STUDIES OF THE SPECIFICITY AND FUNCTION OF IMMUNOGLOBULINS

I. RELATIONSHIP BETWEEN STRUCTURE AND SPECIFICITY IN DINITROPHENYL-BINDING MOUSE MYELOMA IMMUNOGLOBULINS

II. EFFECTOR FUNCTION TRIGGERING IN IMMUNOGLOBULINS

Thesis by

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To

my parents,

Mr. and Mrs. George J. Hardy

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ABSTRACTS

Chapter 1:

This chapter presents the background to part I (chapters one through six) in which the structure of the antibody binding site and the specific interactions that occur between the site and antigen are considered.

Chapter 2:

The relation between structure and specificity of antibodies has been explored by ^{19}F NMR studies of the binding of trifluoromethyl analogues of nitrophenyl haptens to the three mouse myeloma immunoglobulins M315, M460, and X25. Haptens were used with trifluoromethyl groups located at the ortho or para positions of the phenyl ring or attached to the side chain, two atoms removed from the ring (i.e., $-NHCH_2CF_3$). The changes in chemical shift between hapten free in solution and bound to antibody are sensitive to microenvironment and range from 1.7-ppm downfield to 1-ppm upfield. The shifts of p-trifluoromethylnitrophenyl haptens bound to M315 and M460 are both large downfield shifts, which are likely caused by van der Waals interaction and ring-current effects, particularly from tyrosine-34 (L); these haptens do not show similar shifts when bound to X25 which has a deletion of tyrosine-34 (L). Other differences in the binding of the aromatic rings of haptens by M315, M460, and X25 are observed and their origins considered. The importance of hydrogen bonding in the thermodynamic affinity of antibody for hapten has been estimated by comparisons of binding affinities for haptens with trifluoromethyl groups in place of nitro groups.

Chapter 3:

A protein modification reagent, tetranitromethane, has been employed to specifically nitrate a binding site tyrosine residue in three nitrophenyl-specific myeloma proteins. The nitration does not significantly alter the affinity for hapten, which rules out a hydrogen bond from this tyrosine to the hapten. In the case of M315 and X25, ^{19}F resonances on labelled haptens are specifically perturbed from the native protein position, which is interpreted to indicate proximity of the reporter group and the modified tyrosine. A method is described for the introduction of reporter groups into the protein by a reduction of the nitrotyrosine to aminotyrosine.

Chapter 4:

Proton nuclear magnetic resonance has been employed to observe perturbations of methylated nitrophenyl haptens that take place on binding to three nitrophenyl-specific myeloma proteins. A comparison of the previously observed ^{19}F shifts from fluorinated haptens with these proton shifts permits the separation of the proton ring current shifts from the fluorine paramagnetic component. The paramagnetic component, which is large and different for each protein, is ascribed either to van der Waals crowding or to charge transfer interaction or a combination of the two.

Chapter 5:

The contribution of charge transfer to the ^{19}F paramagnetic shift has been examined by the use of model complexes. Hapten with tryptophan methyl ester model complexes allows determination of the total shift by comparison of methyl and trifluoromethyl shifts. This demonstrated that

the para-substituted shift was much larger than the ortho-substituted shift, which is similar to the result found in the protein study. A computer program was employed to determine the intensity and position of the charge transfer absorption band in these model complexes and with the three proteins. The intensity of the charge transfer interaction appears to correlate with the size of the ^{19}F paramagnetic shift. The difference in shift between the ortho- and para-substituted positions is attributed to the particular resonance structures that are important in binding. The association constants of the model complexes were employed to decompose the binding energies of several haptens for the three proteins, and this analysis indicated a hydrogen bond between hapten and antibody for M315 but not M460 or X25.

Chapter 6:

The nitration study (Chapter 3) indicated a particular tyrosine (Tyr-33H) was near the chain reporter group of the hapten when bound to M315. This result, in combination with the charge transfer data, was used to modify a model of the M315 binding site. A computer graphics system was employed to assemble the first heavy chain hypervariable loop with the tyrosine reoriented so as to fit next to the hapten. The resulting structure, when analyzed by a Diamond refinement energy minimization program, was more precisely fit to standard X-ray coordinates than the original structure.

Chapter 7:

This chapter presents background on effector function and effector function triggering, which is the subject of the final two chapters.

The importance of the integration of antigen binding with cellular effector function is described and several effector functions are mentioned. The two major theories of effector triggering are outlined.

Chapter 8:

Complement fixation studies on the nitrophenyl-specific myeloma protein A22 (and IgM) have shown that both mono- and poly-substituted antigens trigger fixation. This result contrasts with previous work on IgG that demonstrated that only antigens capable of forming complexes could trigger complement fixation. This apparent discord is resolved when one considers that the IgM is already aggregated (as compared to IgG) and so does not require further aggregation after the binding of antigen in order to fix complement. The binding of a single monosubstituted antigen per IgM was sufficient for triggering and the triggering could be competitively inhibited by hapten. The fixation did not occur through the alternate pathway. A DNP-specific hybridoma IgG_{2a} protein acted as a control such that, over the entire dilution range employed in this study, no complement was fixed with the monosubstituted antigen, while with polyvalent antigen considerable fixation occurred.

Chapter 9:

A cell culture line of MOPC 460 has been established by alternate growth in animals and in culture. A cell culture line of MOPC 315 has been established by somatic cell fusion of the MOPC 315 cells with a non-secreting myeloma that can grow in culture. Secreting cells were selected by a solid phase radioimmunoassay. Two hybridoma lines secreting DNP-specific IgG were also employed in this study. ^{13}C-enriched

tryptophan was incorporated into large-scale cultures of these cells and their secreted immunoglobulin was thereby enriched in ^{13}C tryptophan. The various proteins were isolated by affinity chromatography of the spent culture fluid and then studied by ^{13}C NMR. NMR of the isolated protein revealed an envelope of resonances near the position of free tryptophan, but shifted over a 10 parts-per-million range. Observations of the proteins with and without monovalent antigen showed large changes in the envelope for the IgG_{2a} protein (a complement fixing class), but only slight changes for the IgG_1 (a non-fixing class). Reduction of the IgG_{2a} produced large changes in the envelope as did papain fragmentation. The results generally support the concept that a conformational change is associated with antigen binding (at least in the IgG_{2a} subclass) and may constitute a major part of the triggering of effector function.

ABBREVIATIONS

ACD	acid-citrate-dextrose anticoagulant
Asn	asparagine
BSA	bovine serum albumin
CD	circular dichroism
DANS	dansyl hapten
$DANS_1$-BSA	monosubstituted dansyl BSA
$DANS_{20}$-BSA	polysubstituted dansyl BSA
DMEM	Dulbecca's modified Engle's medium
DMSO	dimethyl sulfoxide
DNP	2,4-dinitrophenyl hapten
DNP_1-BSA	monosubstituted dinitrophenyl BSA
DNP_{30}-BSA	polysubstituted dinitrophenyl BSA
DNP_1-LBTI	monosubstituted dinitrophenyl lima bean trypsin inhibitor
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-bis-(β-aminoethyl ether) N,N´-tetra-acetic acid
ESR	electron spin resonance
F_{ab}	antigen binding fragments derived from IgG papain digest
F_c	crystallizable fragment derived from IgG papain digest
$Folianic_1$-BSA	monosubstituted folianic acid BSA
gaba	gamma amino butyric acid
Hepes	N-2-hydroxyethylpiperazine-N´-ethanesulfonic acid
IgA	immunoglobulin class A
IgE	immunoglobulin class E
IgG	immunoglobulin class G

IgM	immunoglobulin class M
ip	intra-peritoneally
NMR	nuclear magnetic resonance
PBS	phosphate buffered saline
PEG	polyethylene glycol
Phe	phenylalanine
SDS	sodium dodecyl sulfate
TNP	2,4,6-trintrophenyl hapten
Trp	tryptophan
Tyr	tyrosine

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1

Background on Antibody Structure

The immune system is the means whereby an organism resists infection by a vast number of viruses, bacteria and fungi. Progress in the study of this system has revealed a complex set of soluble molecules (the humoral system) and membrane-bound molecules (the cellular system) both of which are integrated to produce the various immune responses mounted by an organism (Sercarz et al., 1974). Research in immunology touches on very fundamental aspects of biology: molecular recognition by antibody; cellular recognition and differentiation; the genetic mechanism of control and diversity of the response; and the origin of a number of disease states, possibly including cancer.

Historically the existence of antibodies has been known since the work of Emil von Behring in the late 1800's. A more molecular understanding of the antibody-antigen interaction dates from the early twentieth century when Karl Landsteiner (Landsteiner, 1945) demonstrated the existence of antigenic determinants, called "haptens", on the surface of larger molecules. Studies with a series of slightly modified haptens showed how specific antibodies were and also indicated that the repertoire of antibodies expressed by any organism must be very large. In the late 1950's Jerne and Burnet proposed the clonal selection hypothesis (Burnet, 1959) which states that a vast pre-existing library of antibodies is selected by antigenic challenge. The question of the origin of this diverse library is still a subject of intensive study currently being carried on at the DNA level.

The study of the antibody molecule was greatly facilitated by the work of Edelman and Porter in the late 1950's. Porter (Porter,1959) demonstrated that enzymatic cleavage of immunoglobulin produced two distinct fragments: an antigen-binding fragment (called F_{ab}) and a

crystallizable fragment (called F_c). Edelman (Edelman, 1959) showed that immunoglobulin was composed of two sets of chains termed the light (for lower molecular weight) and heavy (for higher molecular weight) chains. The discovery of its multichain structure coupled with its organization into binding and nonbinding regions (Figure 1a) catalyzed the study of antibody at the molecular level.

A major problem in the study of the immunoglobulin structure is the well-known heterogeneity of the response (Haber, 1968). In all but a few cases immunization with any given antigen produces a spectrum of antibody with a variety of sequences and a variety of binding affinities. This problem was solved by taking advantage of a malignant state of plasma cells, the cells which normally produce antibody in response to antigen (Potter, 1972). In multiple myeloma one of these cells undergoes transformation and proliferates in the bone marrow. The serum of a patient with this disease contains a large amount of immunoglobulin with a single sequence which is relatively easy to analyze. Comparison of the amino acid sequences of a number of these proteins from different patients established a remarkable fact about antibodies: the first 108 or so residues from the amino terminal varied from patient to patient, but the rest of the sequence (within any given class) was very similar (Hilschmann, 1965). Thus the terms "variable region" and "constant region" were coined (Figure 2b).

In the mid-1960's the next major step was taken in understanding immunoglobulin structure. Edelman and his colleagues completely sequenced a human IgG antibody (Edelman and Gall, 1969). This work confirmed the existence of variable and constant regions, but moreover demonstrated the existence of internal homologies within the constant

regions. The homology regions within the heavy chain constant region are termed C_H1, C_H2 and C_H3 (Figure 1c) and are also homologous with the light chain constant region, termed C_2. This observation led Hill and Singer (Hill et al., 1966; Singer and Doolittle, 1966) to propose that a primordial gene capable of specifying one homology region had been duplicated several times to give the present-day antibody coding genes.

Work by Milstein (Milstein and Munro, 1970) and Hood (Hood and Talmage, 1970) indicated that there were many variable region genes whereas previous work by Oudin (Oudin, 1960) indicated relatively few constant region genes. This fit in quite well with the hypothesis of Dreyer (Dreyer and Bennet, 1965) that both heavy and light chains are each coded for by two genes, a rather novel notion at that time. Recent work has shown the situation to be even more complex with various coding segments spliced together to give the final coding sequence of a heavy or light chain (Early et al., 1980).

Comparison of the large number of variable region sequences accumulated from human and mouse myeloma proteins showed a further important feature of immunoglobulin structure: the existence of regions within the variable region that were even more variable (Wu and Kabat, 1970). These "hypervariable regions" were three segments in both the heavy and light variable regions of three to ten residues each (Figure 2a). Because of their variability it was reasonable to suspect that these residues formed the combining site of the antibody, a suspicion that was later confirmed.

The most recent major advance in the study of antibody structure has been the analysis of a number of immunoglobulins and F_{ab} fragments by X-ray crystallography (Dickerson, 1964). Low resolution X-ray analysis (Poljak et al., 1972) confirmed the domain hypothesis of Edelman that

corresponding homology regions are associated into globular domains (Figure 1). Thus V_L and V_H form the combining site domain while L_c and C_H1 form the next domain which extends to the huge region separating the F_c and F_{ab} fragments (Figure 3b). The two C_H2 and C_H3 regions each associate to form the F_c domains, important in the so-called "secondary functions" of antibody considered further in part II.

High resolution X-ray studies of binding site domains of a number of myeloma proteins have established a very important concept, that of the "immunoglobulin fold" (Padlan, 1977). The domain is comprised of four segments of antiparallel beta pleated sheet and another layer of three segments (Figure 3a). The hypervariable segments are outside loops of this structure thus forming the binding site residues as predicted earlier. Depending on the antibody studied the site is either a cleft, a shallow groove or a pocket (Figure 3c). There is some indication that antibody to hydrophylic antigens such as polysaccharides have cleft- or groove-type sites while those to hydrophobic antigens such as DNP-conjugated proteins have pocket-type sites (Davies and Padlan, 1975; Winkelhake and Voss, 1970).

For many years, long before the structure of the antibody binding site was known, the chemical and physical properties of the site had been studied. Landsteiner's work (Landsteiner, 1944) mentioned previously was summarized in a book published near the end of his career. Pauling and Pressman (Pauling et al., 1944) brought a more rigorous physical chemical approach to the study of the binding site. Much of this early work involved a comparison of the binding of various similar haptens to antibody raised against one hapten (Figure 2b). For example, antibody raised against paraazophenylarsonate binds paranitrobenzoate maximally,

metanitrobenzoate less well and orthonitrobenzoate only poorly. Work in many systems showed that closeness of fit was a very important parameter of the antibody-antigen interaction. This interaction, due to close contact fitting is termed the van der Waals interaction (Karush, 1962). Other forces found to be involved in hapten binding were ionic interactions (Grossberg and Pressman, 1968) as, for example, a positive charge in a site directed against a negatively charged hapten and hydrogen bonding (Weir, 1963), important in the binding of carbohydrates.

The size of the combining site has been determined by a comparison of the binding affinities of a series of oligosaccharide fragments of the parent dextran antigen (Schlossmann and Kabat, 1962). This work, carried out principally by Kabat, indicated that five or six glucose residues could be bound to a single site. Besides this dextran system a levan-specific myeloma protein has also been investigated (Lundblad et al., 1972) with the result that a trimer is maximally bound. Thus site size may vary somewhat from antibody to antibody. Study of poly-amino acid antigen (Haimovitch et al., 1969) has shown that about six to ten residues are accommodated per site.

Another approach used to study the antibody combining site is the use of reactive haptens known as affinity labels (Singer, 1967). The hapten enters the combining site and reacts specifically with residues in the site. The protein can then be sequenced to determine which residues are labeled. Affinity labeling has implicated tyrosyl (Wofsy et al., 1962), lysyl (Koo and Cebra, 1974) and histidinyl residues (Koyama et al., 1968) in hypervariable regions of the antibody combining site. A particularly interesting study involved two labels with reactive groups at different distances from the haptenic group, DNP (Haimovitch et al.,

1970). With protein M315, a DNP-specific mouse myeloma protein, the labeling reagent with the nearby reactive group labeled a tyrosine on the light chain. The labeling reagent with the more distant reactive group labeled a lysine on the heavy chain. The data imply that the tyrosine is near the DNP ring and that the lysine is somewhat distant.

The kinetics of the reaction of hapten with antibody has also been studied extensively. Methods employed were stopped flow, temperature jump and a magnetic resonance technique, perturbation mixing. Day (Day et al., 1963) employed stopped flow in the study of a DNP-specific antibody population from rabbit. The rate of diffusion into the site was found to be essentially diffusion controlled. The rate of diffusion out of the site depended on the particular antibody preparation and, since the equilibrium binding constant in the ratio of these two, differences in the binding constant in general reflected variation in off rate. Pecht (Pecht et al., 1972) studied the DNP-specific myeloma proteins M315 and M460 by temperature-jump kinetics and found that while binding to M315 was described by one single diffusion controlled rate, binding to M460 was more complex with both a slow and a fast rate. It was suggested that the slow rate might be due to conformational rearrangement induced by binding. A magnetic resonance technique called perturbation mixing (Kooistra et al., 1978) involving the selective perturbation of a "free" signal and then observation of the transfer of this perturbation to the "bound" signal showed that the diffusion rate onto M315 was diffusion controlled.

Electron spin resonance (ESR) has been applied to the study of the antibody-antigen interaction (Stryer and Griffith, 1965). If the ESR free radical probe is attached to a hapten, such as DNP, then the

particular shape of the signal gives information on the relative mobility of the spin label. By varying the distance between the DNP group and the spin label the depth of the combining site could be ascertained since once the label protruded from the binding site it would resemble the solution phase signal (Hsia and Piette, 1969). The site depth was determined to be 10-12 Å. This method also gives information on the relative "tightness" of the site, that is the degree to which the label is immobilized (Willan et al., 1977). A comparison of spin labeled DNP hapten bound to three nitrophenyl-specific myeloma proteins established that one was quite narrow, one was intermediate, and one was quite wide.

Nuclear magnetic resonance (NMR) has also been utilized in the examination of antibody binding sites. Baldeschwieler (Haugland et al., 1967) used $^{35}C\ell$ NMR to observe the immobilization of a parachloro hapten binding to its specific rabbit antibody. In work with homogeneous systems Kooistra and Richards (Kooistra and Richards, 1978) used fluorine NMR to study the interaction between fluorine labeled nitrophenyl haptens and the DNP-specific myeloma protein, M315. They found that irrespective of the side chain the DNP group was always bound in a similar environment. Both ^{31}P NMR and ^{13}C NMR were utilized in studies by Goetze and Richards (Goetze and Richards, 1977) to probe the binding of phosphorylcholine to a number of PC-specific myeloma proteins. One unusual finding was that the binding at the two ends of the hapten was unequal with choline more firmly bound than the phosphate group. They also observed perturbation of the phosphate ionization by binding site residues (Goetze and Richards, 1978).

A different NMR approach to antibody binding site study involves the observation by proton NMR of the entire binding site. This is made

possible because of a special fragmentation scheme that yields the binding site domain for protein M315 (Inbar et al., 1972). Dwek (Dwek et al., 1977) has used this method and studied the difference in spectra depending on whether or not hapten is bound. Most of the resonances perturbed are in the aromatic region and are shifted upfield. This has led to the postulation of an "aromatic box" around the hapten in the M315 binding site.

The 2,4-dinitrophenyl (DNP) group has been very commonly used as a hapten for many years both because of its ease of introduction into carrier protein and because of its simplicity as a haptenic group. Over the years a number of features of DNP-specific antibodies have been noted. Probably the most commonly observed feature is the hydrophobicity of the binding site (Little and Eisen, 1966) which is to be expected for an aromatic hapten like DNP. Affinity labeling has implicated a tyrosine in the binding site of a number of DNP-specific antibodies (Goetzl and Metzger, 1970). A universal feature of nitrophenyl-specific immunoglobulins is the "red shift" experienced by the hapten visible absorption spectrum on binding (Little and Eisen, 1967). This "shift" is due to the formation of a charge transfer complex between the hapten and a binding site tryptophan which produces a new absorption band. This effect has been studied by circular dichroism (Orin et al., 1975) in order to define the geometry between the DNP ring and the tryptophan. Study of the DNP specific myeloma protein, M315, binding a series of similar haptens has shown that besides the usual hydrophobic pocket there is also a positive subsite that will interact with a carboxylate group on the hapten side chain of the proper length (Haimovitch and Eisen, 1971). Hydrogen bonds to the two nitro groups have also been suggested

as a possible interaction in these antibodies, but evidence to date is not definitive (Gettins et al., 1978).

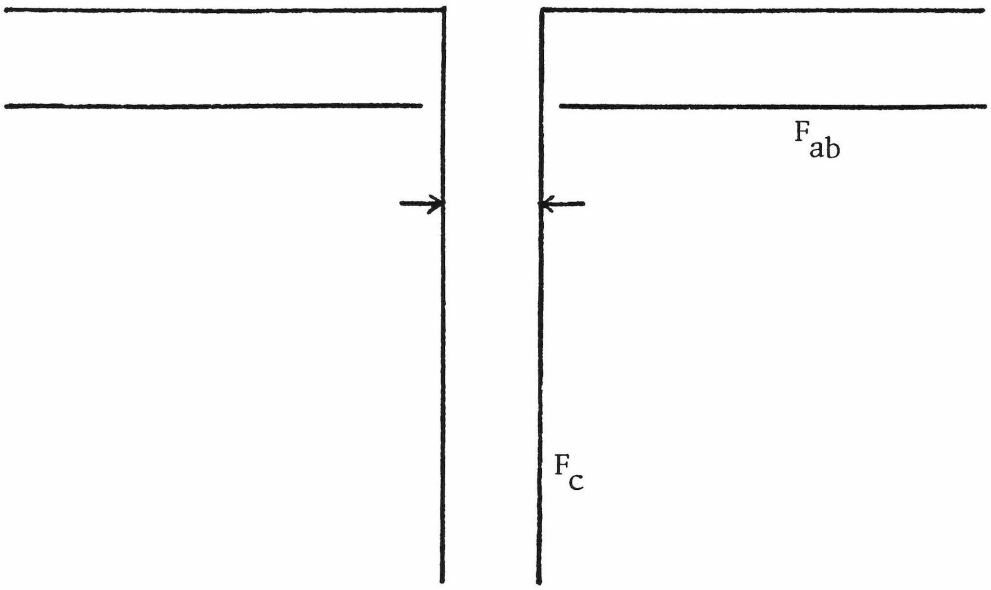
In the following chapters three nitrophenyl-specific myeloma proteins M315, M460 and X25 are studied by a variety of physical techniques. Fluorine NMR is used to compare the way the hapten is bound in the three sites. Protein modification is utilized in conjunction with NMR to locate a binding site residue near the hapten. Proton NMR is employed to dissect ring current shifts from paramagnetic shifts which may be due to the charge transfer interaction. The charge transfer interaction is studied in model systems by visible absorption spectroscopy and by NMR in order to better understand the hapten interaction with the proteins. Finally, most of the information accumulated is synthesized to modify a model of the binding site of M315 and to orient the hapten in the site.

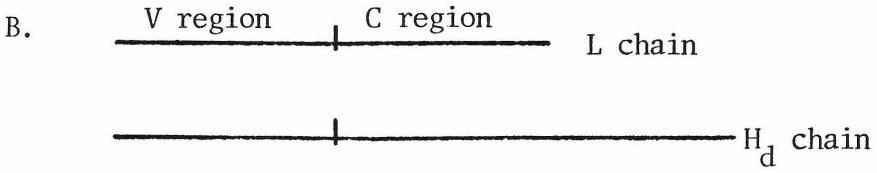
Figure 1

A: The chain structure of IgG
(papain cuts indicated by arrows)

B: F_{ab} chain structure

C: Homology regions in IgG


A.
F_{ab}
F_c


B.
V region
C region
L chain
H_d chain

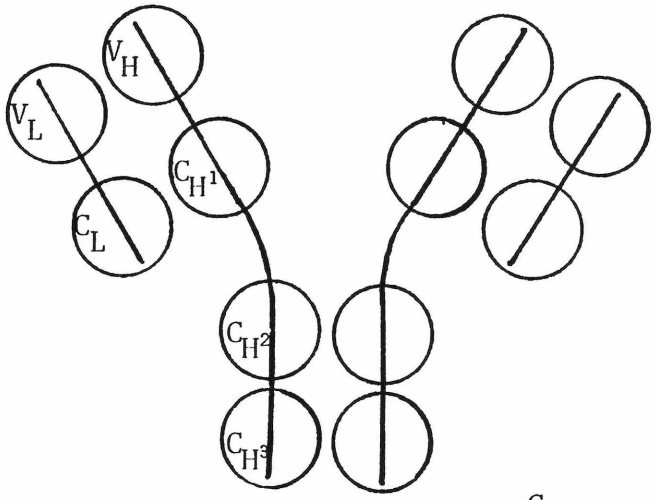

V_H
V_L
C_{H^1}
C_L
C_{H^2}
C_{H^3}
C.

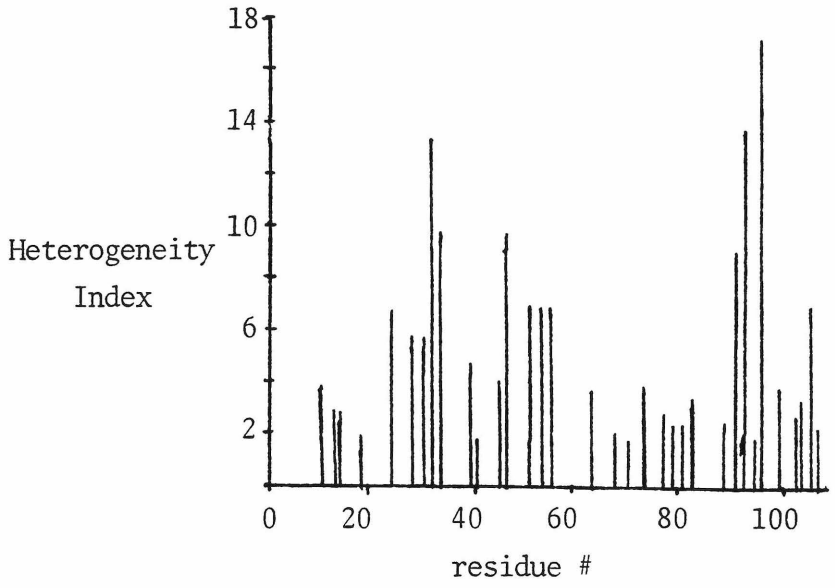
Figure 2

A: Wu and Kabat (1970)

Hypervariable plot

B: Hapten - carrier Antigen

A.


18
14
10
6
2
Heterogeneity
Index
0
20
40
60
80
100
residue #

B.

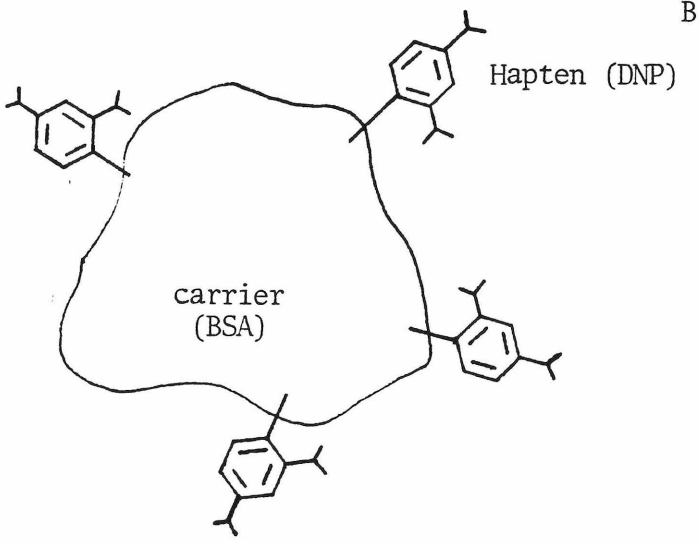
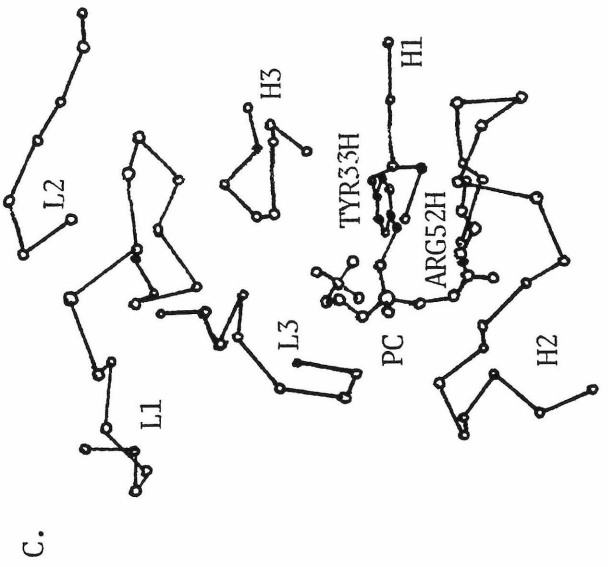

Hapten (DNP)
carrier
(BSA)

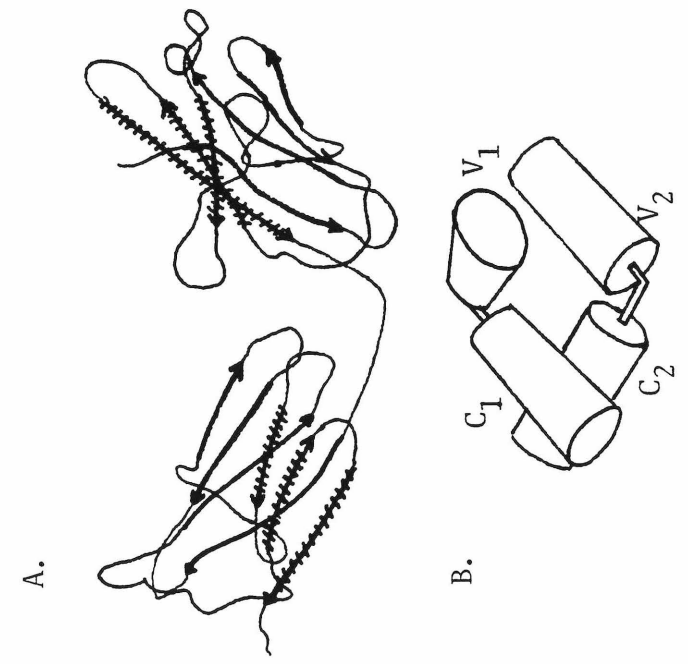
Figure 3

A: Immunoglobulin Fold in Mcg dimer

B: Schematic Diagram of Homology Regions in Mcg

C: M603 Binding Site (with phosphoryl choline)


C.
L2
L1
L3
H3
TYR33H
ARG52H
PC
H1
H2


A.
B.
V_1
V_2
C_1
C_2

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Chapter 2

^{19}F Magnetic Resonance Studies of the Binding Site Interaction Between Nitrophenyl Haptens And Mouse Myeloma Immunoglobulins M315, M460 and X25

In order to examine the precise interactions that take place between three nitrophenyl-specific myeloma proteins and their haptens the technique of fluorine nuclear magnetic resonance (nmr) has been employed. Antibodies are both highly specific and very diverse (Richards and Konigsberg, 1973) so that very close complementarity must play an important role in the antibody-antigen binding interaction (Kabat, 1976). A comparison of the binding interactions of a series of related haptens to a group of nitrophenyl-specific antibody proteins should permit one to compare and contrast the particular features of the binding site common to all or point out some features unique to one. This sort of study should shed some light on the question of multispecificity, the so-called "strange cross reaction" of apparently dissimilar haptens for the same antibody (Michaelides and Eisen, 1974). Additionally, a molecular understanding of the interaction between antibody and antigen (or hapten) should serve as a preliminary basis for subsequent elucidation of the manner in which binding of antigen to certain classes of antibody triggers the various effector functions (Spiegelberg, 1974) such as complement cascade (Müller-Eberhard, 1968) and lymphocyte differentiation (Vitetta and Uhr, 1975).

Homogenous proteins secreted by myeloma tumors (Potter, 1972) provide an ideal subject for molecular studies of antibody-antigen interactions. These tumors either arise spontaneously in susceptible strains of mice or are induced by various agents (mineral oil, x-rays). Many of the

proteins secreted have been screened by precipitin assay against a panel of antigens (Potter, 1971) and groups of immunoglobulin protein have been identified which exhibit specificity for substances related to phosphorylcholine (Leon and Young, 1970), dinitrophenol (Schubert et al., 1968), and a variety of oligosaccharides (Vicari et al., 1970).

The three plasmacytomas employed in this study grow in BALB/c mice and produce antibody of the IgA class which is secreted primarily as oligomers ($[H_2L_2]_n$, n = 2-5) (Nisonoff et al., 1975). M315 (the protein secreted by MOPC315 cells) has a λ_2 light chain (Dugan et al., 1973) whereas M460 (secreted by MOPC460) and X25 (secreted by XRPC25) both have κ light chains (Jaffe et al., 1971; Sharon and Givol 1976). M315 has a high affinity for 2,4-dinitrophenyl (DNP) and 2,4,6-trinitrophenyl (TNP) haptens ($K_a \sim 10^6$ to 10^7 M^{-1}) (Eisen et al., 1968) and also binds menadione (vitamin K_3) with an affinity of 10^5 M^{-1}, an example of the "strange cross reaction" mentioned above. The binding specificity of M315 with a variety of other haptens has also been explored (Haimovich and Eisen, 1971). M460 binds nitrophenyl haptens with moderate affinity ($K_a \sim 10^5$ M^{-1}), exhibits high affinity for 2,4-dinitronaphthol ($K_a \sim 10^7$ M^{-1}) (Haimovitch. et al., 1972) and binds menadione with an affinity comparable to that for nitrophenyl haptens (Johnson et al., 1974). X25 displays moderate affinity for dinitrophenyl haptens ($K_a \sim 10^5$ to 10^6 M^{-1}) but does not bind menadione appreciably ($K_a < 10^3$ M^{-1}) (Vine, 1978).

The complete variable region amino acid sequence of M315 has been determined (Francis et al., 1974; Dugan et al, 1975) as have the first heavy and light chain hypervariable region sequences for M460 and X25 (Hunkapiller et al, 1978). For M315 and X25 fragmentation schemes capable of yielding protein, called F_v, which is comprised of the heavy and light chain variable sequences have been described (Inbar et al., 1972; Sharon and Givol, 1976). For M460, as for the other two, it is possible to prepare a fragment consisting of the variable and first constant region domains called an F_{ab}' fragment.

