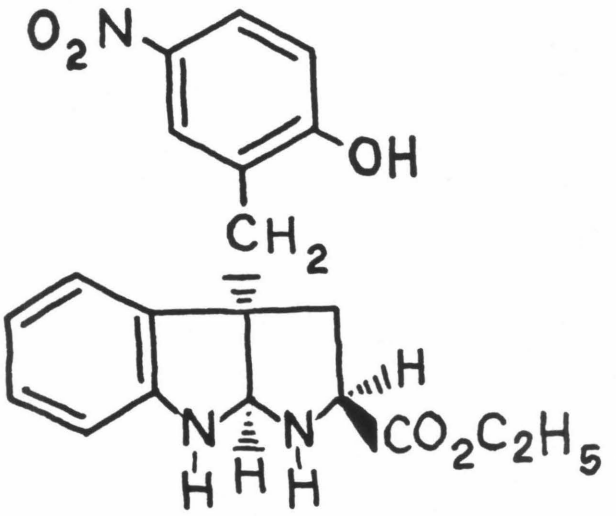
Oza, N.B. and Martin, C.J., *Biochem. Biophys. Res. Comm.* (1967) *26*, 7.

Papa, D., Schwenk, E. and Whitman, B., *J. Org. Chem.* (1942) *7*, 587.

Stevenson, G.T. and Dorrington, K.J., *Biochem. J.* (1970) *118*, 703.

Figure 1. Diastereomers of Tryptophan Ethyl Ester Monobenzylated with Dimethyl (2-hydroxy-5-nitrobenzyl) sulfonium bromide

[Structures are from Loudon and Koshland (1970)]


O2N
OH
CH2
H
N
H
N
H
CO2C2H5

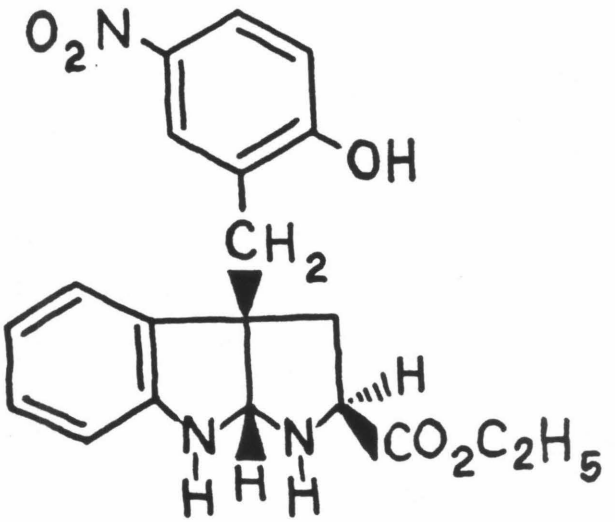
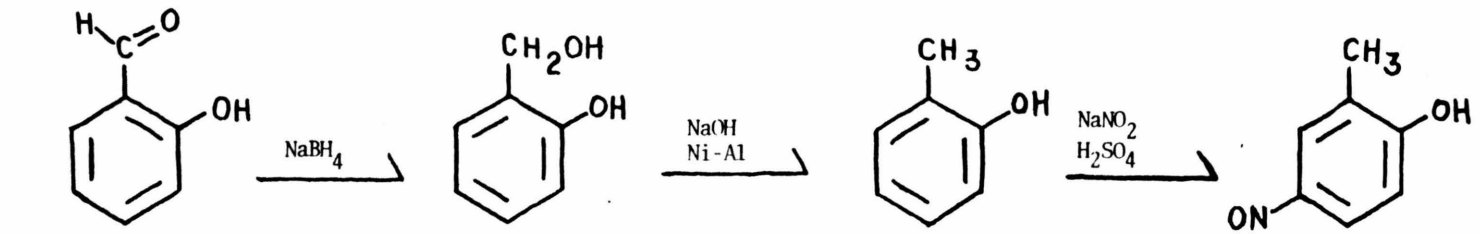

O2N
OH
CH2
H
N
H
N
H
CO2C2H5

Figure 2. 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 occurred in the bromomethylation of 2-methyl-4-nitrophenol with paraformaldehyde and $HBr_{(aq)}$.


H
C=O
OH
NaBH4
CH2OH
OH
NaOH
Ni-Al
CH3
OH
NaNO2
H2SO4
CH3
OH
ON

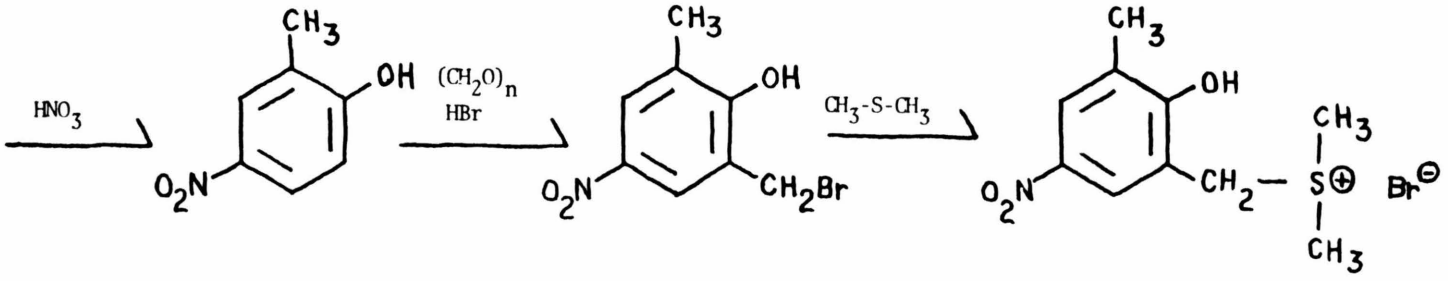

HNO3
CH3
OH
O2N
(CH2O)n
HBr
CH3
OH
O2N
CH2Br
CH3-S-CH3
CH3
OH
O2N
CH2
S⊕
CH3
CH3
Br⊖

Figure 3. NMR Spectrum of 2-hydroxy-3-methyl-5-nitrobenzyl bromide

This spectrum was taken in d_6-acetone. The TMS peak and d_6-acetone peaks occur at $\delta = 0$ and $\delta = 2.1$ ppm respectively. The methyl protons have a downfield shift of 2.3 ppm and the methylene protons have a downfield shift of 4.7 ppm. Peaks for the aromatic protons are at $\delta = 7.95$ ppm and $\delta = 8.15$ ppm.

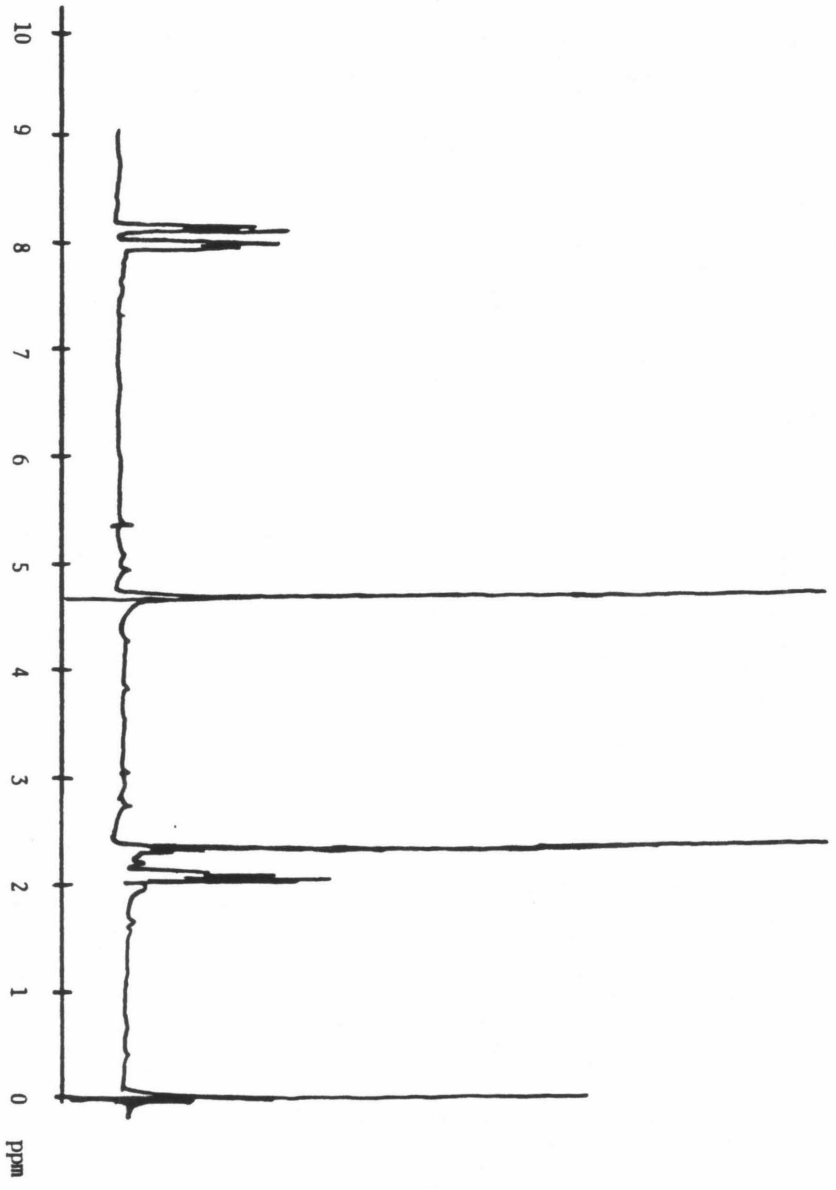

10
9
8
7
6
5
4
3
2
1
0
ppm

Figure 4. Chromatograph of Papain Digestion Fragments from Rabbit IgG

The protein was chromatographed on sephadex G-75 (2.5 x 90 cm) and eluted with 0.01 M NaH_2PO_4, pH 8.5. The left axis of ordinates is the absorbance at 280 nm (——), the right axis of ordinates is the absorbance at 410 nm (—|—|—), and the abscissa is the elution volume in milliliters.

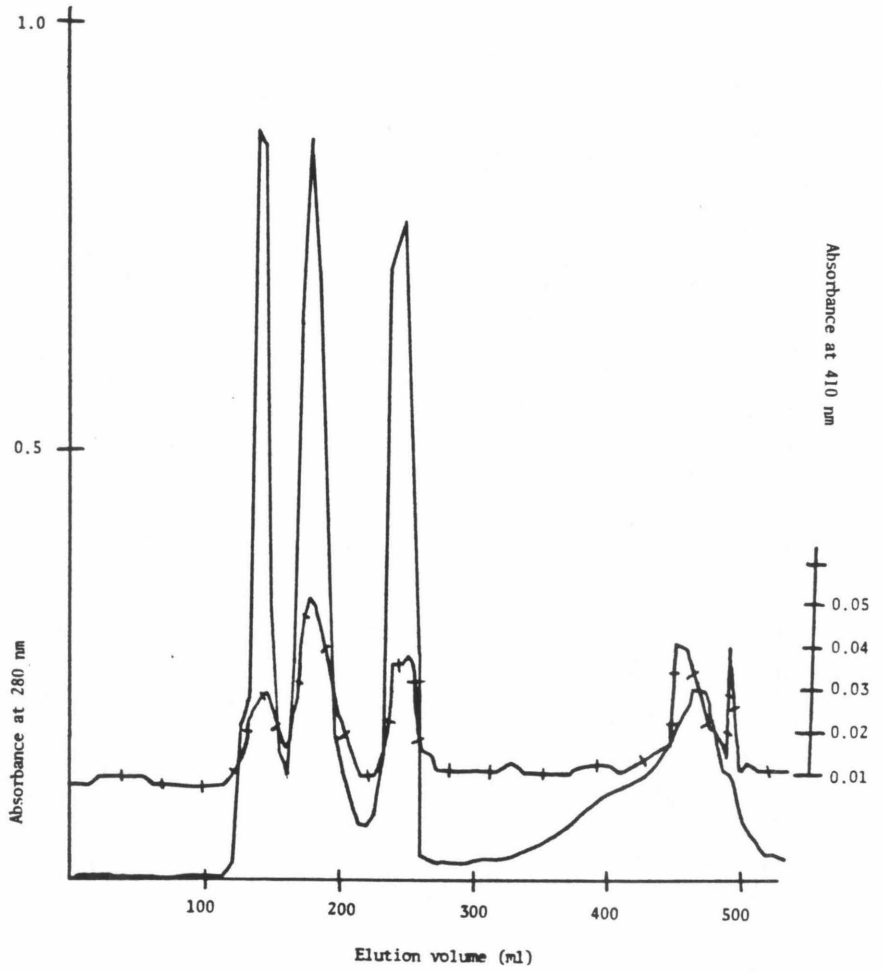

1.0
0.5
Absorbance at 280 nm
Absorbance at 410 nm
0.05
0.04
0.03
0.02
0.01
100
200
300
400
500
Elution volume (ml)

Figure 5. Plot of -[ln(Δ absorbance)] versus -[ln(trp]] for 2-hydroxy-3-methyl-5-nitrobenzyl alcohol and 2-hydroxy-5-nitrobenzyl alcohol

The change in absorbance was measured at 380 nm with the particular benzyl alcohol under study as the reference. The 2-hydroxy-3-methyl-5-nitrobenzyl alcohol was 3.0×10^{-4} M (o) and the 2-hydroxy-5-nitrobenzyl alcohol was 2.7×10^{-4} M (▽). Both benzyl alcohols were in 0.1 M $NaHCO_2$, pH 3.2. Linear regression gave a slope of 1.063 for 2-hydroxy-5-nitrobenzyl alcohol and 1.024 for 2-hydroxy-3-methyl-5-nitrobenzyl alcohol.

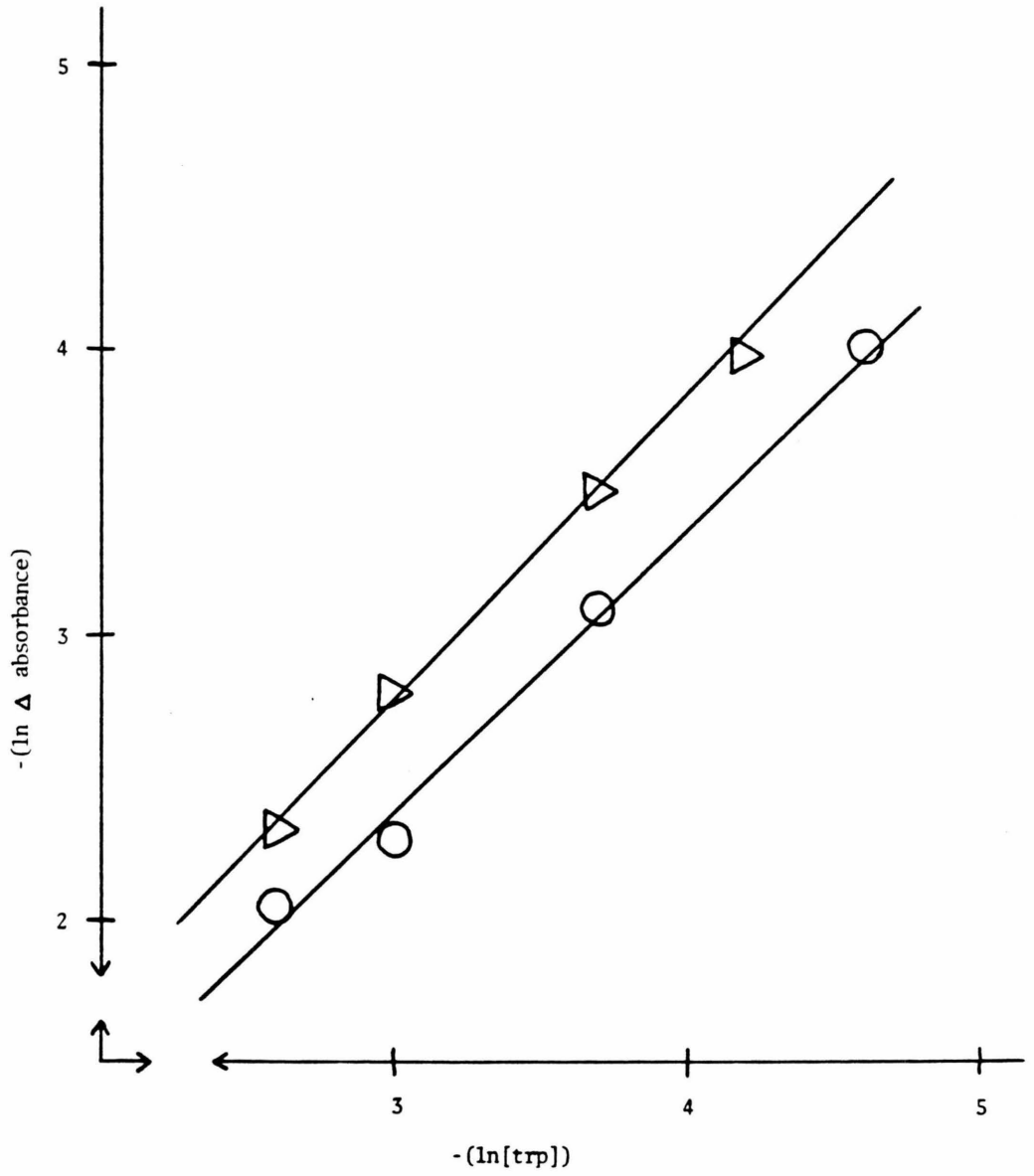

5
4
3
2
-(ln Δ absorbance)
3
4
5
-(ln[trp])

Figure 6. Nitrophenolic Anion of 2-hydroxy-5-nitrobenzyl alcohol

This structure is proposed to account for the large degree of ionization observed for 2-hydroxy-5-nitrobenzyl alcohol at pH 6.8.

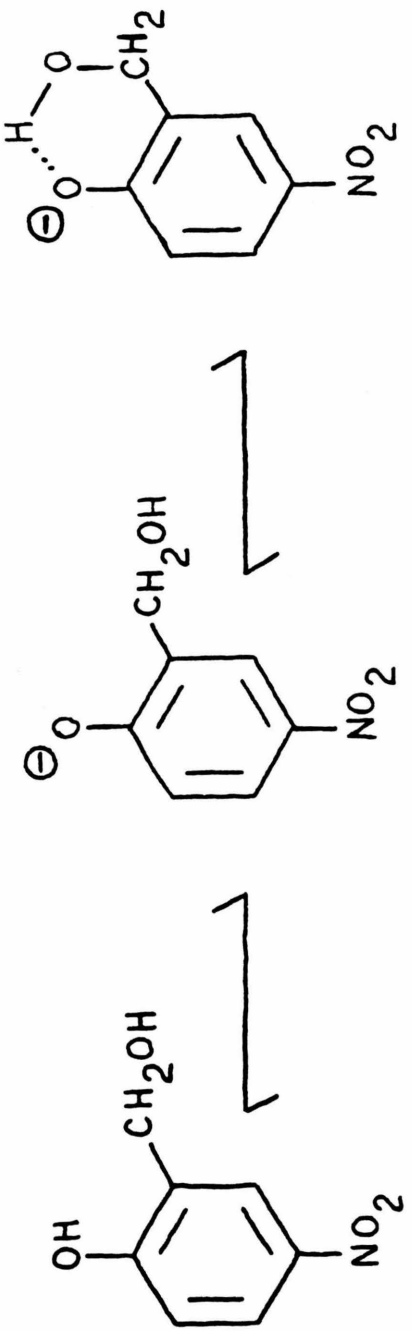
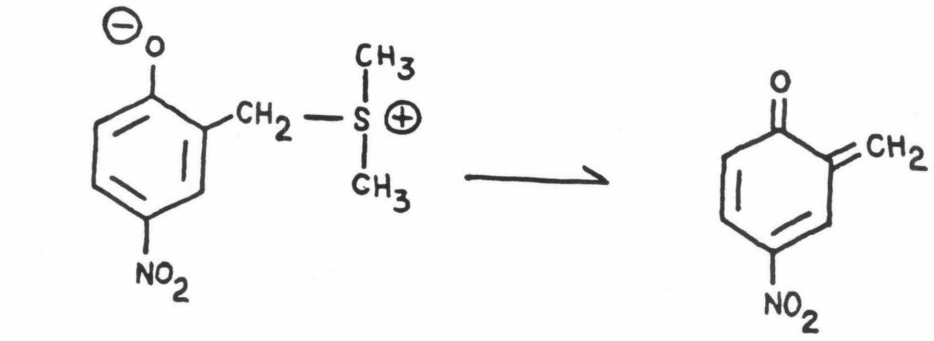

H
O
CH2
NO2
CH2OH
NO2
CH2OH
OH
NO2

Figure 7. Quinone Methide Species for Dimethyl (2-hydroxy-5-nitrobenzyl) sulfonium bromide and Dimethyl (2-hydroxy-3-methyl-5-nitrobenzyl) sulfonium bromide

The structure proposed by Loudon and Koshland (1970) for the sulfonium salt of Koshland's reagent (above) can be applied to the modified Koshland's reagent (below).



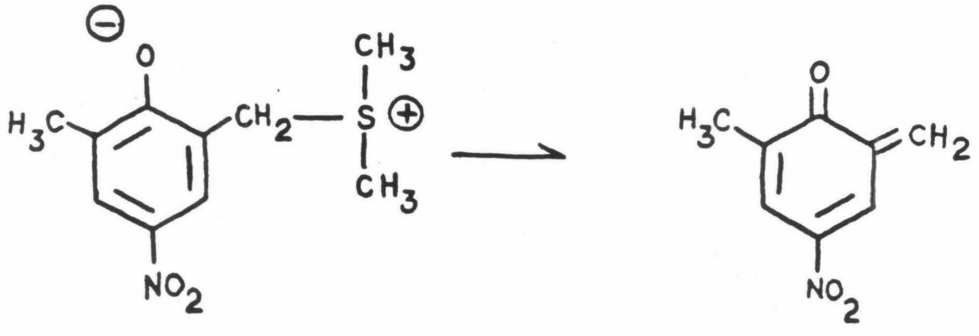


Table I. Analysis of Labeled Free Amino Acid Solution

Amino Acid	0.0 mM HMNB-DMSBr		0.97 mM HMNB-DMSBr		4.9 mM HMNB-DMSBr		9.7 mM HMNB-DMSBr	
	peak area	normalized area[a]	peak area	normalized area	peak area	normalized area	peak area	normalized area
ala	4.37	1	7.11	1	4.12	1	5.16	1
thr	8.31	1.90	12.4	1.74	7.53	1.83	9.04	1.75
tyr	6.82	1.56	8.34	1.17	5.79	1.40	6.56	1.27
phe	5.63	1.28	8.02	1.13	5.58	1.35	6.10	1.18
his	3.57	.817	5.03	.707	2.37	.575	3.88	.752
lys	2.66	.609	4.13	.581	2.53	.614	3.40	.659
arg	2.77	.634	4.45	.626	2.74	.665	3.56	.690
trp	3.45	.802	1.91	.269	-0-	-0-	-0-	-0-

[a]Normalized area refers to the peak area expressed in units of the alanine peak area for a particular HMNB-DMSBr concentration.

Table II. Amino Acid Analysis of Labeled Chicken Lysozyme

amino acid	known[a] composition	unlabeled, reduced and alkylated lysozyme	labeled, reduced and alkylated lysozyme
ala	12	12.1	12.1
arg	11	7.2	6.9
glx	5	6.1	5.9
gly	12	12.6	13.5
his	1	.83	1.1
ile	6	5.4	5.4
leu	8	10.1	9.3
lys	6	4.2	3.9
met	2	1.7	1.7
phe	3	3.3	3.1
ser	10	10.0	10.0
thr	7	6.1	6.6
trp	6	3.3	2.5
tyr	3	3.6	3.7
val	6	5.9	5.7

[a]Composition given by Canfield (1963).

CHAPTER 3

The Dependence of the Reactivity of Tryptophan in IgG with HMNB-DMSBr on the Presence of Antigen

As stated in the first chapter, two mechanistic models have been proposed for the activation of the classical pathway for complement fixation by IgG: an associative and an allosteric model. Much of the rebuttal made against evidence for the allosteric model arises from the inability of current data to ascribe a conformational change to the Fc region of IgG (Metzger, 1974; 1978). Many of the supportive spectroscopic studies do not observe the Fc region directly but draw conclusions from the comparison of the whole immunoglobulin to Fab. Also, the studies showing the appearance of new antigenic determinants in the Fc region upon binding antigen do not adequately deal with the antigen's role in presentation of the determinant to the B cell which makes the immunoglobulin.

This chapter will describe a double radiolabeling technique to measure changes, upon binding antigen, in reactivity of Fc tryptophan residues toward the tryptophan specific reagent, dimethyl (2-hydroxy-3-methyl-5-nitrobenzyl) sulfonium bromide [HMNB-DMSBr]. Observing tryptophan reactivities was first suggested by the work of Allan and Isliker (1974) which found that the modification of

two tryptophan residues in the Fc region of rabbit IgG severely attenuated the ability of aggregates of the immunoglobulin to fix complement. Tryptophan has also been shown to be necessary for polypeptides from the CH_2 domain to interact with C1q (Johnson and Thames, 1976; Lee and Painter, 1980). Finally the data from circularly polarized luminescence supporting conformational changes in actuality reflect the changes in the environments of tryptophan residues (Schlessinger *et al.*, 1975). The CPL technique irradiates the sample at the absorption maxima of tryptophan and it is primarily this amino acid which fluoresces and is observed.

The double radiolabeling technique begins by reacting the tritiated reagent with immunoglobulin in the presence or in the absence of ligand. After all the tritiated reagent has been consumed in the process of benzylating tryptophan or by hydrolysis with water, addition of identical amounts of immunoglobulin derivatized under control conditions with ^{14}C HMNB-DMSBr provides an isotopic counting baseline so that from this point forward the $^{3}H:^{14}C$ ratio does not change. Then, after separation of Fab from Fc, the degree of tritium incorporation under the two conditions can be assayed by determining the relative $^{3}H:^{14}C$ ratios in the intact antibody and Fab and Fc fragments.

MATERIALS AND METHODS

Dimethyl (2-hydroxy-3-methyl-5-nitrobenzyl) sulfonium bromide

Tritiated and ^{14}C dimethyl (2-hydroxy-3-methyl-5-nitrobenzyl) sulfonium bromide were synthesized and purified as described in Chapter 2.

Isolation of UPC-10 IgG

UPC-10 ascites produced in BALBc mice was centrifuged at 8000 x g for one hour to remove any debris. An ammonium sulfate precipitation was performed at room temperature by the slow, dropwise addition of a saturated ammonium sulfate solution (pH 7.4) to the stirring ascites until the final solution was at 45% saturation. The precipitate was stirred for 12 hours at room temperature and after pelleting by centrifugation, the precipitate was redissolved in 0.15 M NaCl, 0.02 M NaH_2PO_4, pH 7.4 buffer. All subsequent purification steps were performed at 4°C. The protein solution was passed through a cibacron-blue column (Pharmacia Fine Chemicals, Upsala, Sweden) eluting with 0.15 M NaCl, 0.02 M NaH_2PO_4 pH 7.4 buffer. The eluted protein was concentrated by ultrafiltration, dialyzed against 0.10 M tris buffer pH 7.4 and applied to a sephadex QAE-50 column (5.0 x 33 cm) previously equilibrated with the tris buffer. After eluting all unbound protein with this buffer, a linear, ionic gradient was used to elute the immunoglobulin. The static chamber

contained 500 ml of 2.0 M NaCl, 0.1 M tris, pH 7.4 buffer and the mixing chamber contained 500 ml of 0.10 M tris with pH 7.4 buffer. UPC-10 IgG was the first peak eluted by the gradient with an absorbance at 280 nm.

Isolation of 29B1 IgG

The mouse hybridoma 29B1 which produces an anti-dinitrophenyl IgG_{2a} (Oi and Herzenberg, 1979) was produced as ascites in SJL x BALBc mice. The ascites fluid was precipitated and the precipitate collected in the same manner as for UPC-10 IgG. Then the precipitate was redissolved in a minimum of 0.05 M tris buffer pH 8.3 and applied to a Whatman DE-sephacel column which had been equilibrated in this buffer. After eluting all unbound protein, a linear ionic gradient was used to elute the immunoglobulin. The static chamber contained 500 ml of 0.5 M NaCl in 0.05 M tris buffer, pH 8.3. The first peak with an absorbance at 280 nm was pooled as the IgG. This peak was concentrated by ultrafiltration, dialyzed against 0.15 M NaCl, 0.02 M NaH_2PO_4, pH 7.4 buffer and chromatographed on LKB Ultragel AcA 34 (2.5 x 100 cm) eluting with this saline-phosphate buffer.

Reduction and Alkylation of UPC-10 and 29B1 IgG

A one mg/ml solution of IgG in 0.10 M tris, 0.002 M EDTA pH 8.0 was made 0.65 mM in dithiothreitol and gently shaken for one hour at room temperature. Then sufficient

iodoacetamide was added to achieve a final iodoacetamide concentration of 6.0 mM. The solution was shaken for 12 hours at room temperature in the dark. The protein was then dialyzed against 0.10 M NaCl, 0.05 M NaH_2PO_4, pH 6.8 buffer. The avidity of reduced and alkylated UPC-10 IgG for bacterial levan antigen was compared to native UPC-10 IgG in the following manner. Aliquots of 25 µl of 8.5 mg/ml UPC-10 IgG or reduced and alkylated UPC-10 IgG in 0.10 M NaCl, 0.05 M NaH_2PO_4, pH 6.8 buffer were placed in microtest tubes. Then 75 µl of a 1.0 mg/ml bacterial levan solution in the same saline-phosphate buffer were added. The solutions were vortexed and stored at 4°C for 24 hours. The resulting precipitate was centrifuged for 30 minutes at 5000 x g and washed four times with cold phosphate buffer. The precipitate was redissolved in 1 ml of 0.1 N NaOH and quantified by the absorbance at 280 nm. The avidity of reduced and alkylated 29B1 IgG for dinitrophenyl moieties was assured by the retention of the protein on a DNP-sepharose column eluted with 0.10 M NaCl, 0.05 M NaH_2PO_4, pH 7.4 buffer (Goetzl and Metzger, 1970).

Synthesis of DNP-antigens

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

The synthesis of DNP_1-cytochrome C began with the chromatographic purification of horse heart cytochrome C

(Sigma Chemical Co.) by dissolving the protein to a concentration of 10 mg/ml in 0.05 M NaH_2PO_4, pH 6.8 and chromatographing on sephadex G-75 (2.5 x 100 cm) eluting with this phosphate buffer. The cytochrome C peak was diluted to a concentration of 1 mg/ml and reacted with a 100-fold excess of freshly recrystalized Koshland's reagent (Aldrich Chemical Co.). After dialyzing in 0.10 M tris pH 8.0, the nitro group was reduced to an amine by making the solution 50 mM in $Na_2S_2O_4$. The protein was dialyzed against 0.05 M NaH_2PO_4, pH 6.3 and a tenfold molar excess of dinitrofluorobenzene was added as a solid and the solution was stirred at room temperature for eight hours. The excess reagent was separated from the protein by chromatography on sephadex G-25 (2.5 x 30 cm) in the 6.3 buffer. The pH of the protein solution was adjusted to 7.4 and the protein concentrated by ultrafiltration. The anti-DNP protein ABPC-22 IgM (Jarvis and Voss, 1981) was coupled to sepharose-4B by the method of Porath *et al.* (1973) and used for affinity chromatography of the DNP_1-cytochrome C. The purified protein was desalted, lyophilized, and stored at -20°C.

DNP_{30}-BSA was synthesized by dissolving 1 gm of BSA (Sigma Chemical Co.) in 10% $NaHCO_3$ and adding 300 mg of dinitrofluorobenzene (Aldrich Chemical Co.). This was stirred at room temperature while protected from light, dialyzed against H_2O, and lyophilized. The extent of

dinitrophenyl incorporation was quantitated using $E^{1\%}_{1cm}$ = 6.1 at 280 nm for BSA (Berrens and Bleumink, 1965) and ε = 16000 at 365 nm for DNP.

Determination of Binding Constant for DNP_1-cytochrome C

Tritiated DNP_1-cytochrome C was synthesized as described above using tritiated dinitrofluorobenzene (Amersham Radiochemicals) diluted with nonradioactive dinitrofluorobenzene to an activity of 73 mCi/mmol. The resulting monovalent DNP_1-cytochrome C had an activity of 61 mCi/mmol. This radiolabeled, monovalent antigen was used to determine the binding constant of DNP_1-cytochrome C with 29B1 IgG by the method of Hummel and Dreyer (1962). A sephadex G-75 column (1 x 40 cm) was equilibrated with a solution of 1.56×10^{-5} M ^{3}H-DNP_1-cytochrome C in 0.15 M NaCl, 0.02 M NaH_2PO_4, pH 6.8 buffer. Then 0.5 ml aliquots of 29B1 IgG at a concentration of 6.7×10^{-5} M were applied to the column at room temperature and 1.1 ml effluent fractions were collected. One ml was taken from each fraction, diluted with 10 ml of Aquasol 2 liquid scintillation fluid (New England Nuclear Radiochemicals) and counted. Then the absorbance at 280 nm was measured to determine the protein concentrations in each fraction. The concentration of bound DNP_1-cytochrome C, 29B1 IgG, and free DNP_1-cytochrome C was thus determined and an association constant calculated.

Isolation of Bacterial Levan from *Aerobacter levanicum*

Aerobacter levanicum (ATCC strain 15552) was grown by initially innoculating 200 ml of media composed of 0.8% nutrient broth (Difco) and 6% sucrose. After three days of growth at 28°C, 18 liter bottles were innoculated with the 200 ml cultures. The bacteria were killed after eight days of growth by adding 36 gm of NaN_3 to the 18 liter cultures. Cell debris was removed from the media by continuous flow centrifugation. The following procedure was used for isolation from two liters of this effluent. The effluent was heated to 95°C, then 50 ml of 0.5 M NaOH and 100 ml of 10% $CdSO_4$ in water were added. After cooling to room temperature the precipitate was removed by centrifugation at 1200 x g for three hours. The supernatant was then passed through a Mallinckrodt MB-3 column (2.5 x 50 cm) and concentrated to 400 ml by rotary evaporation. Then 80 ml of choloroform and 16 ml of 1-butanol were added to 400 ml of concentrated supernatant and the mixture was vigorously shaken for 30 minutes. After centrifuging the mixture at 8000 x g for 30 minutes, the aqueous phase was retained; care was taken to avoid any of the precipitate which formed at the interface. The chloroform/butanol precipitation was repeated until no precipitate formed. The aqueous solution was decolorized with activated charcoal and then added dropwise to four liters of rapidly stirring ethanol. The precipitate was isolated by centrifugation at

8000 x g for 10 minutes and dissolved in a minimal amount of 0.10 M tris base pH 8.5 and passed through a QAE-50 column (5.0 x 25 cm) equilibrated with this tris buffer. The polysaccharide was desalted by dialysis and lyophilized.

Labeling of Immunoglobulins with HMNB-DMSBr

The 29B1 IgG_{2a} was labeled in the presence of hapten or monovalent antigen by first dissolving 1.85 mg DNP-glycine or 41.3 mg DNP_1-cytochrome C in 4 ml of 0.15 M NaCl, 0.02 M NaH_2PO_4, pH 6.8 buffer. Then 1 ml of a 5 mg/ml solution of 29B1 IgG in this phosphate-saline buffer was added. The hapten control was run with 0.5 mg of glycine instead of DNP-glycine in the labeling buffer and the monovalent antigen control had 41.3 mg of cytochrome C which had been treated with Koshland's reagent as in the synthesis of DNP_1-cytochrome C instead of monovalent antigen in the solution. Freshly recrystallized tritiated HMNB-DMSBr (approximately 4×10^{-4} M in 1×10^{-3} N $HCl_{(aq)}$) was added dropwise to the stirring protein solution at 0°C until a sixfold molar excess of reagent to immunoglobulin had been achieved. The labeling solution was then gently agitated at 4°C for 24 hours. After the labeling was complete, 5 mg of IgG, previously labeled in a manner identical to the ^3H labeling procedure in the absence of ligand except using ^{14}C HMNB-DMSBr, was added to each tritium reaction solution. This ^3H-^{14}C labeled antibody solution was then exhaustively dialyzed against 0.10 M tris

buffer pH 8.0. Then 30 mg of unlabeled IgG in 0.10 M tris buffer pH 8.0 was added as protein carrier. Two reactions were performed under each set of conditions using identical reagent solutions.

The UPC-10 IgG was labeled at a lower concentration of immunoglobulin to allow the addition of a large amount of the multivalent antigen, levan. Labeling in the presence of multivalent antigen was performed by dissolving 200 mg of levan in 35 ml of the phosphate-saline labeling buffer. Then 15 ml of a 0.33 mg/ml immunoglobulin solution was added dropwise with stirring at room temperature to prevent the formation of insoluble aggregates. The control solution labeled in the presence of multivalent antigen was UPC-10 IgG in labeling buffer. The rest of the procedure followed that used for 29B1 IgG.

Separation of Antigens from Native or Reduced and Alkylated IgG

Both DNP-glycine and DNP_1-cytochrome C were separated from labeled 29B1 IgG by first dialyzing the protein against 0.05 M tris buffer pH 8.0 and then making the solution 0.03 M in $Na_2S_2O_4$ by the addition of this salt as a solid. The solution was stirred for one hour at room temperature under N_2. Then O_2 was gently bubbled through the solution for 30 minutes to remove any remaining $S_2O_4^{=}$. The resulting diaminophenyl glycine was removed by dialysis against 0.15 M NaCl, 0.02 M NaH_2PO_4, pH 7.4. The derivatized

cytochrome C was removed by chromatography on sephadex G-75 (2.5 x 100 cm) eluting with the same saline-phosphate buffer as for hapten.

The levan/UPC-10 solutions were dialyzed against 8 M urea in 0.10 M tris buffer pH 8.0. These solutions were then applied to QAE-50 (2.5 x 30 cm) previously equilibrated with the urea-tris buffer. After eluting with 500 ml of urea-tris buffer, thereby removing the levan, the column was equilibrated with 0.10 M tris pH 8.0 and the UPC-10 IgG was then eluted with a linear ionic gradient as in the procedure for isolating the immunoglobulin.

Papain digestion of Labeled, Native IgG and Labeled, Reduced and Alkylated IgG

The IgG solutions were dialyzed against 0.05 M sodium acetate pH 5.5 and concentrated to 5 mg/ml by ultrafiltration. The protein was incubated with papain (Putnam *et al.*, 1962) at 37°C. An incubation period of 3 hours was used for UPC-10 IgG and 45 minutes for 29B1 IgG.

Isolation of Fab and Fc Fragments from Papain Digestion of Labeled IgG

The 29B1 fragments were separated by affinity chromatography on a DNP-sepharose column as previously described. The Fc fragment was eluted in the void volume and the Fab fragment was eluted with 0.03 M DNP-glycine in the saline-phosphate buffer.

The papain digest of UPC-10 IgG was concentrated by ultrafiltration and dialyzed against 0.05 M NaH_2PO_4, pH 6.6. Then the digest was chromatographed on a Whatman DE-52 ion-exchange column (2.5 x 30 cm). The Fab fragment was eluted with the starting buffer and the Fc was eluted with a linear ionic gradient established by placing 250 ml of 2.0 M NaCl, 0.05 M NaH_2PO_4, pH 6.6 buffer in the static flask and 250 ml of 0.05 M NaH_2PO_4 pH 6.6 buffer in the mixing flask.

The purity of the protein fragments was checked by polyacrylamide gel electrophoresis. The identity of the Fc fragment was verified by its retention on Protein A-sepharose (Pharmacia Fine Chemicals).

Liquid Scintillation Counting Procedure

The DNP-glycine was separated from 29B1 Fab fragments prior to counting as previously described. The Fab and Fc fragments were desalted by dialysis and concentrated to one ml by ultrafiltration. The concentrated Fab or Fc solutions were then added to 10 ml of Aquasol 2 liquid scintillation counting fluid. Experimental values were averages of duplicate countings on a Beckman LS-250 liquid scintillation counter programmed for a 3% counting error. All samples had counts greater than 10 times background.

Complement Assay of Native or Reduced and Alklated IgG

The complement assay was patterned after the micro-complement assay of Bengali *et al.* (1980). The assay was

carried out in a Hepes buffer containing 0.1% gelatin and consisting of 0.01 M Hepes, 0.15 M NaCl, 0.00015 M $CaCl_2 \cdot 2H_2O$, 0.0005 M $MgCl_2 \cdot 6H_2O$, pH 7.4. Initially the antigen-antibody complex was incubated with two CH_{50} units of guinea pig serum in 75 µl total volume in each well of a microtiter plate for 45 minutes at 37°C. Then 50 µl of a 2% activated sheep erythrocyte cell suspension was added to each well and this was incubated for 45 minutes at 37°C. The microtiter plate was then centrifuged at 1000 x g for 3 minutes at 4°C and 40 µl aliquots from each well were counted on a Beckman Gamma 4000 counter. Each condition was assayed in quadruplicate. A zero percent fixation control was run by incubating two CH_{50} units of guinea pig serum without any antibody or antigen and background for 100% fixation was determined from wells containing 75 µl of Hepes-gelatin buffer with no complement, antibody, or antigen.

RESULTS

Analysis of Monovalent Antigen

Use of 1-fluoro-2,4-dinitro(3,5-^{3}H) benzene (Amersham Radiochemicals) at a specific activity of 20.4 Ci/mmol of reagent allowed liquid scintillation counting to be used as an assay for DNP moieties; while the absorbance at 280 nm was used to determine the cytochrome C concentration (Mayer

and Miller, 1970). The number of DNP moieties incorporated per cytochrome C was 0.98 with a standard deviation of 0.02. An elution profile in counts per minute for tritiated cytochrome C off the sephadex G-75 column used for the Hummel-Dreyer measurement is shown in Figure 8. The association constant for DNP_1-cytochrome C with 29B1 IgG was $K_a = 2.5 \times 10^4$ with a standard deviation of 1.4×10^4 from five independent measurements.

Separation of Fab and Fc Fragments after Papain Digestion of IgG

Figures 9 and 10 show the chromatographic separation of the Fc and Fab fragments after papain digestion of radiolabeled UPC-10 IgG and radiolabeled, reduced and alkylated UPC-10 IgG. The Fab fragment elutes first followed by the Fc fragment, which begins to elute at a conductivity of 17 mS. Figure 11 shows a 7.5% SDS polyacrylamide gel of the Fab and Fc fragments isolated in this way stained with coomassie blue. The Fab and Fc fragments of 29B1 were similar. The Fc fragments of both 29B1 and UPC-10 were retained on a Protein A-sepharose affinity column in 0.15 M NaCl, 0.02 M NaH_2PO_4, pH 7.6 buffer. The Fab fragments of both these immunoglobulins showed no affinity for the Protein A affinity column.