Nuclear magnetic resonance serves as an extremely sensitive technique for the study of microenvironments in proteins (Roberts and Jardetzky, 1970). Both ^{31}P and ^{13}C nmr techniques have been used to investigate myeloma proteins which bind phosphorylcholine and its analogues (Goetze and Richards, 1977a; 1977b; 1978). Binding to nitrophenyl-specific myeloma proteins has been studied by a number of nmr techniques including observation of ^{1}H (Dwek et al., 1975; 1977) and ^{19}F nuclei (Kooistra and Richards, 1978). The former work examined perturbations in binding site and hapten resonances which occurred on binding hapten. The latter work employed fluorinated nitrophenyl haptens in which one nitro group was replaced by a trifluoromethyl group. Such substitution might not greatly change the interaction between hapten and antibody as the electronegativities of both groups are similar (3.35 for trifluoromethyl compared to 3.4 for nitro) (Wells, 1968) and their sizes are comparable (Coles

and Hughes, 1949). In addition, the red shift observed when DNP hapten binds to nitrophenyl-specific antibody (Eisen et al., 1968) is also seen when the trifluoromethyl analogues are bound by these antibodies (Hardy, 1978). A major difference between a nitro group and a trifluoromethyl group is their relative tendency to act as acceptors in the formation of hydrogen bonds (Doddrel et al., 1969) (NO_2 >> CF_3), a difference which can be exploited to probe the importance of hydrogen bonding in the antibody-hapten interaction.

This work examines the binding of ortho- and para-substituted trifluoromethyl nitrophenyl haptens to M460 and X25 and compares the results with those found previously with M315 (Kooistra and Richards, 1978). In addition a hapten with a fluorine label on the aliphatic side chain is employed to examine the environment of the chain when bound to these three antibodies.

Materials and Methods

Tumor Maintenance. MOPC-315 was obtained in solid form from the Salk Institute for Biological Studies, Cell Distribution Center (San Diego, CA). MOPC-460 and XRPC-25 were generously provided by Dr. Michael Potter, National Institutes of Health (Bethesda, MD). The tumors were transplanted by subcutaneous injection into female BALB/c mice of approximately 1 mm^3 pieces of minced tumor tissue (Potter et al., 1972). Mice were obtained from the Texas Inbred Mouse Co. (TIM Co., Houston, TX).

Purification of Myeloma Protein. Protein was isolated from the ascites of mineral oil primed CDF1 hybrid females (BALB/c x DBA/2) which had been injected with a single cell suspension of the tumor (Potter et al., 1972). This suspension was obtained by pressing the solid tumor (1 cm^3) through a fine (200 mesh) screen. Within two or three weeks ascites developed and could be removed through a needle puncture twice weekly. Mice usually survived two or three weeks after the initial tapping. Myeloma protein yields of 2-3 mg/ml were obtained from the pooled ascites fluid. After collection, cells were centrifuged and the supernatant poured off and stored frozen at -20°C.

Prior to reduction and alkylation, the myeloma protein could be assayed by immunoprecipitation with DNP-BGG either by ring test or agar diffusion (Ouchterlony).

Myeloma protein was purified by affinity chromatography according to a modification of a published procedure (Inbar et al., 1971). Pooled ascites was made in 0.2M in Tris by adding one-tenth volume of 2M Tris (pH 8.6). The solution was made 0.01M in dithiothreitol and stirred one hour at room temperature under an argon atmosphere. The pH was then adjusted to 7.8 by adding one-tenth volume of 2M Tris (pH 7.2) and the solution was chilled to 0°C in an ice bath. The solution was made 0.03M in iodoacetamide and stirred 30 minutes under argon at 0°C. The reduced and alkylated protein was then applied to a DNP affinity column.

The affinity column was prepared by coupling

1,6-diaminohexane to cyanogen bromide activated Sepharose 4B. This primary amine spacer arm gel was then reacted with 2,4-dinitrofluorobenzene to yield the DNP Sepharose conjugate.

Bound myeloma protein was eluted from the affinity column with a .05M DNP-glycine solution (Goetzl and Metzger, 1970). Eluate from the column passed directly to a small Dowex 1-X8 ion exchange resin column (2.5 cm x 4 cm) which removed DNP-glycine from the protein. The amount of protein was monitored by its absorbance at 280 nm.; the concentration was determined by using an absorbance value of 1.35 for a 0.1% protein solution (1 cm path length) (Underdown et al., 1971).

SDS gel electrophoresis showed some contamination by albumin and the myeloma protein was further purified on Whatman DE-52 ion exchange cellulose. The solution of 7S monomer was dialyzed against 0.03M potassium phosphate (pH 8.0) buffer and applied to the DE 52 column equilibrated with this buffer. The IgA fraction was eluted with a 0.03 to 0.30M potassium phosphate linear gradient (pH 8.0) at 4°C. The IgA eluted first, followed by the albumin.

Preparation of Fab' Fragments. Fab' fragments were made by a 1% pepsin digest of purified 7S monomer (Inbar et al., 1971). Purity was assayed by 7.5% SDS gel electrophoresis which showed greater than 90% was Fab'.

Preparation of NMR Samples. Purified Fab' protein was dialyzed against buffer (0.15M NaCl, 0.01M NaH_2PO_4, 0.001M EDTA, 0.02% NaN_3, pH 7.40) and brought to 30 mg/ml (0.5 mM) by

pressure dialysis (AMICON Diaflo). Concentration was determined by measuring absorbance at 278 nm (1.4 for a 0.5% protein solution, 1 cm path length) (Inbar et al., 1971). The fluorinated haptens were added in aliquots of 0.05M stock solution. Haptens were dissolved in the buffer described above. The $NHCH_2CF_3$ hapten was insoluble in aqueous solution and was therefore prepared as a stock solution in acetone.

Fluorine nmr spectra were obtained with 2 ml protein samples in 12 mm flat-bottom nmr tubes containing a 5 mm coaxial tube of D_2O for locking. Spectra were observed on a Varian XL-100-15 at 94.1 MHz.

Preparation of Haptens.

1. γ-N-(2,6-Dinitro-4-trifluoromethylphenyl) aminobutyric acid [the para-CF_3 TNP analogue]
2. γ-N-(2-Nitro-4-trifluoromethylphenyl) aminobutyric acid [the para-CF_3-DNP analogue]
3. γ-N-(4-Nitro-2-trifluoromethylphenyl) aminobutyric acid [the ortho-CF_3-DNP analogue]

These haptens were all synthesized by a modification of the published procedure described by Porter (Porter, 1950). Details of synthesis and physical properties were reported previously (Kooistra and Richards, 1978).

4. 2-N-(2,4-Dinitrophenyl) amino-1,1,1-trifluoroethane

This hapten was prepared by reaction of 2,2,2-trifluoroethylamine with 2,4-dinitrofluorobenzene in an ethanol - 10% bicarbonate solution (2:1, pH 9) for 12 hours at room temper-

ature. The ethanol was removed in vacuo and the solution extracted with ether. The ether solution was washed with 10% HCl, then water, dried over $MgSO_4$ and the ether removed in vacuo. The resulting solid was recrystallized from ethanol to give yellow needles, m.p. 116°C.

Analysis: calculated for $C_8H_6N_3O_4F_3$: C, 36.23; H, 2.26; N, 15.88

Found: C, 36.28; H, 2.27; N, 15.67

IR: 3360 cm^{-1} (N-H stretch); 1630, 1540 cm^{-1} (C---C); 1350 cm^{-1} (C-N stretch); 1290 cm^{-1} (CF_3); 1160, 1120, 830 cm^{-1} (aromatic unsymmetrical substitution)

NMR ^1H (in acetone d_6): doublet 9.0 (1H); doublet of doublets 8.4 (1H); doublet 7.55 (1H); multiplet 4.57 (2H); broad N-H under aromatic peak.

^{19}F (in acetone) 3.19 ppm downfield from TFA; triplet J = 8.7 Hz

Absorption maximum 343 nm; Molar Absorptivity $2 \cdot 10^4$ cm^{-1} M^{-1}.

Treatment of Data. For nmr peaks in fast exchange on the nmr time scale, the observed chemical shift is a weighted average of the shift in the two environments (free and bound). Thus:

$$\delta = \frac{[AbHap]}{[Hap_o]} \Delta$$

where δ is the chemical shift difference between the free and

bound peak; [AbHap] is the concentration of bound hapten; and $[Hap_o]$ is the total hapten concentration. By observing the chemical shift at a number of ratios of hapten to antibody one can evaluate both Δ and K (Gammon et al., 1972). This method was used in all cases to determine the bound chemical shifts and the association constants for the fluorinated haptens. An assumed K is used to calculate [AbHap] for a given $[Hap_o]$ and $[Ab_o]$. A plot of chemical shift vs. $[AbHap]/[Hap_o]$ will be linear for the best K with slope of Δ. Figure 4 shows the deviation for linearity when K is varied. Corresponding variation in Δ is also indicated. Relative error is defined as the best r^2 value from a linear least squares fit minus the r^2 value for a given K_D divided by the best r^2 value obtained for that data set. The best r^2 value for a set of 6 or more data points was typically 0.99 or better.

The method is accurate so long as the binding constant is of the same order of magnitude as the protein concentration employed. In all bit two cases, (the $-NHCH_2CF_3$ hapten binding to M315 and X25) this criterion was met. The lower accuracy for the other two values is indicated in Table I by the use of only one significant figure.

Results. Table I lists the affinities of M315, M460 and X25 for the various fluorinated haptens determined in this work by the nmr procedure just described and in earlier work by fluorescence quenching (Kooistra and Richards, 1978). For comparison, affinities for other common haptens are given.

Table II compares the relative affinities of the three antibodies for DNP lysine and the fluorinated haptens in an attempt to assess the relative importance of hydrogen bonding in M315, M460 and X25.

Table III collects the changes observed in the ^{19}F chemical shifts of the haptens between solution and the binding sites of the Fab' fragments of the antibodies. These values of Δ were determined as previously described. Figure 1 shows how values of K and Δ are related to the observed experimental parameters. The values of Δ calculated in this way range from 1 ppm downfield to 1 ppm upfield for bound hapten relative to hapten in solution. The para-trifluoromethyl analogues of both DNP and TNP have the same Δ values when binding to M315; in contrast these two haptens have quite different values of Δ when they bind to M460 and when they bind to X25. The para-trifluoromethyl-TNP analogue is in slow exchange between solution and M315 whereas the para-trifluoromethyl-DNP analogue is in intermediate exchange. Other haptens with M315 and all haptens with M460 and X25 are in fast exchange.

Discussion

Binding Affinities. Formation of hydrogen bonds between antibody (as donor) and the nitro group of the hapten (as acceptor) likely provides to the hapten-antibody complex a significant stabilization in addition to other stabilizing factors such as charge transfer, hydrophobic, and ionic

interactions. Therefore, replacement of a nitro group, which forms moderately strong hydrogen bonds (Baitinger et al., 1964), by a trifluoromethyl group, which forms much weaker hydrogen bonds (Doddrel et al., 1969), should reduce the stabilization of the antibody complex. The degree to which this occurs may provide some measure of the importance of such hydrogen bonding in the affinity of antibody for hapten. Unless the binding site is very narrow, the greater bulk of the spherical trifluoromethyl group compared to the planar nitro group should not, itself, appreciably affect binding (Coles and Hughes, 1949). Also, as mentioned previously, the red shift observed on binding the fluorinated haptens thought to be due to a charge transfer interaction between hapten and tryptophan in the antibody is the same as for corresponding nitrophenyl haptens (Hardy, 1978).

The concept of unitary free energy of binding provides a useful basis for comparing binding interactions. As comparisons of unitary free energies of binding effectively eliminate the term for the entropy of mixing, they are more appropriate for discussion of the energetics of binding than simple free energy values. Equation 1 states the relationship (Utsumi and Karush, 1964) which has previously been used

$$\Delta G_u = -1.365 \log K_A - 2.38 \qquad (1)$$

to compare the binding of inhibitors to papain (Berger and Schechter, 1970) and in the study of antibodies against polyphenyl-

alanine (Schlecter, 1971). Table II summarizes the unitary free energy values for binding DNP lysine and the three trifluoromethyl haptens; the percent binding relative to DNP-lysine is also shown. These values for M315 indicate that hydrogen bonding with a group in the ortho position of the phenyl ring is stronger than that with a group in the para position. Hydrogen bonding interactions from the para position with M460 are similar to those from the ortho position with M315. Table IV summarizes the relative hydrogen bonding interactions for M315, M460 and X25.

Chemical Shifts-Hapten Environments. In the absence of an X-ray structure the explanations of chemical shift data must necessarily be tentative and somewhat speculative. Two major effects are likely to influence fluorine shifts on binding: van der Waals interaction and ring currents. Van der Waals effects arise from crowding of the trifluoromethyl group which distorts the π electron cloud and causes downfield shifts (Farnum and Patton, 1973). Ring currents, which arise from local magnetic fields about π ring systems, can cause either upfield or downfield shifts depending on the orientation of the aromatic ring relative to the probe nucleus (Emsley et al., 1965). Fig. 6 illustrates ring current shifts.

Assignment of fluorine chemical shifts may be approached by an examination of the known facts regarding DNP binding myeloma proteins:

1) Affinity labeling of M315 has localized tyrosine 34L at the DNP ring binding subsite and lysine 52H at the end of

the aliphatic chain (Goetzl and Metzger, 1970; Haimovich et al., 1972).

2) A positive subsite consisting of arginine 95L and/or lysine 52H interacts with the carboxylate end of the hapten (Haselkorn et al., 1974).

3) Circular dichroism and optical absorbance studies indicate that a charge transfer complex is formed between the DNP ring and a tryptophan residue (Freed et al., 1976; Eisen et al., 1968).

4) For a number of immunoglobulins, whose x-ray structures are known, a large ordered tertiary structure (the "immunoglobulin fold") seems always to be present (Padlan and Davies, 1975).

Using such structural data and the known amino acid sequence, Padlan has constructed a model of the M315 binding site (Padlan et al., 1976). Since M315, M460 and X25 show considerable sequence homology for the regions sequenced, this structural model might be extended to these other DNP binding proteins and amino acid substitutions (or deletions/insertions) correlated with the environments experienced by bound haptens.

Comparison of the first hypervariable light chain region (Figure 5a) of M315 and X25 shows only one major difference: the deletion of tyrosine 34L. Both M315 and X25 have a high affinity for DNP and both produce similar red shifts in the hapten absorption spectrum on binding (indicating a charge transfer interaction). If one assumes that the observed

sequence homology between M315 and X25 continues past the 40 residues already sequenced, a similar binding cavity should be formed in the two proteins. Thus the binding site residues near the para-trifluoromethyl group are in the L1 region at the "bottom" of the pocket. Binding sites in both M315 and X25 appear to be rigid so apparently the deletion of tyrosine 34L accounts for the greatly reduced shift of the para-trifluoromethyl group in the hapten bound to X25. Such a shift in M315 could arise either from ring currents or from a combination of ring currents and van der Waals effects.

Further clarification of the origin of the ^{19}F chemical shift observed when hapten binds to antibody can come from simultaneous observation of ^{19}F and ^{1}H shifts (Millet and Raftery, 1972). Preliminary studies of a hapten with a CHF_2 group in the para position (Kooistra et al., 1978) show that, while the ^{19}F resonance is shifted downfield by 1.7 ppm, the ^{1}H signal is shifted upfield by 1 ppm; this result illustrates the important contribution of van der Waals interactions to ^{19}F shifts.

Examination of the L1 hypervariable region of M460 (Figure 5a) tends to support the role of tyrosine 34L. The only significant difference between M315 and M460 is the additional length of the M460 loop. Tyrosine 34L is present in both as is the large chemical shift observed when hapten binds. Another interesting fact shown by the shift data for M460 is that DNP and TNP bind to M460 in somewhat different conformations (unlike binding to M315). Such differences could

reflect small changes in orientations of the aromatic residues in the binding pocket relative to the trifluoromethyl group or changes in the crowding and resultant distortion experienced by the hapten on binding.

In the binding site model, H1 (the first heavy chain hypervariable region) lies toward the rear of the binding site at the edge of the aromatic binding subsite. Figure 5b indicates H1 sequences for M315, M460 and X25 along with the chemical shift data for CF_3 groups at the ortho position and in the side chain.

As was mentioned previously, van der Waals interactions give rise to a downfield shift while ring currents cause shifts in either direction depending on orientation. The ring current induced by an external magnetic field generates a field which opposes the applied field above and below the plane of the aromatic ring, but reinforces it elsewhere. Thus nuclei near the sixfold axis normal to the plane of the ring are shifted upfield while those near the plane of the aromatic ring are shifted downfield (Emsley et al., 1965). Thus a large downfield shift may result from steric crowding, or from ring currents, or from a combination of the two. However, an upfield shift should be due to a domination of the shift by ring currents. Thus for X25 both the ortho and $-NHCH_2CF_3$ upfield shifts are likely due predominantly to ring currents (possibly tyrosine 33H or tryptophan 35H). The $-NHCH_2CF_3$ fluorine shifts caused by binding both to M315 and X25 seem to be dominated by ring current effects and, given

the homology in H1 between M315 and X25, may be due to the same residue (tyrosine 33H or tryptophan 35H). The chain shift for M460 is unusual in that it is large and in the opposite direction from M315 and X25. The M460 site is not as rigid as the M315 site (Hsia and Little, 1973; Wong et al., 1974) so such a large van der Waals shift would not be expected. Possibly the shift of the CF_3 group in the side chain of the hapten on binding to M460 is due to ring current effects. Substitution of methionine for tryptophan (as in the M460 H1 region) might be expected to change nearby tertiary structure so that significant differences may characterize orientation of aromatic residues in the binding pocket of M460 compared to M315 and X25.

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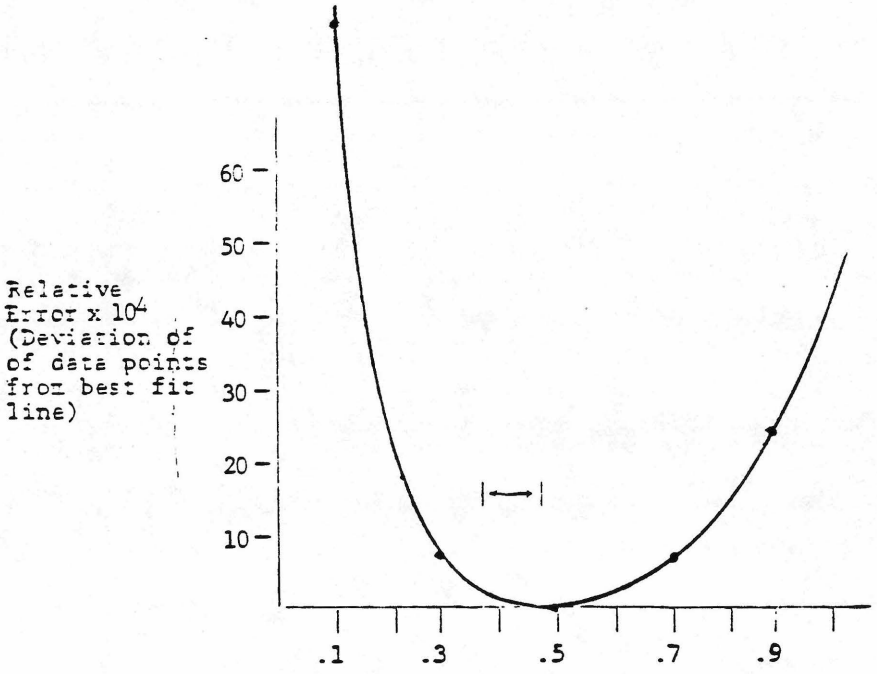
FIGURE 4

Variation of error as a function of the assumed dissociation constant.



error bar represents ± 5% variation in Δ

±15% variation in K_D

FIGURE 5

A. L1 hypervariable sequences and ^{19}F shifts.

B. H1 hypervariable sequences and ^{19}f shifts (- indicates upfield shift).

A.

		para CF_3 shift (ppm)	
		DNP	TNP
M315	Thr-Ser-Asn-Tyr-Ala-Asn-Trp	1.8	1.8
X 25	Asn-Ser-Asn-[ ]-Leu-His-Trp	0.36	0.05
M315	Thr-Ser-[ ]-Asn-[ ]-Tyr-Ala-Asn-Trp	1.8	1.8
M460	Ser-Asn-Gly-Asn-Thr-Tyr-Leu-His-Trp	0.6	1.0

B.

		ortho CF_3 shift	chain CF_3 shift
X 25	Thr-Ser-Gly-Tyr-[ ]-Trp-Asn-Trp-Ile	-1.0	-0.5
M315	Thr-Ser-Gly-Tyr-Phe-Trp-Asn-Trp-Val	0.6	-0.8
M460	Thr-Ser-Gly-Tyr-[ ]-Met-Asn-Trp-Ile	0.3	0.9

Table I

Binding Affinities of DNP Specific Myeloma Proteins

(25°C) $K_A(M^{-1})$

	X25	M460	M315
DNP Lysine	$8.8 \cdot 10^{4*}$	$4.6 \cdot 10^{4*}$	$1.2 \cdot 10^{6}$
Menadione	$<5.0 \cdot 10^{3*}$	$1.0 \cdot 10^{4*}$	$1.6 \cdot 10^{5}$
2,4-Dinitro napthol	$<3.0 \cdot 10^{4*}$	$3.4 \cdot 10^{5*}$	$6.7 \cdot 10^{4}$
p-CF_3 DNP Analogue	$2.0 \cdot 10^{3}$	$7.9 \cdot 10^{3}$	$7.8 \cdot 10^{5\dagger}$
p-CF_3 TNP Analogue	$2.0 \cdot 10^{2}$	$2.3 \cdot 10^{4}$	$3.4 \cdot 10^{6\dagger}$
o-CF_3 DNP Analogue	$1.6 \cdot 10^{4}$	$3.8 \cdot 10^{4}$	$4.0 \cdot 10^{4}$
Chain CF_3 DNP	$8.0 \cdot 10^{4}$	$6.0 \cdot 10^{4}$	$5.0 \cdot 10^{5}$

†fluorescence quenching D. A. Kooistra

*R. Hardy, W. Vine, equilibrium dialysis

remaining determined by ^{19}F nmr data.

[1]due to limited solubility of hapten less certainty in these values determined by nmr

Table II

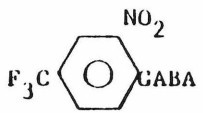
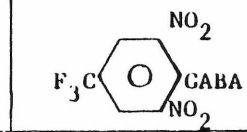
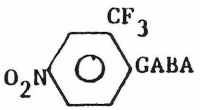
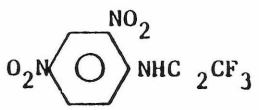
Comparison of Unitary Free Energies of Binding

Fluorinated Haptens with Binding of DNP Lysine (at 25°C)

Hapten	X25		M460		M315	
	$-\Delta G_u$ (kcal/mole)	$\Delta G_u/\Delta G_u^{DNPlys}$	$-\Delta G_u$ (kcal/mole)	$\Delta G_u/\Delta G_u^{DNPlys}$	$-\Delta G_u$ (kcal/mole)	$\Delta G_u/ G_u^{DNPlys}$
DNP Lysine	9.1	1.0	8.7	1.0	10.6	1.0
p-CF_3 DNP Analogue	6.9	0.76	7.7	0.89	10.4	0.98
o-CF_3 DNP Analogue	8.1	0.89	8.6	0.99	8.6	0.81
Chain CF_3 DNP	9.0	0.99	9.2	1.07	10.1	0.95

Table III

Δ Chemical Shifts (bound-free)

Protein				
M315	1.8 *	1.8*	0.6*	-0.77
M460	0.56	1.0	0.33	0.94
X25	0.36	0.05	-0.99	-0.54

Chemical shift in ppm

- indicates upfield relative to Free

GABA = γ-aminobutyric acid

* from Kooistra and Richards (1978)

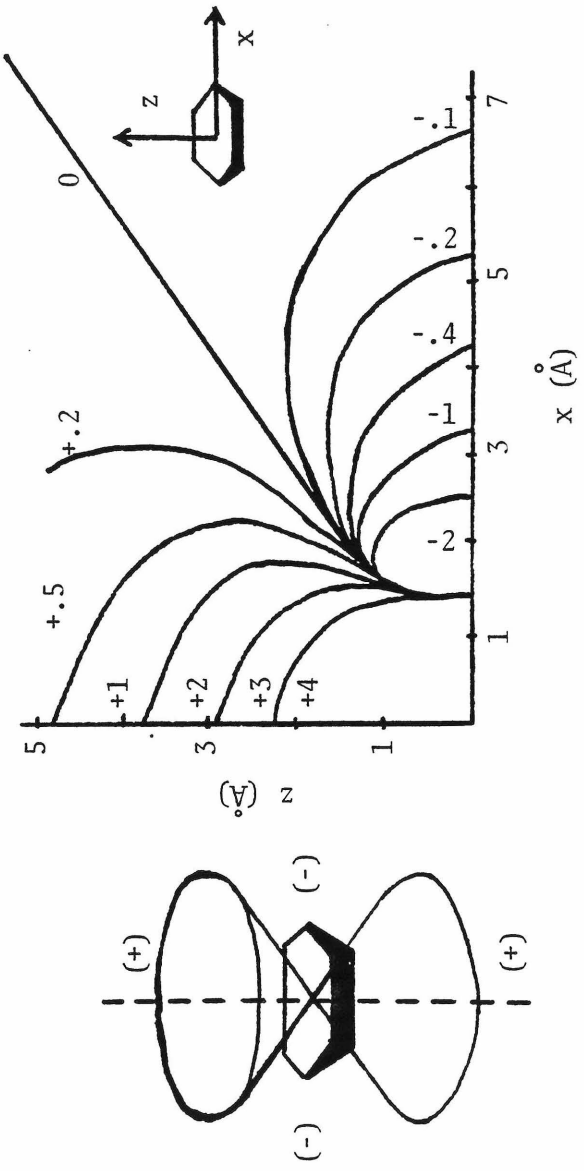
Table IV

Contribution of Hydrogen Bonding to Binding

	M315	M460	X25
para-CF_3	slight	moderate	strong
ortho-CF_3	moderate	slight	moderate
chain-CF_3	slight	slight	slight

FIGURE 6

Ring current shifts as a function of geometry (+ means shielding; - means deshielding; chemical shift values in ppm).


x
z
0
+.2
+.5
+1
+2
+3
+4
-.1
-.2
-.4
-1
-2
5
3
1
7
5
3
1
z (Å)
x (Å)
(+)
(-)
(+)
(-)

Chapter 3

Studies of Chemically Modified Mouse Myeloma Immunoglobulins M315, M460 and X25

In the study of interactions between small molecules and proteins one very useful approach is the chemical modification of protein amino acid residues. A very large number of reagents that react with protein residues are known, some of which are more selective than others (Means and Feeney, 1971). One of the more selective modifying reagents is tetranitromethane which specifically converts tyrosine residues into 3-nitrotyrosine residues (Sokolovsky et al., 1966). The reaction occurs under mild conditions and lowers the pK of the residue modified from 10 (for the tyrosine hydroxyl) to about 7 (in 3-nitrotyrosine). The ionization may be followed spectrophotometrically as the ionized species has an absorption at 430 nm (Riordan et al., 1967). This absorption band also permits quantitation of the degree of derivitization.

The reagent has been used to modify a large number of proteins including carbonic anhydrase (Nilsson and Lindskog, 1967), trypsinogen (Vincent et al., 1970), myoglobin (Atassi, 1968), subtilisin (Johansen et al, 1967), and bovine pancreatic trypsin inhibitor (Meloun et al., 1968). Depending on the ratio of tetranitromethane to protein, a variable number of tyrosines are modified. Often it is found that one or a few residues are modified at low molar excess of reagent while at large excesses many residues are modified. Specifically, the reagent has been used to nitrate rabbit IgG (Fuchs and Givol, 1968) which binds DNP antigen with the result that precipitation with DNP antigens become pH dependent being reduced at high (>8) pH. However, binding affinity to DNP-lysine was unaffected. Tetranitromethane has also been used to nitrate a nitrophenyl-binding human Wäldenstrom macroglobulin (a monoclonal IgM) (Otchin and Metzger, 1971). Increasing nitration reduced the antibody valency, the hapten protected some tyrosine residues from

nitration, and most of the nitration occurred on the heavy chain.

Two approaches to chemical modification have been applied to nitrophenyl-specific myeloma proteins. The first method, known as affinity labeling (Singer, 1967) involves attaching a reactive group to the hapten so that a residue in or near the binding site will be modified. Such studies on M315 and M460 have implicated a tyrosine on the light chain (Goetzl and Metzger, 1970) and a lysine on the heavy chain (Haimovich et al, 1972). A second approach is the use of certain reagents which modify all residues of a certain type such as carboxylate or lysine residues. If, for example, lysine is important to the binding interaction then modification of most (or all) lysine residues should have an effect on the association constant of the antibody (Klostergaard et al, 1977a). Of course, extensive modification may change a protein's conformation so that results are more difficult to interpret unambiguously by this method. Still, such studies have implicated an arginine rather than a lysine as a major contributor to the binding interaction between M315 and DNP-lysine (Klostergaard et al., 1977b).

Previous studies using fluorine nmr suggested that a tyrosine residue on the heavy chain in the first hypervariable region is near the hapten ortho nitro (or trifluoromethyl) group in X25 and near the chain trifluoromethyl reporter group in both M315 and X25 (Hardy and Richards, 1978). Modification of this tyrosine residue (Tyr 33H in M315) would very likely perturb the fluorine chemical shift of any group near this tyrosine. One might expect the perturbation to be pH dependent as the ionized and unionized nitrotyrosyl residue provide quite different environments. Finally, if a hydrogen bond exists between this tyrosine and the hapten then the association constant would become pH dependent,

decreasing at high pH (>8) when the hydroxyl group is deprotonated. Such dependence would be true for the native protein also but the pK of tyrosine is so high that a decrease in association constant near pH 10 might easily be attributable to generalized protein denaturation and not a specific effect.

Thus, tetranitromethane seems a useful reagent with which to study the binding interaction and site conformation of three nitrophenyl specific myeloma proteins M315, M460, and X25. The use of the reagent on three different proteins may show differences in the way that DNP haptens are bound. Also, the reduction of nitrotyrosine by dithionite generates an amino group with a pK much lower than any other in a protein (Sokolovsky et al., 1967) which permits specific reaction with it to attach other labels. This might provide an interesting further probe of the binding site. Finally, nitrotyrosine chelates lanthanide ions (Marinetti et al., 1976) which might be used in later studies to help further map the orientation of the hapten relative to the modified tyrosine using an nmr relaxation probe.

MATERIALS AND METHODS

The procedures for maintenance of tumors, isolation of proteins, and preparation of F_{ab}' fragments have been described previously (Chapter 1).

Nitration of F_{ab}' Fragments

Proteins were nitrated according to the procedure of Malan and Edelhoch (1970). Briefly, a solution of tetranitromethane in ethanol ($8 \cdot 10^{-2}$M) was added to a protein solution ($1 \cdot 10^{-4}$M) previously made 0.1M

in Tris buffer by addition of 1/10 volume of 1M Tris (pH 8.0). The reaction was carried out at room temperature using a twofold excess of reagent (over F_{ab}' concentration). After one hour the solution was applied to a gel filtration column (Sephadex G25) previously equilibrated with 0.01M phosphate - 0.15M NaCl (pH 7.4) and the nitrated F_{ab}' was collected in the void volume. Tetranitromethane was obtained from Aldrich.

Quantitation of Degree of Nitration

As described by Malan and Edelhoch (1970) the number of nitrated tyrosines may be determined spectrophotometrically by measuring the absorbance at 430 nm at pH 3 and 10.6. The ionized nitrotryosyl residue has an extinction coefficient of 4100 M^{-1} cm^{-1}. Protein concentration was determined by monitoring the absorbance at 280 nm which for a 0.1% solution is 1.4. Molecular weight was taken as 55,000 for the F_{ab}' fragment.

Reduction of Nitrated F_{ab}' Fragments

The 3-nitrotyrosyl F_{ab}' proteins could be converted to 3-aminotyrosyl proteins by reduction with dithionite (Sokolovsky et al., 1967). A 20-fold excess of sodium dithionite was added to the nitrotyrosyl F_{ab}' solution buffered with 0.1M Tris (pH 8.0). After 10 minutes the solution was exhaustively dialyzed against 0.01M phosphate, 0.15M NaCl (pH 7.4).

Reaction of 3-aminotyrosyl F_{ab}' Fragments with -ethylthiotrifluoroacetate

A ^{19}F nmr reporter group could be introduced by reaction of S-ethylthiotrifluoroacetate with the reduced nitrotyrosyl F_{ab}' protein

(Schallenberg and Calvin, 1955). The reagent is a well-known protecting group in peptide synthesis specific for unprotinated amines. The unusually low pK of aminotyrosine (4.8) compared to the amino terminal (8), lysine (10), or arginine (> 12) permits specific reaction with aminotyrosine at a pH of 4.5. A fivefold excess of the reagent was added to a solution of the aminotyrosyl F_{ab}' protein (1.10^{-4}M) in 0.05M sodium citrate buffer (pH 4.5) at room temperature. After five hours the solution was dialyzed in a hood against several changes of 0.01M phosphate, 0.15M NaCl (pH 7.4). The reagent was obtained from Pierce Chemical Company.

Determination of the Position of Nitration

Heavy and light chains of the nitrated F_{ab}' proteins were separated on DEAE Sephadex (A50) equilibrated with 4M urea -0.1M Tris (pH 9.0). Chains were reduced with dithionite as described above. The reduced chains were next reacted with (3,5-^{3}H) 2,4-dinitrofluorobenzene obtained from New England Nuclear in 0.05M citrate buffer (pH 4.5) for six hours at room temperature. Unbound label was removed by dialysis against 0.01M phosphate, 0.15M NaCl (pH 7.4). Labeled chains were counted using a scintillation counter to determine extent of labelling on each chain.

Equilibrium Dialysis

2 ml lucite dialysis cells were utilized in all binding studies. Each 2 ml chamber contained 1 ml of protein or hapten solution. Hapten employed was (3,5-^{3}H)2,4-DNP Lysine obtained from New England Nuclear. Protein concentrations were kept within an order of magnitude of the dissociation constant. To avoid nonspecific absorption of very low protein

concentrations one milligram per milliliter of gelatin was included in M315 protein solutions. Typically eight hapten concentrations in quadruplicate were carried out and both sides were counted. Scatchard plots were used to determine the association constant (Scatchard, 1949). The temperature of dialysis was maintained at 25± 1°C and 24 hours were allowed for complete equilibration in all cases. The unitary free energy values were calculated from equation 1 (Utsumi and Karush, 1964).

$$\Delta G_u = -1.365 \log K_A - 2.38 \qquad (1)$$

Determination of the pK of Nitrotyrosyl Residue

As previously mentioned the anionic form of nitrotyrosine has a visible absorption band with a maximum at 430 nm. The pK of nitrotyrosine in the F_{ab}' may therefore be determined by following the change in absorbance at 430 nm as a function of pH. All optical measurements were carried out on a Beckman Acta III. pH measurements were made using a Radiometer 26 pH meter with a combination electrode.

NMR Methods

Protein samples were concentrated via pressure filtration and the buffer used was 0.01M sodium phosphate, 0.15M NaCl, 0.001M EDTA, and 0.02% sodium azide (pH 7.4). Hapten was added from 0.05M stock solutions using a micro pipette. Titrations were carried out by addition of microliter volumes of 1N HCl or NaOH and followed on a Radiometer 26 pH meter. ^{19}F nmr spectra were obtained at 94.1 MHz on a Varian XL-100-15 using 12 mm sample tubes locked on a 5 mm coaxial tube of D_2O. Fourier transform accumulation typically required one to three hours to yield acceptable signal to noise. Protein concentrations were kept near half

millimolar and hapten concentrations were such as to give 70-90% bound signals.

RESULTS

Nitration of F_{ab}' Fragments

The three F_{ab}' proteins, M315, M460, and X25 when nitrated with a twofold excess of tetranitromethane gave an average of one tyrosine derivatized as determined spectrophotometrically. An example of the visible spectrum of one of the nitrated proteins is presented in Figure 7. The pK value of the nitrotyrosine groups in the nitrated protein were determined spectrophotometrically as shown in Figure 8. As hapten has an interfering absorbance band no hapten was included in these samples.

Location of Nitration

The protein was separated into light and digested heavy chains on an ion exchange column which gave the profile shown in Figure 9. The peaks were identified by SDS electrophoresis as is also shown in Figure 9. Tritiated 2,3-dinitrofluorobenzene was reacted with the reduced chains and the fractions counted to determine incorporation of this reagent. Figure 9 also presents the activity per chain and the relative amount of nitration for each chain of the three proteins.

Binding Constants

Table V presents the binding affinities of both nitrated and unnitrated proteins to DNP-lysine at pH values below and above the nitrotyrosyl pK for all three proteins.

NMR Studies

The fluorine resonances of the three haptens bound to the three

proteins were observed as a function of pH. The chemical shift as a function of pH is plotted in Figures 10, 11, and 12. Table VI presents Δ values and pK values for the three haptens bound to the three proteins. Figure 13 shows the ^{19}F nmr spectrum of the fluorine labeled M315 F_{ab}' protein. Table VII summarizes the residues responsible for the shifts of the various haptens. Figure 14 summarizes the various reactions involved in this study.

DISCUSSION

These experiments are a continuation of efforts by our laboratory to define the interactions between combining site residues in three nitrophenyl-binding myeloma proteins and their specific haptens. Previous studies of phosphorylcholine-binding myeloma proteins utilizing ^{13}C and ^{31}P nmr have been quite successful (Goetze and Richards, 1977a,b, 1978) and preliminary studies of the DNP binding proteins indicated one or more aromatic residues in close proximity to certain of the fluorine reporter groups (Kooistra and Richards, 1978; Hardy and Richards, 1978). The reported nitration of tyrosine 33 in the heavy chains (tyr 33H) of M315 (Dwek et al., 1977) suggested a method to test the idea that this residue (Tyr 33H) in M315 and its equivalent in X25 is responsible for the ring current (upfield) shifts observed with the ortho- and chain-haptens. Accordingly, ^{19}F nmr studies have been carried out with these haptens bounds to the three proteins, M315, M460, and X25 nitrated to the extent of one nitrotyrosine per F_{ab}' fragment.

The results support the contention that tyrosine 33H is at least one of the aromatic residues responsible for the upfield shifts of these haptens. The chain trifluoromethyl hapten's ^{19}F signal is shifted further upfield when bound to nitrated M315 F_{ab}' than when bound to the

native protein and titrates with a pK of about 8 which is typical of a nitrotyrosyl residue (Price and Radda, 1969). The ortho-trifluoromethyl hapten's ^{19}F resonance signal when bound to nitrated X25 F_{ab}' is also shifted further upfield than when bound to native protein and again titrates with a pK of about 8. Neither of these haptens' signals is perturbed relative to the native protein when bound to nitrated M460 F_{ab}' which indicates that nitrations alone does not produce a nonspecific change in the resonance signals or a titration curve; that is, the effects observed with M315 and X25 are specific and implication Tyr 33H in the shift of these two haptens. Evidently Tyr 33H in M460 is distant from the hapten.

An important consideration is whether the observed ^{19}F titration curves are due to changes in the environment near the fluorine atoms or are due simply to changes in the affinity of protein for hapten. All the resonances observed are in fast exchange on the nmr timescale so each signal is a weighted average of the free and bound species present in the sample (Pople et al., 1959). In the samples for titration the protein concentrations were always in excess of hapten concentrations so that most of the hapten would be bound. However, changes in the affinity constant would cause either more or fewer haptens to be bound and therefore move the signal toward bound or free.

In order to distinguish whether the observed changes in chemical shift are environmental or due to changes in affinity one must measure the binding constants as a function of pH. Since all effects occur between pH 6 and pH 9 it is sufficient to determine the constants at these two values. The binding constants were measured by equilibrium dialysis and the results show that for both M315 ($1.2 \cdot 10^6 M^{-1}$ at pH 6;

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$1.3 \cdot 10^6 M^{-1}$ at pH 9) and X25 ($4.6 \cdot 10^5 M^{-1}$ at pH 6; $4.8 \cdot 10^6 M^{-1}$ at pH 9) the change in binding constant is too small to account for the observed chemical shift changes thus pointing to environmental changes. Clearly the environment near the nitrotyrosine residue does change since at pH6 the tyrosine hydroxyl group is protonated while at pH 9 it is deprotonated and negatively charged.

The ortho-trifluoromethyl hapten bound to nitrated M315 F_{ab}' experiences a titration similar to the chain trifluomethyl hapten. Since this resonance is shifted downfield on binding it is not clear whether its shift is due to ring currents or not. However, the pH dependence of shift indicates that it is near Tyr 33H. It should be noted that ring current shifts may be either upfield or downfield depending on orientation (Emsley et al., 1965). The resonance of the chain trifluoromethyl hapten bound to X25 F_{ab}' does not shift on nitration or titrate with the expected pK. It does titrate so it appears that another titrable residue, possibly a histidine, is near it.

An interesting fact emerges on comparing the M460 chemical shift data with the equilibrium dialysis results: there is a large variation of chemical shift with pH by the para-trifluoromethyl hapten even though the affinity doesn't change appreciably. Thus this effect is evidently due to an environmental change. The pH dependence of shift is unaffected by nitration and appears to be a titration with a pK around 9.5. This could be an unnitrateable tyrosine in the binding site; for example, Tyr 34L.

The affinity data provide information about the interaction between hapten and binding site residues. If there is a hydrogen bond to an ionizable residue then the affinity will change with pH as the residue

is protonated or deprotonated. A hydrogen bond to a tyrosine hydroxyl group should break above its pK. Thus in M460 although Tyr 34L is probably near the para substituent on the hapten there is no hydrogen bond between them since the affinity actually increases slightly with higher pH. The complete lack of dependence of the X25 affinity with pH indicates no hydrogen bond to the nitrateable tyrosine. The affinity constant of M315 does decrease with increasing pH but the decrease is less with the nitrated protein than with the native protein. If the decrease was primarily due to a hydrogen bond being lost then a larger decrease at pH 9 would be expected with nitrated protein since the pK of nitrated tyrosine is about two pH units lower than for native tyrosine. One explanation for the decrease in affinity with increasing pH is that a lysine residue may be involved in the M315 positive subsite and is being titrated by pH 9.

An unexpected effect of nitration on affinity is the increase in association constant found for all three proteins. The small increase in M315 and M460 (0.1 Kcal) can be attributed to an increased hydrophobic interaction (Karush, 1962) between hapten and nitrated tyrosine. The large increase for X25 (1.1 Kcal) may indicate that the hydrophobic interaction plays a larger role in its binding compared with M315 or M460. Indeed, it has been found that the X25 binding site is considerably "tighter" than either M315 or M460 (Willan et al., 1977). Another effect of nitration is the extra binding energy at higher pH for both M315 and M460. Such a result may reflect a rearrangement of residues in the binding site as the negative charge develops on the nitrotyrosine. Such a reorientation could strengthen a hydrogen bond to the hapten or move the positive subsite residue closer to the carboxylate end of the

hapten. Initial examination of the nitrotyrosine pK values in the protein with and without hapten was somewhat puzzling. The pK value obtained by visible spectroscopy (7.6) was considerably lower than that found by nmr (8.0). At first glance this might be thought to be due to a hydrogen bond between the hapten and the nitrotyrosyl hydroxyl group, but the same high pK (8.0) was also found for the nonhydrogen bonding orthotrifluoromethyl hapten. Both of the experiments were carried out in antibody excess to assure complete binding of the hapten. If the titration is performed with equimolar or excess hapten concentration then the pK found by nmr drops to 7.6. This unusual behavior can be explained if one considers that only 80-90% of Tyr 33H is nitrated (pK of 7.6) while the remaining 10-20% of the sites contain native tyrosine (pK of 9.5-10). At low hapten concentrations if there is preferential binding to the native protein (in contrast to DNP-lysine) then one would observe a higher pK value than expected. At high hapten concentrations more of the nitrated protein would bind hapten and the pK would approach the nitrated tyrosine pK. Of course the visible spectroscopic titration of nitrated protein would only follow the nitrated protein as the unnitrated tyrosine would be invisible at 430 nm.

The chemical shift values of both M315 with the chain hapten and X25 with the ortho hapten change by very similar amounts on nitration. Such a change is likely due to the electron withdrawing effect of the nitro group on the π electrons of the tyrosine aromatic ring which would distort the ring current field surrounding it (Figeys and Flammang, 1967). To test this hypothesis the nitro group was reduced in M315 F_{ab}' nitrotyrosine to an amino group and it was found that the ^{19}F signal returned to the position observed with the native protein. This strongly supports

the idea that the extra upfield shift is an electronic effect since the amine rather than withdrawing electrons would release electrons to the tyrosine π system. The chemical shift change on deprotination of the nitrotyrosine hydroxyl group is different for M315 and X25 which indicates a steric cause rather than simply generation of a negative charge as the origin of the shift. That is, it is likely that the tyrosine ring moves relative to the trifluoromethyl group as the pH is changed.

The fluorine signals observed from trifluoroacetylated aminotyrosine residues in M315 demonstrate the use of this labeling procedure as a means of introducing reporter groups into antibodies. Such labels might be used to probe the binding site or to investigate effector function triggering (Winkelhake, 1978) outside the binding site.

One potential application of the nitrotyrosyl group is its use as a chelating site for lanthanides (Marinetti et al., 1976). Unfortunately, in the case of M315 lanthanides compete with hapten for the binding site, but such is not the case with X25 (Willan et al., 1977). Observation of the fluorine hapten signal as a lanthanide is added to nitrated X25 may provide more information on the orientation of the hapten in the binding site.

In summary, no hydrogen bonds have been found between tyrosine and hapten for the three proteins studied. A nitrateable tyrosine (Tyr 33H) appears responsible for all or part of the shift of the ortho- and chain-hapten trifluoromethyl signals when bound to M315. An analogous nitrateable tyrosine appears responsible for the ortho-hapten trifluoromethyl signal when bound to X25. None of the trifluoromethyl hapten reporter groups are near the suitable tyrosine residue in M460 but a tritateable residue, possibly a tyrosine, is near the paratrifluoro-

methyl reporter group. Nitration is thus a valuable technique for localizing a particular tyrosine near an nmr reporter group because it both changes the ring current field (and therefore the shift near it) and because it lowers the pK into a range over which most proteins are stable.

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FIGURE 7

Absorption spectra of nitrated protein at two pH values.

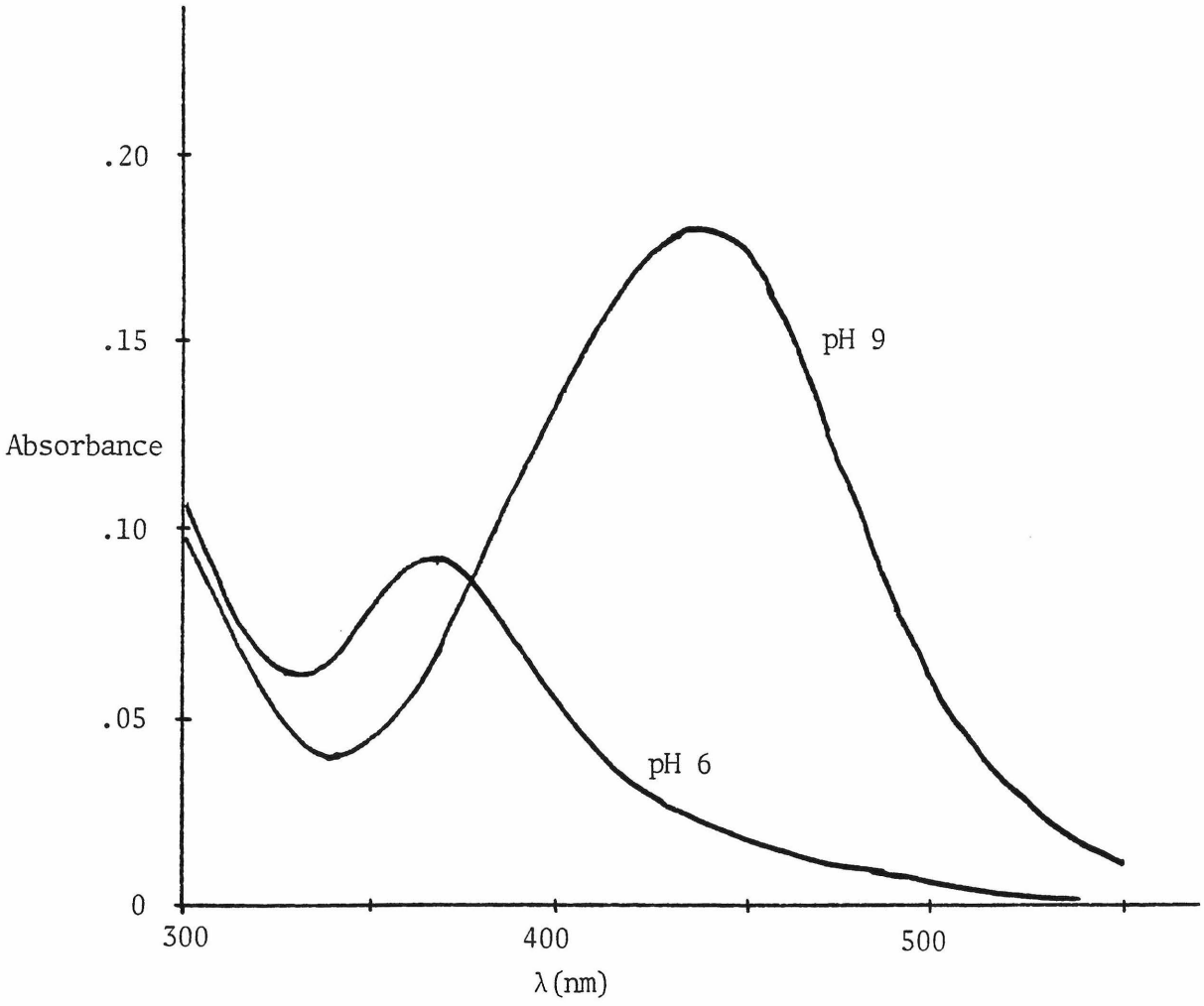

.20
.15
Absorbance
.10
.05
0
300
400
500
λ (nm)
pH 9
pH 6

FIGURE 8

Absorbance of nitrated protein (M315 F_{ab}') as a function of pH.

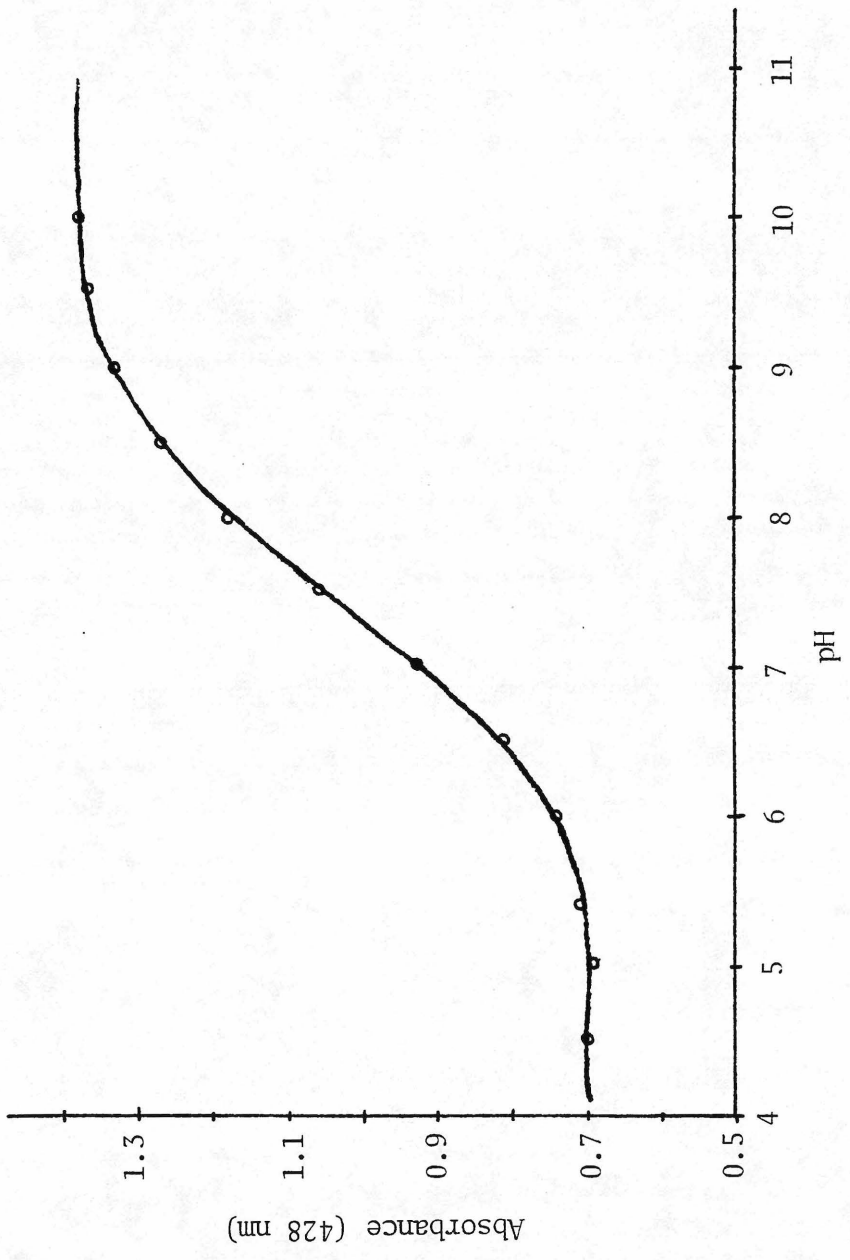

Absorbance (428 nm)
1.3
1.1
0.9
0.7
0.5
4
5
6
7
8
9
10
11
pH

FIGURE 9

A. Column profile of chain separation.

Inset: SDS gels of peaks compared with intact F_{ab}' (reducing 7.5% gels).

B. Counts found from chain separation of tritium labeled F_{ab}.

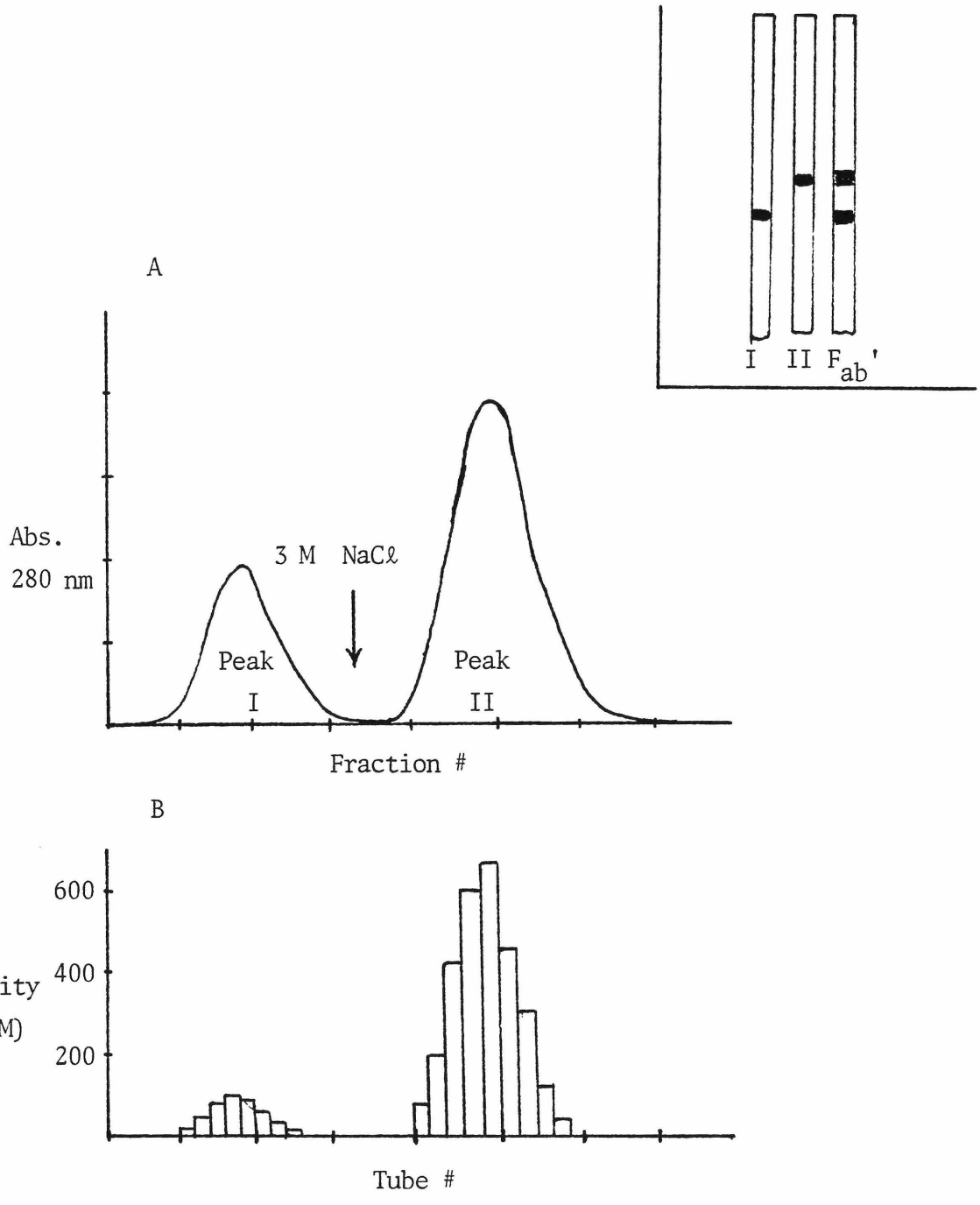

I II F_{ab}'
A
Abs.
280 nm
3 M NaCℓ
Peak
I
Peak
II
Fraction #
B
600
400
200
^{3}H
activity
(CPM)
Tube #

Table V

	Association Constant (M^{-1})		Unitary Free Energy (kcal)	
	pH 6	pH 9	pH 6	pH 9
Protein				
M315	$1.3 \cdot 10^6$	$5.4 \cdot 10^5$	10.7	10.2
M315N	$1.2 \cdot 10^6$	$1.3 \cdot 10^6$	10.7	10.9
X25	$8.8 \cdot 10^4$	$8.8 \cdot 10^4$	9.1	9.1
X25N	$4.6 \cdot 10^5$	$4.8 \cdot 10^5$	10.1	10.1
M460	$5.4 \cdot 10^4$	$5.8 \cdot 10^4$	8.8	8.9
M460N	$6.6 \cdot 10^4$	$1.2 \cdot 10^5$	9.0	9.3

FIGURE 10

Nitrated Myeloma F_{ab}' Proteins with Chain Trifluoromethyl Hapten.

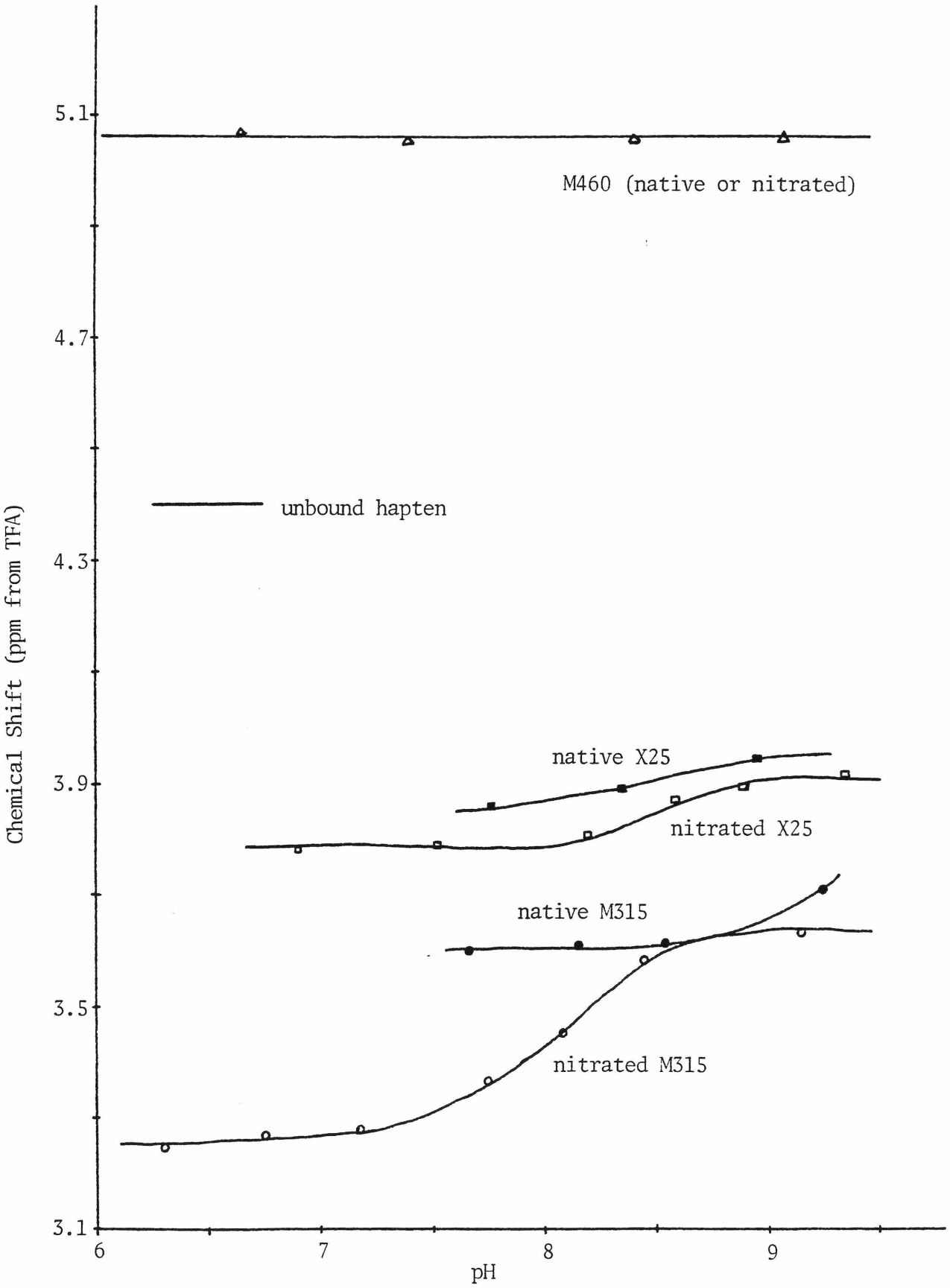

5.1
4.7
4.3
3.9
3.5
3.1
Chemical Shift (ppm from TFA)
M460 (native or nitrated)
unbound hapten
native X25
nitrated X25
native M315
nitrated M315
6
7
8
9
pH

FIGURE 11

Nitrated Myeloma F_{ab}' Proteins with Ortho Trifluoromethyl Hapten.

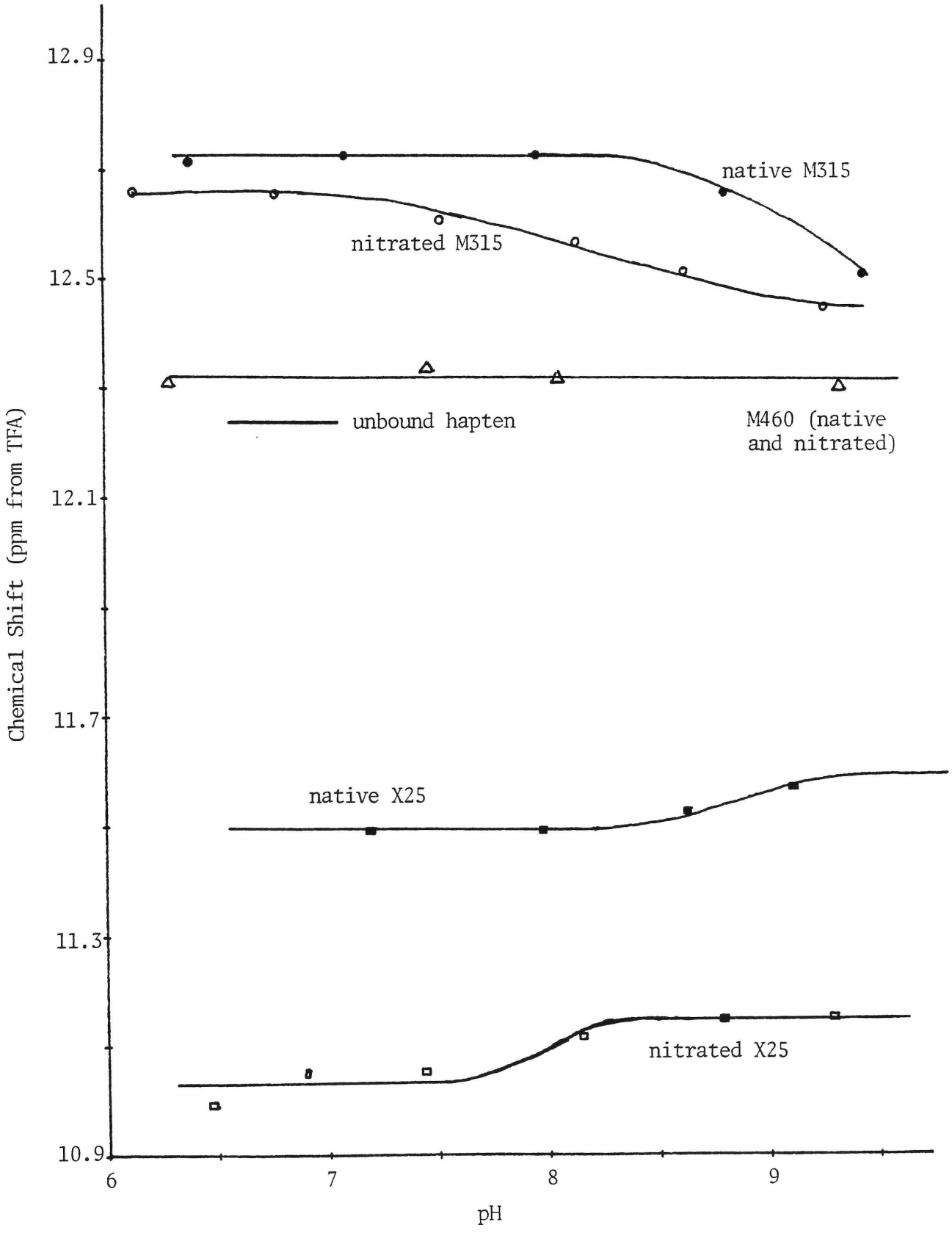

12.9
12.5
12.1
11.7
11.3
10.9
Chemical Shift (ppm from TFA)
native M315
nitrated M315
unbound hapten
M460 (native and nitrated)
native X25
nitrated X25
6
7
8
9
pH

FIGURE 12

Nitrated Myeloma $F_{ab'}$ Proteins
with Para Trifluoromethyl Hapten.