Labeling of Fab and Fc Fragments by HMNB-DMSBr

The covalent incorporation of HMNB-DMSBr labels per IgG molecule was found to be 0.4 and the molar ratio of label to

Fab fragment was 0.12. This analysis was performed spectrophotometrically using ε = 17,500 at 410 nm for the HMNB moiety in 0.1 M NaOH and $E^{1\%}_{1cm}$ = 14 at 280 nm for both whole IgG and Fab in 0.15 M NaCl, 0.02 M NaH_2PO_4 pH 7.4 (Eisen, Simms, and Potter, 1968). The ratio of HMNB moieties to Fc was calculated to be 0.16 using the formula $X_w = 2\ X_{ab} + X_c$ where X_w is the ratio of label incorporation in whole IgG, X_{ab} is the same ratio for Fab and X_c is that ratio for Fc. These values were for native IgG. Reduced and alkylated IgG was found to be 1.14 times more reactive than native IgG but with approximately the same distribution between Fab and Fc.

Table I summarizes the results of labeling done in the presence and absence of antigen under these conditions. The counts incorporated in either DNP_1-cytochrome C or the HNB-cytochrome C were less than 0.4% of the counts incorporated in the immunoglobulin in the same reation. The monovalent antigen concentration was sufficient to occupy greater than 90% of the antigen binding sites. There was no detectable reaction of HMNB-DMSBr with the levan antigen.

Complement Assays of Native or Reduced and Alkylated Immunoglobulins

Figures 12 and 13 compare the difference in complement fixing ability of native 29B1 IgG with DNP_{30}-BSA and UPC-10 IgG with levan to that of the reduced and alkylated immunoglobulin. The assay was performed using either 20 µg of

29Bl IgG or 5 μg of UPC-10 IgG per well and varying amounts of antigen. Reduced and alkylated 29Bl IgG showed essentially no fixation over the range of antigen concentration in which native 29Bl IgG fixation began at 20% and progressed to 100%. Native UPC-10 IgG fixed from 40% to 80% of the available complement as the amount of levan per well was increased from 0.3 ng to 1.2 ng. At the same antigen concentrations the reduced and alkylated UPC-10 IgG fixed at most 20% of the complement.

Figure 14 shows a gel scan at 590 nm of a 7.5% SDS polyacrylamide electrophoresis gel of reduced and alkylated UPC-10 IgG which had been stained with coomassie blue in 10% acetic acid, 25% isopropyl alcohol, and 65% water (v:v:v) solution. Little intact IgG remains and most of the disulfide bonds between the heavy chains have been reduced. Similarly treated 29Bl showed corresponding peaks but in this case no intact IgG was observed. Bacterial levan (75 μg) precipitated 72% as much of the reduced and alkylated IgG as was precipitated by the same amount of antigen acting on native IgG. The reduced and alkylated 29Bl IgG could not be eluted from a DNP-sepharose affinity column by washing with 5 void volumes of 0.15 M NaCl, 0.02 M NaH_2PO_4, pH 7.4 buffer. As with the native form, the reduced and alkylated form was eluted by 0.03 M DNP-glycine in this saline-phosphate buffer.

DISCUSSION

The changes in tryptophan reactivity resulting from antigen binding are summarized in Figure 15. For the native immunoglobulins, hapten only slightly increases reactivity while monovalent and multivalent antigens significantly decrease the reactivity of tryptophan residues in the Fc region. We interpret this decrease as reflecting a change in conformation or motional freedom of regions of the Fc tail of IgG which has bound antigen such that tryptophan residues are, on the average, less available to reagent in solution. The monovalent antigen is probably too small to interfere sterically with the Fc tryptophan residues and the monovalency of the antigen keeps the 29Bl IgG in monomeric form throughout the reaction. Since UPC-10 IgG was slowly added to an excess of antigen, no precipitable complexes formed. Those complexes which may have been created by this process remained soluble and were probably small so that intermolecular Fc interaction as a steric hindrance to reactivity is not a likely cause of the decrease observed in the reactivity of tryptophan in the UPC-10 Fc region. Levan, being a large polymer, could conceivably hinder reactions occurring in the Fc region; this possibility will be discussed later.

The observed dependence of Fc tryptophan reactivity on the presence of antigen correlates well with the complement fixation study performed by Brown and Koshland (1975) on IgM.

They found that while IgM would fix complement when either a monovalent or multivalent antigen was bound, binding hapten was insufficient to cause fixation. This suggests a functional dichotomy between hapten and either monovalent or multivalent antigen which in the present work is manifested as changes in chemical reactivity of tryptophan residues in the Fc region of immunoglobulins.

Reduction and alkylation of the immunoglobulin either negated or completely reversed the decrease in Fc tryptophan reactivity. Thus, the tryptophan residues in the Fc region of reduced 29B1 IgG showed a slight increase in reactivity in the presence of antigen while reduced UPC-10 IgG Fc displayed a marked increase in tryptophan reactivity in the presence of levan. The effects of reduction and alkylation on the observed changes in reactivity correlate with the loss of complement activity after reduction and alkylation of the antibody (Johnson and Hoffmann, 1981). A study of conformational changes of reduced and alkylated IgG (Seegan *et al.*, 1979) found that when small complexes of reduced and alkylated IgG from rabbits were formed by binding a divalent antigen, the CH_2 domain adopted a very open structure (Figure 16). This alteration in structure was not observed for monomeric, reduced and alkylated IgG. Accordingly, the large increase in Fc tryptophan reactivity for reduced and alkylated UPC-10 IgG can be related to this conformational change which provides for greater solvent

exposure of the CH_2 domain because the multivalent antigen may be capable of mimicking the formation of soluble complexes as seen for rabbit IgG. If the observed decrease in reactivity of native UPC-10 IgG Fc were due to steric hindrance of the Fc region by levan then one would have expected the same hindrance in the reduced and alkylated immunoglobulin because the distance from the binding site to the CH_2 domain is only slightly increased by reduction (Seegan *et al.*, 1979). As no decrease in reactivity of Fc tryptophan residues was observed in the reduced and alkylated immunoglobulin, steric hindrance seems unlikely to be the cause of the reactivity changes in native UPC-10 IgG. Presumably a similar increase in Fc tryptophan reactivity was not observed for the reduced 29B1 system because only the monomeric form of the IgG was present in solution so that a conformational change analogous to that in the UPC-10 IgG/levan complexes does not occur.

The reactivity of Fab tryptophan residues in 29B1 IgG did not appear to be dependent on the presence of either hapten or monovalent antigen. UPC-10 IgG Fab showed decreases in reactivity for both the native and reduced and alkylated form when antigen is bound. The agreement in the magnitude of the decrease between the native and reduced and alkylated immunoglobulins supports the results from the quantitative precipitation of UPC-10 IgG with levan and indicates that the binding of antigen by the

protein was relatively unaffected by reduction and alkylation. At least three possibilities explain the observed decrease: (1) a tryptophan residue(s) in the antigen binding site is blocked when levan is bound, (2) a conformational change occurs in the Fab region upon binding which decreases tryptophan reactivity, or (3) the levan sterically blocks tryptophan residues which are not in the binding site. Presently available data do not allow one to choose among these alternative explanations.

The reactivity of Fc tryptophan residues toward HMNB-DMSBr was found to depend on the type of ligand bound and the integrity of the disulfide bonds in the immunoglobulin. This correlates with the dependence of complement activation on both these factors. Since the opening of the structure of the CH_2 region between heavy chains observed by electron microscopy in complexes of reduced IgG was reflected in increased Fc tryptophan reactivity, we believe the decrease in native Fc tryptophan reactivity on binding monovalent or multivalent antigen reflects concomitant conformational changes in the Fc region which allow less solvent exposure of tryptophan residues in this region. The conformational changes may be dynamic in nature and may be thought of as a stabilization of a less exposed conformation (Zavodszdy *et al.*, 1981).

A hypothesis detailing the relationship between conformational changes and complement activation has been formulated by Hoffmann (1976a,b,c). In the terminology

of allosteric conformational equilibria between a T state and an R state (the R state is the one that fixes complement), the binding of antigen shifts the equilibrium toward the R form. The present results support the occurrence of a conformational change in the Fc region and, in terms of Hoffmann's R state-T state model for immunoglobulins, suggest Fc tryptophan residues in the R state have become less accessible and therefore less reactive than they are in the T state.

REFERENCES

Allan, R. and Isliker, H., *Immunochem.* (1974) *11*, 243.

Berrens, L. and Bleumink, E., *Int. Arch. Allergy and App. Immunol.* (1965) *28*, 150.

Bengali, Z.H., Das, S.R., and Levine, P.H., *J. Immunol. Meth.* (1980) *33*, 63.

Brown, J.C. and Koshland, M.E., *Proc. Natl. Acad. Sci. USA* (1975) *72*, 5111.

Eisen, H.N., Simms, E.S., and Potter, M., *Biochemistry* (1968) *7*, 4126.

Goetzl, E.J. and Metzger, H., *Biochemistry* (1970) *9*, 1267.

Hoffmann, L.G., *Immunochem.* (1976a) *13*, 725.

Hoffmann, L.G., *Immunochem.* (1976b) *13*, 731.

Hoffmann, L.G., *Immunochem.* (1976c) *13*, 737.

Hummel, J.P. and Dreyer, W.J., *Biochim. Biophys. Acta* (1962) *63*, 530.

Jarvis, M.R. and Voss, E.W., *Mol. Immunol.* (1981) *18*, 261.

Johnson, B.A. and Hoffmann, L.G., *Mol. Immunol.* (1981) *18*, 181.

Johnson, B.J. and Thames, K.E., *J. Immunol.* (1966) *117*, 1491.

Lee, J.P. and Painter, R.H., *Mol. Immunol.* (1980) *17*, 1155.

Mayer, M.M. and Miller, J.A., *Anal. Biochem.* (1970) *36*, 91.

Metzger, H., *Adv. Immunol.* (1974) *18*, 169.

Metzger, H., *Contemp. Top. in Mol. Immunol.* (1978) *7*, 119.

Oi, V.T. and Herzenberg, L.A., *Mol. Immunol.* (1979) *16*, 1005.

Porath, J., Aspberg, K., Drevin, H., and Axen, R., *J. Chromatography* (1973) *86*, 53.

Porter, R.R., *Methods in Med. Res.* (1950) *3*, 256.

Putnam, F.W., Tan, M., Lynn, L.T., Easley, C.W. and Migata, S., *J. Biol. Chem.* (1962) *237*, 717.

Schlessinger, J., Steinberg, I.Z., Givol, D., Hochmann, J., and Pecht, I., *Proc. Natl. Acad. Sci. USA* (1975) *72*, 2775.

Seegan, G.W., Smith, C.A., and Schumaker, V.N., *Proc. Natl. Acad. Sci. USA* (1979) *76*, 907.

Zavodsky, P., Jaton, J., Venyaminov, S.W., and Medgyesi, G.A., *Mol. Imm.* (1981) *18*, 39.

Table I. $^{3}H:^{14}C$ ratios obtained for various labeled antibody-ligand systems[a]

	Fab	Fc
1. Antibody-hapten		
29B1 IgG + DNP-glycine	0.98 ± 0.06	0.99 ± 0.02
29B1 IgG + glycine	0.95 ± 0.05	0.95 ± 0.04
2. Antibody-monovalent antigen		
29B1 IgG + DNP_1-cytochrome C	2.46 ± 0.05	2.10 ± 0.01
29B1 IgG + HNB-cytochrome C	2.40 ± 0.05	2.47 ± 0.05
3. Reduced antibody-monovalent antigen[b]		
R29B1 IgG + DNP_1-cytochrome C	1.96 ± 0.01	1.98 ± 0.05
R29B1 IgG + HNB-cytochrome C	1.92 ± 0.02	1.95 ± 0.02
4. Antibody-multivalent antigen		
UPC-10 IgG + levan	2.45 ± 0.05	2.14 ± 0.03
UPC-10 IgG	2.79 ± 0.05	2.77 ± 0.02
5. Reduced antibody-multivalent antigen		
RUPC-10 + levan	2.03 ± 0.03	2.80 ± 0.06
RUPC-10	2.31 ± 0.05	2.23 ± 0.05

[a]Each value is the average of duplicate experiments. The error indicated is half the difference between the duplicate values. [b]Antibody which has been both reduced and alkylated is prefixed by an R.

Figure 8. Elution Profile of ^{3}H-DNP_1-cytochrome C for the Hummel-Dreyer Measurement of Binding Constants

DNP_1-cytochrome C at 1.56×10^{-5} M in 0.15 M NaCl, 0.02 M NaH_2PO_4, pH 6.8 was used to elute 0.5 ml of 29B1 IgG which was at a concentration of 6.7×10^{-5} M in the same buffer. The counts per minute are for 1 ml aliquots from each fraction collected. Sephadex G-75 (1 x 40 cm) was used for the gel filtration.

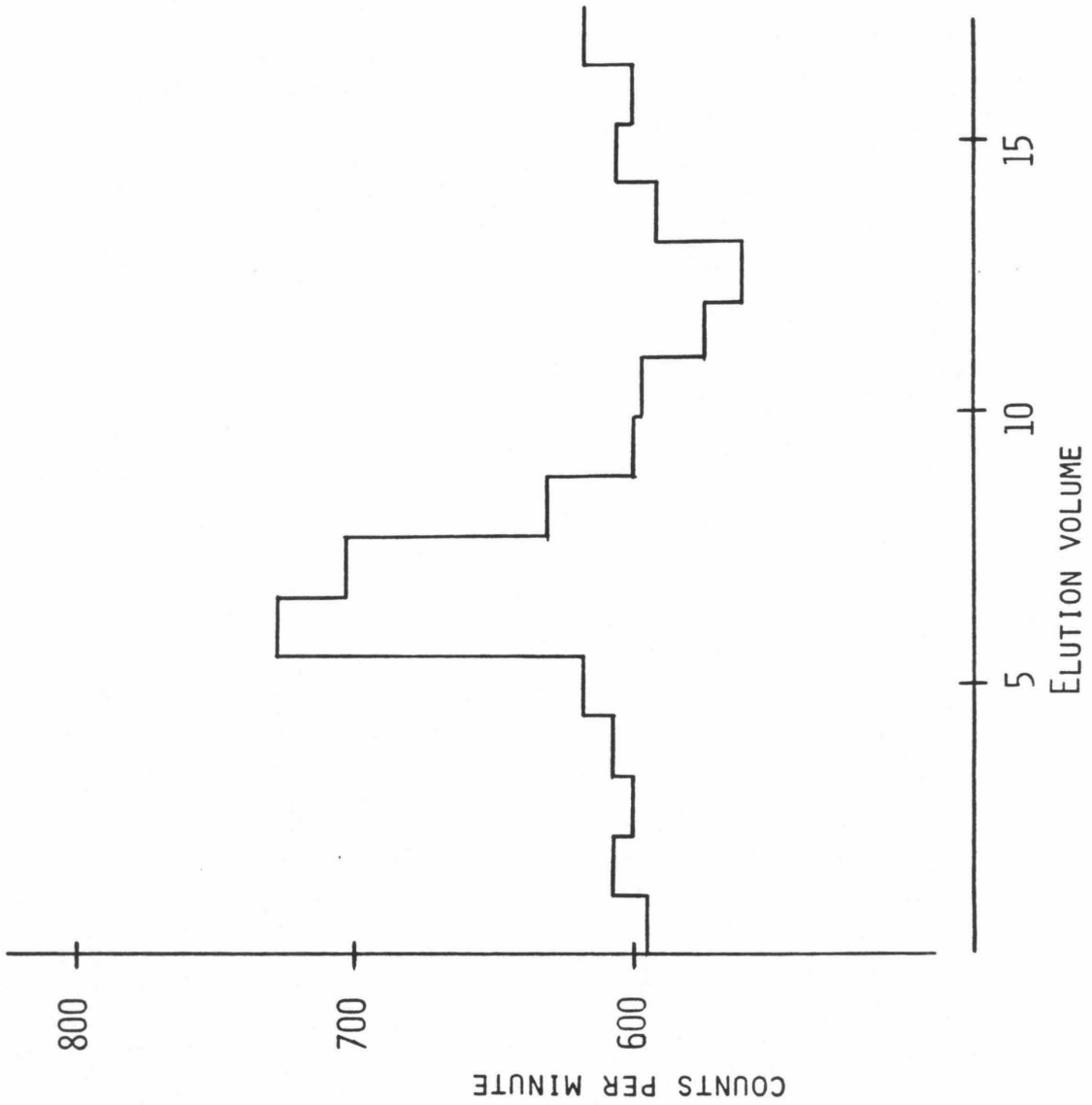

800
700
600
COUNTS PER MINUTE
5
10
15
ELUTION VOLUME

Figure 9. Chromatograph of the Papain Digest of Native UPC-10 IgG

The papain digest of UPC-10 IgG was chromatographed on Whatman DE-52 in a 0.05 M NaH_2PO_4, pH 6.6 at room temperature. The Fab fragment was not retained on the column; the Fc fragment was eluted with a linear ionic gradient. The left axis of ordinates is the absorbance at 280 nm, the right axis of ordinates is the conductivity and the abscissa is the elution volume.

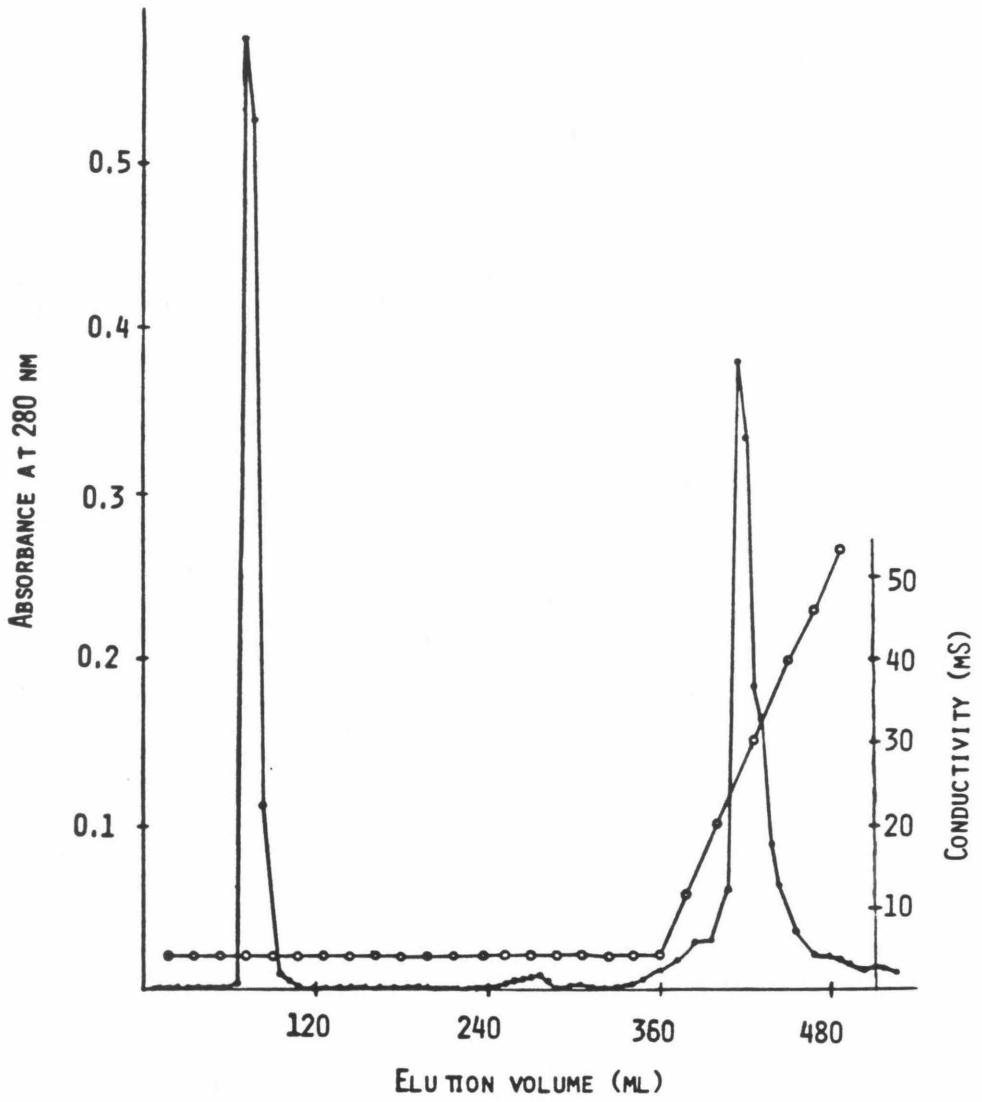

0.5
0.4
0.3
0.2
0.1
ABSORBANCE AT 280 NM
50
40
30
20
10
CONDUCTIVITY (MS)
120
240
360
480
ELUTION VOLUME (ML)

Figure 10. Chromatograph of the Papain Digest of Reduced and Alkylated UPC-10 IgG

The papain digest of reduced and alkylated UPC-10 IgG was chromatographed on Whatman DE-52 in 0.05 M NaH_2PO_4, pH 6.6 at room temperature. The Fab fragment was not retained on the column; the Fc fragment was eluted with a linear ionic gradient. The left axis of ordinates is the absorbance at 280 nm, the right axis of ordinates is the conductivity and the abscissa is the elution volume.

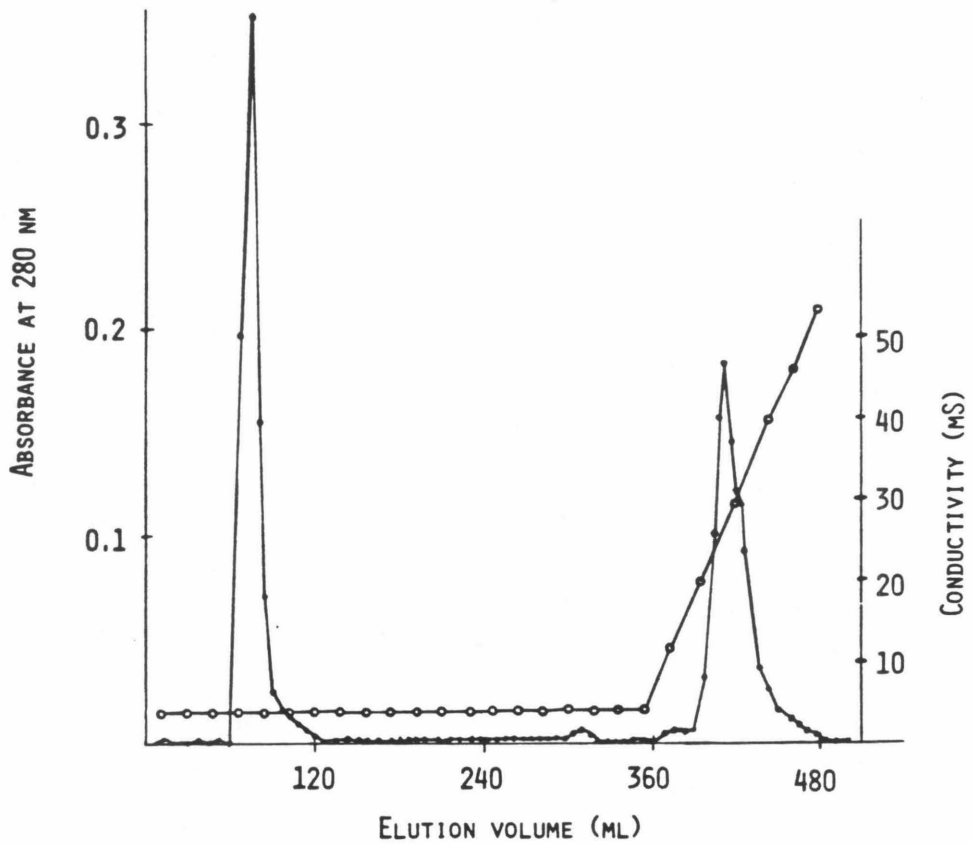

0.3
0.2
0.1
ABSORBANCE AT 280 NM
50
40
30
20
10
CONDUCTIVITY (MS)
120
240
360
480
ELUTION VOLUME (ML)

Figure 11. SDS Polyacrlyamide Gels of Fab and Fc Fragments

The percentage acrylamide of the gel was 7.5%. Track 1 is Fab from native UPC-10 IgG; track 2 is Fc from native UPC-10 IgG. Tracks 3 and 4 are respectively Fab and Fc from reduced and alkylated UPC-10 IgG. Track 5 shows bovine serum albumin, alcohol dehydrogenase and cytochrome C. The gel was run under reducing conditions.

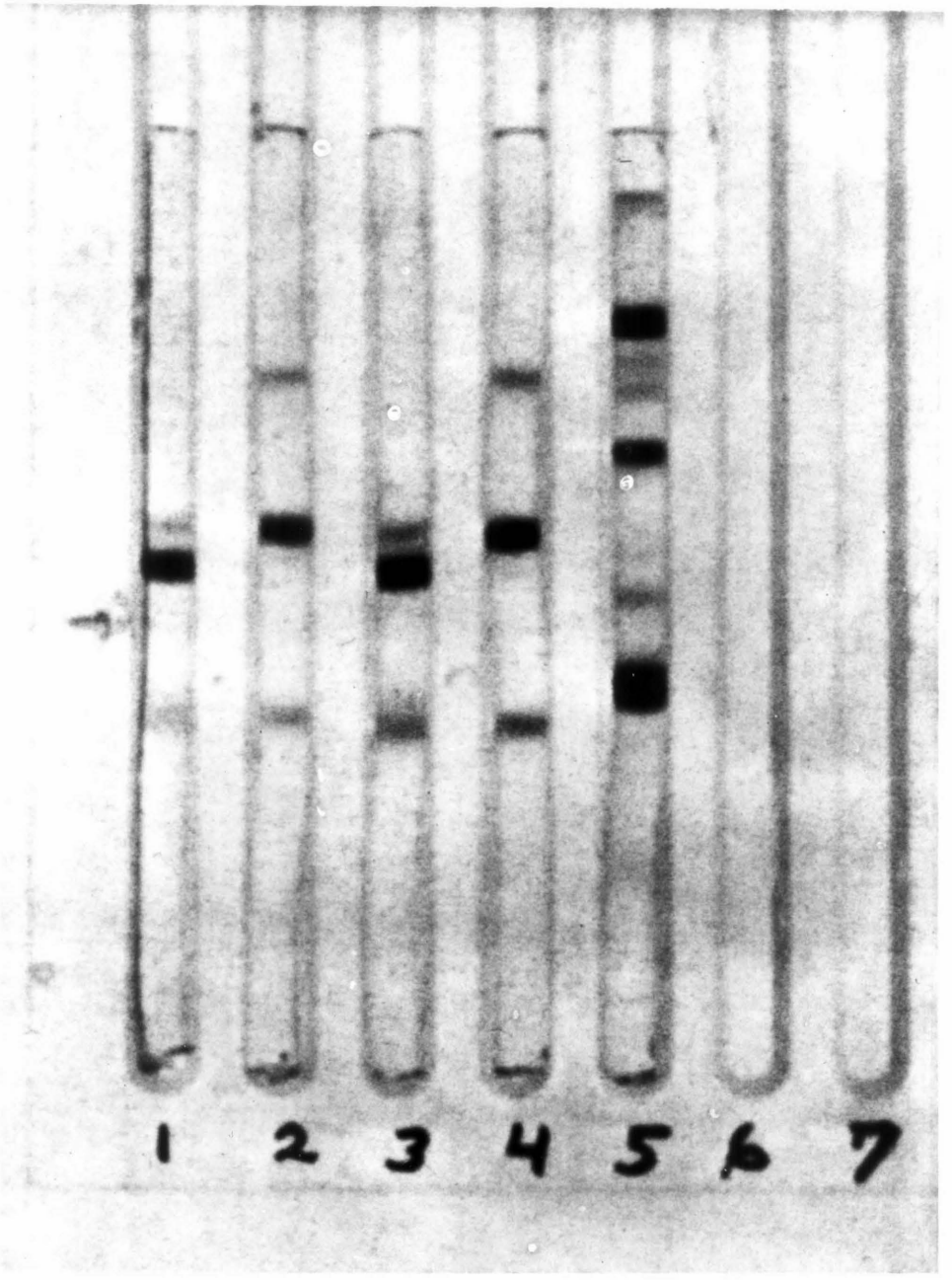

1
2
3
4
5
6
7

Figure 12. Complement Fixation Assay Comparing Native 29B1 IgG to Reduced and Alkylated 29B1 IgG

Varying amounts of DNP_{30}-BSA were incubated with 20 µg per microtitre well of native 29B1 IgG (▽) or with 20 µg per microtitre well of reduced and alkylated 29B1 IgG (O). The ordinate is the percent fixation of two CH_{50} units of guinea pig serum and the abscissa is the amount of DNP_{30}-BSA per well.

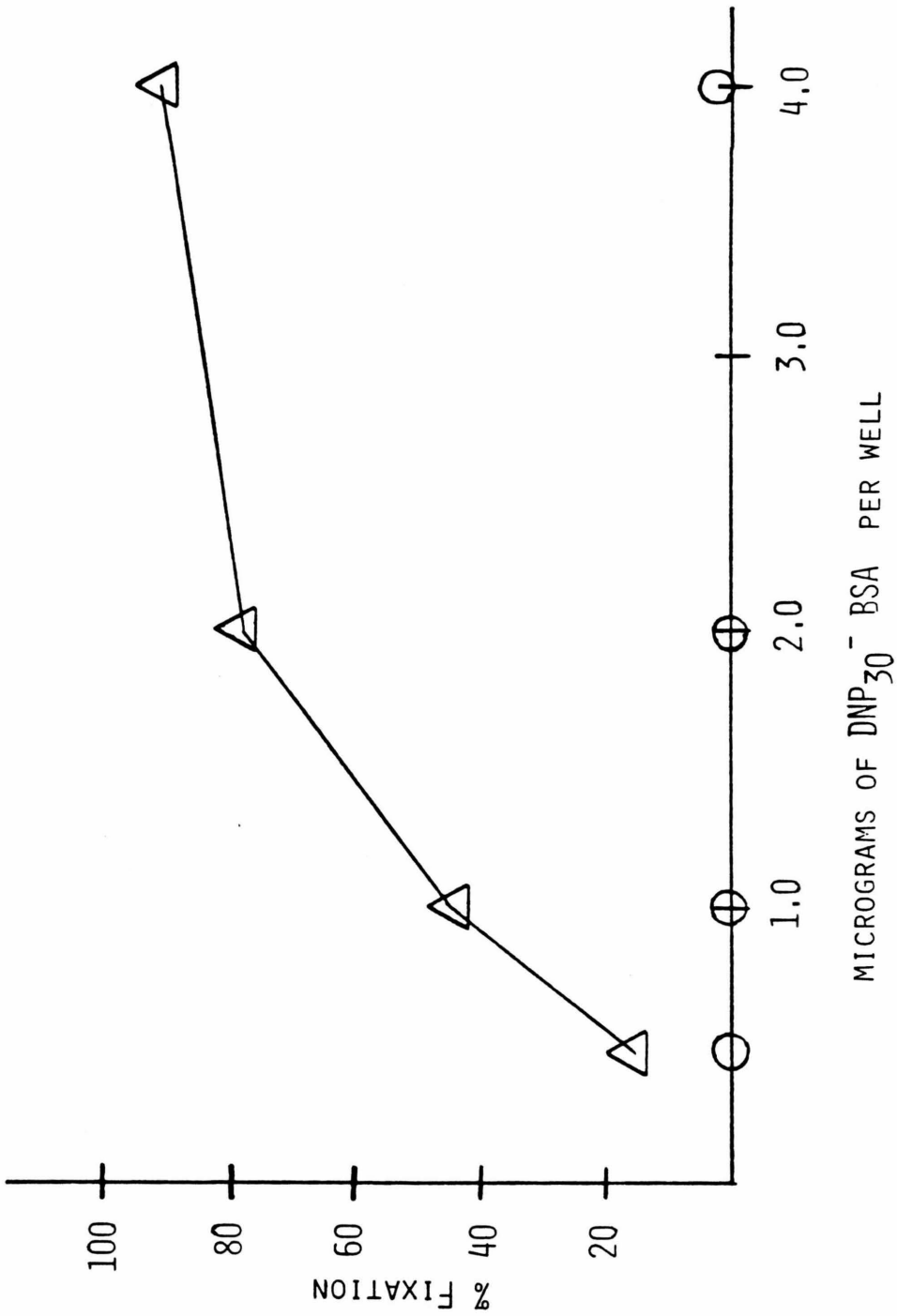

% FIXATION
100
80
60
40
20
1.0
2.0
3.0
4.0
MICROGRAMS OF DNP_{30}^{-} BSA PER WELL

Figure 13. Complement Fixation Assay Comparing Native UPC-10 IgG to Reduced and Alkylated UPC-10 IgG

Varying amounts of bacterial levan were incubated with 5 µg of native UPC-10 IgG (▽) or 5 µg of reduced and alkylated UPC-10 IgG (○). The ordinate is the percent fixation of two CH_{50} units of guinea pig serum and the abscissa is the amount of levan per well.

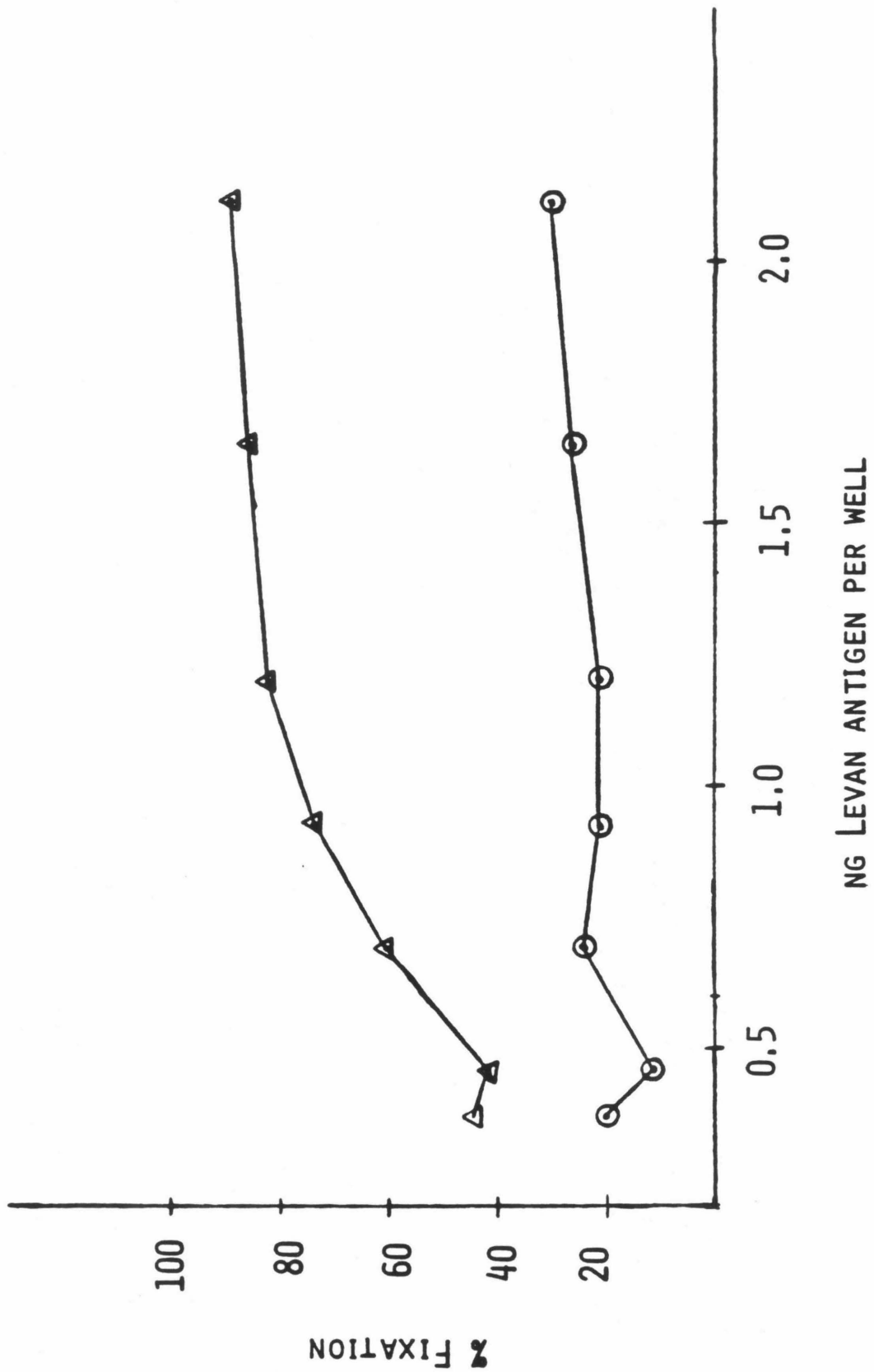

% FIXATION
100
80
60
40
20
0.5
1.0
1.5
2.0
NG LEVAN ANTIGEN PER WELL

Figure 14. Gel Scan of SDS-polyacrylamide Gel of Reduced and Alkylated UPC-10 IgG

The scan of reduced and alkylated UPC-10 IgG on 7.5% polyacrylamide was made at 590 nm observing the coomassie blue used to visualize the products of reduction. Peak A is light chain and peak B is heavy chain. The molecular weights for the rest of the peaks were determined to be C = 81 kilodaltons, D = 108 kilodaltons, E = 126 kilodaltons, and F = 150 kilodaltons by the plot of ln[molecular weight] versus R_f using A and B as standards.

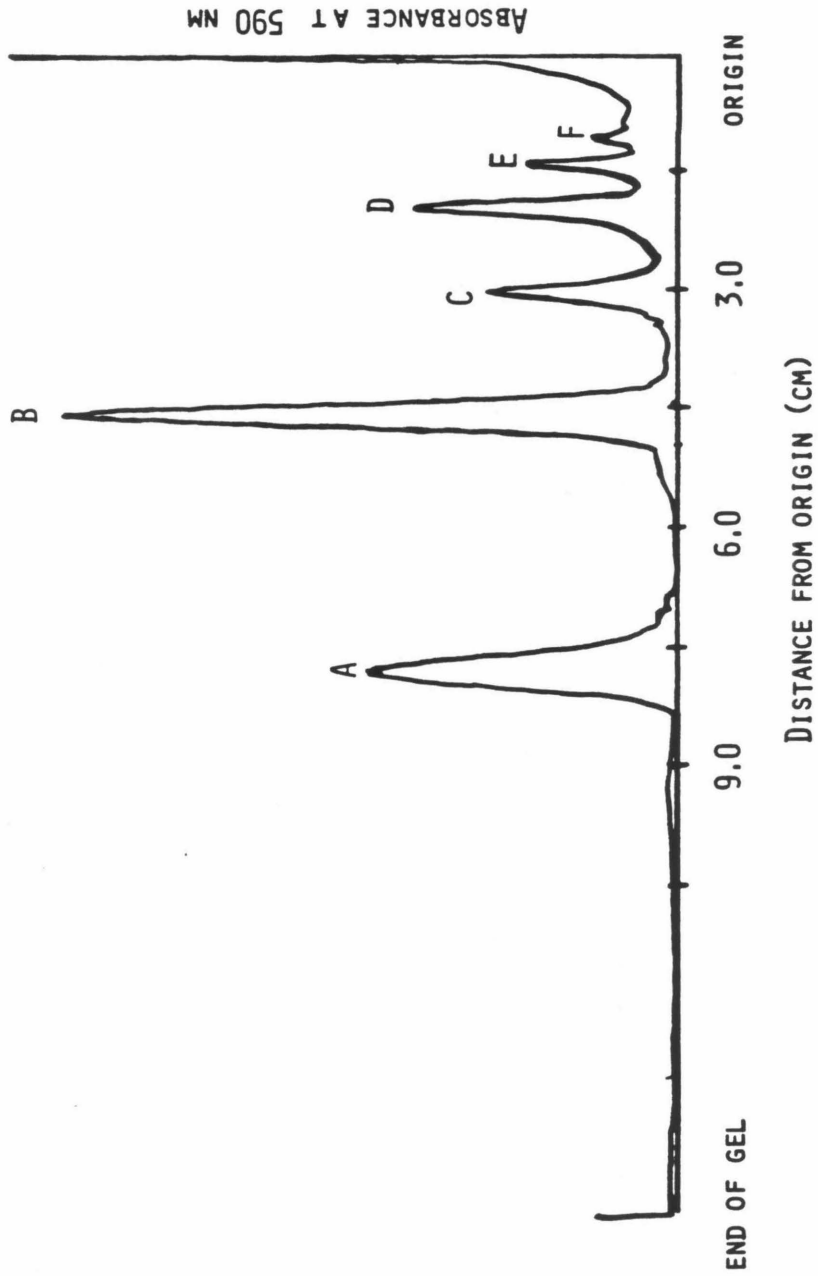

ABSORBANCE AT 590 NM
A
B
C
D
E
F
END OF GEL
9.0
6.0
3.0
ORIGIN
DISTANCE FROM ORIGIN (CM)

Figure 15. Summary of Changes in Tryptophan Reactivities for Various Antibody/Ligand Systems

The upper 5 bars are for Fab fragments and the lower 5 bars are Fc fragments. The changes are expressed in percentage change of incorporation of label in the presence of ligand compared to incorporation of label in the absence of ligand.