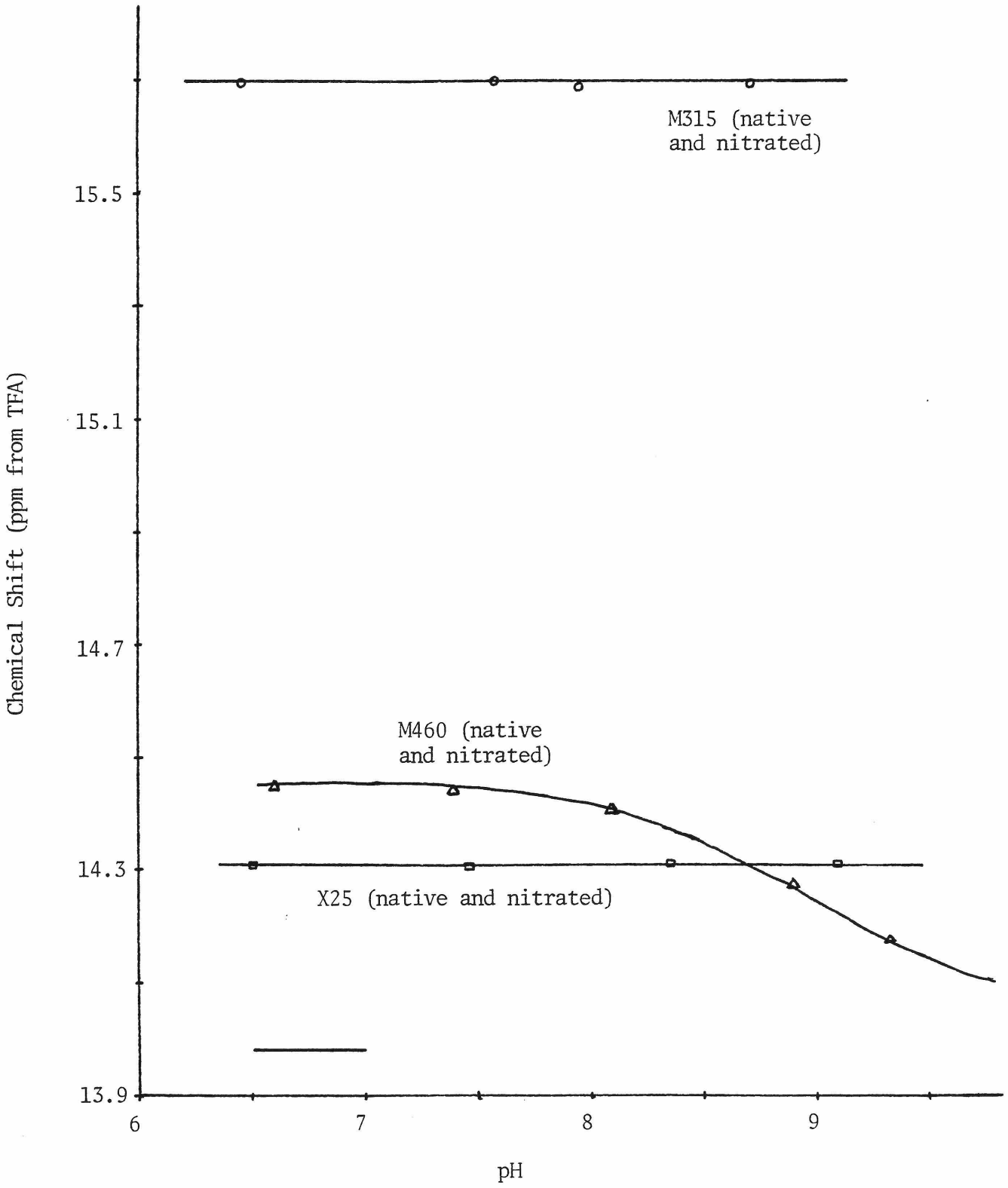

M315 (native
and nitrated)
15.5
15.1
14.7
M460 (native
and nitrated)
14.3
X25 (native and nitrated)
13.9
Chemical Shift (ppm from TFA)
6
7
8
9
pH

Table VI: Chemical Shift pH Dependence

Protein		Hapten para	ortho	chain
M315	native	none	0.1; $\geq$ 9	0.1; > 9
	nitrated	none	0.2; 8.1	0.4; 8.0
M460	native	Δ > 0.25; pK $\geq$ 9	none	none
	nitrated	same	none	none
X25	native	none	0.1; 8.8	0.1; 8.5
	nitrated	none	0.1; 8.0	0.1; 8.5

first value: total chemical shift change

second value: pK of titration

FIGURE 13

^{19}F Labeled M315 F_{ab}'

Fluorine Magnetic Resonance Spectrum.

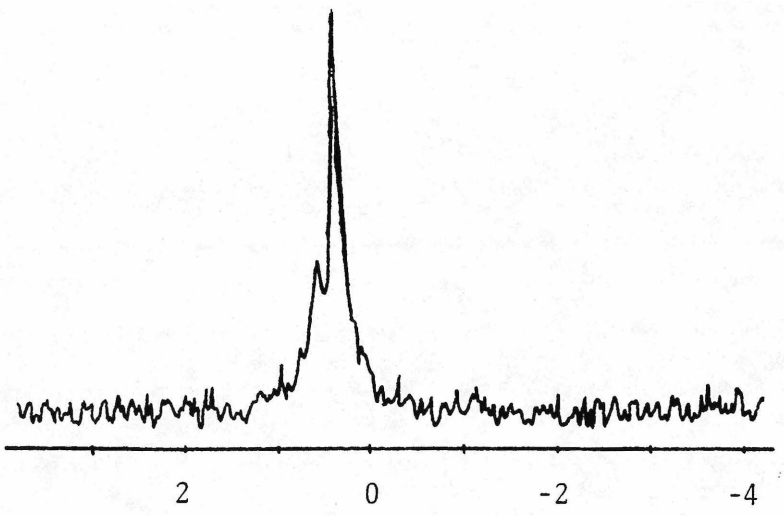
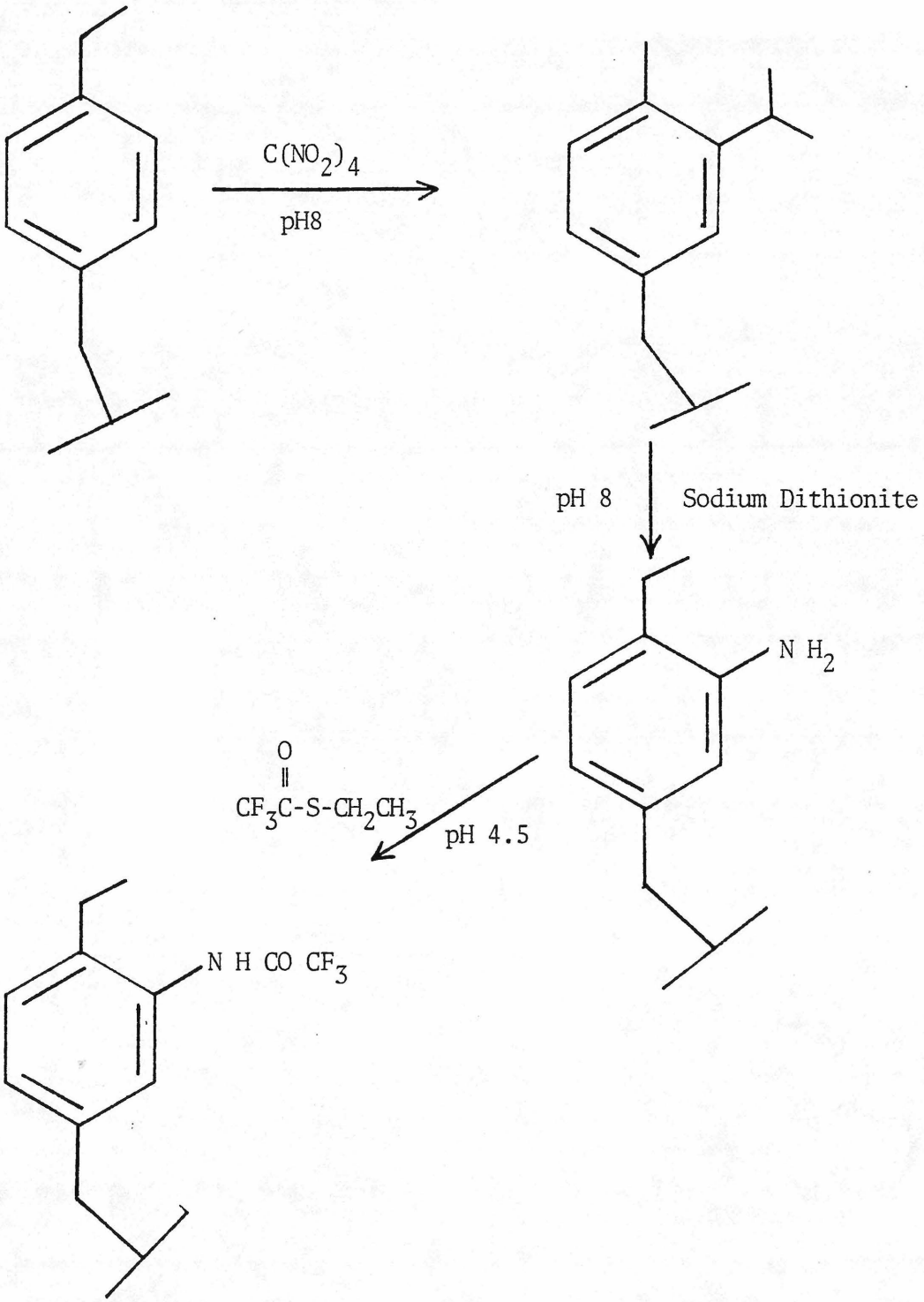

2
0
-2
-4
Chemical Shift (ppm relative to TFA)

Table VII: Group Responsible for pH Dependence

	Hapten		
Protein	para	ortho	chain
M315	----	Tyr 33H	Tyr 33H
M460	Tyr 34L	----	----
X25	----	Tyr 33H	?

FIGURE 14

Reaction involved in protein modification of nitrophenyl-specific F_{ab}'


C(NO2)4
pH8
pH 8
Sodium Dithionite
N H2
O
CF3C-S-CH2CH3
pH 4.5
N H CO CF3

Chapter 4

^{1}H Magnetic Resonance Investigation of the Binding of Methyl Nitrophenyl Haptens to Mouse Myeloma Immunoglobulins M315, M460, and X25

Prior studies involving the use of fluorine NMR to study the binding of fluorinated nitrophenyl haptens to three nitrophenyl specific myeloma proteins, M315, M460 and X25, showed that in at least some cases ring currents played a dominant role in the changes in chemical shift observed on binding (Hardy and Richards, 1978). The upfield shifts which occur on binding can unambiguously be ascribed to ring currents as no other interaction in the site could yield such shifts (Johnson and Bovey, 1958). On the other hand, the downfield shifts could be due entirely to ring currents or possibly to hydrogen bonding (Hauge and Reeves, 1966), to hydrophobic interactions (Hull and Sykes, 1976) to van der Waals crowding (Kimber et al., 1977) or to some combination of these effects. A method whereby the diamagnetic component of the shift (due to ring currents) may be isolated from the paramagnetic component has been described (Millet and Raftery, 1972). The procedure involves replacing the fluorine reporter group which is subject to both components by a proton group whose shift is completely due to the diamagnetic component. Thus in this work nitrophenyl haptens have been synthesized with methyl groups in the place of trifluoromethyl groups and their binding studied by proton NMR.

The use of proton NMR to study the binding of small molecules to a protein is considerably more difficult than the analogous fluorine NMR experiment. This is because of the large "background" signal arising from the large number of protein protons (Bradbury and Chapman, 1972). This problem can be overcome by a combination of two techniques: use of a fast exchange system and use of a special relaxation technique.

If a particular reporter group is in fast exchange on the NMR timescale then the observed signal position is a weighted average of the

mole fractions of the free and bound species (Dwek, 1973). This is in contrast with the slow exchange case in which both the free and the bound resonances are observed. Thus in the fast exchange limit even at relatively large excesses of unbound hapten, i.e., tenfold, the observed signal is still shifted toward location of the bound resonances. The mole fractions of each species may be calculated if the association constant is known and, since the position of the free signal is already known, the position of the bound resonance can be determined. In theory only one spectrum at a given ratio of hapten to antibody should suffice for such a determination, but in practice several spectra at varying molar ratios permit a linear least squares analysis and thus greater precision.

The association constants are determined separately by the technique of competition equilibrium dialysis. In simple equilibrium dialysis (Eisen and Karush, 1949) hapten and antibody are each placed in a chamber separated from each other by a semipermeable membrane which permits free diffusion of the hapten but not the protein. After a suitable equilibration time the hapten will be distributed between the two sides with more on the side containing antibody to which it is bound. A determination of the amounts of hapten on each side, commonly achieved through the use of radiolabeled hapten, permits the amount of hapten bound at that particular concentration to be calculated. Such determinations are made at several hapten concentrations and the data are plotted according to the method of Scatchard (Scatchard, 1949) which yields the antibody valency and the association constant.

In a competition study a constant amount of labeled hapten is added to each compartment so as to yield a binding of about 50%. Then

increasing concentrations of the competing hapten are added to each compartment which yields data on the amount of labeled hapten no longer bound at any given inhibitor hapten concentration. If the binding constant of the labeled ligand is known then a suitable plot (Cheng and Prusoff, 1973) gives the association constant of the competing hapten. This method has been used to study the binding of inhibitors to enzymes and hormones to their receptors (Jacobs et al., 1975).

Thus if the binding constant is known in a fast exchange system then one may employ large excesses of hapten and obtain spectra at ten-, fiften- or twentyfold excesses of hapten over antibody. At these levels the hapten resonance should be visible even in the presence of the large protein background and so the bound position may be extrapolated.

Another technique which is useful for the observation of resonances of small molecules in the presence of background resonances from a large molecule involves a special pulse sequence known as "inversion recovery spin echo" (Rabenstein et al., 1979). The technique takes advantage of the fact that resonances from a large molecule are quite broad due to its long correlation time. Such signals relax very quickly (have short T_2 values) and may be selectively reduced relative to resonances with longer relocation times (sharp signals) typical of small molecules. Thus by selecting an appropriate delay time in the pulse sequence sharp resonances may be relatively enhanced compared to the protein background resonance envelope. This approach is especially important in the case of a hapten with limited solubility such that large excesses are not attainable.

In this study spectra have been obtained with the two water soluble methyl nitrophenyl haptens at various large molar excesses binding to the

three nitrophenyl-specific myeloma proteins employed in previous work. The water insoluble chain methyl hapten was observed at low molar excesses with the proteins using the spin echo pulse sequence. In addition spectra were obtained with this hapten using a normal pulse sequence and signals were obtained using the procedure of difference spectroscopy. In this method the spectrum of antibody is subtracted by computer from the spectrum of hapten plus antibody yielding the hapten resonances. It is somewhat less reliable than direct observation of the signals.

Materials and Methods

Protein Preparation

F_{ab}' fragments of M315, M460 and X25 were obtained by pepsin digestion as described in Chapter 1.

NMR Sample Preparation

Deuterated buffer used in the study was obtained by lyophylization of the standard buffer (0.15 M NaCℓ, 0.01 M $N_aH_2PO_4$, 0.001 M EDTA, 0.02% NaN_3, pH 7.40) followed by reconstitution with 99% D_2O several times. Protein was transferred into this buffer by pressure dialysis (AMICON Diaflo) and all samples were concentrated to about 65 mg/ml (1.2 mm) at the same time. The two haptens with ionizable side chains were made up in deuterated buffer as 0.05 M stock solutions. NMR samples were prepared by addition of aliquots of these stock solutions to the protein solution. The aliphatic side-chain hapten was insoluble in aqueous buffer and so was prepared as a 0.05 M stock solution in acetone. Proton NMR spectra were accumulated using 5 mm round bottom tubes locked on the deuterium signal of the buffer. Spectra were observed on a

JEOL-FX-90Q spectrometer at 89.56 MHz or on a Bruker WM-500 spectrometer at 500 MHz.

Acquisition of Spectra

As mentioned above spectra were acquired on samples with large hapten excess using a spin echo pulse sequence. Methyl peaks were observed at greater than fivefold excess to twentyfold excess. In the case of the aliphatic chain hapten such excesses were not attainable so peaks were located by difference spectroscopy subtracting spectra in the computer.

Synthesis of N-(2,6-dinitro-4-methyl phenyl) glycine

This hapten was prepared by reaction of 4-chloro-3,5-dinitrotoluene (ICN-K and K Labs., Plainview, N. J.) with glycine (Sigma, St. Louis, Mo.) in an ethanol-10% bicarbonate solution (1:1, pH9) for three hours on a steam bath. The ethanol was removed in vacuo and the solution was extracted twice with ethyl ether. The solution was acidified and the product precipitated. Recrystallization from ethanol-water yielded yellow needles : mp 187°C. NMR: 7.27(2H); 2.35(3H); 1.70(2H) (in deuterated PBS).

Synthesis of γ-N-(2-methyl-5-nitrophenyl) aminobutyric acid.

This hapten was prepared by reaction of 1-fluoro-4-nitrotoluene (Pfaltz and Bauer, Stamford, Conn.) with γ-amino butyric acid (Calbiochem-Behring Corp., San Diego, Ca.) in an ethanol-10% bicarbonate solution (1:1, pH9) for 16 hours at reflux on an oil bath. The ethanol was removed in vacuo and the solution extracted twice with ethyl ether. The solution was treated as above. Recrystallization from ethanol-water yielded yellow needles: mp 197°C. NMR: multiplet centered at 7(3H); triplet at 2.1(2H); multiplet from 2.3 to 1.7(4H); superimposed singlet

at 2.1(3H) (in deuterated PBS).

Synthesis of N-(2,4-dinitrophenyl) aminoethane.

This hapten was prepared by reaction of ethyl amine (70% in water; Pfaltz and Bauer, Stamford, Conn.) with 2,4-dinitrofluorobenzene (Aldrich, Milwaukee, WI.) in an ethanol-10% bicarbonate solution (2:1, pH9) for 16 hours at room temperature. The ethanol was removed in vacuo and the solution extracted with chloroform. The chloroform layer was dried over magnesium sulfate, the chloroform evaporated and the resulting solid recrystallized from ethanol: mp 114°C. NMR: multiplet centered at 8(3H); multiplet centered at 3.3(2H); triplet centered at 1.1(3H) (in acetone-d_6).

Hapten Binding Assay

ε-N-(3,5-^{3}H-2,4-dinitrophenyl) lysine was obtained from New England Nuclear (Boston). Equilibrium dialysis was carried out in lucite wells of 2 ml capacity containing 1 ml per side separated by cut tubular dialysis membrane (V.W.R., Los Angeles). The membrane was prepared by boiling it in a 0.1% carbonate-EDTA solution followed by several changes of water and boiling again in distilled water. Prepared tubing was stored at 4°C in the dialysis buffer (0.15 M NaCℓ, 0.01 M NaH_2PO_4, 0.001 M EDTA, 0.02% NaN_3 pH 7.4). All protein was F_{ab}' fragment and concentration was determined spectrophotometrically at 280 nm using a value for the absorbance of a 0.1% solution (1 cm path) of 1.4 and a molecular weight of 55,000. All equilibrations were carried out on a rocker table for 24 hours at 22°C. Aliquots (0.8 ml) were withdrawn from each side and added along with 10 ml of Aquasol cocktail (New England Nuclear, Boston) to Beckman plastic scintillation vials. All samples were counted on a Beckman LS9000 Counter to an average error of

2%. Competition binding experiments were carried out at a DNP-lysine concentration giving 50% bound to obtain maximum sensitivity. At high inhibitor concentration a correction was made for quenching of the cocktail by the inhibitor. Direct equilibrium dialysis binding DNP-lysine to the antibodies was analyzed by a Scatchard plot (Scatchard, 1949) least squares analysis while competition binding was analyzed according to Cheng and Prusoff (1973). Protein concentration for M315, M460, and X25 were 0.05, 0.5 and 1.0 mg/ml respectively. 0.1% gelatin (Difco, Detroit) was included in all buffer solutions to minimize nonspecific binding.

Results

Table VIII presents the binding constants determined for the three methyl haptens to each protein as found by competition equilibrium dialysis. Figure 15 provides an example of typical fast exchange proton spectra with increasing amounts of hapten in the presence of 1 mM antibody. Figure 16 shows the different spectrum technique useful for the water-insoluble hapten. Table IX collects the proton shifts determined in this study and also indicates the fluorine shifts found previously with the analogous fluorine haptens. Table X gives the values calculated for the paramagnetic component of the fluorine shift.

Table VIII: Association Constants (M^{-1})

	p-CH_3 gly	O-CH_3 gaba	chain CH_3
M315	$5.9 \cdot 10^3$	$5.8 \cdot 10^4$	$4.4 \cdot 10^5$
M460	$2.0 \cdot 10^4$	$2.9 \cdot 10^4$	$6.0 \cdot 10^4$
X25	$5.9 \cdot 10^2$	$1.5 \cdot 10^4$	$7.0 \cdot 10^4$

Table IX: NMR Shifts (Bound-Free)

(ppm)

	para TNP		ortho DNP		chain DNP	
	^{19}F	^{1}H	^{19}F	^{1}H	^{19}F	^{1}H
M315	1.8†	-1.2	0.6†	-1.2	-0.8	~-0.8
M460	1.0	-0.8	0.2	-0.5	0.6	~ 0.4
X25	0.1	-1.0	-0.8	-1.1	-0.6	~-0.6

†Kooistra and Richards, 1978.

Table X: Non-Ring Current Fluorine Shifts (Paramagnetic Component) (ppm)

	P-TNP	O-DNP	chain DNP
M315	3.0	1.8	slight
M460	1.8	0.7	<0.3
X25	1.1	0.3	slight

Figure 15

Proton spectra: increasing para-methyl hapten with M315.

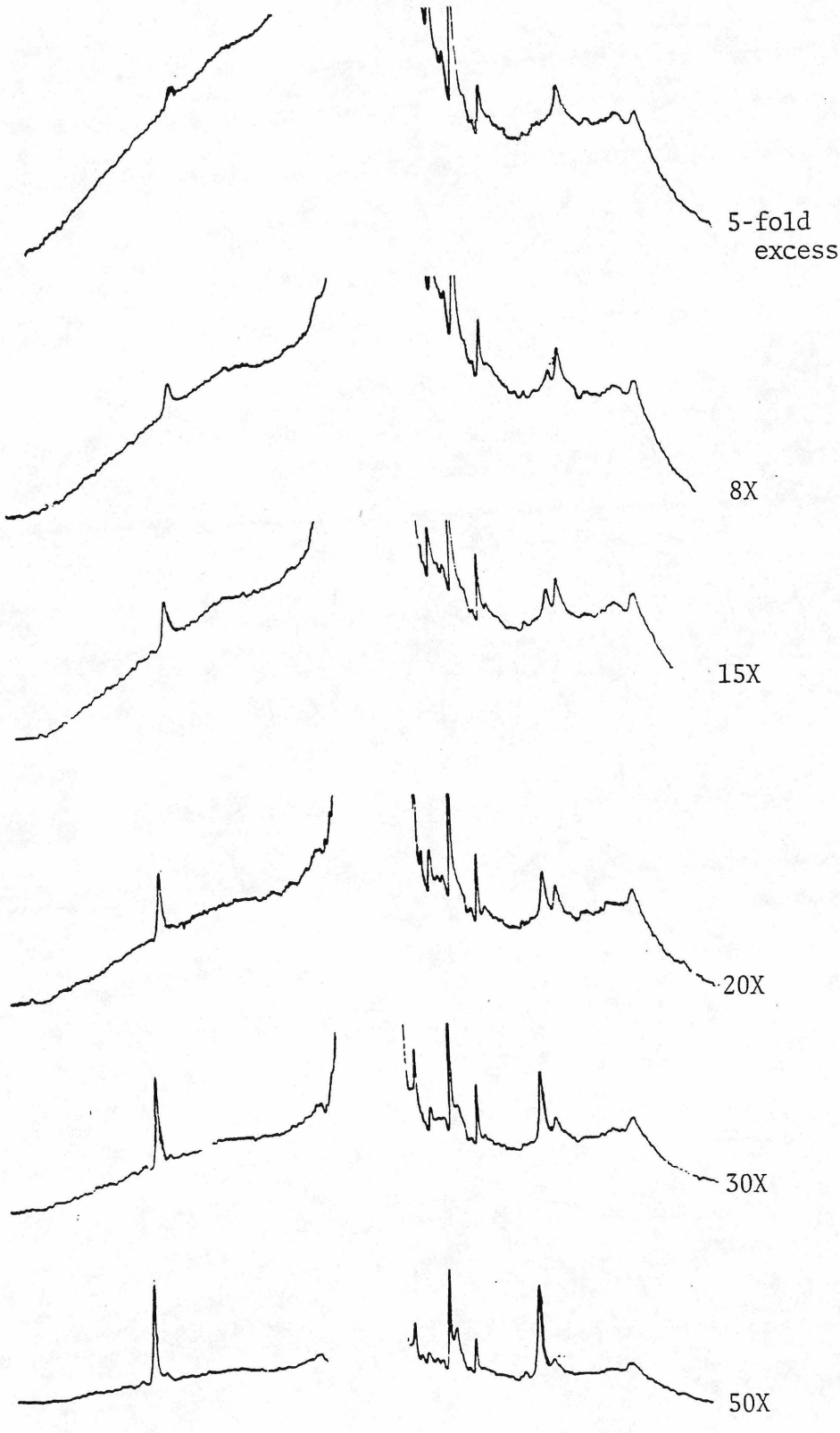
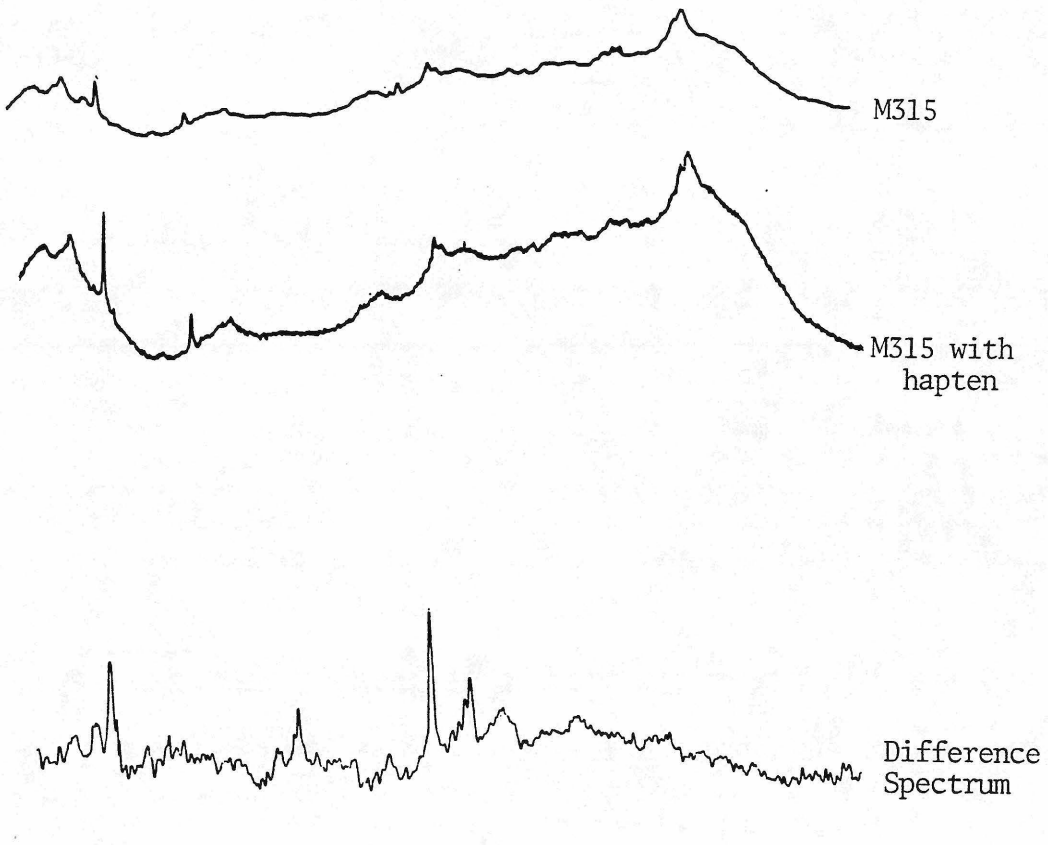

5-fold
excess
8X
15X
20X
30X
50X

Figure 16

Proton difference: spectrum of M315 and M315 with chain methyl hapten.


M315
M315 with hapten
Difference Spectrum

Discussion

The methods of hapten excess in fast exchange systems or the spin echo acquisition sequence provide a relatively reliable means for obtaining the bound chemical shifts of methyl hapten in the presence of antibody background resonances. The results showed that, without exception, all the resonances of the methyl groups on the nitrophenyl ring shifted upfield on binding typically by one part per million. Such large shifts have been observed in hapten binding to M315 previously (Dwek et al., 1977) and are clearly due to ring currents in these very aromatic binding sites. In the case of M315 some nearby aromatic residues include Tyr 34L, Tyr 33H, Phe34H and Trp 93L (Padlan et al., 1976). An interesting result emerges on calculation of the fluorine paramagnetic shifts. They are all downfield and appear to progress in magnitude from the smallest shift on the chain to the largest shift for the para substituted methyl group. This systematic variation may be explained most readily by two potential effects. First, it is well known that paramagnetic shifts due to van der Waals crowding are downfield (Kimber et al, 1977) and as one progresses deeper into the site (toward the para position) such crowding would probably increase. A problem with this explanation accounting for the entire shift is that independent experiments using electron spin resonance (Willan et al., 1977) showed that X25 most completely immobilizes the hapten, M460 immobilizes it least and 315 is intermediate. On the other hand, the largest paramagnetic shifts are found with M315, the least with X25 and M460 is intermediate.

A second possible cause of these shifts is a charge transfer interaction between the nitrophenyl hapten and a tryptophan in the binding site. Charge transfer interactions are a common feature of nitrophenyl-

specific antibodies (Little and Eisen, 1967) and take place in each of these three proteins as shown by a red shift in the hapten visible absorption spectrum on binding. Large fluorine shifts have been observed in charge transfer complexes (Foster and Fyfe, 1965), but the trifluoromethyl group isn't a part of the π electron system (which accepts the electron) so that it might not be expected to experience as large shifts as a directly bonded fluorine. Thus any shift would be a secondary effect. However, as shifts of over 10 parts per million for directly bonded fluorine nuclei have been observed in strong charge transfer complexes (Taft and Carten, 1964) such a secondary effect might still yield a shift of the order observed here. Depending on which resonance forms of the hapten are the most important, one site (i.e., the para position) might localize more of the transferred charge than the others. Naturally, the chain group, being separated by three atoms from the aromatic ring, would experience the least effect.

One further observation might be made concerning the values of the methyl hapten association constants. The magnitudes of the binding constants are essentially the same for the trifluoromethyl and methyl homologues. Thus it would appear that the trifluoromethyl group does not significantly withdraw electron density from the π electron system (in the manner that nitro clearly does) since the methyl group certainly isn't electronegative. Therefore replacement of a nitro group by either a methyl or trifluoromethyl group changes the electron deficiency of the hapten and may weaken the charge transfer interaction with the antibody. This casts some doubt on previous attempts to define hydrogen bond interactions by replacing nitro groups with trifluoromethyl groups (Hardy and Richards, 1978) because these assume that since the electronegatives of

$-CF_3$ and $-NO_2$ are comparable then the haptens will interact similarly with the antibody in every way except for hydrogen bonding.

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CHAPTER 5

Nuclear Magnetic Resonance and Visible Absorption Studies of Charge-Transfer in Model Complexes and in DNP-Binding Antibodies

The existence of charge transfer complexes has been recognized in organic chemistry for many years (Andrews, 1954). The complex consists of a donor molecule or moiety that transfers all or part of its highest orbital electron to an acceptor molecule or moiety (Person and Mulliken, 1962). Such an interaction is often accompanied by the emergence of a new electronic absorption band due to transitions of the transferred electron (Brackman, 1949). The first examples of charge transfer complexes were recognized on the basis of the development of unexpected colors on mixing the donor and acceptor (Benesi and Hildebrand, 1949). Various organic complexes have been extensively characterized by their visible absorption spectra (Foster, 1969). Studies have also been carried out with fluorescence (Rosenberg and Eimutis, 1966), and infrared spectroscopy (Lake and Thompson, 1967). The extent of the interaction has been related to the intensity of the charge transfer absorption band (Emslie et al., 1965). The association constant between donor and acceptor has been determined by a variety of physical techniques, one of which is nuclear magnetic resonance spectrometry (Hanna and Ashbaugh, 1964).

Charge transfer interactions are known to occur in a number of biological systems such as the interaction between nicotiniamide adenine dinucleotide and tryptophan in glyceraldehyde-3-phosphate dehydrogenase (Cilento and Tedeschi, 1961). A well-known example of this interaction in antibodies is the binding of dinitrophenyl haptens by DNP-specific immunoglobulins (Little and Eisen, 1967).

A number of spectropscopic techniques have been applied to study the interaction between hapten and antibody binding site. Among these

are uv-visible absorption spectroscopy (Eisen, 1966), fluorescence (McGuigan and Eisen, 1968), and circular dichroism (Conway-Jacobs et al., 1970) applied either to the hapten, to the antibody, or to both. In particular, visible spectroscopy has been used to observe hapten electronic spectral changes that occur on binding (Eisen et al., 1970) which is especially facilitated if the absorption studied is outside the protein's absorption envelope. This greatly simplifies the problem of distinguishing hapten perturbations from changes in the antibody. Dinitrophenyl haptens, with their intense yellow color, have long been popular subjects of visible absoprtion studies. Very early it was found that the spectrum of a DNP-chromophore is considerably perturbed on binding to a nitrophenyl-specific antibody experiencing what was termed a "red shift" - the maximum absorption was shifted to longer wavelength (Little and Eisen, 1967).

The "red shift" of a nitrophenyl hapten's absorption band which takes place on binding has been interpreted as due to the formation of a charge transfer complex between the electron-deficient nitroaromatic ring and an electron-rich tryptophan indole group in the binding site (Eisen and Siskind, 1964). Thus the "red shift" is actually due to the appearance of a new absorption band at longer wavelength than the hapten band. Very similar spectra can be obtained from solutions of the DNP-hapten in large excesses of tryptophan (so that the hapten is fully complexed) (Dewar and Lepley, 1961).

A study to define more precisely the charge transfer interaction in the nitrophenyl-specific myeloma protein M315 has been carried out

using the technique of circular dichroism (Inbar et al., 1973). The position and magnitude of the CD bands indicated that the DNP hapten and indole ring were likely parallel and separated by 3 1/2 Å. Furthermore, only a limited number of relative orientations were allowed in a later more detailed study utilizing CD (Freed et al., 1976). Even in solutions of model complexes it appears that certain stable configurations are preferred so that a specific part of the DNP ring is next to a specific part of the indole system (Foster and Fyfe, 1966).