Fab fragments

29Bl IgG + DNP-glycine

29Bl IgG + DNP_1-cytochrome C

R29Bl IgG + DNP_1-cytochrome C

UPC-10 IgG + levan

RUPC-10 IgG + levan

Fc fragments

29Bl IgG + DNP-glycine

29Bl IgG + DNP_1-cytochrome C

R29Bl IgG + DNP_1-cytochrome C

UPC-10 IgG + levan

RUPC-10 IgG + levan

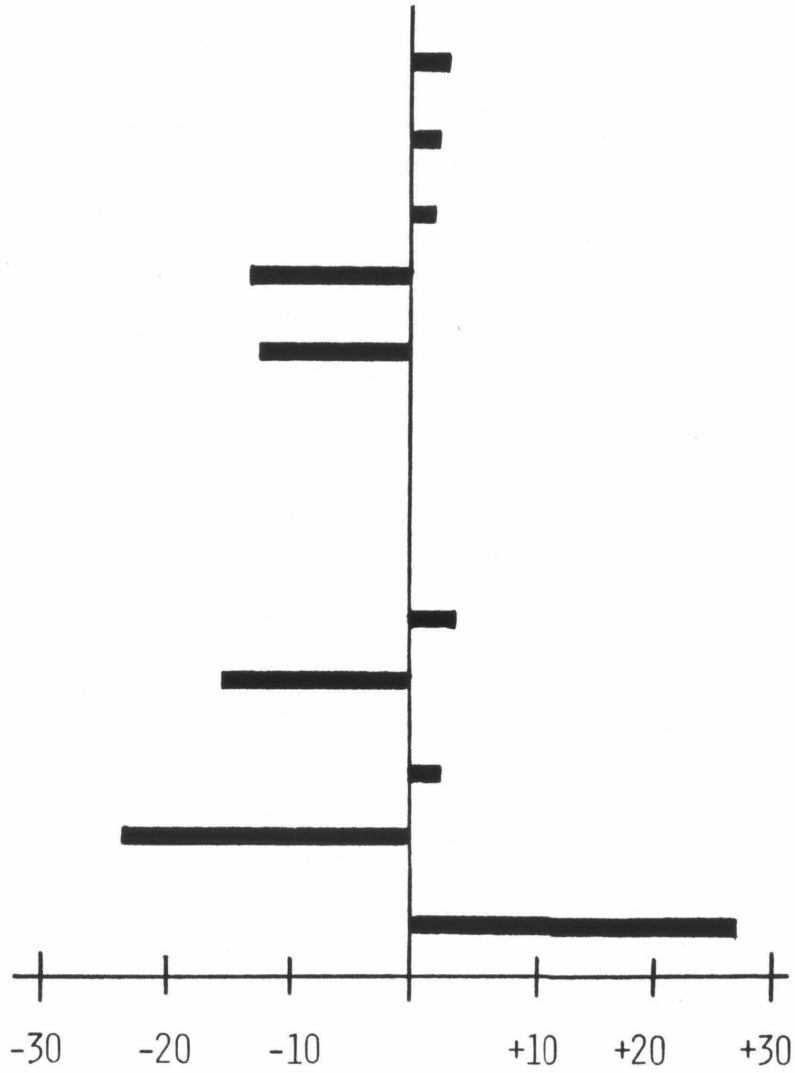
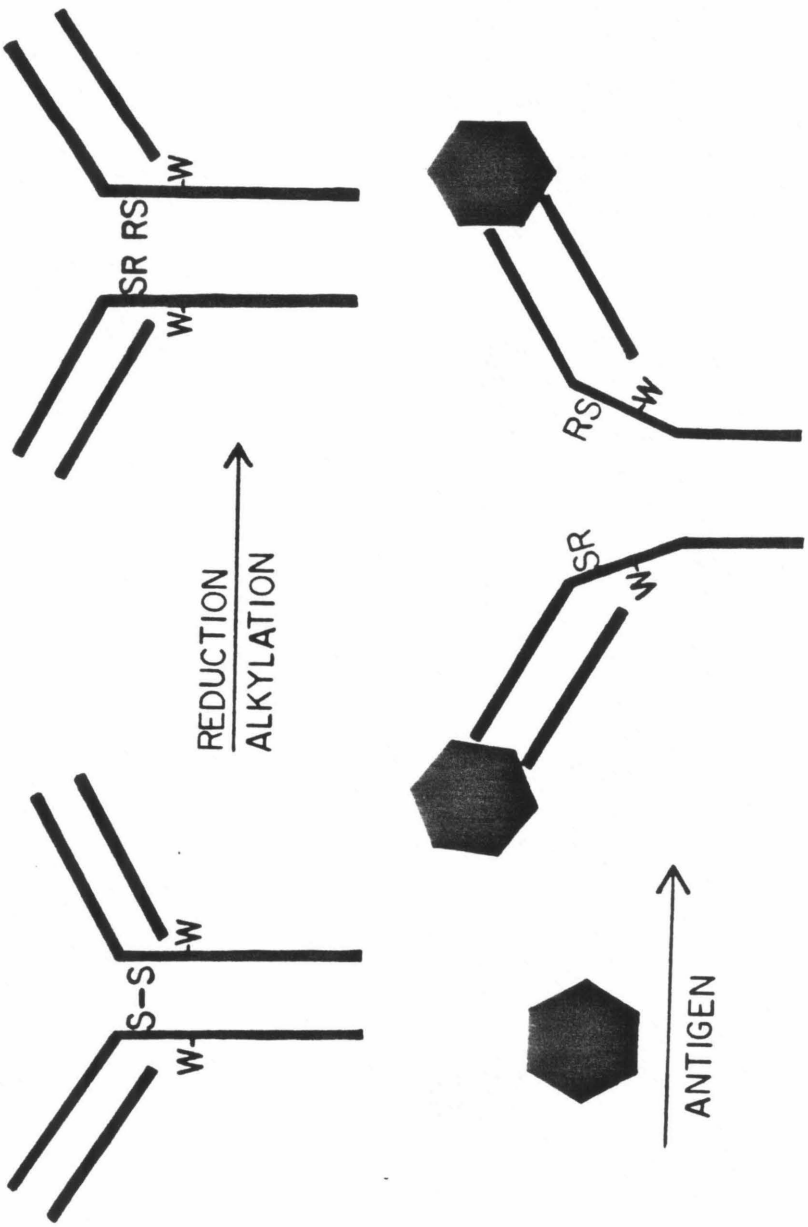


Figure 16. Schematic of Structural Change Observed for Reduced and Alkylated Rabbit IgG (Seegan *et al.*, 1979)

The binding of multivalent antigen gives rise to an open structure in the CH_2 domain. RS represents alkylated cysteine and W represents tryptophan 277.


S–S
W
W
REDUCTION
ALKYLATION
SR RS
W
W
ANTIGEN
SR
W
RS
W

CHAPTER 4

Complement Fixation by Dimers and Trimers of 29B1 IgG Produced by Chemical Crosslinking

One central piece of evidence for the aggregation theory of complement activation is the ability of aggregates of IgG produced by heat denaturation (Ishizaka and Ishizaka, 1960) or chemical crosslinking (Ishizaka *et al.*, 1962; Wright *et al.*, 1980a) to activate complement. Any conformational requirements on the IgG molecule in the aggregate appear to be restricted only to the size of the aggregate because the ability to activate complement seems independent of the method used to generate the aggregate and therefore independent of any specific conformational change in the IgG molecule (Metzger, 1974).

The aggregation model can account for the cooperativity of C_1^- binding to antigen-antibody complexes observed by Thompson and Hoffmann (1974), if one allows for multiple C1 sites on IgG which are masked while free in solution but are revealed by aggregation (Metzger, 1974; Hoffmann, 1976). This does not account for complement activation studies using rabbit IgG and monovalent antigen (Goers *et al.*, 1977). This study found that monovalent antigen increased the binding of C1 with IgG; this result indicated the occurrence of some structural alteration to produce this increased affinity. However it is important to note that

the increased affinity was not observed for Clq binding and that the monovalent antigen used, 2,4-dinitrophenyl poly-L-lysine$_{20}$, interacted with the Cl components Clr and Cls in a manner that was not completely defined.

Furthermore, the ability of IgG aggregates produced by heat denaturation to activate complement components subsequent to Cl has been examined. By monitoring histamine release from granulocytes as an assay for C3a production, Hartman and Glovsky (1981) found that aggregates produced by heat denaturation were much less efficient at producing C3a than antigen-antibody aggregates containing the same amount of immunoglobulin. Likewise Dodds and Porter (1979) found that 100 times more immunoglobulin in aggregates produced by heat denaturation is required to activate C4 via the classical pathway compared to immunoglobulin in antigen-antibody aggregates. Although chemically crosslinked Fc fragments appear to activate complement (Ishizaka *et al.*, 1962); evidence has accumulated which indicates that IgG interaction with C4 and possibly C3 in addition to Cl may be required for cytolytic capability (Campbell *et al.*, 1980). This result may indicate that a reasonably precise relationship between the Fc region, C_1^- , and the Fab region must exist for C4 to be activated in such a manner as to proceed with subsequent proteolysis.

One method of aggregation by chemical crosslinking has been developed in which dithiobissuccinimidyl propionate is used to crosslink rabbit IgG and produce oligomers of the

immunoglobulin which can then be separated into dimers, trimers, and tetramers by gel filtration chromatography (Wright *et al.*, 1980b). This study demonstrated an affinity between these oligomers and Clq by analytical ultracentrifugation. This same work used the assay system of Levine and van Vunaki (1967) to assay for complement fixing ability of these oligomers of IgG. By this assay system, oligomers of crosslinked rabbit IgG fixed complement with tetramer requiring 100 times less protein than trimer to achieve 50% fixation and trimer requiring 10 times less protein than dimer for this level of fixation. Monomeric IgG did not fix complement by this assay.

The assay of Levine and van Vunakis may be categorized as a complement depletion assay which, with various modifications, has been used for determining the activity of aggregated IgG and aggregated Fc from IgG (Ishizaka and Ishizaka, 1960; Ishizaka *et al.*, 1962). In this type of assay the material in question is incubated with a source of complement, typically guinea pig serum, for a fixed amount of time under specific environmental conditions. Then a fixed amount of sheep erythrocytes which have been sensitized by exposure to anti-sheep red blood cell antiserum is added to the incubated guinea pig serum suspension and this is again incubated under specific conditions. By comparing the amount of sheep red blood cells incubated in control guinea pig serum which contains none of the test

material, one can obtain a measure of the amount of complement inactivated by the test material. Lysis of the sheep red blood cell will directly relate to the assembly of the $C\overline{5b6789}$ complex on the surface of the cell. The ambiguity in the assay is that the diminution of any complement component in the serum either by degradation or complexation will result in the attenuation of $C\overline{5b6789}$ complex formation. As an example, if the substance under study were to degrade C6 without affecting any other complement components, then a decrease in cell lysis might be observed, though this observation would not directly relate to biological complement activation.

This chapter will describe an assay system which distinguishes effects on the complement cascade preceding and succeeding the activation of C3. This new double assay system will be used to more narrowly localize the activity observed for oligomers of IgG produced by chemical crosslinking.

MATERIALS AND METHODS

Classical Pathway Assay

The assay of complement fixation by the classical pathway was adapted from the method of Bengali *et al.* (1980). Antigen-antibody complexes or oligomers of crosslinked immunoglobulin were diluted into a buffer of 0.01 M Hepes, 0.15 M NaCl, 0.00015 M $CaCl_2 \cdot 2H_2O$, 0.0005 M $MgCl_2 \cdot 6H_2O$,

pH 7.4 with 0.1% gelatin (w:v) [Hereafter referred to as classical buffer]. Guinea pig serum was diluted 30-fold with classical buffer and 10 µl of this complement source were incubated in wells of a microtitre plate with 65 µl of the immunoglobulin solution for 45 minutes at 37°C in a Lab-line Incubator which maintained a constant temperature, humidity, and atmospheric CO_2 content. Then 50 µl of a 2% suspension of sensitized sheep red blood cells (cells and hemolysin from Flow Labs Inc.) which had been loaded with ^{51}Cr-Na_2CrO_4 were added to each well and this suspension was incubated for an additional 45 minutes at 37°C. After centrifuging the microtiter plate at 1000 x g for 3 minutes at 4°C, 40 µl aliquots of supernatant were removed and counted on a Beckman 4000 Gamma counter. The zero percent fixation level was determined by the incubation of guinea pig serum in classical buffer for 45 minutes at 37°C prior to the addition and incubation of 50 µl of the 2% sensitized sheep red blood cell suspension. This control showed that the concentration of guinea pig serum was sufficient to lyse 92% of the total number of cells added. The background for 100% fixation was established by incubating 50 µl of the 2% erthrocyte suspension in 75 µl of classical buffer and counting 40 µl of this supernatant. Other controls were preincubation of monomeric IgG in the absence of antigen with guinea pig serum and preincubation of antigen with guinea pig serum prior to the addition of sensitized erythrocytes.

Each antibody-antigen concentration, oligomer concentration, and control were assayed in quadruplicate.

Combined Classical-Alternate Pathway Assay

This assay was based on the classical pathway assay of Bengali *et al.* (1980) and the alternate pathway assay of Riches and Stanworth (1980). The source of complement was human serum diluted by a factor of 2.5 with classical buffer. Rabbit erthrocytes obtained under sterile conditions by cardiac puncture were repeatedly washed and suspended in a buffer of 0.01 M Hepes, 0.15 M NaCl, 0.007 M $MgCl_2 \cdot 6H_2O$, 0.02 M EGTA pH 7.2 with 0.1% gelatin [hereafter referred to as alternate buffer] and loaded with $^{51}Cr\text{-}Na_2CrO_4$ in the same manner as was used for sheep erythrocytes. Antigen-antibody complexes, oligomers of crosslinked immunoglobulin G, monomeric antibody, and antigen were diluted to various concentrations with classical buffer. Then to each well of a microtitre plate was added 50 µl of the immunoglobulin solution followed by 50 µl of the diluted human serum. The resulting activation solution was incubated for 75 minutes at 37°C continually maintaining constant temperature, humidity, and atmospheric CO_2 content. Then 25 µl of a 2% suspension of rabbit red blood cells were added and a second incubation at 37°C was performed for 45 minutes. Sensitized sheep erythrocytes (25 µl of an 8% suspension in classical buffer) were added to wells which corresponded to each experimental condition assayed for by the rabbit erythrocytes

and these suspensions were also incubated for 45 minutes. Following the second incubation period the microtitre plates were centrifuged at 1000 x g for 3 minutes at 4°C and 40 µl aliquots of the supernatant removed and counted as previously described. Control conditions like those included for the classical pathway assay were included in this assay and the zero percent fixation control showed 90% lysis of rabbit erythrocytes under these conditions and 73% lysis of the sensitized sheep erythrocytes.

Time Course of Classical Pathway Complement Fixation

Guinea pig serum was diluted as described in the Classical Pathway assay. The diluted guinea pig serum (0.27 ml) was incubated with 1.73 ml of 29B1 dimer (1.1 mg/ml) or 29B1 IgG trimer (0.06 mg/ml) at 37°C. Then at various times 75 µl aliquots of this solution were added to 50 µl of a 2% suspension of sheep erythrocytes in classical buffer which had been loaded and sensitized as described for the Classical Pathway assay. The erythrocyte suspension was incubated at 37°C for 45 minutes, centrifuged at 1000 x g for 3 minutes at 4°C and 40 µl of supernatant were removed and counted.

Crosslinking IgG

Immunoglobulin G from the hybridoma 29B1 (Oi and Herzenberg, 1979) was produced and isolated by the method described in Chapter 3 of this thesis. The procedure used to crosslink 29B1 IgG was a modification of "Method B" by

Wright *et al.* (1980b). Immediately after the crosslinking reaction had proceeded for two hours, the protein-reagent solution was chromatographed on Sephadex G-25 (5.0 x 20 cm) eluted with 0.15 M NaCl, 0.02 M NaH_2PO_4, pH 7.4 in order to rapidly separate the immunoglobulin from any unreacted reagent. Then the protein solution was applied to an LKB Ultragel AcA 34 column (2.5 x 120 cm) and the protein was eluted with the same saline-phosphate buffer used for the previous G-25 column. After separately pooling monomeric and dimeric IgG fractions, the protein peak which contained trimeric and larger oligomers was applied to an LKB Ultragel AcA 22 column (2.5 x 140 cm) and the same saline-phosphate buffer was again used as the eluent. The dimer was rechromatographed on the Ultragel AcA 34 column and trimer was rechromatographed on the Ultragel AcA 22 column. The purity of these oligomers was determined by SDS electrophoresis on a 4% acrylamide slab gel which was stained with coomassie blue to visualize the proteins. The dimer and trimer were stored at -70°C until used in the complement assay; the oligomers had previously been shown to be stable for as long as two months under these conditions (Wright *et al.*, 1980a).

Synthesis of DNP-antigens

DNP_1-cytochrome C and DNP_{30}-BSA were prepared as described in Chapter 3.

RESULTS

Classical Pathway Complement Assay of 29Bl IgG Dimer and Trimer

A 4% acrylamide gel of 29Bl monomer, dimer, and trimer showed some contamination of dimer with trimer and trimer with tetramer. The yields of dimer, trimer, and higher oligomers were 20%, 8%, and less than 4%, respectively. The classical pathway complement assays using guinea pig serum are shown in Figure 17 for 29Bl IgG dimer and Figure 18 for 29Bl IgG trimer. The 50% fixation point for the dimer occurred at an immunoglobulin concentration of 9.5×10^{-6} M while the same level of fixation was achieved for the trimer at 2.0×10^{-6} M. It should be made clear that the amount of immunoglobulin required to achieve 50% fixation varied by as much as 20% from preparation to preparation. When the oligomers were reduced with dithiothreitol by a published procedure (Wright *et al.*, 1980b), no fixation was observed over the same range of immunoglobulin concentrations.

Classical-Alternate Pathway Combined Assay of 29Bl Oligomers

The results of the combined assay are shown in Figure 19 for 29Bl IgG dimer and Figure 20 for 29Bl IgG trimer. As the concentration of either oligomer increased, the lysis of sensitized sheep red blood cells decreased but no change occurred in the level of alternate pathway lysis of rabbit red blood cells. When the same assay system was applied to

antigen-antibody complexes, a concomitant decrease in both sheep and rabbit erythrocyte lysis was observed. Figure 21 shows the complement fixation of 6.7×10^{-7} M IgG with increasing amounts of DNP_{30}-BSA and Figure 22 plots the same results for an IgG concentration of 6.7×10^{-6} M. At the antigen concentrations used in this assay, DNP_{30}-BSA alone did not cause observable fixation. To insure that no lysis by the classical pathway was observed for rabbit erythrocytes in alternate buffer, an 8% suspension of sensitized sheep erythrocytes in alternate buffer was added to the zero fixation control wells and less than 2% lysis of these cells occurred. The oligomers of immunoglobulin were assayed over the same range of DNP_{30}-BSA concentrations that was used for monomeric 29Bl IgG. The alternate pathway lysis of rabbit red blood cells under these conditions is shown in Figures 23 and 24. Oligomers of 29Bl IgG are capable of diminishing lysis of rabbit red blood cells when further complexation of the oligomers by polyvalent antigen has occurred. In the presence of a large excess of DNP_1-cytochrome C (2×10^{-3} M) the oligomers did not show any significant decrease in the amount of rabbit red blood cells lysed by human serum as seen in Figures 25 and 26.

Time Course of Classical Pathway Complement Fixation by Oligomers of 29Bl IgG

The fixation of guinea pig serum as a function of time by dimers of 29Bl IgG is shown in Figure 27. A similar result was obtained for 29Bl IgG trimer as seen

in Figure 27. The zero percent fixation control showed that guinea pig serum diluted with classical buffer does not spontaneously degrade at 37°C. Both oligomers produce, over a period of hours, continuous decreases in the ability of the incubated guinea pig serum to lyse sensitized sheep erythrocytes.

DISCUSSION

A greater amount of 29Bl IgG oligomer was required to fix guinea pig serum complement in the classical pathway assay of this work as compared to oligomers of rabbit IgG by the assay system of Levine and van Vunakis (Wright *et al.*, 1980b). Several factors probably contribute to this difference. Among these factors are: (1) the possibility that the species differences between rabbit IgG and mouse IgG_{2a} give rise to differing abilities to fix guinea pig complement, and (2) the time and temperature of the preincubation in the absence of erythrocytes are different in these two assays.

The combined classical-alternate pathway assay simultaneously gauges the cytolytic ability of the C1-C9 system and of the C3-C9 system after preincubation of the test material with human complement. The results of this assay on oligomers of 29Bl IgG show an effect on some component or components of the classical pathway by these oligomers. Furthermore, the component affected does not

seem to participate in the alternate pathway which focusses attention on C1, C2, and C4 as the components which might be affected by oligomers of 29B1 IgG. The positive control of this assay which uses antigen-antibody aggregates insures that an observed fixation of the C1-C9 system by a biologically competent complex is concomitant with fixation of the C3-C9 system. Additionally the assay of the complexes comprised of antigen and chemically crosslinked IgG indicates that the immunoglobulin has not become impotent as a result of chemical modification. However, no increase in the inactivation of the C3-C9 system was observed for oligomers of 29B1 IgG in the presence of the monovalent antigen DNP_1-cytochrome C.

The apparent fixation of the classical pathway system by 29B1 IgG oligomers could arise from either binding some combination of components C1, C2, and C4 thereby decreasing their contribution to cytolysis; or activating the first component in a manner resembling the system in vivo but failing to achieve the formation of $C\overline{}_{14b2a}$; that is C3 convertase, thereby leaving the components involved in the alternate pathway unchanged. The time course of classical pathway fixation gives more credence to the latter hypothesis. If complement components were merely being sequestered then one would expect a plateau in the time course of fixation once the binding equilibrium had been established. But even in the case of the trimer followed for four hours, the

remaining cytolytic capability of the solution continues to decrease. This continued decline probably results from the enzymatic proteolysis, activation, and spontaneous inactivation of components in the cascade preceeding the formation of C3 convertase.

The evidence obtained from analytical ultracentrifugation that oligomers of rabbit IgG bind Clq seems reliable. However the binding of Clq and even the production of $C\overline{1}$ should not be considered equivalent to activation of the cascade. The work of Allan and Isliker (1974) found that tryptophan derivatization may be manipulated in such a way as to considerably reduce complement activation by rabbit IgG while only slightly diminishing Clq binding by the modified IgG. The Dodds and Porter study (1979) found that aggregates produced by heat denaturation activated Cl which went on to proteolyze C4, but at a rate much less than that of antigen-antibody complexes. These authors speculated that the enzymatic activation of C3 was not achieved by the formation of C3 convertase but instead as executed by $C\overline{1}$ in a manner analogous to the activation of complement by proteases, such as trypsin, which are exogenous to the cascade (Arroyave, 1972).

The overall implication of the complement assays performed on oligomers of 29Bl IgG is that chemically crosslinked IgG does not closely duplicate the biological activity of antigen-antibody aggregates; therefore

mechanistic arguments based on studies of such synthetic oligomers should be cautiously formulated. While the process of IgG oligomerization is important to complement activation, it does not seem to be sufficient for the activation of complement.

One possible explanation for the functional difference between aggregates produced by chemical crosslinking versus those produced by antigenic crosslinking is that the antigen molds the orientation of C_1^- and IgG to proceed with activation. The covalent attachment of C4b to the Fd portion of IgG appears to be rather specific in that no attachment to the light chain has been observed (Campbell *et al.*, 1980). If antigen serves to provide the proper conformation and structural rigidity of the $IgG_nC_1^-$ complex, then the results of the complement assays of chemically crosslinked oligomers of IgG and such IgG oligomers in the presence of monovalent antigen described in this work are not surprising because neither system appears to provide structural rigidity.

A hypothesis of this sort requires the examination of the effects antigen has on the interaction of IgG not only with C1 but also those components succeeding C1 in the complement cascade. This broadened scope generates increased complexity in the formulation of a molecularly detailed mechanism for complement activation by the classical pathway.

REFERENCES

Allan, R. and Isliker, H., *Immunochem.* (1974) *11*, 243.

Arroyave, C.M., *Fed. Proc.* (1972) *31*, 659.

Bengali, Z.H., Das, S.R., and Levine, P.H., *J. Immunol. Meth.* (1980) *33*, 63.

Campbell, R.D., Dodds, A.W., and Porter, R.R., *Biochem. J.* (1980) *189*, 67.

Dodds, A.W. and Porter, R.R., *Mol. Immunol.* (1979) *16*, 1059.

Goers, J.W., Ziccardi, R.J., Shumaker, V.N., and Glovsky, M.M., *J. Immunol.* (1977) *118*, 182.

Hartman, C.T., Jr. and Glovsky, M.M., *Int. Arch. Allergy and Appl. Immunol.* (1981) *66*, 274.

Hoffmann, L.G., *Immunochem.* (1976) *13*, 737.

Ishizaka, K. and Ishizaka, T.J., *J. Immunol.* (1960) *85*, 163.

Ishizaka, K., Ishizaka, T., and Sugahara, T., *J. Immunol.* (1962) *88*, 690.

Levine, L. and van Vunaki, H., *Meth. Enzymol.* (1967) *11*, 928.

Metzger, H., *Adv. Immunol.* (1974) *18*, 169.

Oi, V.T. and Herzenberg, L.A., *Mol. Immunol.* (1979) *16*, 1005.

Riches, D.W.H. and Stanworth, D.R., *Immunol. Lett.* (1980) *1*, 363.

Thompson, J.J. and Hoffmann, L.G., *Immunochemistry* (1974) *11*, 431.

Wright, J.K., Tschopp, J., Jaton, J.-C., and Engel, J., *Biochem. J.* (1980a) *187*, 775.

Wright, J.K., Tschopp, J., and Jaton, J.-C., *Biochem. J.* (1980b) *187*, 767.

Figure 17. Classical Pathway Complement Assay of $(29Bl\ IgG)_2$

The ordinate is the % lysis of sensitized sheep erythrocytes by guinea pig serum which had been preincubated with varying amounts of $(29Bl\ IgG)_2$. The abscissa is the micromolar concentration of IgG.

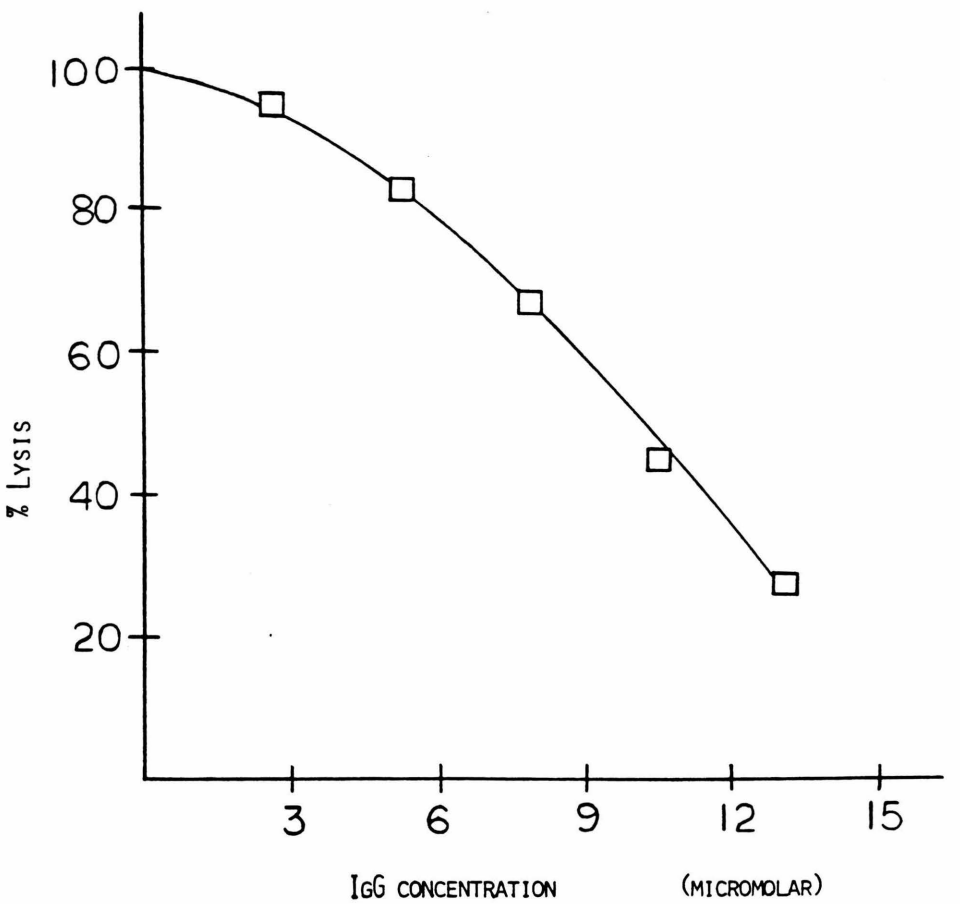

100
80
60
40
20
% Lysis
3
6
9
12
15
IgG CONCENTRATION
(MICROMOLAR)

Figure 18. Classical Pathway Complement Assay of $(29Bl\ IgG)_3$

The ordinate is the % lysis of sensitized sheep erythrocytes by guinea pig serum which had been preincubated with varying amounts of $(29Bl\ IgG)_3$. The abscissa is the micromolar concentration of IgG.

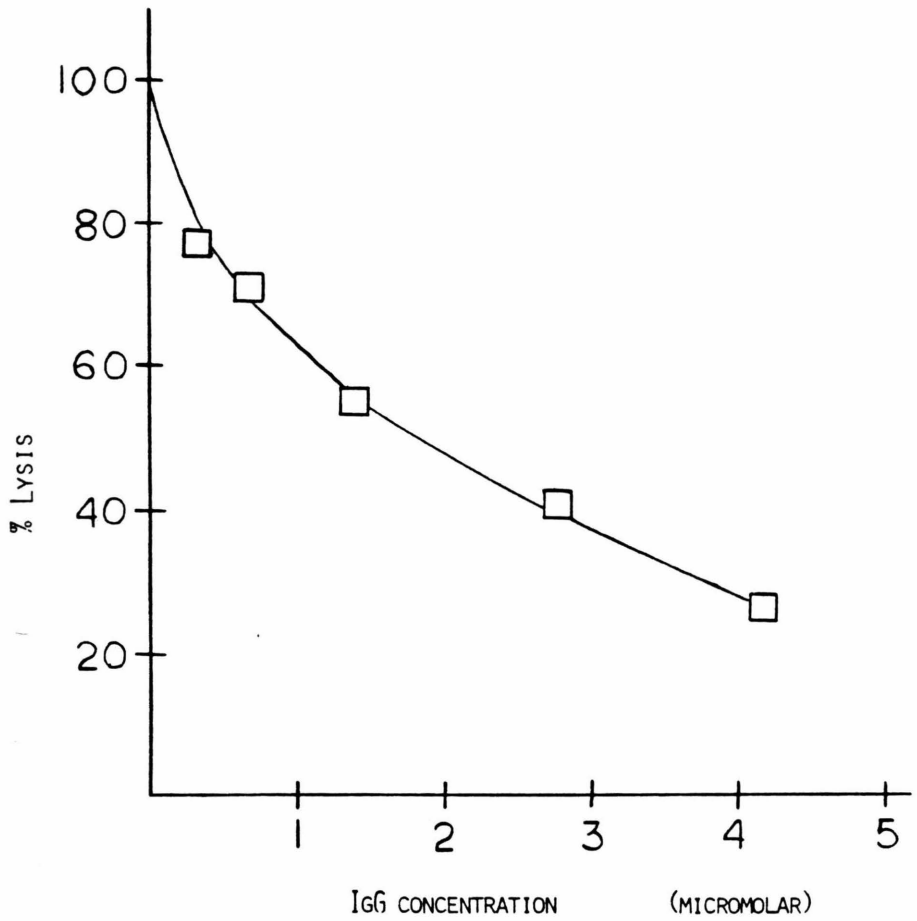

100
80
60
40
20
% LYSIS
1
2
3
4
5
IGG CONCENTRATION (MICROMOLAR)

Figure 19. Combined Classical-Alternate Pathway Assay for (29Bl IgG)$_2$

The ordinate is the % lysis of rabbit erythrocytes by the alternate pathway (O) or sheep erythrocytes by the classical pathway (□). The source of complement was human serum which had been preincubated in classical buffer with varying amounts of (29Bl IgG)$_2$. The abscissa is the micromolar concentration of IgG.

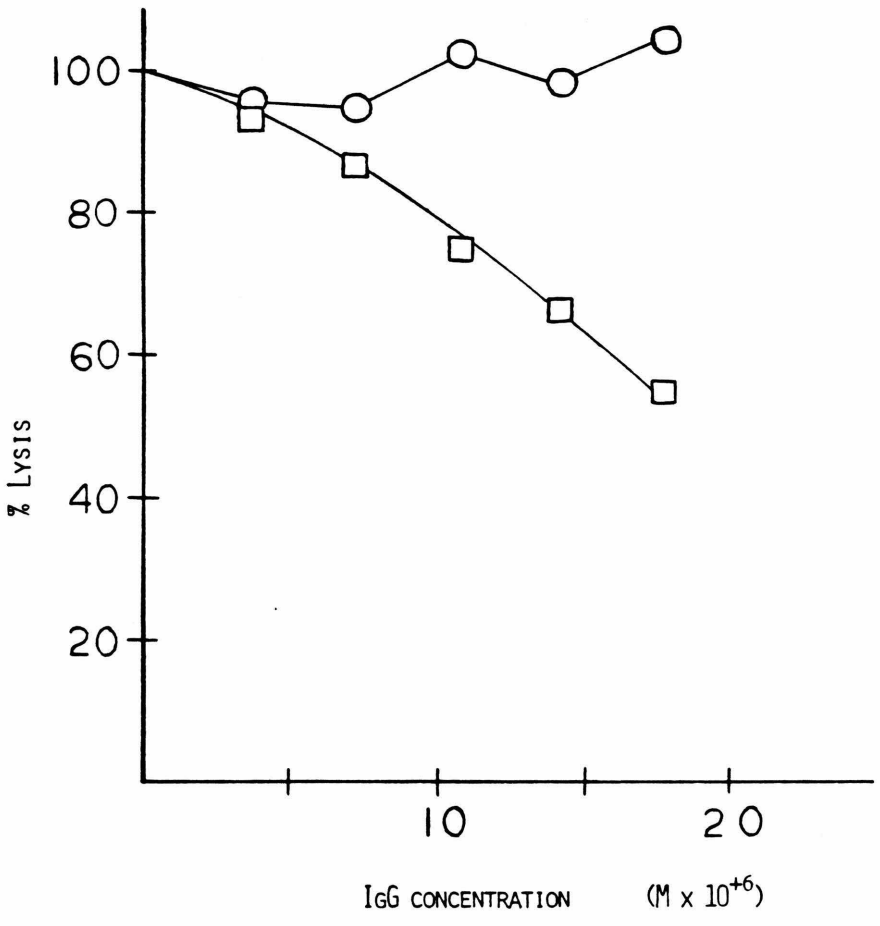

100
80
60
40
20
% Lysis
10
20
IgG CONCENTRATION (M x 10^{+6})

Figure 20. Combined Classical-Alternate Pathway Assay for $(29Bl\ IgG)_3$

The ordinate is the % lysis of rabbit erythrocytes by the alternate pathway (O) or sheep erythrocytes by the classical pathway (□). The source of complement was human serum which had been preincubated in classical buffer with varying amounts of $(29Bl\ IgG)_3$. The abscissa is 1×10^7 times the molar IgG concentration.

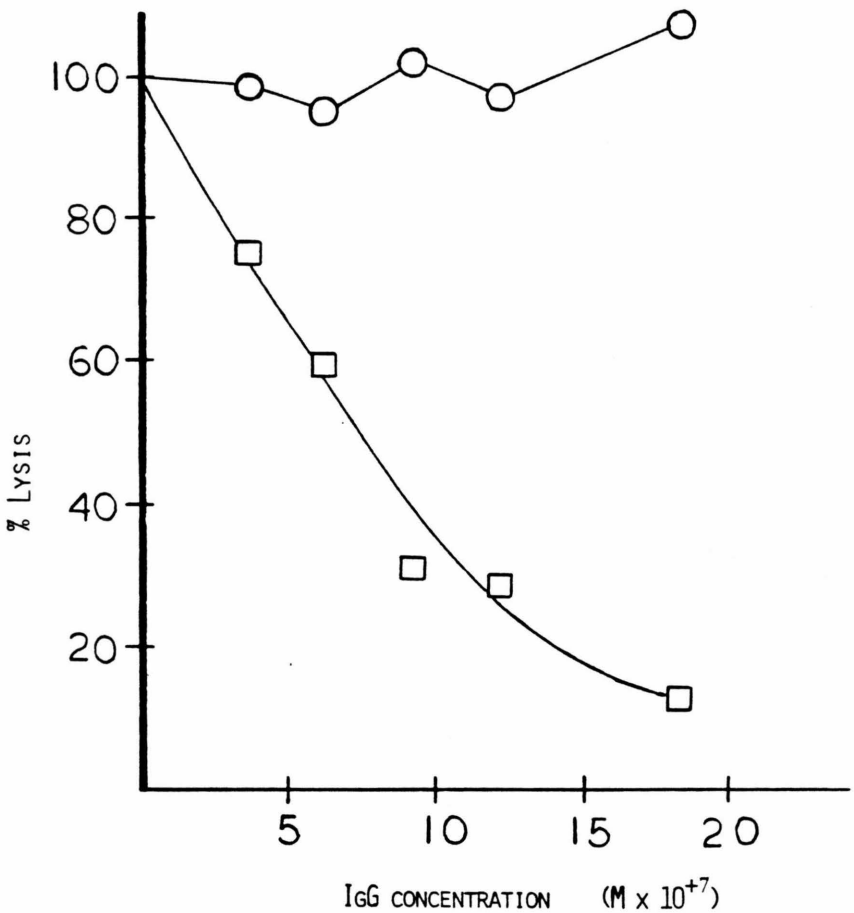

100
80
60
40
20
% LYSIS
5
10
15
20
IgG CONCENTRATION (M x 10^{+7})

Figure 21. Combined Classical-Alternate Pathway Assay of 29B1 IgG and DNP_{30}-BSA

The ordinate is the % lysis of rabbit erythrocytes by the alternate pathway (O) or sensitized sheep erythrocytes by the classical pathway (□). The source of complement was human serum which had been preincubated with 29B1 IgG (6.7×10^{-7} M) and varying amounts of DNP_{30}-BSA. The abscissa is the amount of DNP_{30}-BSA per well in micrograms.

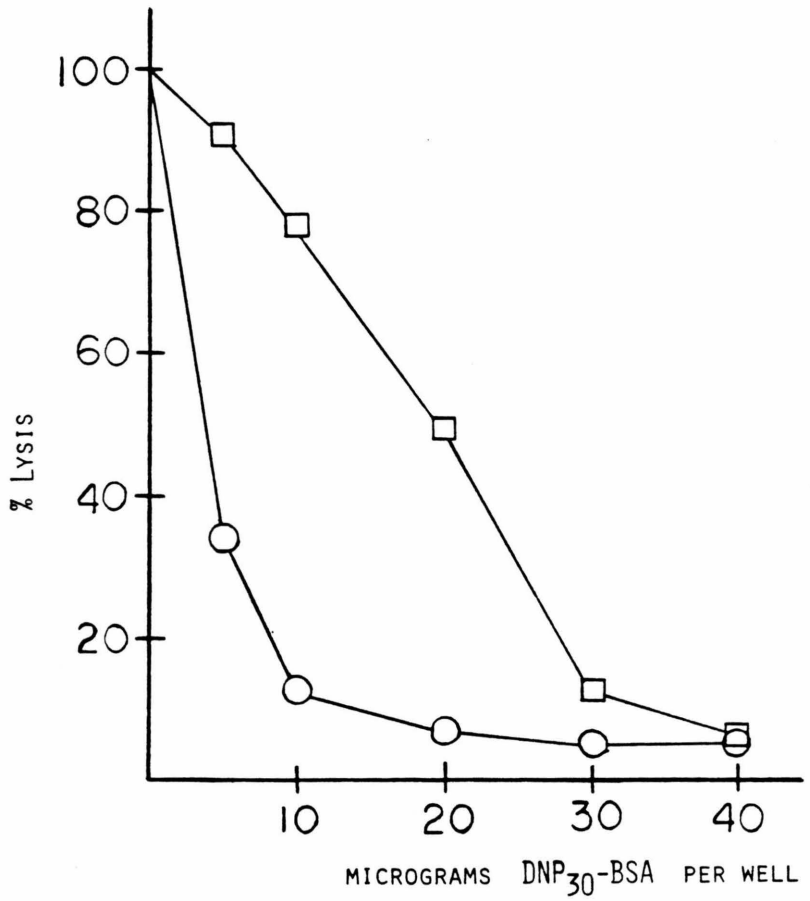

100
80
60
40
20
% LYSIS
10
20
30
40
MICROGRAMS DNP30-BSA PER WELL

Figure 22. Second Combined Classical-Alternate Pathway Assay of 29B1 IgG and DNP_{30}-BSA

The ordinate is the % lysis of rabbit erythrocytes by the alternate pathway (O) or sensitized sheep erythrocytes by the classical pathway (□). The source of complement was human serum which had been preincubated with 29B1 IgG (6.7×10^{-6} M) and varying amounts of DNP_{30}-BSA. The abscissa is the amount of DNP_{30}-BSA per well in micrograms.

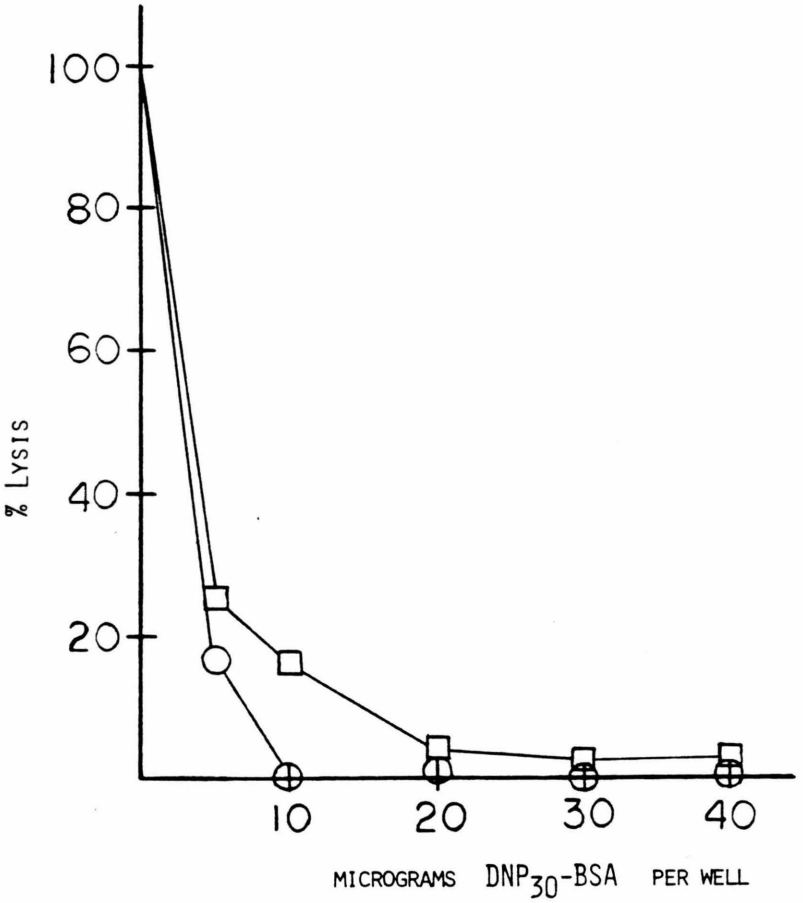

100
80
60
40
20
% LYSIS
10
20
30
40
MICROGRAMS DNP30-BSA PER WELL

Figure 23. Combined Classical-Alternate Pathway Assay of $(29B1\ IgG)_2$ and DNP_{30}-BSA

The ordinate is the % lysis of rabbit erythrocytes by the alternate pathway. The source of complement was human serum which had been preincubated in classical buffer with $(29B1\ IgG)_2$ and varying amounts of DNP_{30}-BSA. The IgG concentration for each well was 2.7 μM. The abscissa is the amount of DNP_{30}-BSA per well in micrograms.