Fluorine magnetic resonance studies of the binding of trifluoromethyl nitrophenyl haptens to several DNP-specific myeloma proteins indicated large downfield shifts on binding in many instances (Hardy and Richards, 1978). A comparison of the proton shifts occurring on binding of methyl analogues with the fluorine shifts of the trifluoromethyl haptens permitted the determination of the paramagnetic component of the fluorine shift (as distinct from the ring current dominated diamagnetic component) (Millet and Raftery, 1972). These shifts were found to be uniformly larger in the para-substituted group than in the ortho position. This seemingly systematic variation suggests the hypothesis that most, if not all, of the paramagnetic component of the fluorine shift observed on binding is due to the charge transfer interaction. Such an interaction could specifically change the charge distribution in the π electron cloud of the nitrophenyl hapten which would cause a shift of the trifluoromethyl fluorines, albeit secondhand. However, the sensitivity of fluorine to such perturbation is such (Saika and Slichter, 1954) that a one

to three parts per million shift (as was observed here) could easily be due to a secondary effect.

The contribution of charge transfer to the change of chemical shift that takes place on binding may be investigated by carrying out NMR studies on charge transfer model complexes. Such complexes consist of the various haptens interacting with a methyl ester of tryptophan in a suitable organic solvent. This solvent permits the high concentrations of tryptophan necessary to favor complete complexation of the hapten. These studies will permit determination of the association constant of each complex and the position of the fully complexed fluorine chemical shift.

Materials and Methods

Visible Spectral Sample Conditions

50 μℓ of a 0.05 M stock solution of the hapten in acetone was added either to 450 μℓ of acetone or to 450 μℓ of a 0.50 M tryptophan methyl ester in acetone solution. UV-VIS spectra were obtained with a Beckman Acta III at 25°C using 1 mm pathlength matched cells. For protein spectra all antibody was approximately 1 mM and hapten was added so that most would be bound.

Magnetic Resonance Sample Conditions

100 μℓ of 0.05 M hapten stock solution was added to 900λ of acetone-d_6. Aliquots of a 0.50 M tryptophan methyl ester solution (in acetone-d_6) were added and spectra accumulated after temperature equilibration (at 25°C). Excellent signal to noise was achieved after

a five minute accumulation. Samples were run in 5 mm round bottom tubes on a Bruker WM500 at either 500 MHz (for ^{1}H) or at 470 MHz (for ^{19}F) locked on the acetone-d_6 resonance.

Gaussian Fitting Procedure

Hapten uv-visible spectra were fit to a simple gaussian curve according to the equation:

$$A(\lambda) = ae^{-h_1^2(L_1-\lambda)^2}$$

using a fitting algorithm programmed on a Commodore Pet microcomputer. This gave values for the parameters a, h_1, and L_1 where a is the maximum intensity of the absorption band, h is related to the width of the band and L is the wavelength of the maximum absorption. Then the charge transfer spectra which consist of the original absorptions, slightly diminished, with the added charge transfer bands were fit using the same program according to the equation:

$$A(\lambda) = abe^{-h_1^2(L_1-\lambda)^2} + ce^{-h_2^2(L_2-\lambda)^2}.$$

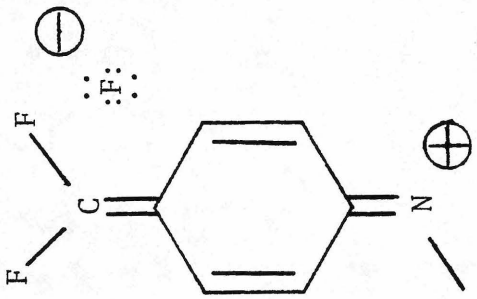
The values of h_1, a, and L_1 were obtained from the hapten spectrum and the parameters c, h_2, and L_2 are found by varying b so as to minimize the rms error of the fit.

Results

Figure 17 is a drawing of the resonance structures in these haptens important to the charge transfer interaction. Figure 18 shows the effect on the hapten's absorption spectrum produced by addition of DNP specific antibody. Figure 19 demonstrates that the charge transfer band can be deconvoluted from the mixture of hapten and charge transfer absorptions. Table XI collects the charge transfer binding and shift data determined by NMR while Table XII collects the charge transfer parameters found by the gaussian fitting of the visible absorption spectra. Table XIII gives the binding energies of a number of haptens to M315, M460, X25, and tryptophan. Table XIV decomposes the total binding energies into components. Table XV compares the ^{19}F paramagnetic shifts of hapten-antibody association with hapten-tryptophan association for the three major haptens. Figure 20 presents two views of the DNP-tryptophan charge complex most likely in these DNP-specific antibodies.

Figure 17

Resonance structures important to the binding of haptens to nitrophenyl-specific antibodies


F
F
C
N

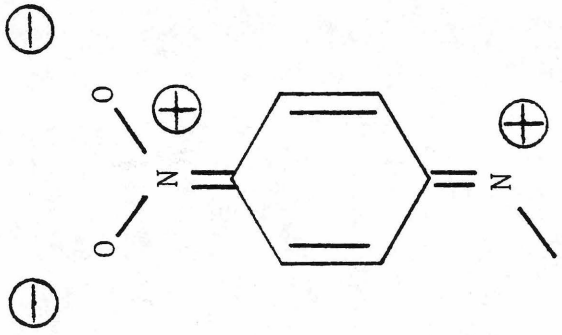
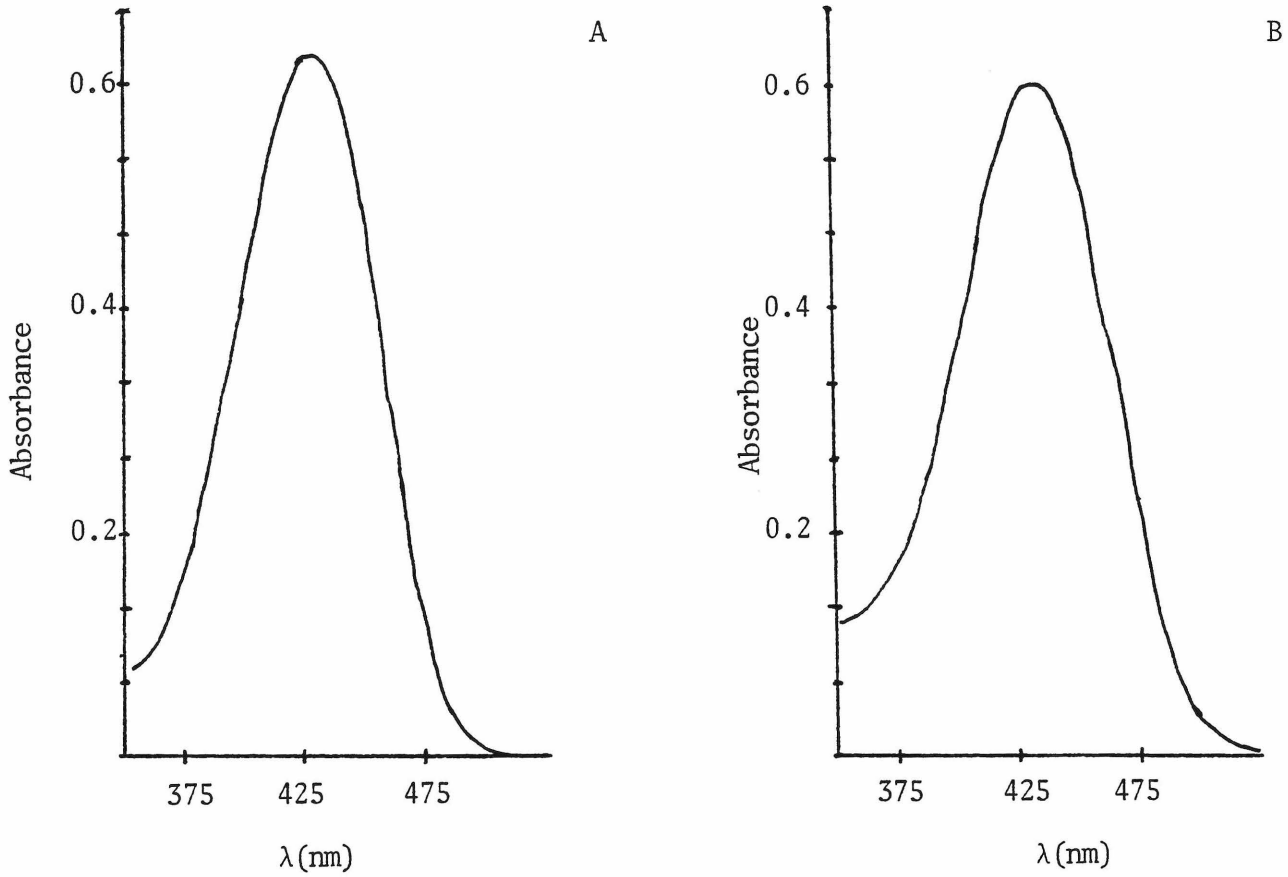

O
O
N
N

Figure 18

A: DNP hapten alone

B: DNP hapten with M315

C: Difference spectrum between A and B.


A
B
0.6
0.4
0.2
Absorbance
375
425
475
λ(nm)

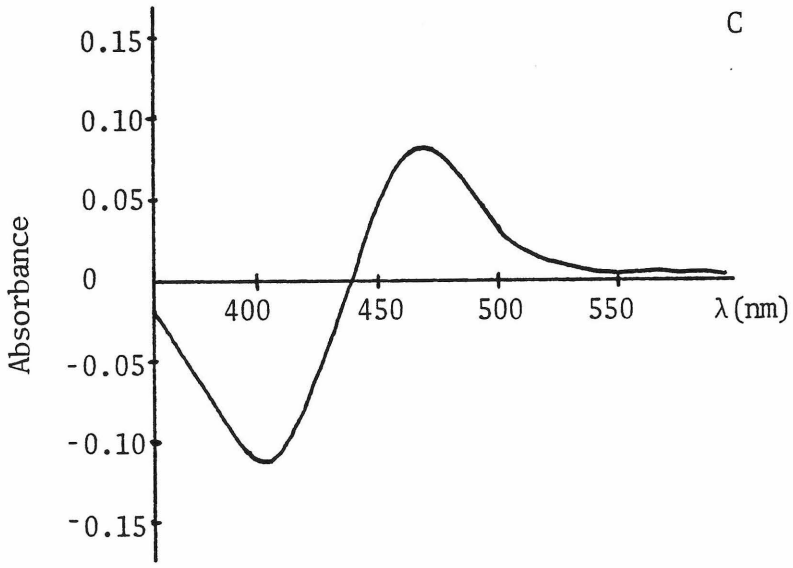

C
0.15
0.10
0.05
0
-0.05
-0.10
-0.15
Absorbance
400
450
500
550
λ(nm)

Figure 19

A: DNP hapten alone

B: DNP hapten with M315

C: Charge transfer band fit by computer.

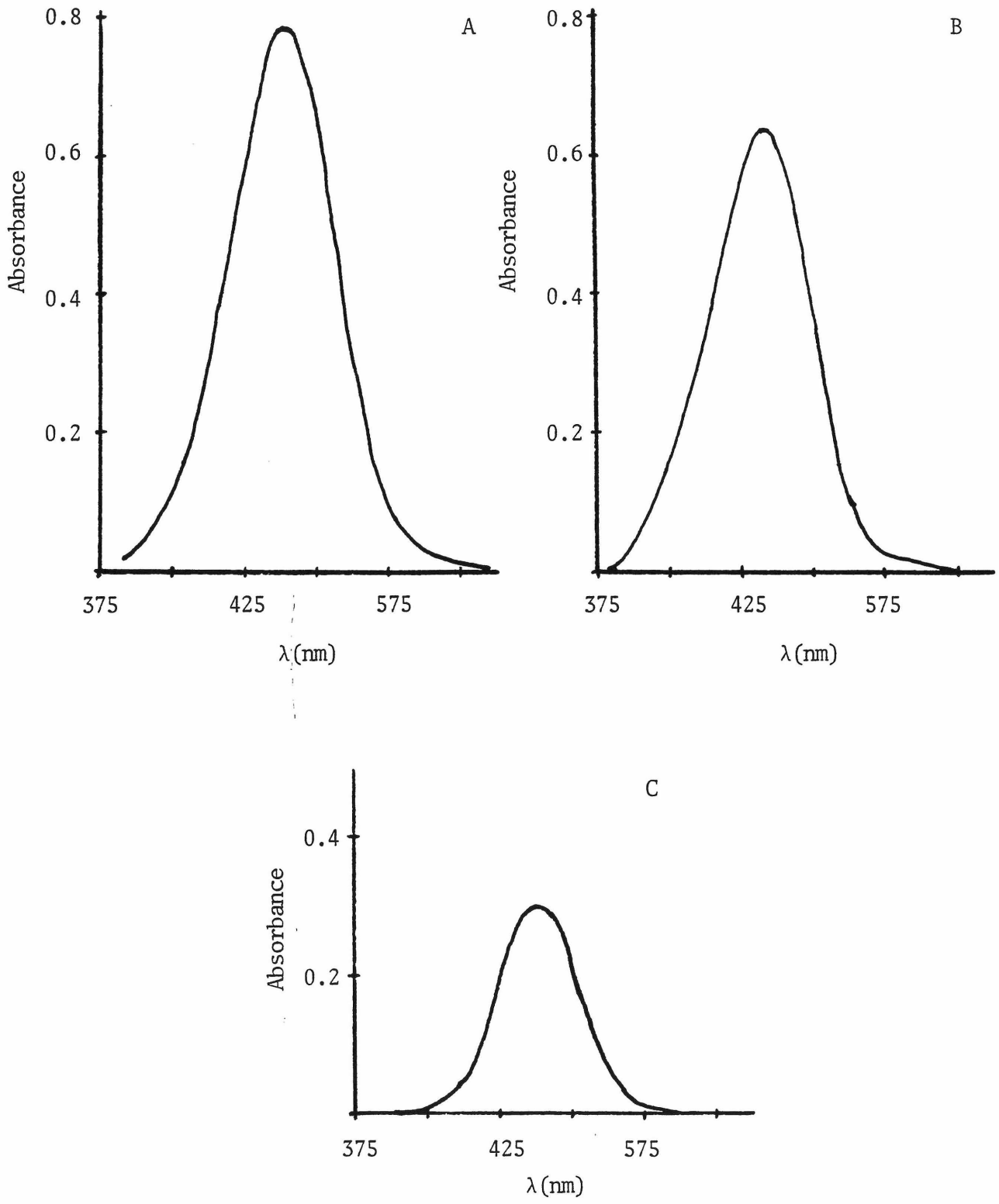

A
0.8
0.6
0.4
0.2
Absorbance
375
425
575
λ(nm)
B
0.8
0.6
0.4
0.2
Absorbance
375
425
575
λ(nm)
C
0.4
0.2
Absorbance
375
425
575
λ(nm)

Table XI Charge Transfer Model Complex Association Constants and Chemical Shifts

		$K_a(M^{-1})$	Δ(Bound-Free)(ppm)
p-CF_3TNP	Trp	25	0.35
p-CF_3DNP	Trp	5	0.33
o-CF_3DNP	Trp	50	0.07
p-CF_3DNP	5-F-Trp	15	-0.40
o-CF_3DNP	5-F-Trp	12	-0.40
p-CH_3TNP	Trp	20	0.30
o-CH_3DNP	Trp	50$^{\neq}$	0.00

$^{\neq}$assumes the same value as for the ^{19}F analogue

Table XII

	Decrease in Hapten Intensity	Charge Transfer Parameters			$\Delta\lambda$	rms error
		ε	n	λ_{max}(nm)		
p-CF_3 plus Trp (acetone)	0.40	875	0.028	424	25	0.0086
o-CF_3 plus Trp (acetone)	0.65	1120	0.039	374	24	0.0095
p-CF_3 plus M315 (PBS')	0.50	250	0.027	452	27	0.0038
p-CF_3 plus M460 (PBS')	0.68	190	0.025	459	34	0.0120
p-CF_3 plus X25 (PBS')	no observable charge transfer band					

Table XIII Binding Energies (kcal M^{-1})

	M315	M460	X25	Trp
p-CF_3DNP gaba	10.4	7.5	6.9	1.0
p-CF_3TNP gaba	11.3	8.4	5.5	
p-CF_3TNP gly	7.4	8.4	6.3	1.9
p-CH_3TNP gly	7.5	8.3	6.2	
o-CF_3DNP gaba	8.7	8.6	8.1	2.3
o-CH_3DNP gaba	8.9	8.5	8.1	
chain CF_3DNP	10.1	9.2	9.0	
chain CH_3DNP	10.1	9.2	9.0	
DNP-lysine	10.6	8.7	9.1	

Table XIV Components of Binding Energies (kcal M^{-1})

	M315	M460	X25	Trp
p-CF_3DNP gaba	6.9 + 0.6 + 2.9	6.9 + 0.6	6.9	1.0
p-CF_3TNP gaba	6.9 + 0.6 + 2.9 + 0.9	6.9 + 0.6 + 0.9	6.9 + 0.9 + 2.3	1.9
p-CF_3DNP gaba	8.1 + 0.6	8.1 + 0.6	8.1	2.3

6.9 contributed by p-charge transfer and hydrophobic interaction

8.1 contributed by o-charge transfer and hydrophobic interaction

0.9 extra charge transfer interaction from extra NO_2 group

2.9 M315 hydrogen bond

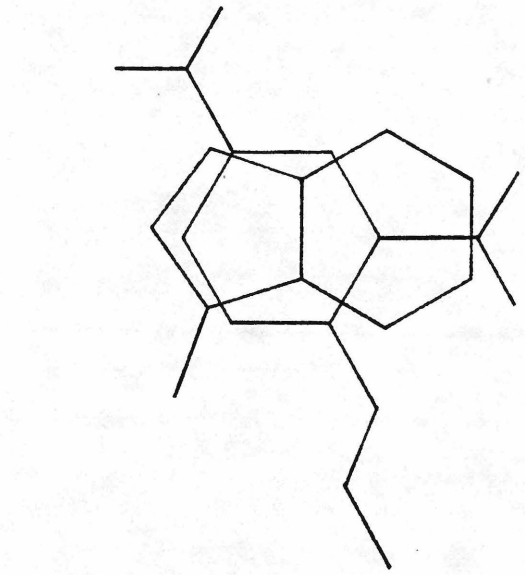
-2.3 X25 steric interaction with TNP haptens

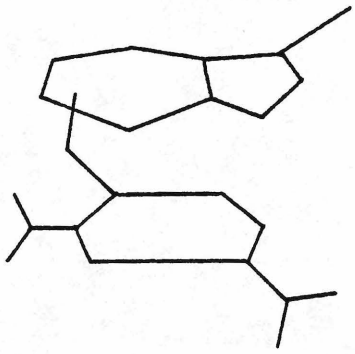
Table XV ^{19}F Paramagnetic Shift (ppm)

	M315	M460	X25	Trp
p-CF_3	3	1.8	1.1	0.65
o-CF_3	1.8	0.7	0.3	0.07
chain CF_3	slight	$\leq$ 0.3	slight	0.00

Figure 20

Two views of the charge transfer
complex likely in DNP-specific antibodies.





Discussion

A gaussian fitting algorithm permitted calculation of the position and size of the charge transfer band from the visible spectral data. The energy of the charge transfer band is related to the difference between the ionization potential of the donor (tryptophan) and the electron affinity of the acceptor (the DNP hapten) (Mulliken, 1952) so it is not surprising to find the differences in wavelength between the charge transfer band and the original hapten absorption band are all about 25 to 30 nm. The intensity of the band is related to the amount of interaction between donor and acceptor (the amount of overlap between filled donor orbital and vacant acceptor orbital) (Kroll, 1968). The intensity is often solvent dependent, but one can make relative comparisons if all studies are carried out in the same solvent. The data indicate that in acetone the ortho-substituted trifluoromethyl hapten has a stronger interaction with tryptophan than the para-substituted hapten. In buffer the para-substituted hapten interacts maximally with M315, less with M460 and has no detectable charge transfer band with X25.

The association constants for several complexes were obtained from fluorine magnetic resonance experiments. As expected from the intensity data the ortho trifluoromethyl substituted hapten binds much more strongly to tryptophan than the para trifluoromethyl substituted hapten. The difference in association constants between these two systems is most readily explained by the better resonance structures possible with the para-nitro group compared to the para-

trifluoromethyl group. In this way it more completely disperses the negative charge transferred in the complex. An extra nitro group (making a TNP analogue) increases the association constant as would be expected since this adds another electron withdrawing group to the electron acceptor half of the complex. Interestingly, both the para trifluoromethyl and para methyl haptens have similar affinities indicating that the trifluoromethyl group is much less able than nitro to accept electrons from the π electron cloud.

Chemical shifts of the methyl and trifluoromethyl groups when completely complexed have been derived. In the case of the fluorine shifts an atom on the donor has its resonance shifted upfield while a resonance of a fluorine on the acceptor is shifted downfield. This is in the opposite direction expected if the shifts were due to simple changes in electron density: transfer of electron density from tryptophan to the hapten increases electron density about the fluorine shielding it to a greater extent and causing an upfield shift in the fluorine resonance (Foster and Fyfe, 1969). The converse should apply to a fluorine on the donor, tryptophan. The observed shift of the fluorotryptophan is most easily explained as a ring current shift (Dwek, 1973) predominating over the direct electron density produced shift. This has already been found for proton shifts in a similar system (Dwek et al., 1977). The ring current field seen by the fluorine on the tryptophan is composed of both the new ring current of the hapten and the diminished field of the indole system (decreased because of the loss of electron density on complexation). Both of these

effects would tend to give an upfield shift which could easily cancel out any downfield shift due to change in electron density about the fluorine.

An explanation of the shift found with the trifluoromethyl group on the acceptor ring is more complicated and requires consideration of the proton shift observed with a methyl group in its place. The methyl group's proton resonance moves upfield on complexation while the fluorine resonance moves downfield. The shift of the para trifluoromethyl group is much greater than the shift found at the ortho position. The proton shift is sensitive principally to ring currents while the fluorine is sensitive both to ring currents and to electronic effects (the paramagnetic component). The ring current of the hapten would increase on complexation yielding a downfield shift, but this is presumably overcome by the extra ring current field of the tryptophan system to yield a net upfield shift. A similar ring current field surrounds the trifluoromethyl group so that the fluorine paramagnetic shift may be calculated as downfield by 0.65 parts per million. This shift is most readily explained by a consideration of the resonance structure possible in the para trifluoromethyl hapten (Roberts et al., 1950). This structure would be destabilized on complex formation and would tend to give a downfield shift on binding. As this structure is unavailable to the ortho trifluoromethyl hapten it is much less shifted on complexation with presumably all due to changes in the ring current field.

These data have considerable bearing on the binding of similar haptens to nitrophenyl-specific myeloma proteins both with regard to

affinity and to chemical shift on binding. A comparison of the binding energies of methyl and trifluoromethyl haptens to three nitrophenyl specific myeloma proteins and to tryptophan alone allow a number of conclusions to be made concerning the binding of these haptens to these antibodies. First, both methyl and trifluoromethyl groups have similar effects on the binding constant. Secondly, addition of an extra nitro group (yielding a TNP analogue) increases binding simply by increasing the charge transfer interaction except in the case of X25. The large decrease in binding to X25 is due to steric crowding in this protein's tighter site (Willan et al., 1977). A comparison of the para trifluoromethyl and ortho trifluoromethyl hapten association constants shows that in the case of X25 and M460 there is an increase in binding the ortho hapten very similar to that found with the model complex, indicating that this difference in binding reflects a change in the charge transfer interaction. However, the unexpectedly low binding constant of the ortho hapten to M315 indicates that something besides charge transfer is being affected by the replacement of a nitro group by a trifluoromethyl group. This reduced affinity very likely reflects the loss of a hydrogen bond to the ortho nitro group. A final comparison among the three proteins shows that X25's affinity constant is consistently lower when binding to hapten with the gamma amino butyric acid side chain, which indicates that while M315 and M460 have a binding interaction with this chain through a positive subsite, X25 does not.

Conclusions regarding the chemical shift changes observed on binding may be drawn from an examination of the results with the model complexes. It was previously shown that the paramagnetic component of the fluorine chemical shift change observed on binding is largest in the para position and successively less in the ortho and chain positions. This is consistent with a large component of this shift arising from the charge transfer interaction. As was pointed out earlier, to the extent that the para trifluoromethyl resonance structure is destabilized by accepting electron density the fluorine signal will move downfield. If the uv-visible spectra are taken as a guide then the greatest interaction occurs with M315, less with M460, and the least with X25. The paramagnetic shift follows this trend with probably over one part per million of M315's shift due to charge transfer, slightly less for M460 and very little with X25. Presumably, the rest of the downfield paramagnetic shift is due to van der Waals crowding (Kimber et al., 1977) which would be greatest in X25 (the "tightest site") compensating for its lack of charge transfer shift. As expected the ortho position shifts are less than the para position shifts except for the case of M315 which is larger than expected. Quite possibly this reflects the hydrogen bound interaction noted earlier as hydrogen bond formation is known to shift fluorine resonances downfield (Hauge and Reeves, 1966). The paramagnetic shifts with the chain reporter groups are still less, which is in accord with a lack of charge transfer interaction and less crowding further from the bottom of the binding site.

In conclusion, a comparison of the association constants, visible absorption spectra, and chemical shifts between model complexes and hapten-antibody complexes has illuminated several of the interactions taking place in the antibody binding sites. Among these are charge transfer, ionic interactions, hydrogen bonding, and steric crowding. The three binding sites have been shown to possess both common and unique features which lead to their particular fine specificities. Presumably such a combination is a common factor of all antibody binding sites.

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Chapter 6

Computer Modeling the M315 Binding Site

The binding interaction that takes place between antibody and antigen depends on a number of factors including a general complementarity (Pauling et al., 1944) such that the shape of the antigenic determinant fits relatively precisely in the site and on specific forces such as ionic interactions (Grossberg and Pressman, 1968), hydrogen bonds (Weir, 1963) or charge-transfer (Ohta and Gill, 1970). The clonal selection hypothesis proposed by Burnett (Burnett, 1959) states that an enormous diversity of such sites exists in an organism and that presentation of antigen triggers cells with corresponding receptors (identical to the antibody binding site) to proliferate and differentiate into antibody secreting cells. This pre-existing diversity is composed of heavy and light chain variable regions (Nisonoff et al, 1975), one per cell, but the origin of this diversity is still not completely understood (Smith, 1973). It appears likely that it arises from a combination of germ line genes, recombination events and point mutations as the information is transferred from gene to antibody (Early et al., 1980). An understanding of the relationship between the structure of an antibody binding site and its hapten specificity should help to define the "preciseness" of fit required to give a functional antibody. This has important implications to the question of diversity since multispecificity (one antibody binding several haptens) could considerably reduce the required number of coding sequences necessary to generate the enormous diversity of the immune response (Richards and Konigsberg, 1973).

Much progress has been made in recent years toward an understanding of antibody binding site structure. It has been known for some time that the three hypervariable regions of the heavy and light chain are responsible for antibody specificity (Wu and Kabat, 1970). X-ray crystallo-

graphic studies on a number of myeloma proteins defined a common feature of antibody domains consisting of two layers of antiparallel β pleated sheet (Padlan, 1977). This structure termed the "immunoglobulin fold" forms the framework in the variable domain for the hypervariable loops that make a binding site. The precise fit between antibody and hapten was particularly well defined in the X-ray structure of the binding site of the phosphorylcholine-specific mouse myeloma proteins M603 (Segal et al., 1974).

Unfortunately, crystallization of variable region fragments and subsequent diffraction analysis are arduous tasks which clearly can be carried out on only a few of the myeloma proteins. Thus a study of the relationships between antibody structure and specificity which certainly requires the structures of a number of sites with related specificities is not possible using X-ray diffraction analysis alone. However, the existence of the conserved immunoglobulin fold permits a solution of this difficulty. That is, one can model an unknown site if its amino acid sequence is known by arranging it to form this conserved fold. Thus a model of the combining site can be constructed from sequence alignments reasoning by analogy from a known structure such as that of M603.

This method has been employed by workers at the National Institutes of Health to model the nitrophenyl-specific mouse myeloma, M315 (Padlan et al., 1976). A computer graphics system permitted many different conformations to be considered and special programs that avoided unfavorable contacts eliminated unlikely structures. Such a model still requires the input of a great deal of data in order to distinguish between possible orientations of the hypervariable loops and to orient the hapten in the

site. Fortunately, considerable work has been carried out in an attempt to define the forces important to binding in this site. Thermodynamic studies which involved the determination of the binding affinities of a number of related haptens to M315 defined several subsites within the binding site (Haselkorn et al., 1974). Affinity labeling studies pinpointed residues near the hapten in the binding site (Givol et al., 1971). Proton magnetic resonance studies of the hapten and protein indicated many aromatic residues surround the hapten forming a so-called "aromatic box" (Gettins and Dwek, 1977).

Fluorine magnetic resonance studies of trifluoromethyl-substituted nitrophenyl haptens binding to M315 indicated the likely presence of aromatic residues near the probes (Kooistra and Richards, 1978) and the existence of a hydrogen bond to the hapten (Hardy and Richards, 1978). Further work with chemically modified protein localized one of the aromatic residues near a specific fluorine reporter group. A comparison of fluorine binding shifts with protein shifts occurring on binding analogous methyl haptens indicated that charge transfer played a large role in the binding interaction, a point already suspected from visible absorption studies (Eisen et al., 1968).

The original model of the M315 binding site (Padlan et al., 1976) based on the structure of M603 was modified by Dwek (Dwek et al., 1977) to fit ring current shift data. However, the precise orientation of the hapten in the combining site is not completely established so such changes in the model may be premature. Accordingly, the original model was utilized and new data mentioned above were employed to obtain an orientation of the hapten in the site. Then, data from the protein modification study was used to reorient the first hypervariable loop of

the heavy chain. All this modeling was carried out on a computer graphics system, the MMS-X, at the Washington University computer laboratory (Perry, 1978).

Materials and Methods

Computer Modeling

The X-ray coordinates for M603 and the model coordinates for M315 were obtained from Dr. Davies at the National Institutes of Health. The MMX-S interactive computer graphic system, designed and built at Washington University, was used at the Washington University computer laboratory. The system consists of a Texas Instruments 980B central processing unit interfaced with the MMS-X controller, coordinate transformer and display driver. The software consisted principally of BUILD, the molecular model building system, which produces the display from the input coordinates and permits manipulation of the display under joystick control. A stereo pair display is also possible. The master program calls on various other programs such as GROW which permits the construction of new amino acid chains one residue at a time. It is also possible to hold sections of a structure fixed while independently varying other segments or small molecules (such as the hapten).

A Diamond coordinate refinement program was obtained from Dr. Takano at the California Institute of Technology. The program was run on an IBM 370 in the Caltech Computer Center. Input coordinates were fit to ideal X-ray coordinates via an energy minimization algorithm and output consisted of the refined coordinates along with rms deviations between the original and refined coordinates.

Structural Data

The hapten orientation was based on the importance of charge transfer in the binding interactions (Freed et al., 1976), on affinity labeling studies (Givol et al., 1971), and on the NMR data presented in the previous chapters. The reorientation of the first heavy chain hypervariable region was based on NMR results with chemically modified protein presented in Chapter 3.

Results

Table XVI presents fluorine and proton shift data used in orienting the hapten in the M315 model binding site. Table XVII collects association constants and energies of binding for a series of haptens used in determining the presence of hydrogen bonding. Table XVIII shows shift and binding constant data for haptens binding to native and nitrated M315 which were used to reorient the first hypervariable loop of the heavy chain. Figure 21 is the old model. Figure 22 is the new structure with the hapten in place.

Table XVI: ^{19}F and ^{1}H Shifts (in ppm)

	^{19}F	^{1}H	Paramagnetic Component (^{19}F-^{1}H)
para substituent	1.8	-1.2	3.0
ortho substituent	0.6	-1.2	1.8
chain group	-0.8	~-1	~0.2

Table XVII: Binding Data

	Association Constant (M^{-1})	Energy of Binding (Kcal)
p-CF_3 TNP + M315	$3.4 \cdot 10^6$	11.3
p-CF_3 DNP + M315	$7.8 \cdot 10^5$	10.4
O-CF_3 DNP + M315	$4.0 \cdot 10^4$	8.7
chain CF_3 DNP + M315	$4.5 \cdot 10^5$	10.1
p-CF_3 TNP + Trp	25	1.9
p-CF_3 DNP + Trp	5	1.0
O-CF_3 DNP + Trp	50	2.3

Table XVIII: ^{19}F Shift Data: Native and Nitrated M315 (at pH 7.40)

	Native M315	Nitrated M315
p - CF_3	1.8	1.8
O - CF_3	0.6	0.6
chain - CF_3	-0.8	-1.2

pH Dependence of Association Constants (to ε DNP lysine) (M^{-1})

	Native M315	Nitrated M315
pH 9	$2.7 \cdot 10^5$	$6.6 \cdot 10^5$
pH 6	$6.5 \cdot 10^5$	$6.0 \cdot 10^5$

Figure 21

Original model of the
M315 binding site.