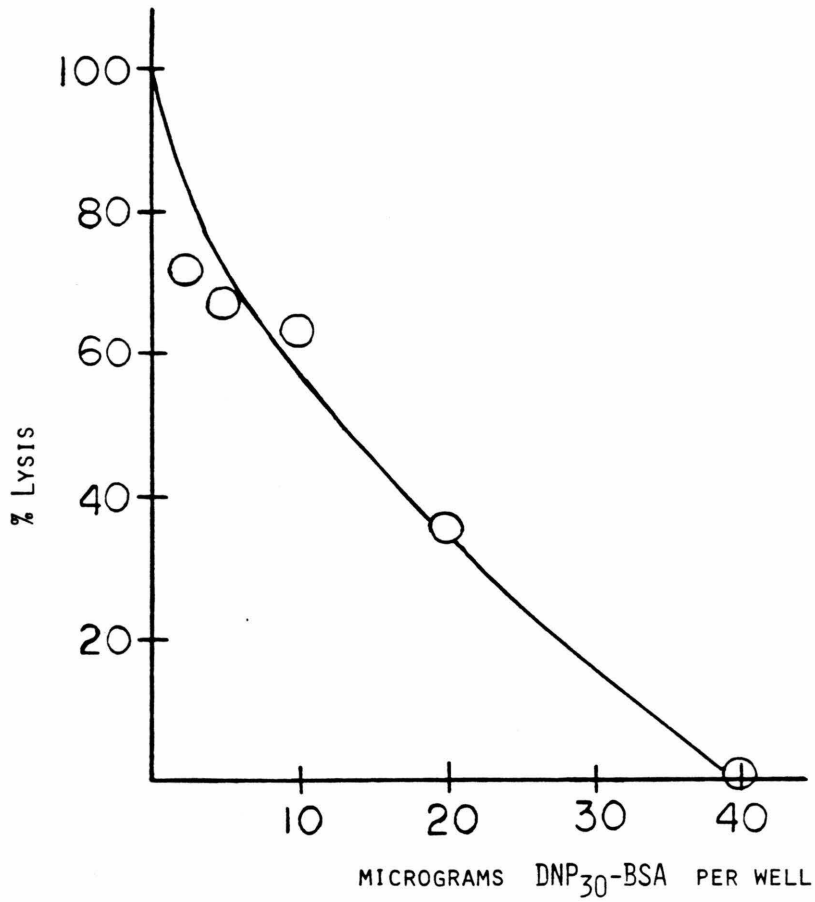

100
80
60
40
20
% LYSIS
10
20
30
40
MICROGRAMS DNP30-BSA PER WELL

Figure 24. Combined Classical-Alternate Pathway Assay of $(29Bl\ IgG)_3$ and DNP_{30}-BSA

The ordinate is the % lysis of rabbit erythrocytes by the alternate pathway. The source of complement was human serum which had been preincubated in classical buffer with $(29Bl\ IgG)_3$ and varying amounts of DNP_{30}-BSA. The IgG concentration for each well was 2.7 μM. The abscissa is the amount of DNP_{30}-BSA per well in micrograms.

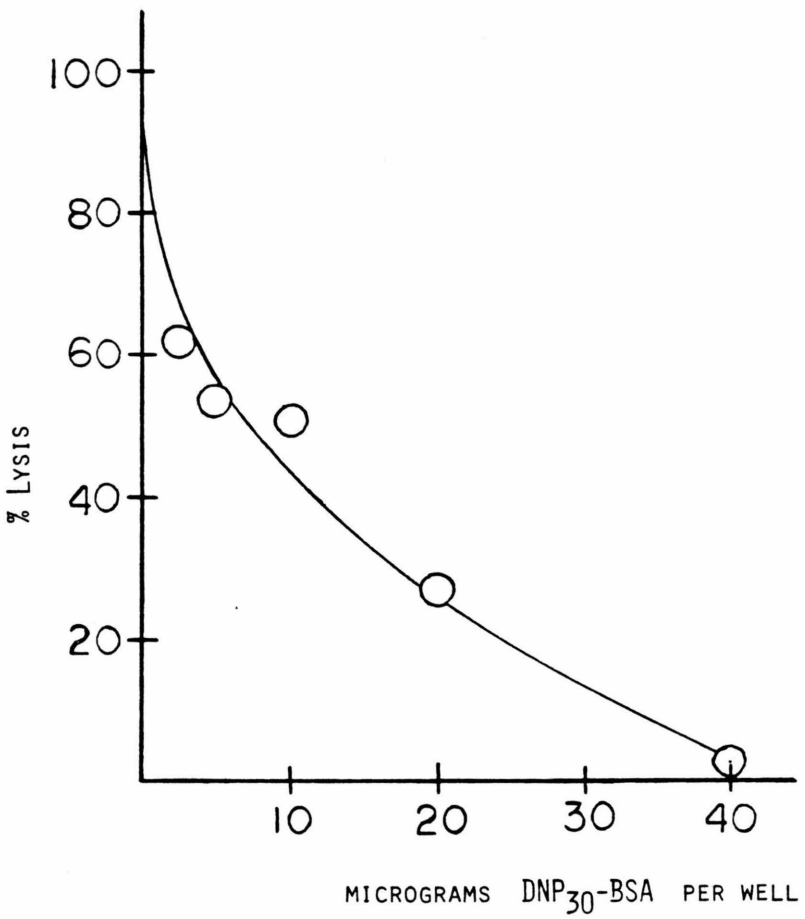

100
80
60
40
20
% LYSIS
10
20
30
40
MICROGRAMS DNP30-BSA PER WELL

Figure 25. Combined Classical-Alternate Pathway Assay of $(29Bl\ IgG)_2$ and DNP_1-cytochrome C

The ordinate is the % lysis of rabbit erythrocytes by the alternate pathway. The source of complement was human serum which had been preincubated in classical buffer with varying amounts of either $(29Bl\ IgG)_2$ (O) or $(29Bl\ IgG)_2$ and DNP_1-cytochrome C at a concentration of 2 mM (▽). The abscissa is the micromolar concentration of IgG.

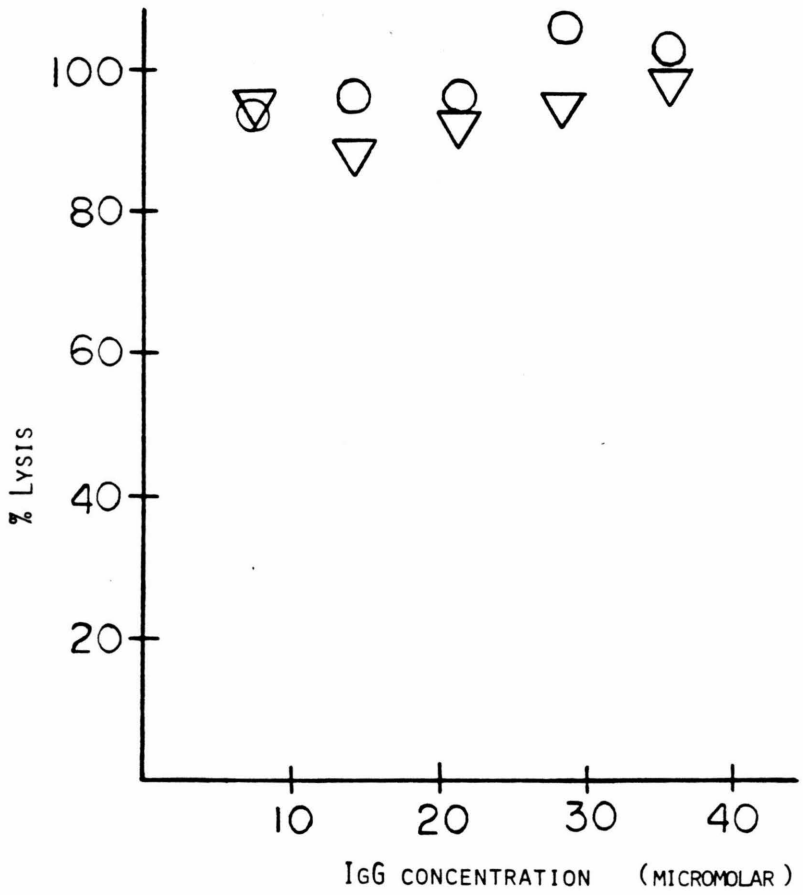

100
80
60
40
20
% LYSIS
10
20
30
40
IgG CONCENTRATION (MICROMOLAR)

Figure 26. Combined Classical-Alternate Pathway Assay of $(29B1\ IgG)_3$ and DNP_1-cytochrome C

The ordinate is the % lysis of rabbit erythrocytes by the alternate pathway. The source of complement was human serum which had been preincubated in classical buffer with varying amounts of either $(29B1\ IgG)_3$ (O) or $(29B1\ IgG)_3$ and DNP_1-cytochrome C at a concentration of 2 mM (▽). The abscissa is the micromolar concentration of IgG.

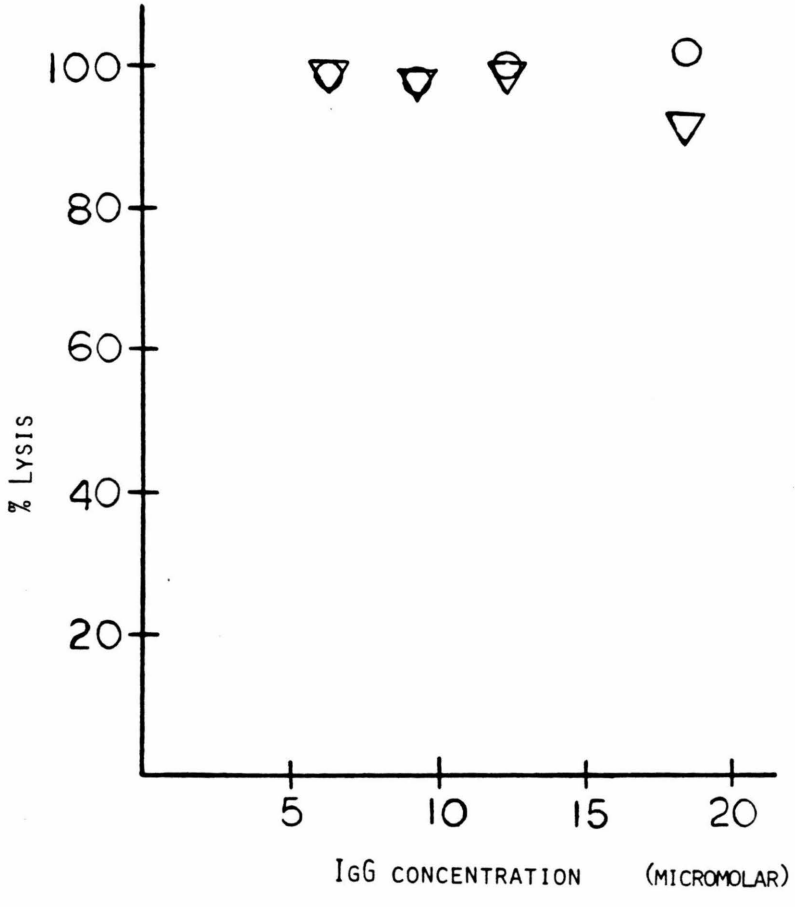

100
80
60
40
20
% LYSIS
5
10
15
20
IgG CONCENTRATION (MICROMOLAR)

Figure 27. Time Course of Complement Fixation by $(29Bl\ IgG)_2$

The ordinate is the % lysis of sensitized sheep erythrocytes by the classical pathway. The source of complement was guinea pig serum which had been preincubated with $(29Bl\ IgG)_2$ at a concentration of 11 μM (O) or 29Bl IgG and DNP_{30}-BSA at concentrations of 4.4 μM and 0.14 μM (□) respectively. Guinea pig serum which was incubated in classical buffer with no test material present was used to determine the 100% lysis level for each time point. The amount of lysis for these controls compared to lysis at t = 0 is shown (▽). The abscissa is the time of preincubation in hours.

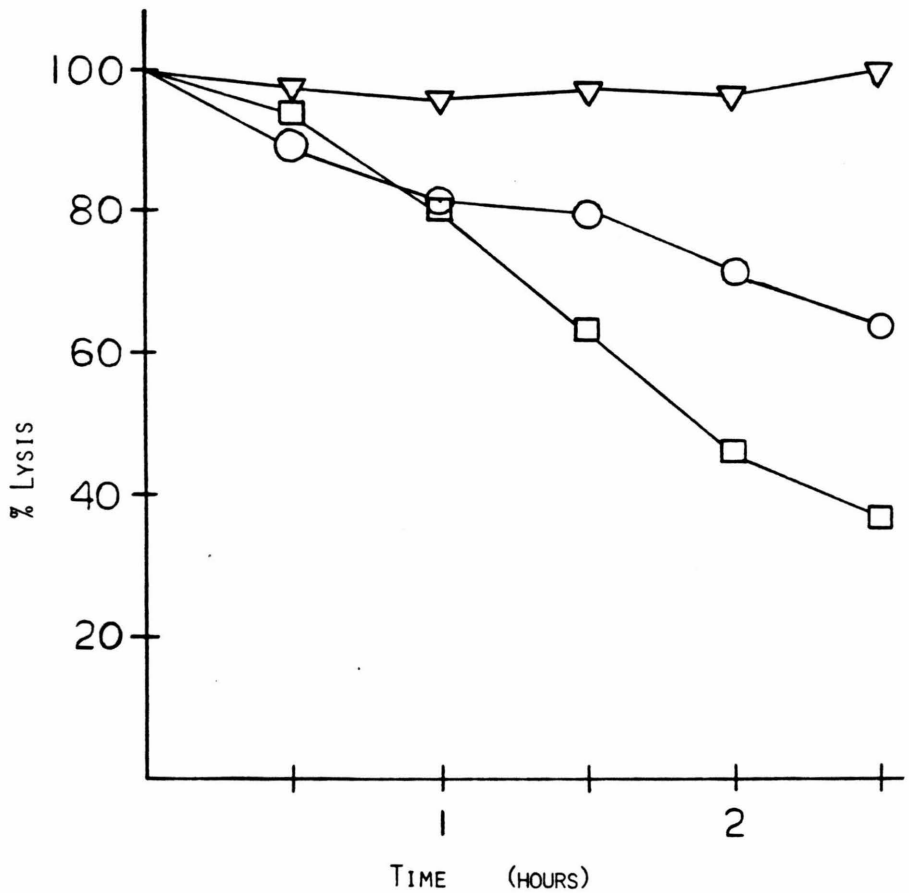
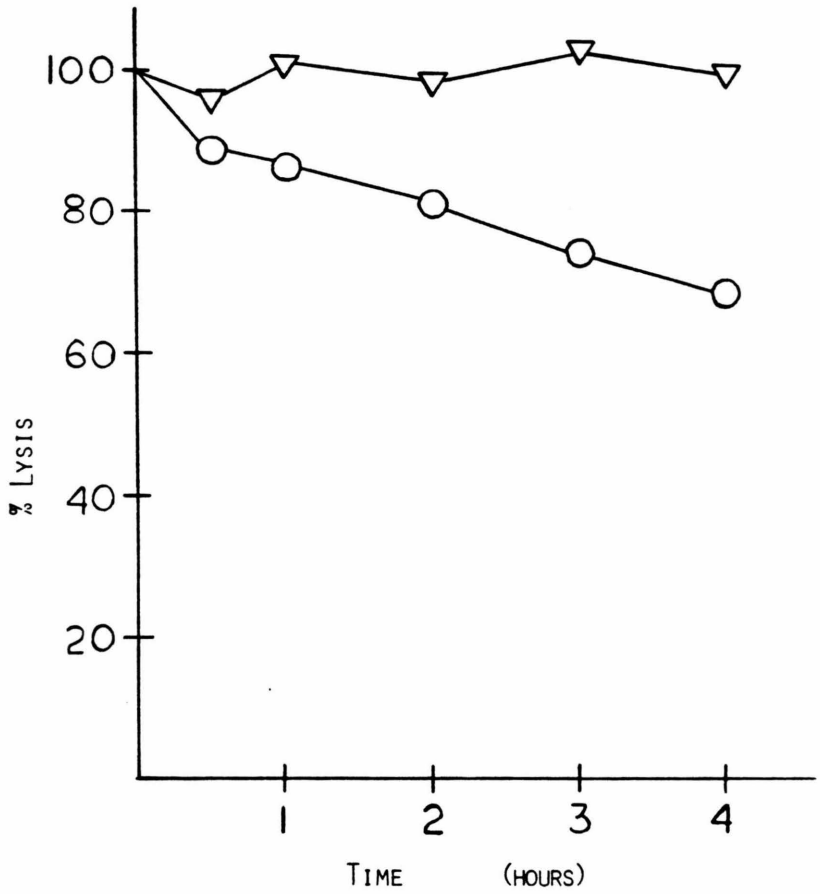

100
80
60
40
20
% Lysis
1
2
Time (hours)

Figure 28. Time Course of Complement Fixation by $(29Bl\ IgG)_3$

The ordinate is the % lysis of sensitized sheep erythrocytes by the classical pathway. The source of complement was guinea pig serum which had been preincubated with $(29Bl\ IgG)_3$ at a concentration of 0.32 μM (O). Guinea pig serum which was preincubated in classical buffer with no test material present was used to determine the 100% lysis level for each time point. The amount of lysis for these controls compared to lysis at t = 0 is shown (▽). The abscissa is the time in hours.


100
80
60
40
20
% Lysis
1
2
3
4
Time (hours)

Proposition I

Specific Fluorescence Quenching of Tryptophan 277 in IgG

Circularly polarized luminescence (CPL) has been used to study conformational changes in immunoglobulin G upon binding antigen (Schlessinger et al., 1975). One limitation of this study is that conclusions about the Fc region of IgG, which is the region that activates conplement, are inferred from differences between the CPL spectra of the whole immunoglobulin and the Fab fragment (Metzger, 1974). To overcome this objection, a method for observing Fc region fluorescence without the modfication of the protein's tertiary structure by enzymatic cleavage must be found.

The basis for such a method lies in the ability to selectively quench tryptophan residues in the Fc region of immunoglobulin G. Amino acid sequences of human and rabbit IgG (Edelman et al., 1969; Fruchter et al., 1970) show a tyrosine residue juxtaposed to tryptophan 277 (Eu numbering). This particular tryptophan has been implicated by several researchers (Johnson and Thames, 1976; Lee and Painter, 1980) as being the site of C1q binding or as being relevant to complement activation (Allan and Isliker, 1974). This is the only trp-tyr sequence which has been observed in the Fc region. Iodination of this tyrosine may quench the fluorescent emission of the tryptophan. When tryptophanyl tyrosine dipeptides are iodinated by the method of Azari and Feeney (1961), the tryptophan fluorescence is quenched to a large extent (figure 1). Presumably this quenching arises from the large p orbitals of iodine providing a facile mechanism for inter-system crossing and the subsequent radiationless transition from the triplet state to the ground state (Schulman, 1977). If such

quenching is observed in the protein then difference spectra between native IgG and iodinated IgG should provide a means for observing those tryptophan residues that are quenched. Thus the only chemical modification necessary for this observation is the iodination of tyrosine 278 in IgG.

The possible shortcomings of this technique stem from two experimental considerations. First if only two tryptophans are quenched per IgG, then the difference between native and iodinated IgG will be small; thereby requiring high sensitivity to be observed. This problem can be overcome by current signal averaging techniques (Longworth et al., 1976). Secondly sequence variability in IgG may lead to additional tryptophans which are juxtaposed to tyrosines. Definitive results would require that the difference spectra be consistent in the dependence on antigen binding for several IgG-antigen systems.

Allan, R. and Isliker, H., *Immunochem.* (1974) *11*, 243.

Azari, P.R. and Feeney, R.E., *Arch. Biochem. Biophys.* (1961) *92*, 44.

Edelman, G.M., Cunningham, B.A., Ball, W.E., Bottlieb, P.P., Rutishauser, U., and Waxdal, M.J., *Proc. Natl. Acad. Sci. USA* (1969) *63*, 78.

Fruchter, R.G., Jackson, S.A., Moles, L.E., and Porter, R.R., *Biochem. J.* (1970) *116*, 249.

Johnson, B.J. and Thames, K.E., *J. Immunol.* (1976) *117*, 1491.

Lee, J.P. and Painter, R.H., *Mol. Immunol.* (1980) *17*, 1155.

Longworth, J.W., McLaughlin, C.L., and Solomon, A., *Biochemistry* (1976) *15*, 2953.

Metzger, H., *Adv. Immunol.* (1974) *18*, 169.

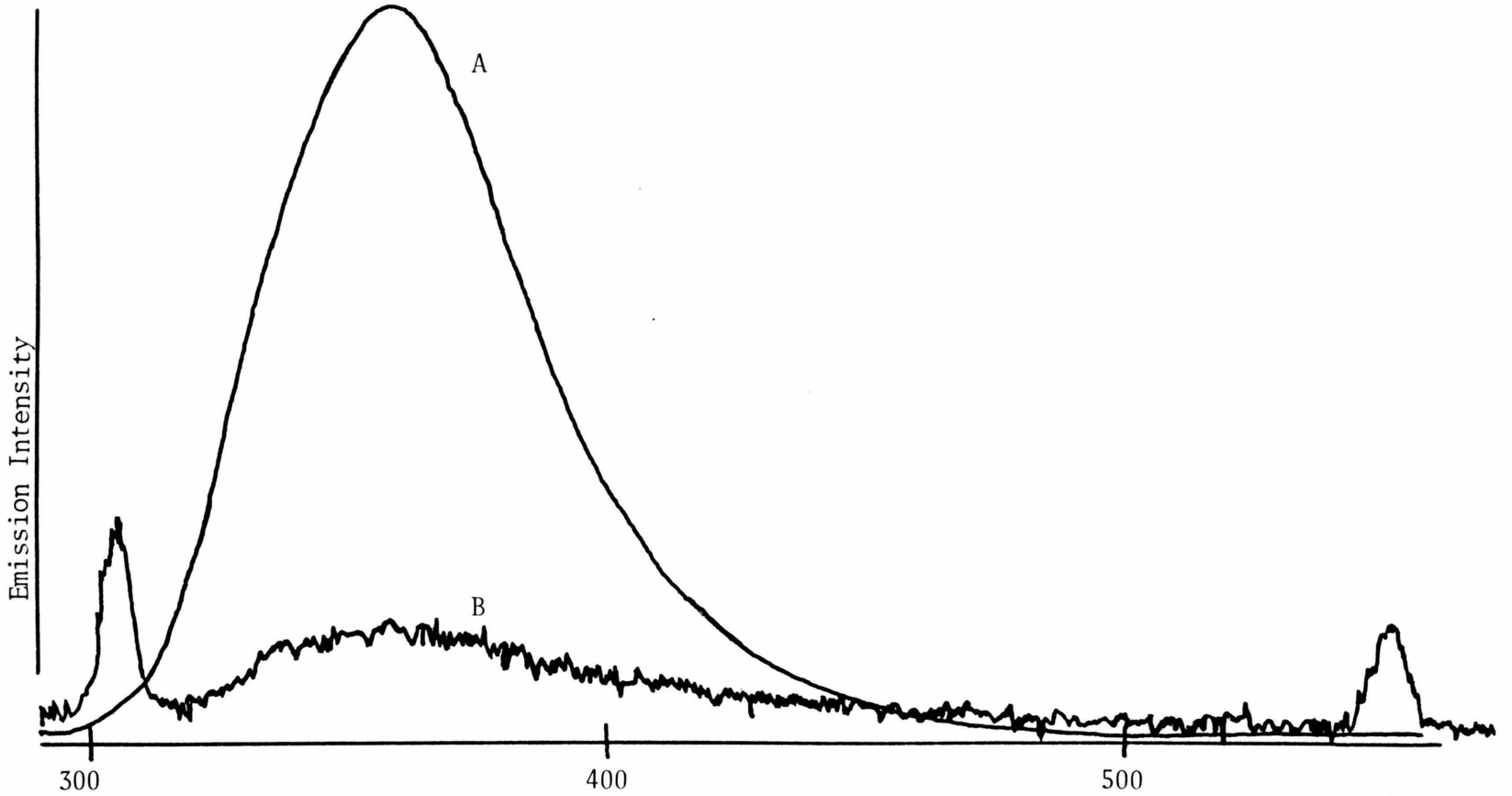
Schlessinger, J., Steinberg, I.Z., Givol, D., Hochman, J., and Pecht, I., *Proc. Natl. Acad. Sci. USA* (1975) *72*, 2775.