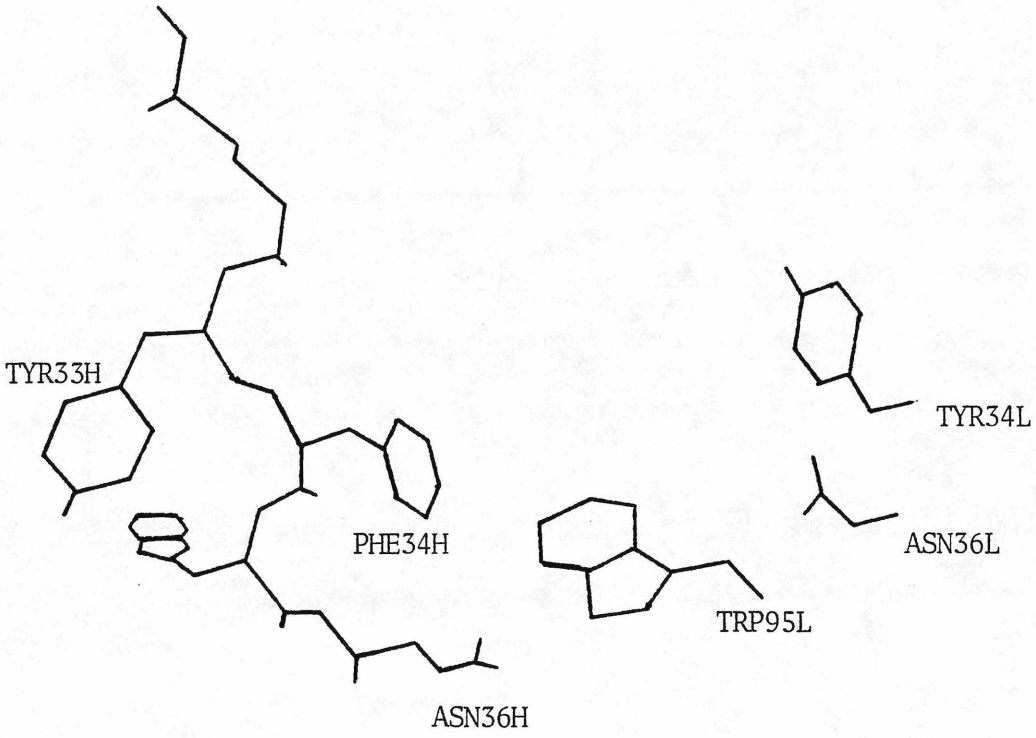
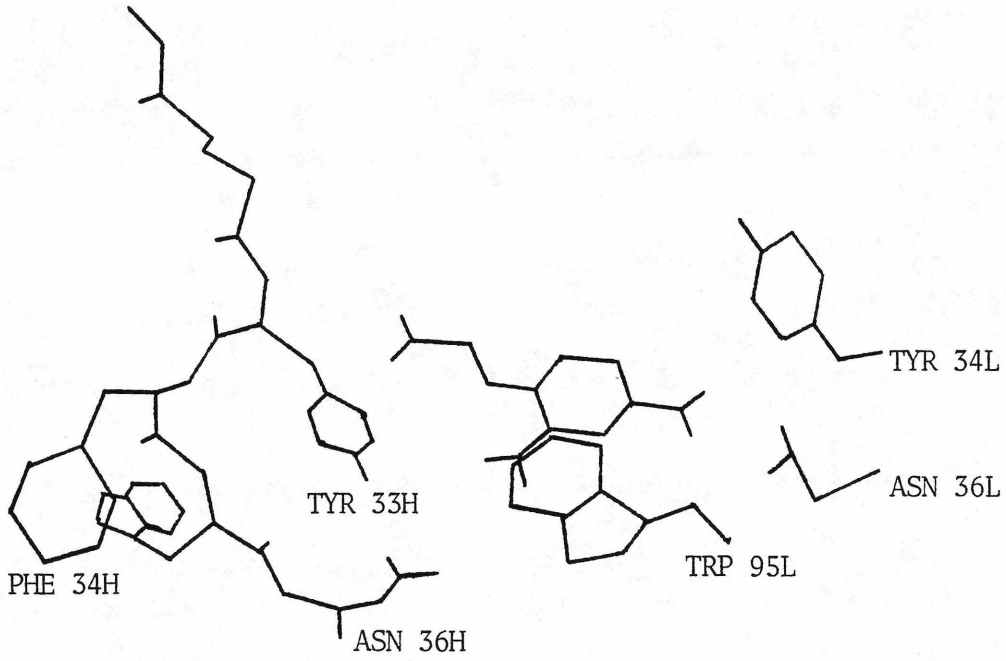

TYR33H
PHE34H
ASN36H
TRP95L
TYR34L
ASN36L

Figure 22

Modified model of the M315 binding site (with hapten in place)


TYR 34L
ASN 36L
TYR 33H
TRP 95L
PHE 34H
ASN 36H

Data summarized in the results section indicate a number of features of the M315 combining site. One major feature is the strong charge transfer interaction with a nearby tryptophan (Freed et al., 1976) which has a considerable effect on the fluorine chemical shift of the para substituted trifluoromethyl hapten. Replacing the hydrogen bonding nitro group by a non-hydrogen bonding trifluoromethyl group and taking into consideration the decrease in binding due to a weaker charge transfer interaction indicates that a moderately strong hydrogen bond exists between the antibody and the ortho nitro group of the hapten. No such interaction is found at the para position. Proton chemical shift data are similar to the results obtained by Dwek (Dwek et al., 1977) in that large upfield shifts are observed on binding which are characteristic of ring currents from nearby aromatic residues.

A more specific result concerning the orientation of such aromatic residues has been obtained by a protein modification study. This work, in which a tyrosine in the first hypervariable region of the heavy chain was labeled, showed that such labeling perturbed the chain trifluoromethyl resonance indicating it was in close proximity. The study also eliminated this residue as a potential hydrogen bonding group as titration of the tyrosine hydroxyl group to a pH at which it was not protonated had little effect on the binding constant.

The MMS-X computer graphics system at Washington University was utilized to orient the dinitrophenyl hapten in a model of the M315 binding site originally devised by analogy with the M603 site (Segal et al., 1974). The geometry of the charge transfer complex provided strict constraints on possible orientation (Orin et al., 1976) as did the location of an affinity labeled residue (Tyr 34L) and the previously defined

positive subsite (Haselkorn et al., 1974). It was possible to make a hydrogen bond to the ortho nitro group from a nearby asparagine, but the ring current at the chain position was due to a phenylalanine, not the nitrated tyrosine.

In the light of the nitration results the first heavy chain hypervariable region was reoriented as this sequence contained the nitrated tyrosine. It became clear that a relatively simple movement of the chain would swing the phenylalanine mentioned above away from the hapten and the modified tyrosine into the pocket near the hapten. The new conformation of the hypervariable loop was constructed by fixing the two ends of the sequence so as to align it with the M603 structure and then building the intermediate sequence one residue at a time. At each step unfavorable contacts were minimized. The coordinates of the newly oriented sequence were then refined according to the method of Diamond (Diamond, 1966). Interestingly, the original structure when refined by the Diamond procedure was changed quite a bit so that it clearly contained a number of unfavorable orientations. The new structure was changed only slightly by the refinement indicating very good fit to standard X-ray coordinates.

Thus the reoriented structure is consistent with all the data available including the protein modification results. The ring current shift at the chain trifluoromethyl group is due to a tyrosine (Tyr 33A) and not the phenylalanine (Phe 34H) proposed in the original structure. The tyrosine is in the correct position relative to the hapten necessary to produce the shift observed. The similarity of phenylalanine and tyrosine ring currents illustrates the danger of relying too heavily on ring currents to establish orientation. Finally, an asparagine residue

near the end of the reoriented segment (Asn 36H) is conveniently placed to form a hydrogen bond to the ortho nitro group of the DNP hapten. As it appears that there is a moderately strong interaction at this position then this is probably the residue contributing the hydrogen bond.

In summary, it is likely that while a number of specific interactions have allowed the specification of the hapten orientation and of some residues about the hapten the model of the site still contains errors. Obviously, if a chain has little participation in the binding interaction then data derived from binding or from the hapten will say little about that chain's orientation. The importance of the model is in orienting the important, specificity-conferring residues around the hapten and in suggesting other possible interactions. In addition, it may serve as a prototype DNP-combining site for the study of other nitrophenyl-specific myelomas such as M460 and X25 and for DNP-specific hybridoma antibodies yet to be studied.

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CHAPTER 7

Background on Effector Function

The binding of antigen by immunoglobulin is the most readily recognized feature of the immune system (Nisonoff et al., 1975), but unless such binding is coupled to the cellular immune system little in the way of host defense will be accomplished (Katz, 1977). These so called "secondary functions" of immunoglobulins are an equally important aspect of the integrated immune response of which antigen binding is only the first step. This integration of antigen binding site and the various proteins and cells of the immune system is accomplished by the immunoglobulin constant region (Spiegelberg, 1974). Thus the Fc domains act as an interface between the process of antigen binding and the various effector functions mediated by the immune system.

Probably the most characterized effector function of immunoglobulins is the triggering of the complement cascade (Porter and Reid, 1980). Complement consists of a group of nine proteins (Figure 23) which when triggered by antibody-antigen complexes can produce a variety of results including the elaboration of chemotactic factors, cell lysis and viral inactivation (Fearon and Austen, 1976). $C1_q$, the first component of complement, interacts with the C_H2 domain of IgG (Ellerson et al., 1972) and probably of IgM (Fust et al., 1976) and on formation of immune complexes initiates the sequence (Reid and Porter, 1975). The initial events include enzymatic steps so that considerable amplification of the response occurs, hence the term "cascade". The precise mechanism whereby the binding of antigen

triggers this system is controversial. One of the two theories holds that simply forming aggregates of immunoglobulins generates an assemblage of Fc regions necessary for initiation (Metzger, 1974, 1978). The other theory postulates a conformational change in the Fc region caused by binding of antigen that acts as a trigger for the cascade (Cathou, 1978). It may be that the truth lies intermediate between these two views.

Quite a bit of evidence has accumulated to support the contention that binding of antigens has an effect on antibody at some distance from the binding site. Circular dichroism studies (Steiner and Lowey, 1966) comparing intact human IgG with Fab and Fc fragments derived from papain digest show that while the Fab is similar in structure to the original IgG, the Fc undergoes considerable change in secondary structure. X-ray scattering of IgG solution (Pilz et al., 1970) indicated that on binding antigen the movement of the Fab arms is restricted and the entire molecule becomes more compact. Additionally, it has been found that reduction of the inter-heavy chain disulfide bonds, which is known to eliminate complement fixation (Press, 1975), also abolishes circular dichroism changes due to the Fc region which takes place when antigen is bound (Chan and Cathou, 1977). A study utilizing the technique of circularly polarized luminescence found changes in the CPL spectrum of rabbit antibodies to pneumococcal type III polysaccharide when large fragments of the antigen were bound, but not with smaller fragments (Jaton et al., 1975).

Convincing evidence has been accumulated, on the other hand, that, at least for IgG, crosslinking of antibody is necessary for complement fixation. It has been reported that at a minimum dimers of IgG are necessary for effector triggering (Wright et al., 1980; Strader, 1980). Studies with antigen fragments of various size show that monovalent antigen bound to IgG will not initiate the complement cascade whereas polyvalent antigen is quite efficient (Jaton et al., 1976). Contrary results for IgM have emerged from one lab (Brown and Koshland, 1977). In their system it appears that conformational changes distant from the site occur on binding of monosubstituted antigen and this is sufficient for complement fixation. Thus the already "aggregated" IgM, a pentamer of IgG-like subunits (Figure 24), does not require crosslinking for effector triggering. It may be that several adjacent Fc regions are necessary for the initiation of the complement cascade, but that a conformational change is also required.

Whether or not aggregation or conformational change or both are necessary for certain effector function triggering it is very likely that specific regions on the constant domains are responsible for particular functions. This view is supported by the large degree of conservation of sequence in these domains (Kabat et al., 1975). The framework of the combining site which presumably must interact with the constant domains has been found by x-ray diffraction study to have a conserved "immunoglobulin fold" tertiary structure (Padlan, 1977). Within the various classes the sequences around the carbohydrate attachment sites are highly conserved (Torano et al., 1977) and the positions

of the intra-chain disulfide bonds are conserved so as to give similar three dimensional domains (Edelman and Gall, 1969). It is likely that the extensive homology within each of the various immunoglobulin classes represents the evolutionary preservation of effector function structure specific to that class (Low et al., 1976).

Besides the activation of complement by the C_H2 domain a number of other effector functions should be noted. One intensively studied system is the triggering of mast cell degranulation by antigen binding to IgE (Segal et al., 1977). These cells possess receptors that bind the carboxyl terminal domain of IgE and mediate the release of histamine by the cell. In this case crosslinking of the IgE is necessary for degranulation, either by multivalent antigen or by anti-IgE antibody.

Macrophages (Diamond et al., 1978), B lymphocytes (Dickler, 1976) and T lymphocytes (Klein et al., 1977) also have Fc receptors on their surface. Although the details of the interactions of these cells are not well understood the immunoglobulin bound to their surfaces acts to regulate the immune response both at the level of humoral antibody and at the level of cell killing. Thus through mediation of the constant region the binding site serves to regulate its own production probably by a feedback regulation mechanism as proposed by Jerne (Jerne, 1974) in his network hypothesis.

The discovery that a single variable region can be associated with a variety of constant sequences provides an intriguing mechanism for modulation of the immune response (Sledge et al., 1976). That is,

the same antigen can trigger a number of different effector functions depending on which constant region is associated with the binding site. For example, early in the response to antigenic challenge IgM is secreted (Bellanti, 1971) which is easily triggered by low concentration of antigens because of its high avidity, due to multivalency. Later in the response IgG becomes more prominent which is less easily triggered by low concentrations, but is readily triggered by higher concentrations and which can mediate phagocytosis.

In the following sections effector function triggering is investigated at two levels. In the first, the biological effector system of complement mediated cell lysis is studied as a variety of antigens are bound by a monoclonal IgM. This establishes clearly that further crosslinking by antigen is not necessary for this effector triggering in IgM thus strongly suggesting that a conformational rearrangement in the Fc domains is occurring. In the second section, a physical technique exquisitely sensitive to conformation (^{13}C nuclear magnetic resonance) is applied to specifically study the tryptophan residues in antibodies as antigen is bound. The changes observed support the contention that a conformational change, while not sufficient, in IgG is nevertheless a necessary constituent of the process of effector function triggering.

Figure 23

The classical and alternate complement pathways

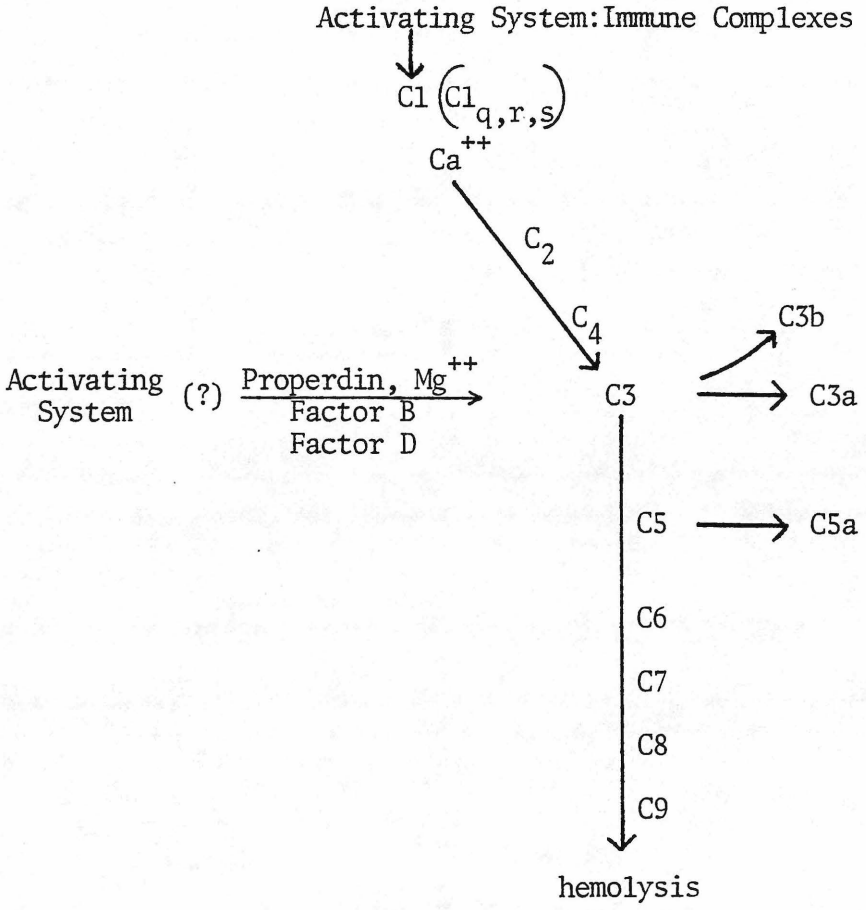
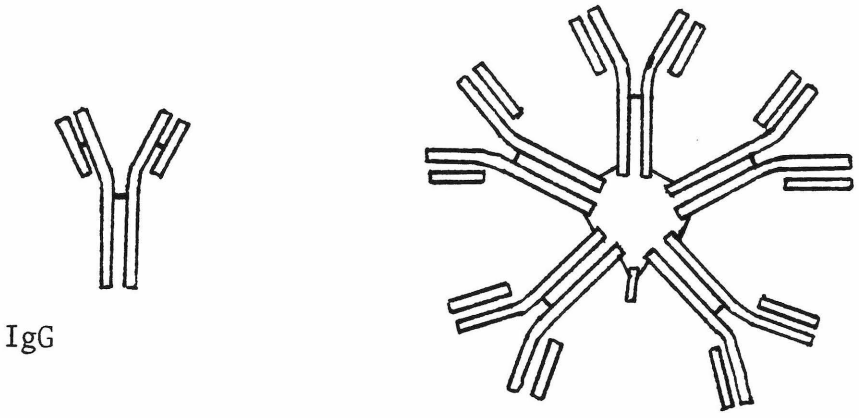

Activating System: Immune Complexes
C1 ($C1_{q,r,s}$)
Ca^{++}
C_2
C_4
C3b
Activating System
(?)
Properdin, Mg^{++}
Factor B
Factor D
C3
C3a
C5
C5a
C6
C7
C8
C9
hemolysis

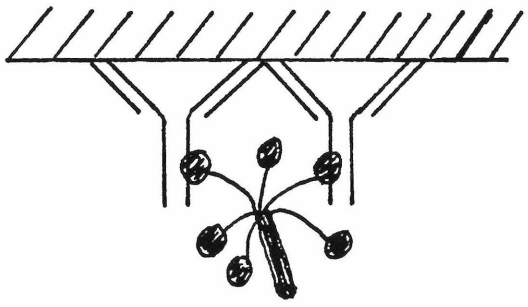
Figure 24

A. A comparison of IgG and IgM

B. The interaction of $C1_q$ with IgG



A.



B.

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CHAPTER 8

Complement Fixation by Monovalent Antigen: A Study of the Mouse Myeloma Protein A22

Antibody tertiary structure is characterized by specific domains (Edelman and Gall, 1969): two in each Fab arm, one of which interacts with antigen; and two or three others in the Fc region which mediate various effector functions (Winkelhake, 1978). Among these effector functions are activation of complement (Rapp and Borsos, 1970), triggering of B lymphocyte differentiation (Taylor et al., 1971), and initiation of histamine release from mast cells (Ishizaka et al., 1971). All of these processes have one feature in common: binding of the antibody's specific antigen somehow affects interactions in the distant Fc domains.

Of the three effector functions mentioned above the activation of complement is by far the most extensively studied (Gigli and Austen, 1971). The complement system is composed of a number of proteins participating in a sequence of events (several of which are chemical reactions) that produce an amplification of the initial triggering event, hence the term "cascade". The events include production of chemotactic factors, membrane lysis, and viral inactivation (Ruddy et al., 1972). Two sequences of reactions early in the pathway can lead to lysis: 1) the "classical pathway" involving the interaction of antibody-antigen complexes with certain complement components (Müller-Eberhard, 1969); and 2) the "alternate pathway" involving the interaction of antibody aggregates or certain polysaccharides with other components (Müller-Eberhard and Götze, 1976). The former has been extensively characterized while the latter is still not well understood.

It is well established that "fixation" of complement, a term used for depletion of the components by participation in the triggered pathway, varies with antibody class in each species. In the rabbit IgG and IgM classes fix complement by the classical pathway (Ishizaka et al., 1968). Considerable work has shown that for IgG to fix complement it must be crosslinked by antigens into at least dimers (Wright et al., 1980). On the other hand, there is evidence that for IgM monovalent antigen is sufficient to fix complement (Brown and Koshland, 1975).

Two principal theories have been proposed to account for the triggering of complement by antibody-antigen complexes. One holds that the simple aggregation of antibody enhances the binding of the first component of complement to the Fc domains and thereby initiates the cascade (Metzger, 1974). The other theory, often called the "distortive" or "allosteric" theory, holds that the binding of antigen in the Fab domain produces a conformational change in the distant Fc domain which leads to initiation of the cascade (Hoffmann, 1976a, 1976b, 1976c). A slight modification of this theory is that such a conformational change is necessary but not sufficient in the case of IgG which also must be aggregated before triggering the pathway. IgM is already "aggregated" so it only requires the conformational change.

Previous studies of the activation of complement by antigen-antibody complexes have been complicated by the use of heterogenous antibody (Brown and Koshland, 1977) or by the use of complex antigens

(Jaton et al., 1975). The system can be greatly simplified by the use of a monoclonal myeloma immunoglobulin. Unfortunately, until recently most of the myeloma proteins with known specificities were of the IgA class which does not fix complement by the classical pathway. Exceptions are M104E which secretes an IgM antibody specific for dextran (Leon et al., 1970) and UPC-10 which secretes an IgG_{2a} specific for levan (Cisar et al., 1974). However both these antigens are somewhat complex and difficult to work with. An IgM antibody secreted by the myeloma ABPC22 which binds Dansyl- and DNP- derivatized proteins has recently been obtained. This provides both the homogenous antibody and the simple hapten necessary for a thorough study of complement fixation by monovalent antigen. Large quantities of antibody can be produced by standard techniques in mice (Riesen et al., 1975) and purified by affinity chromatography (Goetzl and Metzer, 1970). The protein so isolated can then be subjected to rigorous physical characterization. In this chapter the isolation and characterization of this protein is described as is its complement fixation behavior. The complement fixing results are compared with those for two IgG hybridoma proteins specific for DNP which serve as controls on crosslinking of antigen.

Materials and Methods

Isolation of A22

The tumor, ABPC22, was maintained by subcutaneous transplantation in BALB/c mice. Intraperitoneal inoculation of a tumor cell suspension (in Locke's solution) into CDF_1 hybrid female mice primed two weeks

earlier with 1/2 ml mineral oil i.p. produced ascites which could be repeatedly removed by an 18 gauge needle puncture. A22 was precipitated from pooled ascites by addition of an equal volume of saturated ammonium sulfate solution dropwise with stirring at 25°C. The precipitate was collected by centrifugation (5000XG for 10 min), redissolved in saline and dialyzed against phosphate buffered saline (0.01 M sodium phosphate, 0.15 M NaCl, pH 7.40). The protein solution was applied to a DNP-Sepharose 4B affinity column equilibrated with the same buffer and the column was washed with this buffer until the effluent was protein-free (as determined by the absorbance at 280 nm). A22 IgM was specifically eluted by a 0.05 M DNP-glycine solution and the hapten was removed by passing the output of the affinity column directly onto a DOWEX 1X8 ion exchange column (OH^- form). The yield of A22 averaged 5 mg per ml of ascites.

Characterization of A22

The resulting protein was greater than 95% pure as assayed by SDS gel electrophoresis. Molecular weights of the various chains and subunits were determined by a standard log plot with BSA, alcohol dehydrogenase (yeast) and cytochrome c as standards. The protein was assayed with anti μ antisera raised against M104E (provided by Dr. M. Kehry). The ability of the protein to precipitate with antigen was assayed by ring test with DNP_{30}-BSA, $DANS_{20}$-BSA, sulfonated polystyrene, and heparin.

Production of IgM_S, Fab_μ Fragments

IgM_S subunits were produced by the standard reduction with 2-mercaptoethanol (Seon and Pressman, 1976). Trypsin digest of A22 using standard conditions (Miller and Metzger, 1966) produced Fab_μ which were isolated by affinity chromatography as described above.

Preparation of Antigens

DNP_{30}-BSA and $DANS_{20}$-BSA were prepared by standard methods (Eisen, 1964; Chen, 1968). Heparin was purchased from Sigma Chemical Co. (St. Louis). Sulfonated polystyrene was a gift of Mr. David Strader. Monovalent antigens were prepared by reaction of crystalline bovine serum albumin (Sigma) with a two-fold excess of tetranitromethane (Sokolovsky et al., 1966) in 0.05M Tris buffer (pH 8.0) for 1 hour at 25°C. The nitrated protein was isolated on a Sephadex G-25 column and reduced from nitrotyrosine to aminotyrosine (Sokolovsky et al., 1967) with a ten-fold excess of sodium dithionite in 0.05 M Tris buffer (pH 8.0). After dialysis overnight against the same buffer, the solution was made 0.05 M in acetate (pH 4.5) and a ten-fold excess of reactive hapten (2,4-dinitrofluorobenzene, dansylchloride, or flavianic acid) was added. The reaction was allowed to proceed 12 hours at room temperature and then the protein was isolated on a Sephadex G-25 column. From previously determined extinction coefficients the degree of incorporation was found to be one hapten per BSA molecule in such case.

Lima bean tryspin inhibitor (fraction IV), a protein containing only one tyrosine (Tan and Stevens, 1971) was isolated by gel filtration followed by ion exchange chromatography according to an established

procedure (Jones et al., 1963). The crude lima bean extract was purchased from Sigma (St. Louis). The isolated protein (pure by SDS electrophoresis) was nitrated, reduced and labeled with fluorodinitrobenzene following a procedure analagous to that used for the preparation of DNP_1-BSA except that dialysis using 3K molecular weight cut-off tubing (Spectrum Medical Products, Los Angeles) replaced column separations. The extinction coefficient of the DNP group ($1.6 \cdot 10^4$ M^{-1} cm^{-1}) verified one DNP group per 9K protein molecule.

Haptens

ε-N-2,4-dinitro-(3,5-^{3}H)phenyl lysine was purchased from New England Nuclear (Boston, Mass.) Dansyl-γ-amino butyric acid was synthesized by reaction of dansyl chloride with γ-amino butyric acid using standard conditions (Weber, 1952). DNP-ε-aminohexanoic acid was synthesized by reaction of fluorodinitrobenzene with ε-aminohexanoic acid in a 1:1 mixture of 1% sodium carbonate solution with ethanol. Folianic acid was purchased from Aldrich Chemical Co. (Milwaukee, Wis.).

Binding Measurements

The affinity of A22 for small haptens was determined by equilibrium dialysis (Eisen and Karush, 1949) in lucite 2 ml cells containing 1 ml solutions of A22 at 10 mg/ml and various hapten concentrations. The buffer used was phosphate buffered saline with 0.001 M EDTA, 0.02% NaN_3, and 0.1% gelatin. Equilibration times were on the order of a day at 22°C. The protein concentration was determined by absorbance measurement assuming an absorbance of 1.25 for 0.1% A22 (280 nm;

1 cm path). Binding of DNP-lysine was determined directly by scintillation counting of both sides of the dialysis chamber. Binding to dansyl-γ-amino butyric acid was found by competition (Cheng and Prusoff, 1973). Binding of monovalent antigens was approximated by competition complement assays with DNP-lysine or DNP-γ-amino butyric acid.

Complement Assay

A standard microcomplement ^{51}Cr release assay (Bengali et al., 1980) was employed in the complement fixation studies with a few modifications. The buffer used in all studies was hepes-buffered saline (0.01 M HEPES, 0.15 M NaCl, 0.15 mM $CaCl_2$, 0.50 mM $MgCl_2$, 0.1% gelatin, pH 7.40). Hemolysin and sheep red blood cells were purchased from Flow Labs (Inglewood, Calif.) Hemolysin incubation was for 1/2 hour at 37°C followed by 1/2 hour at 0°C. Cells were then washed three times with hepes-buffered saline and standardized as in the original reference. As has been found with some other IgM preparations (Brown and Koshland, 1975) above a certain concentration large amounts of complement were spontaneously fixed which limited concentrations in these assays to 10 μg/well (or 80 μg/ml).

Briefly, the antibody in 25 μl was added to the various dilutions of antigen in 25 μl, complement (2 CH_{50} units) was added in 25 μl and the 96 well plate was incubated at 37°C for 45 minutes. Controls included wells containing just the antigen dilutions plus complement, just the antibody plus complement, just complement, just the buffer, and a 100 percent lysis standard of water. After the 45

minute incubation 50 µl of 2% activated-^{51}Cr loaded-erythrocytes were added to each well and the plate was again incubated for 45 minutes at 37°C. At the end of this time the plates were centrifuged using plate holders (Flow Labs, Inglewood, Calif.) in a GLC-2 centrifuge (Sorvall) for 10 minutes at 2,200 rpm at 4°C. Aliquots of 50 µl per well were withdrawn and counted in gamma counter vials on a Beckman Gamma 4000 Counter using the standard ^{51}Cr isoset.

Competition assays were carried out by addition of various dilutions of hapten to pre-formed antibody-antigen complexes, then assaying the amount of complement fixed by each solution. Because of background fixation by these haptens, control wells of hapten alone were used to normalize the wells with antibody-antigen complexes yielding percent inhibition values.

All complement assays and titrations were carried out in Microtiter 96 well plates (Flow Labs, Inglewood, Calif.). Pipetting was carried out with Gilson automatic pipettors of 20, 200 and 1000 µl maximum capacity and a Pipetteman pipettor of 5000 µl maximum capacity. Incubations were carried out in a Lab Line 715 CO_2-100% humidity incubator.

Alternate Pathway Complement Assay

An assay adapted from one described previously (Riches and Stanworth, 1980) was employed to determine if any of the fixation observed was occurring by the alternate pathway (Müller-Eberhard and Götze, 1976). Rabbit blood was collected in acid-citrate-dextrose (ACD) anticoagulant at a ratio of 1 to 4 ACD to blood and the erythro-

cytes were washed three times with the alternate pathway complement buffer (Hepes gelatin complement buffer plus 10 mM EGTA and 16 mM MgCl). Human serum was collected and stored frozen at -70°C in 100 microliter aliquots. The serum was standardized by adding dilutions of the human serum to 1% ^{51}Cr-rabbit erythrocytes previously loaded with chromium in a manner analogous to that used for sheep red blood cells. The assay was carried out by first incubating the immune complexes for 45 minutes at 37°C in the wells of a microtiter plate with two CH_{50} units of human complement. Then the ^{51}Cr-rabbit erythrocytes were added and the wells incubated another 45 minutes at 37°C. The cells were pelleted as in the standard assay and aliquots of the well supernatants counted on a gamma counter. Controls included antibody plus complement, antigen dilutions plus complement, complement alone, buffer alone, and water for a 100% lysis standard. Lippopolysaccharide (a potent activator of the alternate pathway) was used as a positive control to test the assay's sensitivity. The lippopolysaccharide was isolated from E.coli:Serotype No 0127:B8 (TCA extract) obtained from Sigma (St. Louis).

Results

Table XIX presents the association constants between A22 and several hapten and monovalent antigens. Figure 25 collects many complement fixation curves on a single plot. Figure 26 demonstrates the "background fixation" of A22 without antigen. Figure 27 shows that reduction and alkylation which removes the inter-heavy chain disulfide bond decreases complement fixation. Figure 28 provides the

IgG (an IgG_{2a} and an IgG_1) control complement fixation plots and compares them with the plot of A22. Figure 29 indicates that the IgG fixation curves are similar to precipitation curves with polyvalent antigen. This implies that crosslinking is necessary for fixation by IgG and the simple increase in A22 again demonstrates that single site occupation is sufficient for fixation. Figure 30 provides examples of hapten inhibition curves. Figure 31 shows that the fixation observed with A22 is not due to the alternate pathway. In this assay the addition of EGTA chelates all calcium in the solutions and as the first component of the classical pathway requires calcium to function (to associate C_{1q}, C_{1r} and C_{1s} into C_1) then the only fixation seen with EGTA is the alternate pathway.

Table XIX Binding Constants (M^{-1} at 25°C)

ε DNP Lysine	1.0×10^3
Dansyl gaba	1.5×10^3
DNP_1-BSA	4.4×10^3 ≠
$DANS_1$-BSA	7.2×10^3 ≠
$Folianic_1$-BSA	8.1×10^3 ≠

≠competition complement assay

Figure 25

Complement fixation by A22
with various monosubstituted antigens.

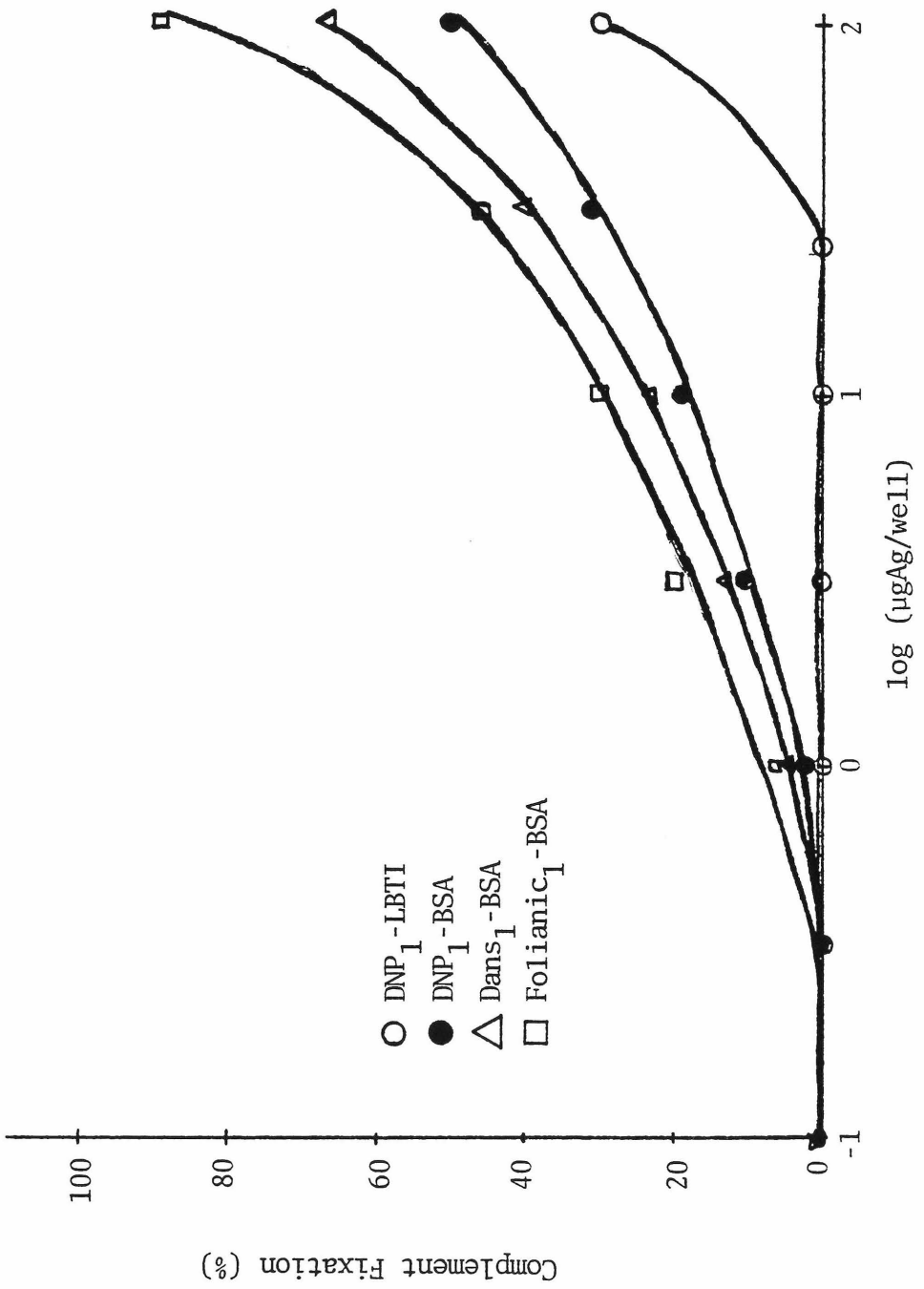

Complement Fixation (%)
100
80
60
40
20
0
-1
0
1
2
log (μgAg/well)
○ DNP1-LBTI
● DNP1-BSA
△ Dans1-BSA
□ Folianic1-BSA

Figure 26

Spontaneous fixation by A22.

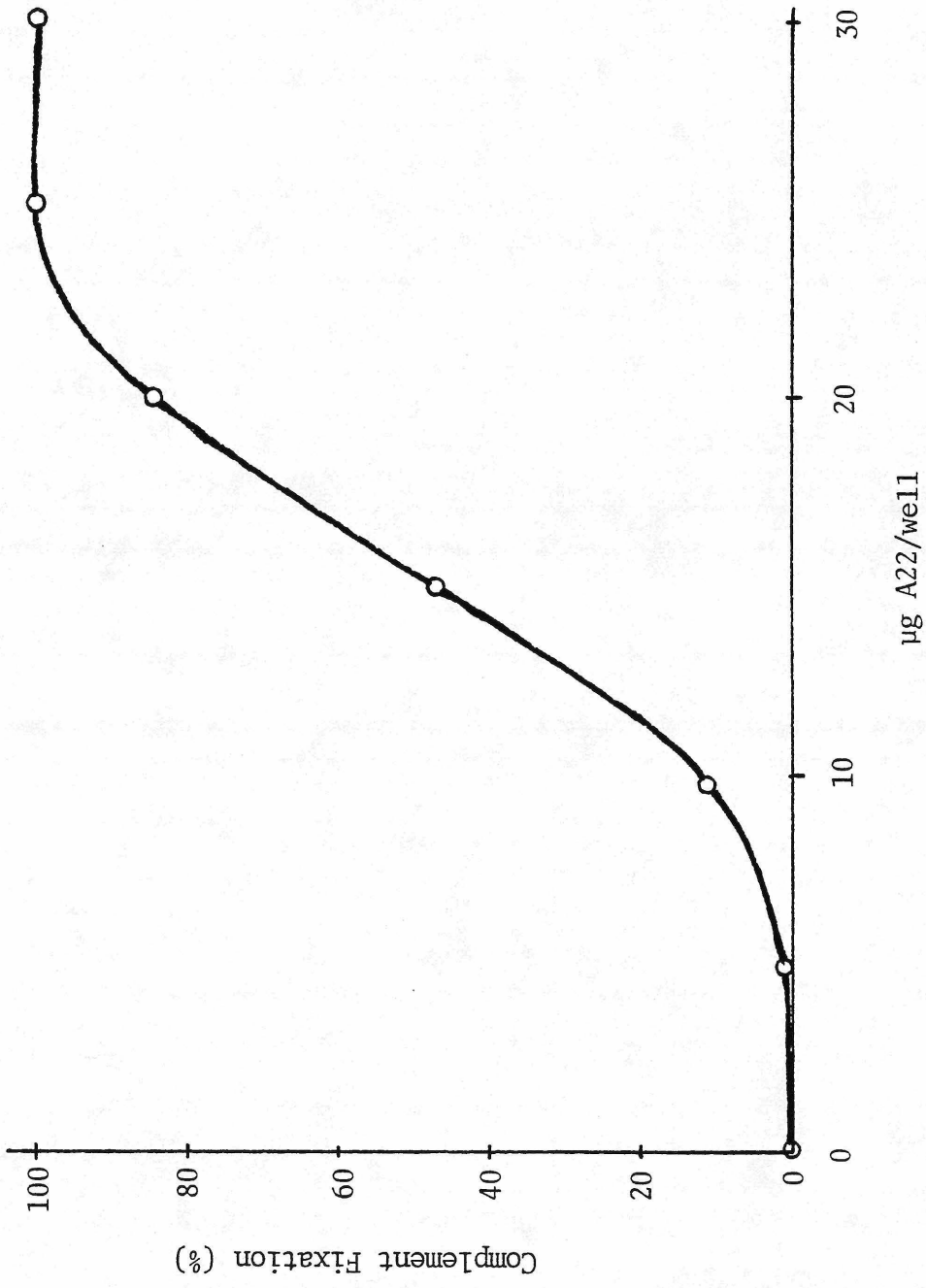

Complement Fixation (%)
100
80
60
40
20
0
0
10
20
30
µg A22/well

Figure 27

Complement fixation by native and reduced/alkylated A22.

inset: SDS gels of native, mildly reduced, and completely reduced A22.

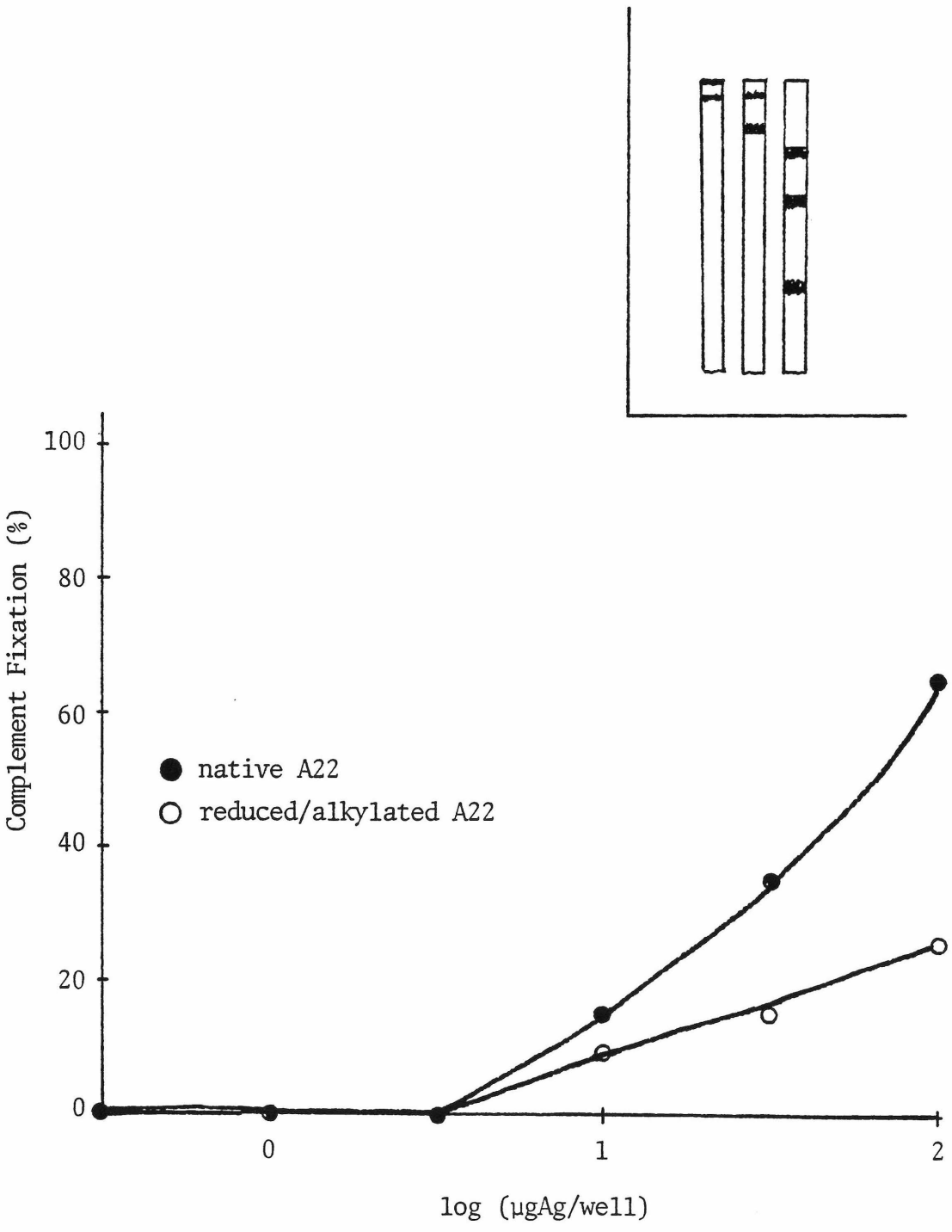

100
80
60
40
20
0
Complement Fixation (%)
native A22
reduced/alkylated A22
0
1
2
log (µgAg/well)

Figure 28

Complement fixation by A22 and
two IgG antibodies with monovalent antigen.

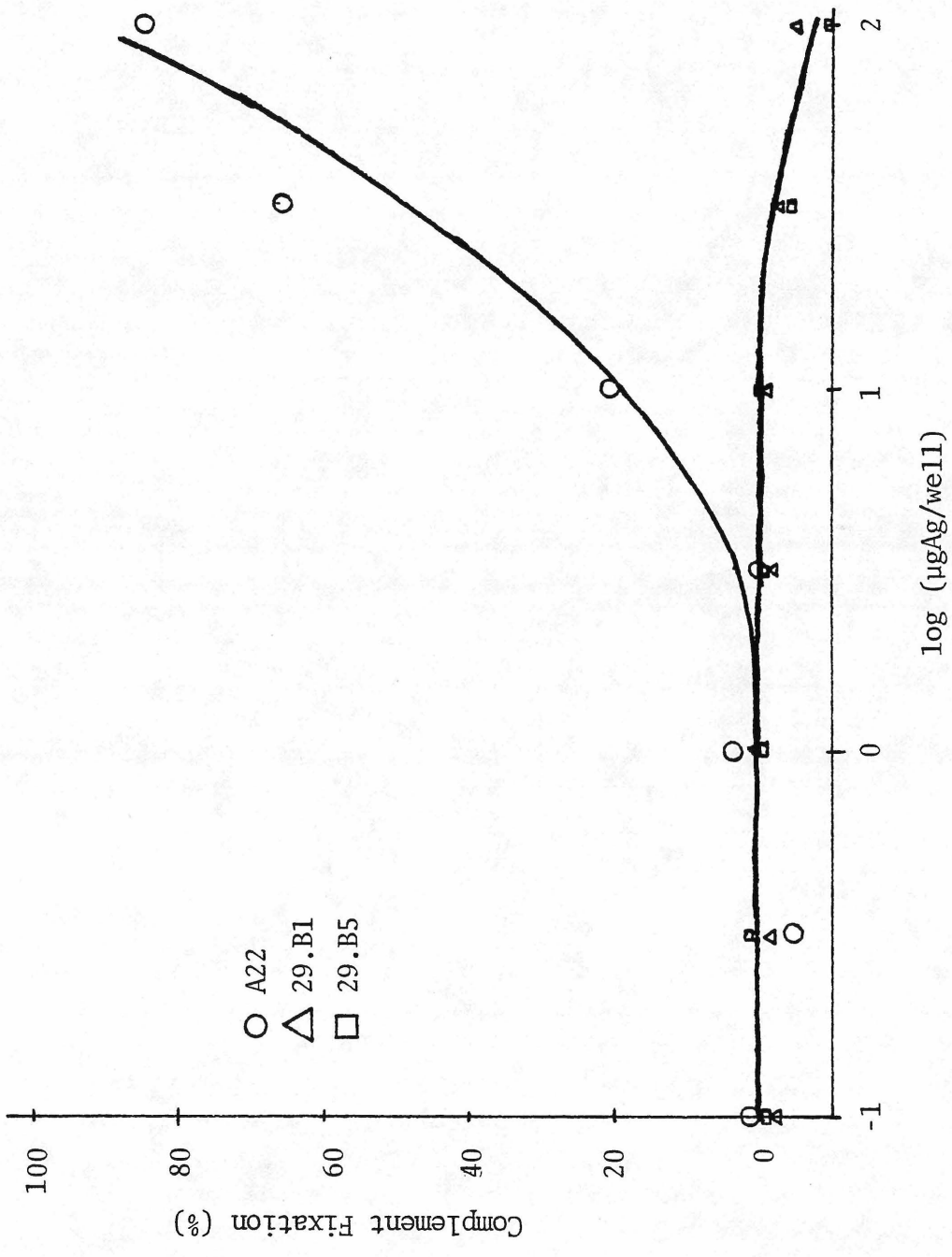

Complement Fixation (%)
100
80
60
40
20
0
-1
0
1
2
log (µgAg/well)
A22
29.B1
29.B5

Figure 29

Complement fixation by A22 and two IgG antibodies with DNP_{30}-BSA.

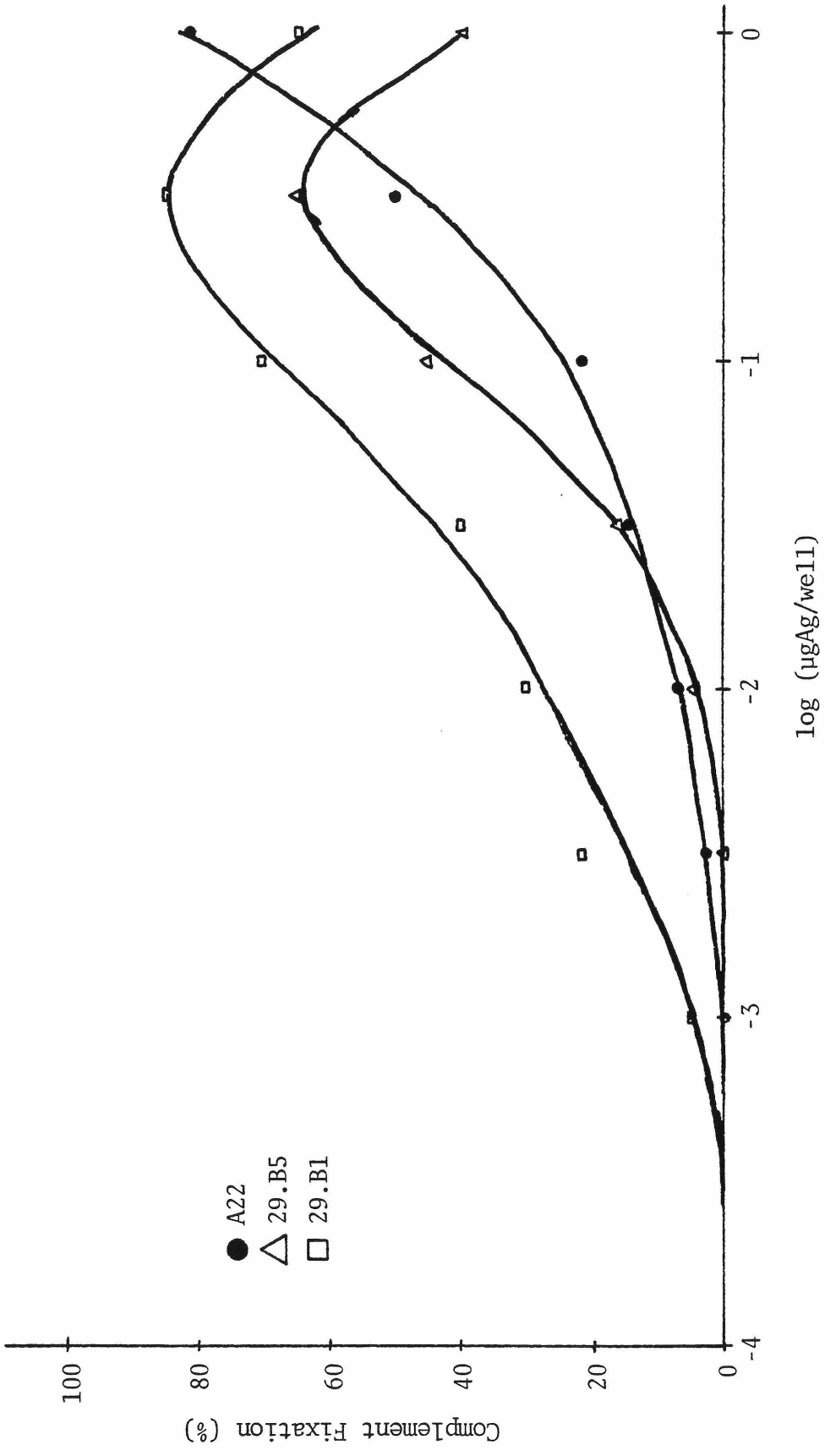

Complement Fixation (%)
100
80
60
40
20
0
-4
-3
-2
-1
0
log (µgAg/well)
● A22
△ 29.B5
□ 29.B1

Figure 30

Hapten inhibition of complement fixation by A22.

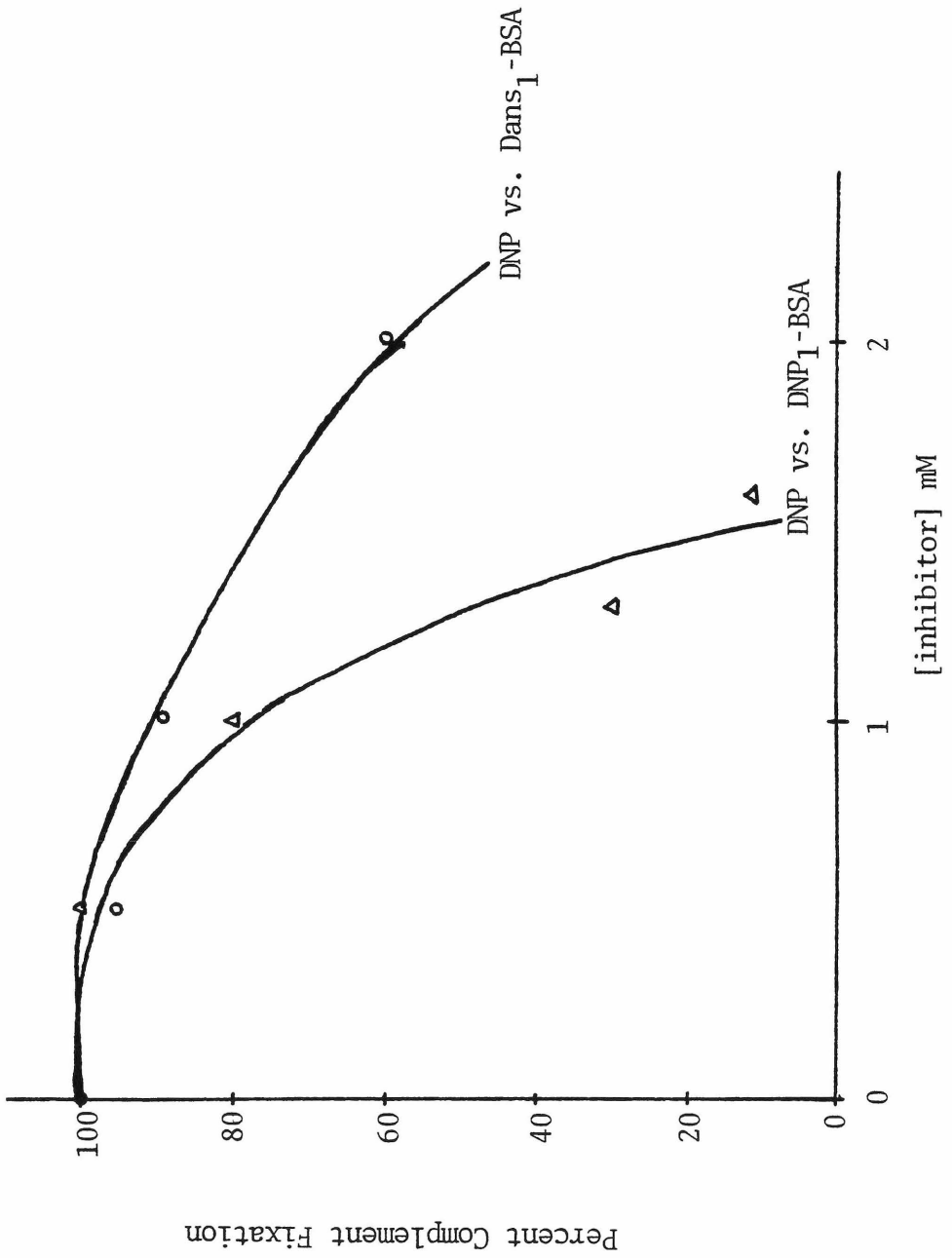
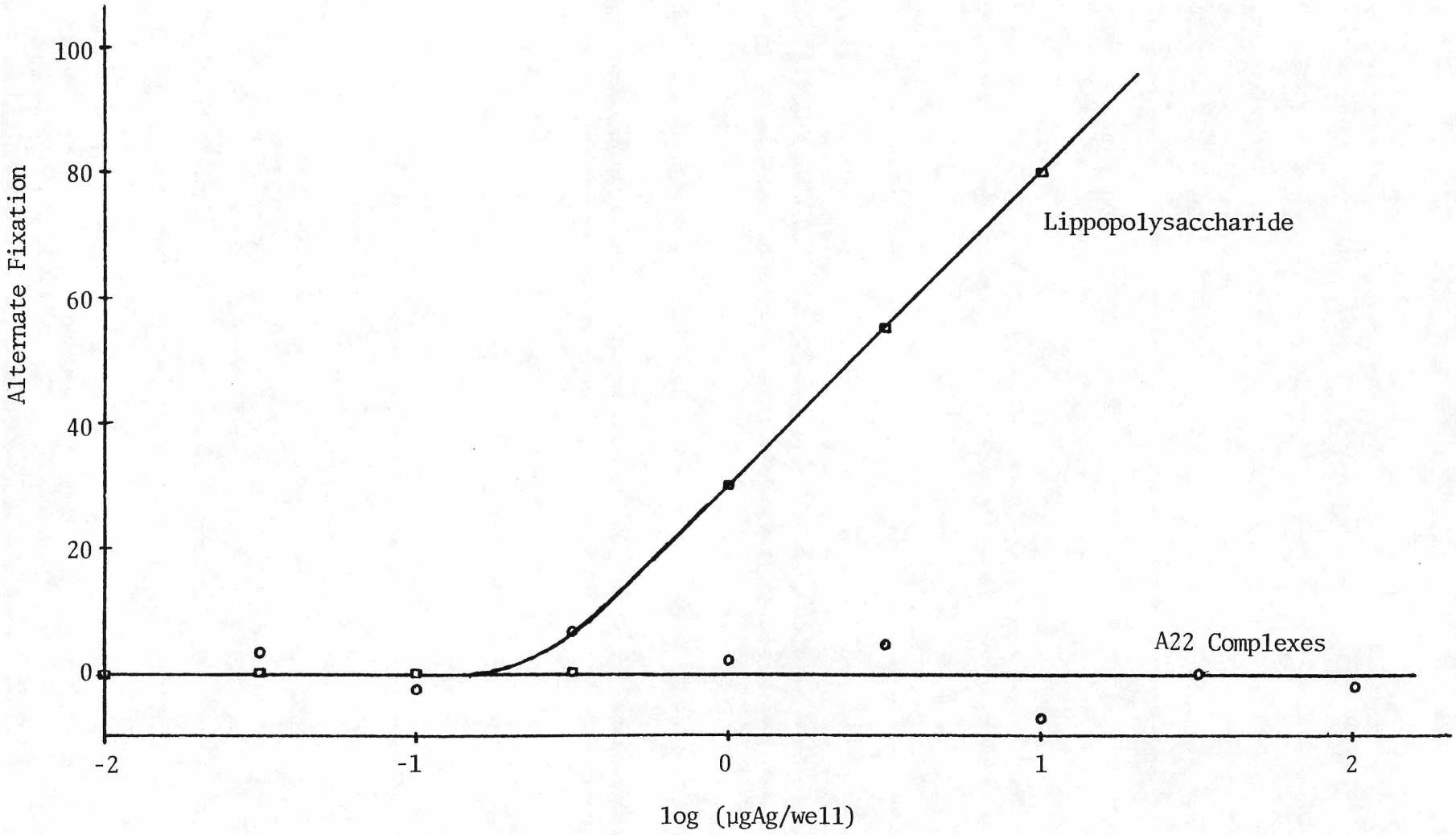

Percent Complement Fixation
100
80
60
40
20
0
0
1
2
[inhibitor] mM
DNP vs. Dans$_1$-BSA
DNP vs. DNP$_1$-BSA

Figure 31

Alternate Pathway Assay with
A22 - monovalent antigen complexes.
Lippopolysaccharide control curve is
also shown.


100
80
60
40
20
0
Alternate Fixation
Lippopolysaccharide
A22 Complexes
-2
-1
0
1
2
log (µgAg/well)

Discussion

The results of this study clearly demonstrate that A22 is a complement-fixing cryoglobulin. It is capable of binding a variety of aromatic haptens including the DNP- and dansyl- groups. More significantly, complement fixation takes place not only with polyvalent antigens such as DNP_{30}-BSA or sulfonated polystyrene but also with monosubstituted antigens such as DNP_1-LBTI or DANS-BSA. This result indicates that at least for this IgM and probably for most IgM antibodies crosslinking of the Fab arms is not necessary for the triggering of some effector function (complement fixation). It is simply necessary that the binding site be occupied by a sufficiently large antigen.

The triggering of complement fixation by A22 is not limited to the binding of a single antigenic determinant but rather by any hapten that is bound significantly by A22 conjugated to various carriers. This indicates that the fixation observed is not an artifact of the hapten functional groups or of peculiar properties of the carrier. Reduction and alkylation of the native IgM considerably reduces its complement fixing ability as the inter-heavy chain disulfide bonds are broken. In this way it behaves similarly to IgG in which removal of the inter-heavy chain disulfide bonds reduces or eliminates complement fixation (Isenman, 1975). Such mild reduction and alkylation does not hinder the ability of the protein to precipitate with polyvalent antigen.

Complement fixation with A22 while not requiring crosslinking of its binding sites nevertheless does require a minimum size of antigen as both hapten-BSA and hapten-LBTI when bound trigger complement fixation while hapten alone does not. Care must be taken at high hapten concentrations to subtract out spontaneous fixation by hapten, but if this is done one finds that hapten will inhibit the fixation of complement by A22-monovalent antigen complexes. As one might expect it is easier to inhibit the weakly binding DNP_1-BSA and more difficult to inhibit the strongly binding $Dansyl_1$-BSA. With the same antigen dansyl hapten competes with antigen better than DNP hapten, again as might be expected. From calculation of the binding affinity and relative degree of complement fixation it appears that 50% of the fixation occurs with only one antigen bound to 50% of the IgM molecules. It is impossible to carry the complement fixation curve out to 100% fixation because of the low affinity of A22 for these antigens due to insolubility of the antigens and the spontaneous fixation by antigen and antibody at really high concentrations.

As a control to establish that the monovalent antigen really is monovalent under the conditions of the assay the study of two DNP-specific IgG hybridoma antibodies was undertaken. The antibodies are of the IgG_1 and IgG_{2a} classes and possess high affinity for DNP (Herzenberg et al., 1980). The antibody typed as an IgG_{2a} fixes complement with DNP_{30}-BSA as expected but the other antibody, the IgG_1, also appears to fix complement with DNP_{30}-BSA. IgG_1 is commonly considered to be a nonfixing species in the mouse, but a recent report

(Ey et al., 1979) suggests that a large subclass of IgG_1 does dix complement. Thus it would appear that this IgG_1 belongs to that subclass. A definitive experiment would be the lysis of DNP derivatized erythrocytes by the IgG_1 in the presence of complement as this would show that the entire cascade was triggered. Such an experiment is included in the final chapter of this thesis.

The important point here is that both antibodies fail to fix complement with monovalent DNP-BSA at concentrations sufficient for A22 to fix. Considering the large difference in binding affinities between these two antibodies and A22 (29.B1 is $\sim 10^7$ M^{-1}, 29.B5 is 10^8 M^{-1}, A22 is 10^3 to 10^4 M^{-1}) this shows that there is not a small amount of divalent or aggregated DNP_1-BSA which would invalidate the results with A22. It also confirms the work of Ishizaka in rabbit (Ishizaka et al., 1965) and Strader in a monoclonal levan-specific system (Strader and Richards, 1980) which showed that aggregates of antibody were required for complement fixation.

The fixation of complement by monovalent antigen bound to IgM has previously been demonstrated by Koshland in rabbit anti-lactose IgM binding monosubstituted-Lac-RNAse (Brown and Koshland, 1975). The antibody was raised conventionally in rabbit and was clearly heterogenous as shown by Sips's index of 0.6 (where homogenous is 1.0) (Sips, 1949). Metzger has observed (Metzger, 1978) that considering the stated affinity the amounts of antigen and antibody required for complement fixation are not consistent with a homogenous population of IgM associating with monovalent antigens. He suggests a small

amount of very high affinity IgM is binding a small amount of polyvalent antigen and that these crosslinked complexes are responsible for the observed fixation. Analytical ultracentrifugation showed no significant increase in aggregation of IgM on addition of antigen but Metzger maintains that the amount of complex necessary to produce the fixation observed may be below the level of detection by this method.

The objection that the extreme sensitivity of a complement fixation assay makes the elimination of aggregation impossible has been answered in this study by the use of the high affinity monoclonal IgG antibodies. Thus an assay equal in sensitivity to the IgM assay detected no dimerization or aggregation of antigen. Also, in working with a myeloma IgM the binding constant is well defined and another problem is eliminated. Considering the expected binding affinities of A22 for antigens the complement fixation data is consistent with one antigen binding to an IgM triggering that IgM to fix complement. This is reinforced by the hapten competition experiments. The unfortunate aspects of this system are the low affinity of A22 necessitating large antigen concentrations and the background fixation of complement by aromatic ligands (Loos and König, 1977). However if one uses the results with the DNP hybridoma antibodies as controls then it certainly appears that the simplest explanation of the data with A22 is that occupation of a binding site by a sufficiently large molecule triggers complement fixation in IgM antibodies.

Of the two principal theories mentioned in the introduction these data support the allosteric or distortive model of effector triggering in IgM antibodies. The data also show that cross-

linking is necessary for effector triggering in IgG. Thus it may be that both theories are partially true: a conformational change transmitted from binding site to the Fc region may be necessary but not sufficient for effector triggering. In the case of IgG the allosteric change would occur but since the molecules are unaggregated no triggering would take place. In IgM which is already "aggregated" only the conformational change would be required for triggering. There is some evidence for a conformational rearrangement of the Fc occurring in IgG under conditions such that no complement is fixed (Jaton et al., 1975).

Further studies on the DNP-specific IgG hybridoma antibodies by various physical methods are currently being carried out in order to observe the predicted conformational changes therein. One approach is to study the fixation by dimers of IgG chemically crosslinked as monovalent antigen is bound. If the results are similar to those with A22 then this is strong support for the combined aggregation/ allosteric model outlined above. Another approach is to place conformationally sensitive NMR probes at the putative $C1_q$ binding site (where initiation occurs) and to observe the antibody both ligated and unligated. This has been carried out by the biosynthetic incorporation of ^{13}C labeled tryprophan into the two hybridoma antibodies and is described in the next and final chapter.

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CHAPTER 9

NMR Studies of Biosynthetically Enriched Immunoglobulins: Search for Conformational Change

Work with a monoclonal dansyl-specific IgM (Chapter 8) showed that effector function triggering, at least in the form of complement fixation, could be initiated by the binding of monosubstituted antigen to that immunoglobulin. This result provides support for the allosteric model of effector function triggering (Hoffman, 1976) which states that binding of antigen in the Fab region leads to a conformational change in the Fc domains which mediate effector functions. In the same study (Chapter 8) it was found that IgG did not fix complement with monosubstituted antigens, but rather required crosslinking by polyvalent antigen to initiate fixation. This result is consistent with previous work on IgG (Jaton et al., 1976) and such data have been employed to support the other major model of effector triggering, the associative model (Metzger, 1974). In this model it is postulated that crosslinking of IgG by antigen generates aggregates of Fc region which display greater affinity for the first component of complement simply on the basis of avidity. Such binding is proposed as the initiating event in the pathway.

The results with IgM and IgG may be reconciled if both a conformational change and aggregation are necessary for complement fixation. Since IgM is already an "aggregate" of five IgG-like subunits it can fix complement with monosubstituted antigen which is consistent with a previous study on IgM (Brown and Koshland, 1975). On the other hand IgG when binding monovalent antigen would presumably undergo the conformational changes, but as it would not be aggregated the first component of complement wouldn't be bound and so fixation wouldn't be initiated. This interpretation of the IgG data is supported by a pre-

vious study (Jaton et al., 1975) which demonstrated conformational change in the Fc region while binding fragments of antigen incapable of fixing complement.

With this in mind an attempt has been made to observe conformational change in IgG on binding monosubstituted antigen. The highly sensitive technique of ^{13}C nuclear magnetic resonance (Stothers, 1972) provides a very useful probe of protein microenvironments (Wüthrich, 1976). One approach might be to attach a probe onto the IgG antibody at some well-defined site (Means and Feeney, 1971), but such a study must be interpreted with care as any label may perturb the effect one is attempting to observe. For this reason on attempt by Goetze (Goetze, 1978) to label cysteins after reduction with fluorine probes was destined to fail.