Schulman, S.G., *"Fluorescence and Phosphoresence Spectroscopy: Physicochemical Principles and Practice"*, Pergamon Press: Oxford (1977) p. 40.

Proposition I, figure 1.

Emission specra of Tryptophanyl tyrosine and Tryptophanyl diiodotyrosine

Tryptophanyl tyrosine (A) at a concentration of 1.55×10^{-5}M and tryptophanyl diiodotyrosine (B) at a concentration of 1.15×10^{-4}M in 0.10M borate buffer, pH 9.5 were irradiated at 295 nm. Spectrum B was amplified 100 times that of spectrum A.


A
B
Emission Intensity
300
400
500
Emission wavelength in nanometers

Proposition II

A Heterobifunctional Photoactivated Reagent with Specificity for Tryptophan

Several photoreactive heterobifunctional reagents are currently used for crosslinking proteins (Ji, 1979). These reagents have diazo or azido functionalities which, upon irradiation, can produce reactive carbenes or nitrenes. Typically the other functionality in the reagent is targetted for amino, carboxyl or sulfhydryl residues (Das and Fox, 1979). Koshland's reagent (Horton and Koshland, 1965) may provide the basis for a heterobifunctional photoactivated reagent which has a specificity for tryptophan. An azido group can be placed meta to the benzyl bromide group in the synthetic route outlined in figure 1. Several characteristics considered to be essential for the specificity of Koshland's reagent are retained in the photoactivated crosslinker. Among these characteristics are a hydroxyl group ortho to the benzyl bromide and a degree of hydrophobicity (Loudon and Koshland, 1970).

One protein which provides possible applications for 3-azido-2-hyroxy-5-nitrobenzyl bromide is immunoglobulin G. Allan and Isliker (1974) have shown that careful modification of two tryptophan residues in the CH_2 domain of IgG with Koshland's reagent only slightly decrease Clq binding while considerably decreasing complement activation. Evidence supporting the assignment of tryptophan 277 to the Clq binding site is reasonably strong (Johnson and Thames, 1976; Lee and Painter, 1980). If an immunoglobulin G modified with 3-azido-2-hydroxy-5-nitrobenzyl bromide in the dark is used in an aggregated form to bind Clq, then the photoactivation of this complex might link the IgG to the Clq. The resulting linkage would in itself be

Further evidence that tryptophan 277 is in the C1q binding site. Also subsequent enzymatic and acidic hydrolyses could elucidate which amino acid residues of C1q are involved in the interaction with IgG.

Presumably 3-azido-2-hydroxy-5-nitrobenzyl bromide would be sufficiently hydrophobic to be lipid soluble. This would allow the reagent to be used to chemically crosslink protein complexes in a cell membrane. However the retention of specificity for tryptophan by Koshland's reagent in a membrane would have to be demonstrated.

Allan, R. and Isliker, H., *Immunochem.* (1974) *11*, 243.

Buehler, C.A., Kirchner, F.K., and Deebel, G.F., *"Org. Syn. Coll. Vol."* (1967) *3*, 468.

Das, M. and Fox, C.F., *Ann. Rev. Biophys. Bioeng.* (1979) *8*, 165.

Horton, H.B. and Koshland, D.E., Jr., *J. Am. Chem. Soc.* (1965) *87*, 1126.

Ji, T.H., *Biochim. Biophys. Acta* (1979) *559*, 39.

Johnson, B.J. and Thames, K.E., *J. Immunol.* (1976) *117*, 1491.

Lee, J.P. and Painter, R.H., *Mol. Immunol.* (1980) *17*, 1155.

Loudon, G.M. and Koshland, D.E., Jr., *J. Biol. Chem.* (1970) *17*, 2247.

Mikkelson, R.B. and Wallach, D.R.H., *J. Biol. Chem.* (1976) *251*, 7413.

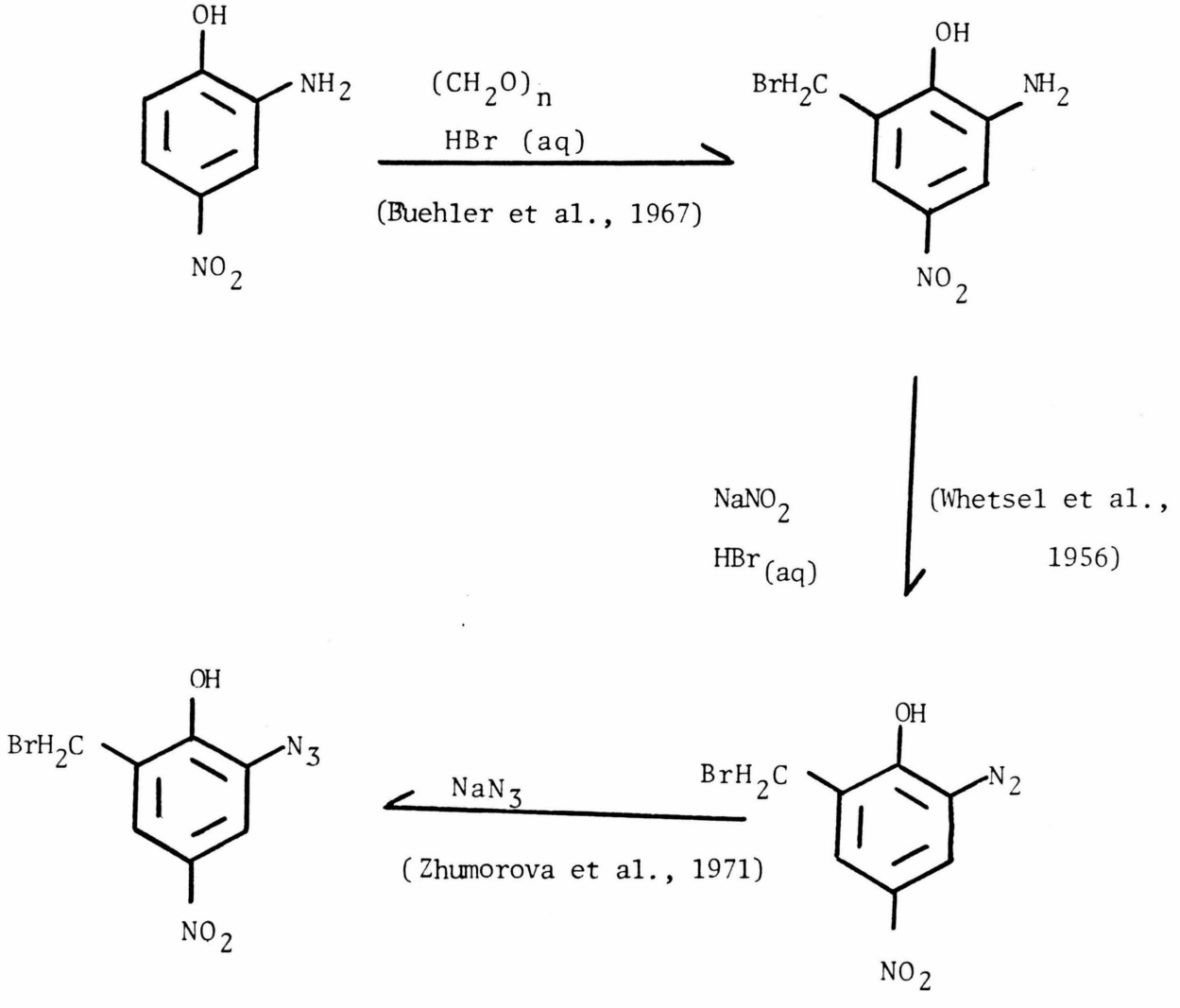
Toussaint, N.F. and Hennion, G.F., *J. Am. Chem. Soc.* (1940) *62*, 1145.

Whetsel, K.B., Hawkins, G.F., and Johnson, F.E., *J. Am. Chem. Soc.* (1956) *78*, 3360.

Zhumorova, I.N., Yurchenko, R.I., and Kirsanov, A.V., *Zh. Obsh. Khim.* (1971) *41*, 787.

Proposition II, figure 1.

Synthetic Route to 3-azido-2-hydroxy-5-nitrobenzyl bromide


OH
NH2
NO2
(CH2O)n
HBr (aq)
(Buehler et al., 1967)
OH
BrH2C
NH2
NO2
NaNO2
HBr(aq)
(Whetsel et al., 1956)
OH
BrH2C
N2
NO2
NaN3
(Zhumorova et al., 1971)
OH
BrH2C
N3
NO2

Proposition III

Concentration Dependence of Products
from Laser and Conventional Photolysis

It has recently been reported that the products from photolysis of diphenyldiazomethane with a laser are considerably different from those produced by photolysis with a conventional lamp (Turro et al., 1980). Although the production of fluorene from the diphenylcarbene is an internal conversion, the major product from the photolysis of diphenyldiazomethane with a conventional lamp is benzophenone azine (Reimlinger, 1964). The major products from laser photolysis are fluorene, 9,10-diphenylanthracene, and 9,10-diphenylphenanthracene (Turro et al., 1980). Previous work by flash photolysis gave tetraphenylethylene as the major product from diphenyldiazomethane and this result was the basis for the postulation of an equilibrium between the singlet and triplet states of diphenylcarbene (Closs and Rabinow, 1976).

However it is possible that the difference in the products from laser versus conventional photolysis is not related to the equilibrium between the singlet and triplet states, but is due to the concentration of intermediates during the course of the reaction. As diagrammed in figure 1, diphenyl carbene can react with diphenyldiazomethane to give the conventional azine (Zimmerman and Paskovich, 1964). If the intensity of the laser is sufficient to convert essentially all the diazo precursor into the carbene intermediate, then the major products would necessarily be derived from the interaction of carbenes and not the reaction of the carbene with the diphenyldiazomethane.

While an increase in diphenyldiazomethane concentration did not increase the amount of azine formed (Turro et al., 1980), the authors were attempting to keep the reagents in a range of concentrations where Beer's Law remained applicable. Presumably one could achieve

increased diphenyldiazomethane concentrations in the presence of diphenylcarbene by attenuating the laser output. Such attenuation could lead to the production of the benzophenone azine and thereby duplicate the results of conventional photolysis. If the condition of increased diphenyldiazomethane concentration in the presence of diphenylcarbene were to be achieved by increasing the diazo concentration then concentrations considerably greater than those obtained by Turro and his coworkers (1980) seem to be required.

Closs, G.L. and Rabinow, B.E., *J. Am. Chem. Soc.* (1976) *98*, 8190.

Reimlinger, H., *Chem. Ber.* (1964) *97*, 339.

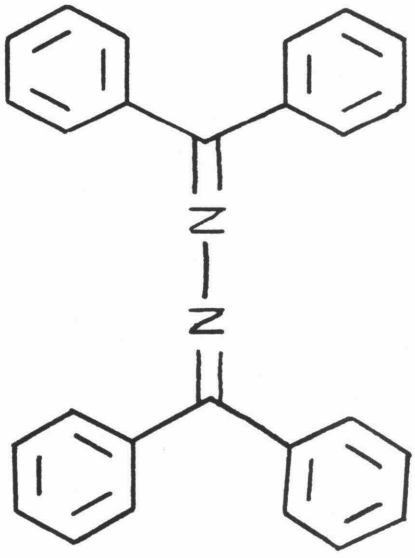
Turro, N.J., Aikawa, M., Butcher, J.A., and Griffin, G.W., *J. Am. Chem. Soc.* (1980) *102*, 5127.

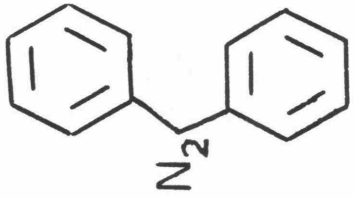
Zimmerman, H.E. and Paskovich, D.H., *J. Am. Chem. Soc.* (1964) *86*, 2149.

Proposition III, figure 1.

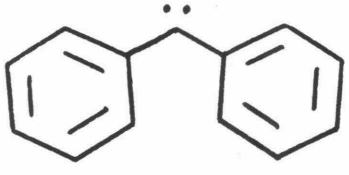
Reaction of Diphenylcarbene with Diphenyldiazomethane

1

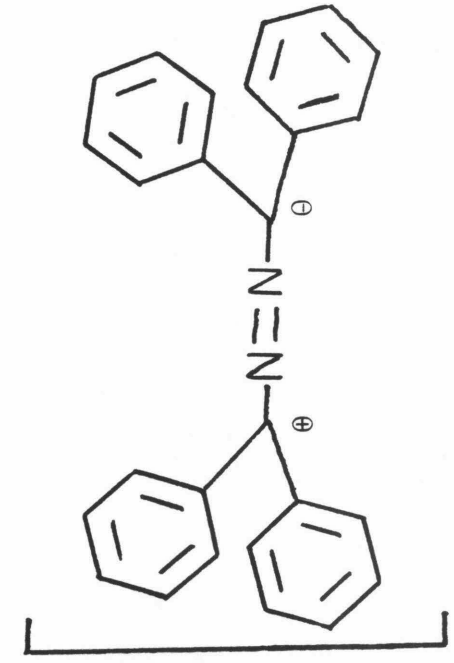




+



1



Proposition IV

Specific Protein Modification using a Polymeric Support

Because of their ease of handling, specificity, and ability to be regenerated; polymer-supported reagents have been used in organic synthesis for many years (Gainelli et al., 1981). The derivatization of proteins might be made more selective by having the reagent on a support and allowing the support to define the microenvironmental characteristics of charge, steric hindrance, and solvation. For example, the microenvironment of the support can be made more hydrophobic by introducing hydrocarbon chains in the monomer of the support (Dubin and Levy, 1981). The selectivity of a support might be made more specific if an affinity for a particular region of the protein to be derivatized can be imparted to the support.

Such affinity might be developed from the concept of surface simulation (Atassi, 1978). Surface simulation is the process of modeling the three dimensional structure of a protein by a linear sequence of amino acids. Atassi and his coworkers located the antigenic site of chicken lysozyme and then simulated this site with a polypeptide consisting of a linear sequence of those amino acids which appear to be juxtaposed in the tertiary structure of the protein. The resulting polypeptide cross-reacted with anti-lysozyme sera; thereby indicating that the three dimensional antigenic site was simulated by the linear polypeptide.

This technique of linear simulation could be used to give a polymeric support an affinity for a specific region of a protein. Given a system of protein to be derivatized (abbreviated PrD) and a second protein (abbreviated PrA), such as an antibody to or receptor for PrD; a surface analysis of PrA should lead to a poly-

peptide sequence which would likely have an affinity for PrD. Then by linking this linear polypeptide to the support of the modifying reagent, a specific region of PrD would be brought into close proximity with the reagent. A similar concept would apply to the derivatization of enzymes in the vicinity of binding sites by covalently linking substrate or cofactor analogs to the support.

If no PrA is known or for some reason cannot be analyzed, then surface complementarity (Atassi, 1978) might enable one to deduce an appropriate sequence for the linear polypeptide. An analysis of the three dimensional surface of PrD would lead to a linear polypeptide which simulates that surface. Then a complementary polypeptide might be made by matching positive charges of the original polypeptide to negative charges on the complementary polypeptide, negative charges on the orginal polypeptide to positive charges on the complementary polypeptide, hydrophobic residues to hydrophobic residues, and hydrogen bonding functionalities to hydrogen bonding functionalities. For example, Atassi found that the polypeptide FGKKNTD simulated the antigenic site of lysozyme. A complementary polypeptide might have W correspond to F, E correspond to K, and K or H correspond to D. Such a complementary structure might have an affinity specifically for the antigenic site of chicken lysozyme. To continue this example further, if one wished to specifically reduce the disulfide at the antigenic site [C(72)-C(94)] then a support such as styrene derivatized with 2-hydroxyphenol and the polypeptide WGEEAHH might provide the proper selectivity to reduce only this disulfide by virtue of the affinity of the complementary polypeptide for the antigenic site.

Atassi, M.Z., *Immunochem.* (1978) *15*, 909.

Dubin, P.L. and Levy, I.J., *Polymer Preprints* (1981) *22*, 132.

Gainelli, G., Manescalchi, F., and Contento, M., *"Organic Synthesis Today and Tomorrow"* (1981) *1*, 176.

Kun, K.A. and Cassidy, H.G., *J. Polymer Sci.* (1962) *56*, 83.

Proposition V

The Association of Lipids with the Terminating Complex of Complement

Several models for the mechanism of cytolysis by $C_{\overline{5b6789}}$ have been proposed. Three of these are: (1) the leaky patch model, (2) the doughnut model (Mayer, 1972) and (3) the localized disorder model (Esser et al., 1979). The leaky patch model proposes an enzymatic mechanism similar to phospholipase as a mechanism cytolysis. The doughnut model is based largely on electron microscopic evidence and concludes that lesions observed on the membrane are ionophoric channels which allow the transport of water and salts; thereby disrupting the membrane with osmotic pressure. The localized disorder model states that the lytically active $C_{\overline{5b6789}}$ complex has the ability to disorder the phospholipid bilayer in the vicinity of the complex. Esser and his coworkers (1975) observed such disordering in the ESR spectra of lipid spin-labels in the membrane and they speculated that disarrangement of the bilayer would be sufficient to degrade its integrity and allow lysis without invoking an enzymatic degradation of the phospholipid components of the membrane.

The association of detergent with C_{5b6789} in solution has been demonstrated (Podack et al., 1979) and a recent photolabeling study (Hu et al., 1981) indicates a similar association in membranes. There is a decided functional difference between C_{5b6789} in solution and $C_{\overline{5b6789}}$ in the membrane and this difference is that $C_{\overline{5b6789}}$ can be removed from the membrane of a lysed cell and will retain its lytic activity (Bhakdi et al., 1978) while a stable complex of C_{5b6789} with lipid formed in solution has no cytolytic capability (Podack et al., 1978) which indicates that a very stable

$\overline{C_{5b6789}}$ complex is formed in the membrane (Boyle and Borsos, 1980).

It is possible that the stability of the complex may extend beyond its protein components to include lipids from the membrane being lysed. In terms of the localized disorder model those lipids which are disarranged by $\overline{C_{5b6789}}$ are in strong association with the protein complex. One technique which could give insight into this possibility is a modification of the spin-label study which serves as a basis for the localized disorder model (Esser et al., 1979). If the concentration of the spin-label analog of lecithin is sufficiently high then the exchange rate will be sufficient to coalesce the triplet of the nitroxide radical to a single, broad peak. Then after isolating the $\overline{C_{5b6789}}$ complexes and introducing them into new membranes, the ESR spectrum of the new membranes would provide information as to the quantity of lipid transferred with the complex from the original membrane. Furthermore since the 1 cal concentration of spin-label would be high about the newly inserted complexes, the dissipation of rapid spin-exchange would provide a measure of the stability of the complex in the membrane by an analysis similar to that used for the dissipation of rapid spin-exchange by phospholipid diffusion (Devaux and McConnell, 1972). As the lipids in the original complex began to exchange with those in the new membrane the spin-exchange rate would decrease and the dinetics of the exchange of lipids could be followed.

Bhakdi, S., Bjerrum, O.J., Bhakdi-Lehnen, B., and Tranum-Jensen, J., *J. Immunol.* (1987) *121*, 2526.

Boyle, M.D.P. and Borsos, T., *Mol. Immunol.* (1980) *17*, 425.

Devaux, P. and McConnell, H.M., *J. Am. Chem. Soc.* (1972) *94*, 4475.

Esser, A.F., Kolb, W.P., Podack, E.R., and Müller-Eberhard, H.J., *Proc. Natl. Acad. Sci. USA* (1979) *76*, 1410.

Hu, U.W., Esser, A.F., Podack, E.R., and Wisnieski, B.J., *J. Immunol.* (1981) *127*, 380.

Mayer, M.M., *Proc. Natl. Acad. Sci. USA* (1972) *69*, 2954.

Podack, E.R., Kolb, W.P., and Müller-Eberhard, H.J., *J. Immunol.* (1978) *120*, 1841.

Podack, E.R., Biesecker, G., and Müller-Eberhard, H.J., *Proc. Natl. Acad. Sci. USA* (1979) *76*, 897.