Another approach is biosynthetic incorporation of a ^{13}C enriched amino acid (Hunkapiller et al., 1973) utilizing myeloma cells growing in culture (Horibata and Harris, 1970). This certainly produces a minimal perturbation of the antibody and greatly increases the chances of observing the signals. The natural abundance of ^{13}C is so low that coupled with the inherent low intensity of the signals the observation of a few carbons at a concentration of 0.1 millimolar would be extremely difficult (Chance and Clark, 1976). In addition, the background signal from all the other protein carbons would tend to obscure any specific resonance (Eakin, 1975). Enrichment serves both to improve the signal to noise ratio and to diminish background relative to the enriched signal.

An important point to be considered is that observation should be carried out on a position likely to sense any conformational changes that take place. Thus placing a probe at the end of the carbohydrate (Nezlin et al., 1978) is probably not the best method of detecting small reorientations of the protein backbone. The carbohydrate is already in such a free environment that changes in the protein would likely not be transferred through the oligosaccharide linkages to any extent. Thus labeling an amino acid, preferably one of the less frequently occurring ones in order to minimize spectral complexity, would be a preferable approach.

The least common amino acid residue in most proteins is tryptophan (Dayhoff et al., 1976). Also, because of its bulky indole group one would expect it to be sensitive to changes in the peptide chain. More importantly, previous studies (Isenman et al., 1977) have already shown that the solvent exposure of tryptophan in IgG changes depending on whether antigen is bound or not. Very recent studies show that labeling of tryptophan is different for antibody and for complexes (Sand, 1980). Finally, it appears that tryptophan is an important residue in the activation of complement as reagents that specifically modify tryptophan reduce an antibody's ability to initiate complement fixation (Allan and Isliker, 1974). Thus enriching a carbon of the tryptophan residues seems to be the best choice for this study.

Careful consideration should be given to the specific carbon chosen for enrichment. The resonance should be relatively sharp and sensitive to conformation. Such a carbon is the gamma carbon in

tryptophan. It is a quaternary carbon and so has a very sharp resonance and is known to shift over five parts per million depending on environment from studies carried out at natural abundance on very concentrated samples (Oldfield et al., 1975). Also, because it has no directly bonded hydrogens one may accumulate a spectrum without proton decoupling and so diminish the background resonances relative to the quaternary carbons (Norton et al., 1977). As these carbons have very long relaxation times one may also adjust the acquisition parameters to favor observation of these quaternary carbons at the expense of the proton background resonances. An additional advantage of the gamma carbon is that its resonance is outside both the aliphatic and aromatic envelopes of resonances which further reduces interference by either antibody or antigen background resonances.

This study thus involves the growth of liter volumes of various myeloma cells which secrete homogenous antibody into the medium (Horibata and Harris, 1970). The substitution of ^{13}C gamma carbon enriched tryptophan for normal tryptophan results in the production of antibody with every tryptophan enriched at that position. Since tryptophan is an essential amino acid all tryptophan in the antibody is labeled. Over the time course of the labeling (ca 4-5 days) there should be minimal reutilization of tryptophan to synthesize other amino acids, especially in a well-formulated medium (Roberts et al., 1976).

The immunoglobulins chosen for this study are a group of DNP-binding proteins of the IgA, IgG_1, and IgG_{2a} classes. DNP is a simple

antigenic determinant which can easily be introduced into a variety of carrier proteins (Eisen, 1964). Also, a monosubstituted antigen developed for a previous study (Chapter 8) is ideal for this work where soluble complexes are a necessity. As two proteins are of classes that do not fix complement these can act as controls on the IgG_{2a} that does fix.

Materials and Methods

Culture Conditions

Myeloma cells were cultured either in Dulbecco's modified Eagle's medium (DMEM) or in RPMI1640 medium and with 10% fetal calf serum (Flow Labs, Inglewood, Calif.). The medium was made up with double glass distilled water using a commerical powder formulation (Grand Island Biological Co., Santa Clara, Calif.) and then sterilized by pressure filtration through a 0.2 micron pore filter (Bio-Rad, Richmond, Calif.). Labeling medium was made up from individual components omitting L-tryptophan using either formulation (DMEM or RPMI1640). Just prior to use 20 milligrams of enriched L-tryptophan (KOR Isotopes, Cambridge, Mass.) was dissolved in 5 milliliters of saline solution and pressure filtered into one liter of medium. Prior to labeling cells were grown in 250 milliliter screwcap glass erlenmeyer flasks at 37°C in a humidified, 10% carbon dioxide gassed incubator (Lab line 715). Large suspension cultures were grown in 2.8 liter flasks containing 1.5 liter of medium and stirred with a "floating" stir bar (Nalge, Ace Scientific, Linden, N.J.).

Cell Lines

Cells of the MOPC460 tumor cell line were adapted to grow in culture by the serial passage technique (Periman, 1971). Briefly, tumor cells from ascites of tumor-bearing mice were removed aseptically by a 10 milliliter syringe. Erythrocytes were lysed by a one minute exposure to 0.3% KCl solution followed by restoration of isotonicity with an equal volume of twice concentrated saline and centrifugation at 150 xg for five minutes. Cells were resuspended and incubated in medium at 37°C in the CO_2 incubator. After four days in culture the surviving cells were injected intraperitoneally into mineral oil primed CDF_1 mice and allowed to grow and yield ascites. Three repetitions of this cycle produced cells that would grow in culture with a doubling time of 18 hours.

M315 secreting cells were produced by somatic cell fusion with the NSP_2 cell line. Standard polyethylene glycol fusion (Köhler and Milstein, 1975; Peters and Wille, 1977) yielded hybrids that would grow in selective medium and secrete the DNP-binding IgA protein.

29.B1 and 29.B5 were generously provided by Dr. Leonard Herzenberg. 29.B1 is a DNP-specific IgG_{2a} with a high association constant ($\sim 10^7\ M^{-1}$) while 29.B5 is a DNP-specific IgG_1 with an extremely high affinity constant ($\sim 10^8\ M^{-1}$) (Herzenberg et al., 1980). It has been previously determined that both fix complement by the classical pathway (Chapter 8).

Single Cell Agar Cloning

In order to insure homogenous protein production the cells were first cloned before large-scale protein production. The soft agar cloning method was used (Coffino et al., 1972). On a base coat of 0.2% SeaChem agar (Marine Colloids Inc., Rockland, Maine) cells suspended in 0.2% agar were layered and incubated at 37°C in a CO_2 incubator. Typically, after five to ten days about 10% of the cells would form colonies on the agar. These colonies could be removed by aspirating them with a drawn pasteur pipette. The cells could then be cultured in the wells of a microtiter plate (Flow Labs, Inglewood, Calif.). After five days plate wells were assayed for production of DNP-binding antibody by a solid phase radioimmunoassay (Marier et al., 1979).

Cell Fusion

A relatively simple procedure for the production of well-characterized proteins (such as M315) in culture was employed when serial animal passage failed to establish a line. The myeloma tumor cells were isolated from ascites as described above and then fused with a non-secreting azaguanine resistant cell line (NSP2) using 50% PEG (Köhler and Milstein, 1975; Peters and Wille, 1977). Both fusion partners were washed three times with serum-free medium (RPMI1640) and CO-pelleted at 500 xg for five minutes. After exposure to 50% PEG for two minutes serum-containing medium was added and the cells pelleted again. The supernatant was removed, the cells gently resuspended in serum-containing medium, and the suspension aliquoted out into the wells of a 96 well microtiter plate (Flow Labs, Inglewood, Calif.).

After 24 hours the medium was removed and replaced by selective medium (HAT-RPMI1640) (Galfré et al., 1977). Within ten days colonies of hybrid cells were observed in most of the wells. Secreting cells were selected by a solid phase radioimmunoassay (Marier et al., 1979) and after repeated subclonings secretion was stabilized in the hybrid cells.

^{125}I Protein A Labeling

A standard chloramine T labeling procedure (Hunter and Greenwood, 1962) was employed to produce radioactive protein A. Protein A (Sigma, St. Louis, Mo.) at one milligram per milliliter in 50 millimolar phosphate buffer (pH 7.40) was aliquoted out and stored frozen in snap-cap centrifuge tubes at -70°C. An aliquot was mixed with 25 microliters of 40 millimolar phosphate buffer and one milliCurie of Na^{125}I (in 10 microliters; Amersham, Arlington Heights, Ill.) was added. Next, the chloramine T solution was added (10 microliters of a 2.5 milligram per milliliter phosphate buffer solution) and the mixture stirred for 60 seconds. Then 25 microliters of sodium metabisulfite solution was added (2.5 milligrams per milliliter in phosphate buffer) followed by 100 microliters of a 1% BSA in PBS solution. The solution was then applied to a 5 milliliter syringe containing Sephadex G10 (Pharmacia, Sweden) previously washed with 20 milliliters of 1% BSA in PBS. The syringe was inserted in a 15 milliliter centrifuge tube and the combination spun at 1000 xg for five minutes. Essentially the entire volume of applied solution was eluted leaving the unreacted Na^{125}I in the gel. The activity of the protein solution

was determined by counting an aliquot and then diluted with 1% BSA in PBS to yield 3 x 10^6 counts per minute per 50 microliter aliquot. The aliquots were stored frozen at -70°C in 1.5 ml snapcap centrifuge tubes. Prior to use in the solid phase radioimmunoassay an aliquot was diluted by a factor of 100 with 1% BSA in PSA. Thus one aliquot was sufficient to carry out one 96 well plate assay.

Screening Assay

A solid phase radioimmunoassay similar to that described by Marier (Marier et al., 1979) was employed to detect secreting cells. DNP_{30}-BSA was incubated in the wells of a microtiter plate overnight at 37°C (50 microliters per well of a 4 milligram per milliliter solution in PBS). The antigen solution was removed and replaced by a 5% BSA in PBS buffer solution (125 microliters per well) to block any sites not yet saturated. After two hours the BSA solution was removed and culture supernatants (50 microliters per well) were transferred to the assay plate wells in a replicate fashion. In the case of IgA secreting cells after a 45 minute incubation the supernatant was replaced by rabbit anti-mouse IgA (Nordic Immunology, London). In the case of IgG secreting cells this step could be skipped. Following three rinsings with 1% BSA, ^{125}I labeled protein A was applied to the wells (30,000 counts per minute in each well) and incubated for 45 minutes. At the end of this time the protein A solution was removed and the wells washed three times with 1% BSA in PBS. Finally, the plate was sealed with an adhesive plate cover and autoradiographed. Preflashed x-ray film (Kodak X-Omat R film:XR5) with intensifying

screen (DuPont Cronex Lightning-Plus) was employed with exposure times of 12 to 48 hours at -70°C. On development positive wells were visualized as intense dark spots on the film.

Production of Enriched IgA

A small culture of myeloma cells was centrifuged at 150 xg for five minutes, the supernatant removed, and the cell pellet resuspended in enriched medium to give a cell density of about 5×10^4 cells per milliliter. After maximal growth (to about 10^6 cells per milliliter) 0.02% azide was added to the medium and the cells removed by centrifugation. The medium was concentrated to one-tenth volume by ultrafiltration (Amicon Corp., Lexington, Mass.) and an immunoglobulin-containing fraction isolated by addition of an equal volume of saturated ammonium sulfate solution. After centrifugation the pellet was redissolved in PBS and dialyzed against PBS overnight. The solution was reduced and alkylated by standard methods (Goetzl and Metzger, 1970) and applied to a DNP-Sepharose 4B column. The immunoglobulin was specifically eluted from the column by a 0.05 molar DNP-glycine solution and the glycine removed by passage down a DOWEX 1 x 8 ion exchange column (OH^- form). The resulting protein was concentrated by ultrafiltration to 15 milligrams per milliliter and dialyzed into deuterated PDS buffer. Purity was checked by 7.5% SDS gel electrophoresis.

Isolation of Enriched IgG Antibodies

The spent culture medium was centrifuged to remove cells and then applied directly to a DNP-Sepharose 4B column. Prior to each use the column was washed with 20% acetic acid and then equilibrated with PBS. After the effluent was protein-free (as determined by the absorbance at 280 nm) the antibody was specifically eluted with 0.05 M DNP glycine. Most of the hapten was removed by a DOWEX 1X8 ion exchange column (OH^- form). The protein fractions were pooled and a ten-fold molar excess of sodium dithionite was added with stirring to the solution buffered at pH 8.0 with 50 millimolar Tris. The dithionite reduced the bound DNP as observed by the disappearance of the yellow color and was immediately removed by pressure dialysis using PBS as the replacement buffer. The solution was concentrated to 20 millgram per milliliter in deuterated PBS and then centrifuged to remove precipitated protein (5000 xg for 10 minutes) just prior to NMR study. SDS gel electrophoresis verified that this material still contained intact molecules and that dithionite had not reduced disulfide bonds.

Direct Lysis Complement Assay

Sheep red blood cells (Flow Labs, Inglewood, Calif.) were washed three times in gelatin Hepes-buffered saline (10 millimolar Hepes, 150 millimolar NaCl, 0.15 millimolar $CaCl_2$, 0.50 millimolar $MgCl_2$, pH 7.40, 0.1% gelatin) and then labeled with 2,4-dinitrofluorobenzene (Aldrich, Milwaukee, Wis.) according to the procedure of Levine and

Levytska (Levine and Levytska, 1967). After the reaction the cells were washed three times in the above mentioned buffer and then loaded with ^{51}Cr chromate by the standard one hour incubation procedure (Chapter 8). Then the cells were washed three times to remove residual chromium and adjusted to a 1% suspension (200 microliters of a 1% suspension when lysed into 2800 microliters water yields an absorbance at 541 nm of 0.170).

For the assay 50 microliter aliquots of cell suspension were distributed into the wells of a 96 well microtiter plate (Flow Labs, Inglewood, Calif.) followed by 25 microliter aliquots of the antibody dilutions. The plate was incubated 45 minutes at 37°C and then 25 microliters of a one to 20 dilution of guinea pig serum was added to the wells. After 45 minutes more at 37°C the cells were pelleted using plate holders in a GLC-2 centrifuge (Sorvall) at 2000 xg for 10 minutes. Controls included complement alone, buffer alone and a 100% lysis standard of distilled water. After centrifugation 50 microliter aliquots from each well were counted on a Beckman 4000 gamma counter using the standard chromium isoset.

The possibility that lysis might be due to alternate pathway was checked by addition to all solutions of EGTA which chelates calcium, a requirement for the function of C1 (the initiator of the classical pathway). Addition of 12 microliters of an 83 millimolar EGTA, 133 millimolar Mg^{++} stock solution per 100 microliters of standard hepes buffer suffices to completely inhibit classical fixation

while leaving the alternate pathway unaffected.

NMR Conditions

Two machines were employed to observe ^{13}C spectra: a Bruker WM-500 at 125 MHz and a Varian XL-200 at 50 MHz. Both utilized 10 millimeter sample tubes with 2 milliliter volume and were locked to the internal sample D_2O signal. Typical protein concentrations were 0.1 to 0.3 millimolar and spectra were accumulated for five to 12 hour periods. As the ^{13}C atom enriched has no directly bonded hydrogens no decoupling was used.

Cell Storage

After production of secreting cloned cells, aliquots of cells were frozen in 10% DMSO in fetal calf serum and stored in a liquid nitrogen refrigerator (Weiner, 1976). Cells were pelleted and resuspended at 5×10^6 to 1×10^7 cells per milliliter in the freezing medium. One milliliter aliquots were dispensed into 2 ml polypropylene freezer vials and the vials placed in a styrofoam block (approximately one centimeter of insulation around each vial). The block was placed in a -70°C refrigerator and after 24 hours the vials were transferred to the liquid nitrogen refrigerator. Warming the vials in a water bath to 38°C and washing the cells to remove the DMSO medium restored the cells to a viable state. The average recovery is on the order of 70 to 90% depending on storage time.

IgG Reduction

29.B1 was reduced in the 10 mm NMR tube by addition of a 100-fold excess over antibody of 2-mercaptoethanol. The spectrum was accumulated immediately thereafter.

IgG Fragmentation

29.B1 was digested with papain in the 10 mm NMR tube by addition of 1/10 volume of digest buffer (0.5 M sodium acetate, 0.1 M cysteine, 0.02 M EDTA, pH 5.5) followed by an adjustment of the pH to 5.5 with 20% acetic acid and then addition of papain solution (1:100, enzyme: antibody by weight). The digestion was carried out for three hours at 37°C and terminated by increasing the pH to 7.5 with 1 M sodium hydroxide. The spectrum was accumulated immediately thereafter.

Results

Figure 32 illustrates that the cell culture derived protein is identical in molecular weight with protein derived from ascites. Figure 33 shows sketches of a cloning plate and the film from a radioimmunoassay used to check secretion. Figures 34, 35, and 37 present spectra of three antibodies both with and without antigen bound. Figure 38 presents the IgG_{2a} with and without hapten bound demonstrating that the changes observed in Figure 37 are not produced by binding of a small molecule. Figure 39 compares the IgG_{2a} bound to hapten in native and papain digested form. Figure 40 compares the IgG_{2a} bound to antigen in native and reduced form. Figure 41 shows the IgG_{2a} both with and without antigen at lower field. Figure 42 demon-

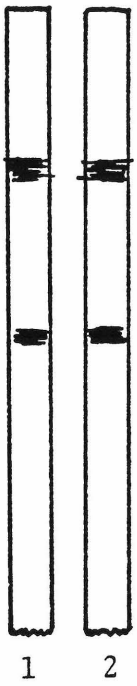
strates that the IgG_{2a}-fixes complement more efficiently, the IgG_1 less and that both fix via the classical pathway. Figure 36 compares the spectrum of 29.B1 with that of free tryptophan (γ-carbon).

Figure 32

SDS gels of cell culture- and ascites-derived antibody proteins.

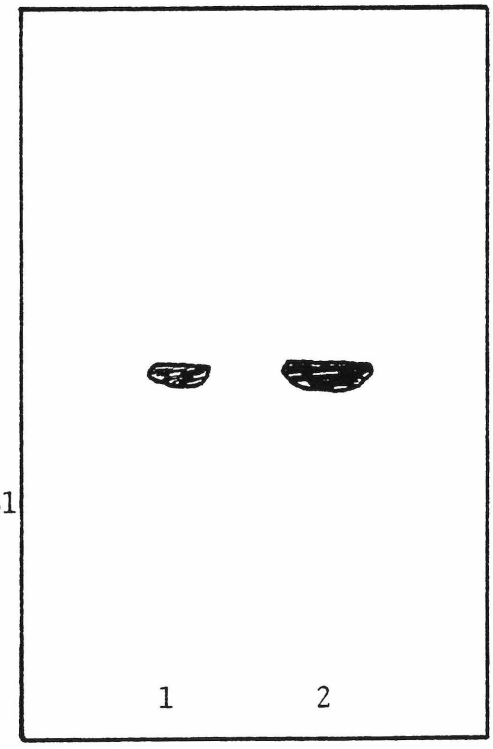
Upper: 7.5%; reducing buffer

Lower: 4%; non-reducing buffer



Gel 1: Ascites derived M460

Gel 2: Cell culture derived M460



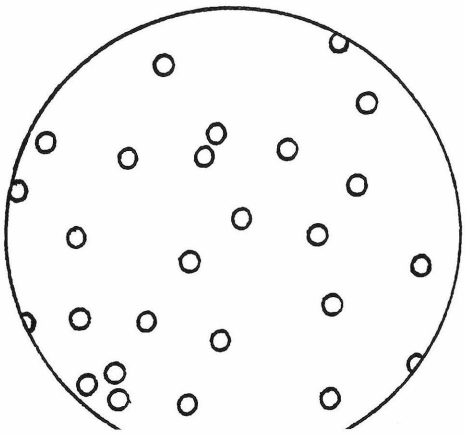
Lane 1: UPC10 (Ascites)

Lane 2: Cell culture 29.B1

Figure 33

Upper: Sketch of cloning plate

Lower: Typical secretion radioimmunoassay film



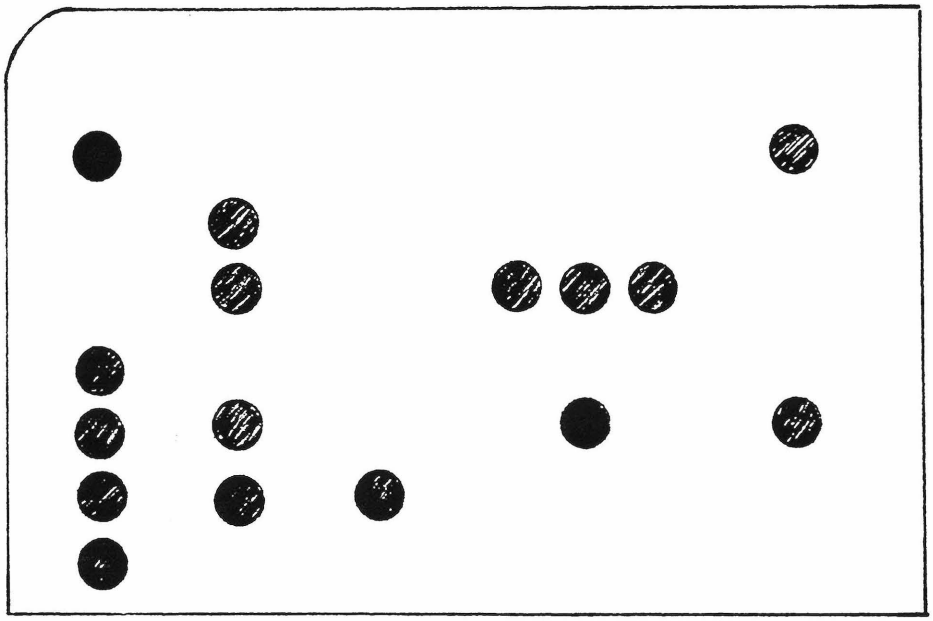


Figure 34

Upper Spectrum: γ-^{13}C-Trp enriched M460 antibody (0.06 mM)
119 K transients at 125 MHz

Lower Spectrum: γ-^{13}C-Trp enriched M460 antibody (0.04 mM)
with DNP_1-BSA (0.05 mM)
85 K transients at 125 MHz

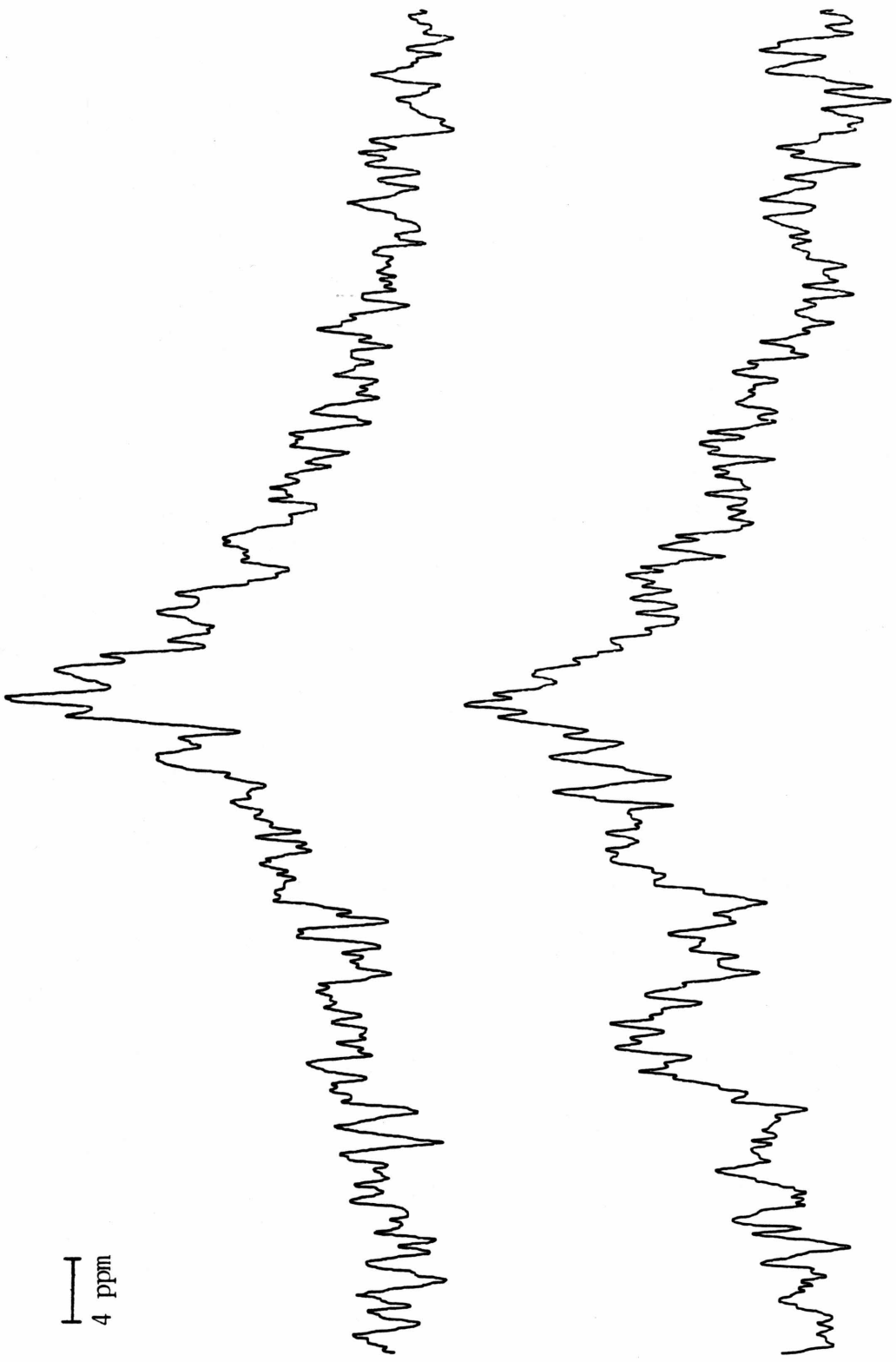

4 ppm

Figure 35

Upper Spectrum: γ-^{13}C-Trp enriched 29.B5 antibody (0.1 mM)
75 K transients at 125 MHz

Lower Spectrum: γ-^{13}C-Trp enriched 29.B5 antibody (0.07 mM)
with DNP_1-BSA (0.08 mM)
93 K transients at 125 MHz

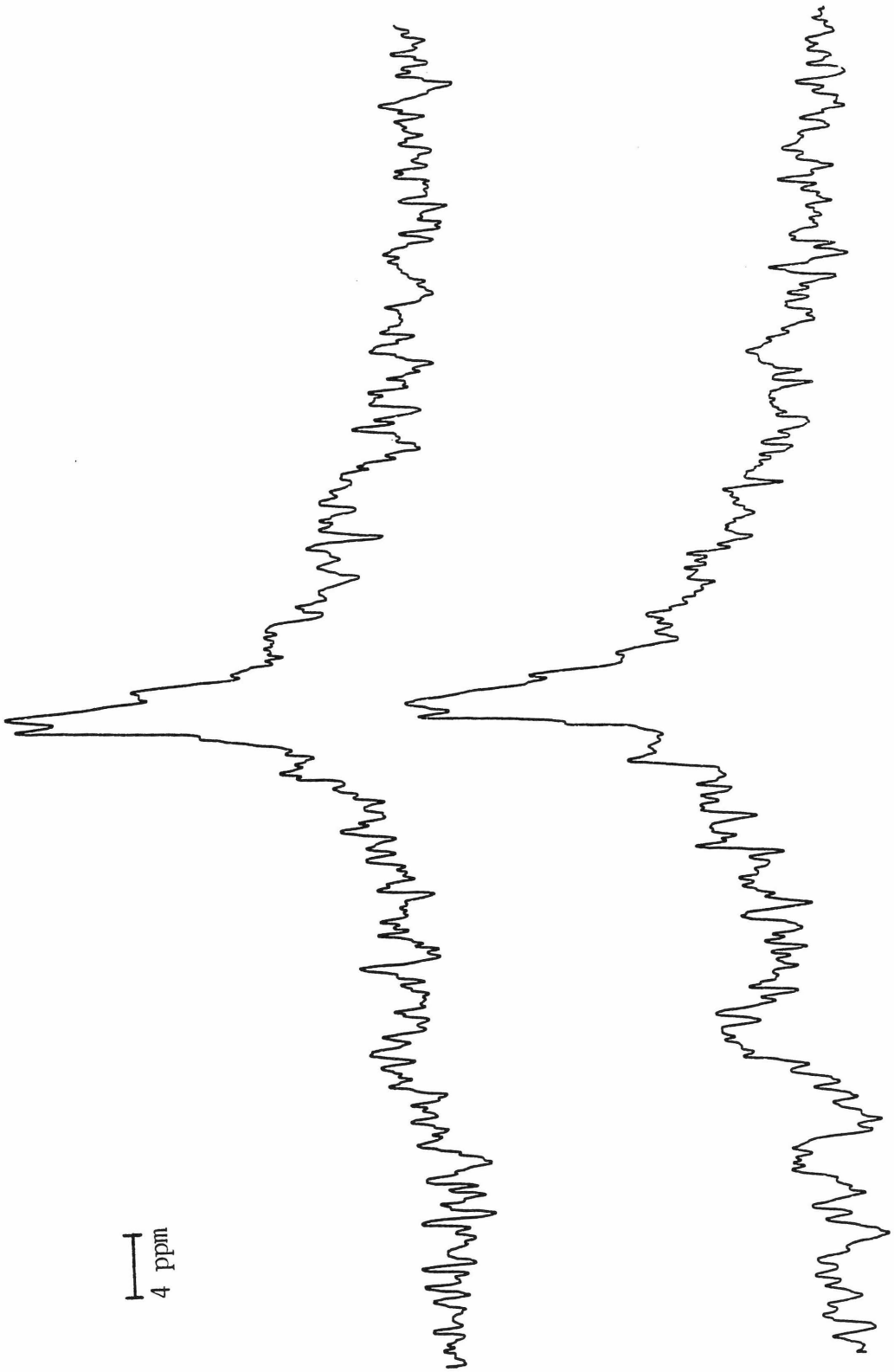

4 ppm

Figure 36

Upper Spectrum: γ-^{13}C-Trp enriched 29.B1 antibody (0.1 mM)

132 K transients at 125 MHz

Lower Spectrum: γ-^{13}C-Trp (20 mM)

2 K transients at 125 MHz

Chemical Shift: 110 ppm downfield of TMS

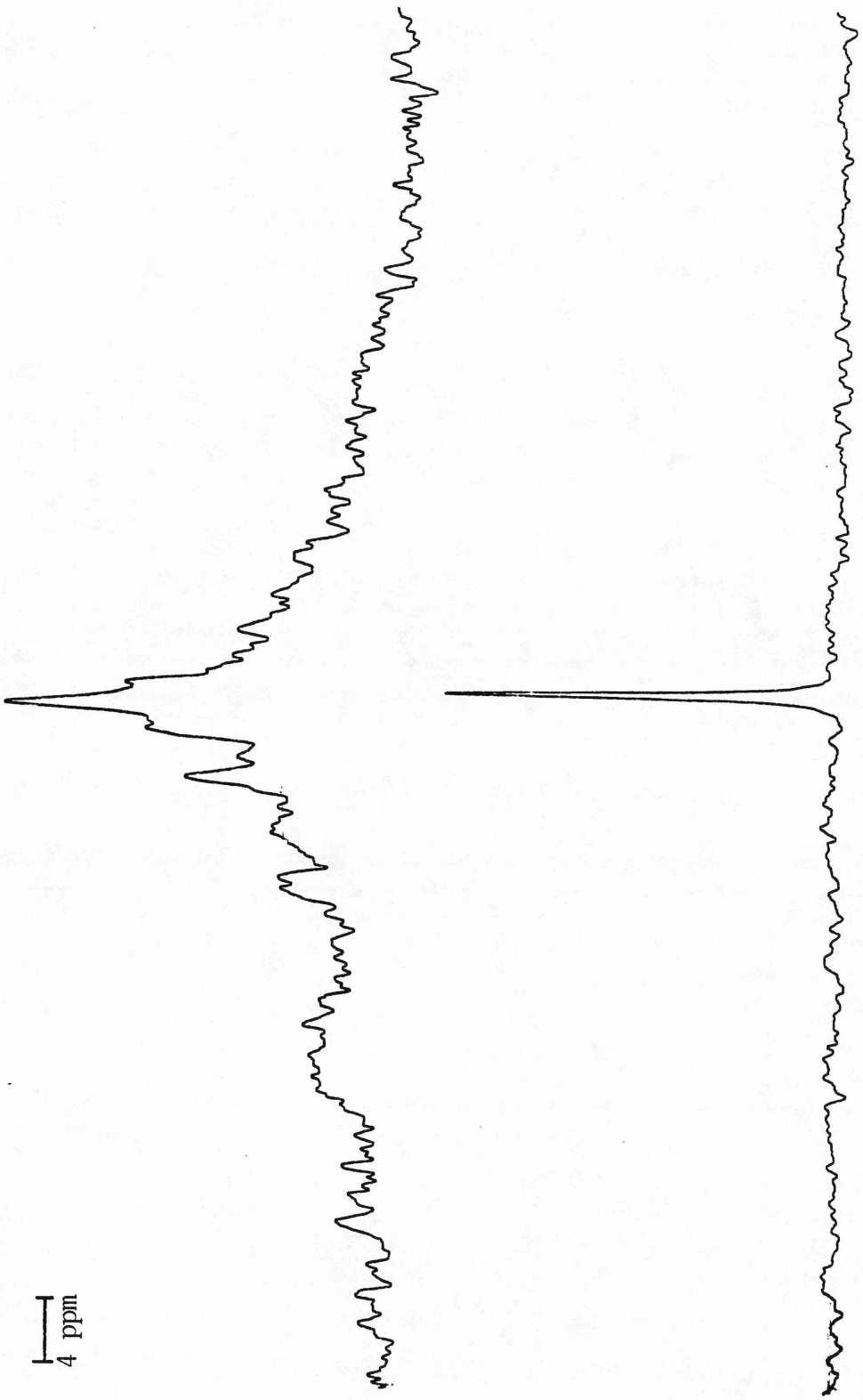

4 ppm

Figure 37

Upper Spectrum: γ-^{13}C-Trp enriched 29.B1 antibody (0.1 mM)
132 K transients at 125 MHz

Lower Spectrum: γ-^{13}C-Trp enriched 29.B1 antibody (0.06 mM)
with DNP_1-BSA (0.07 mM)
167 K transients at 125 MHz

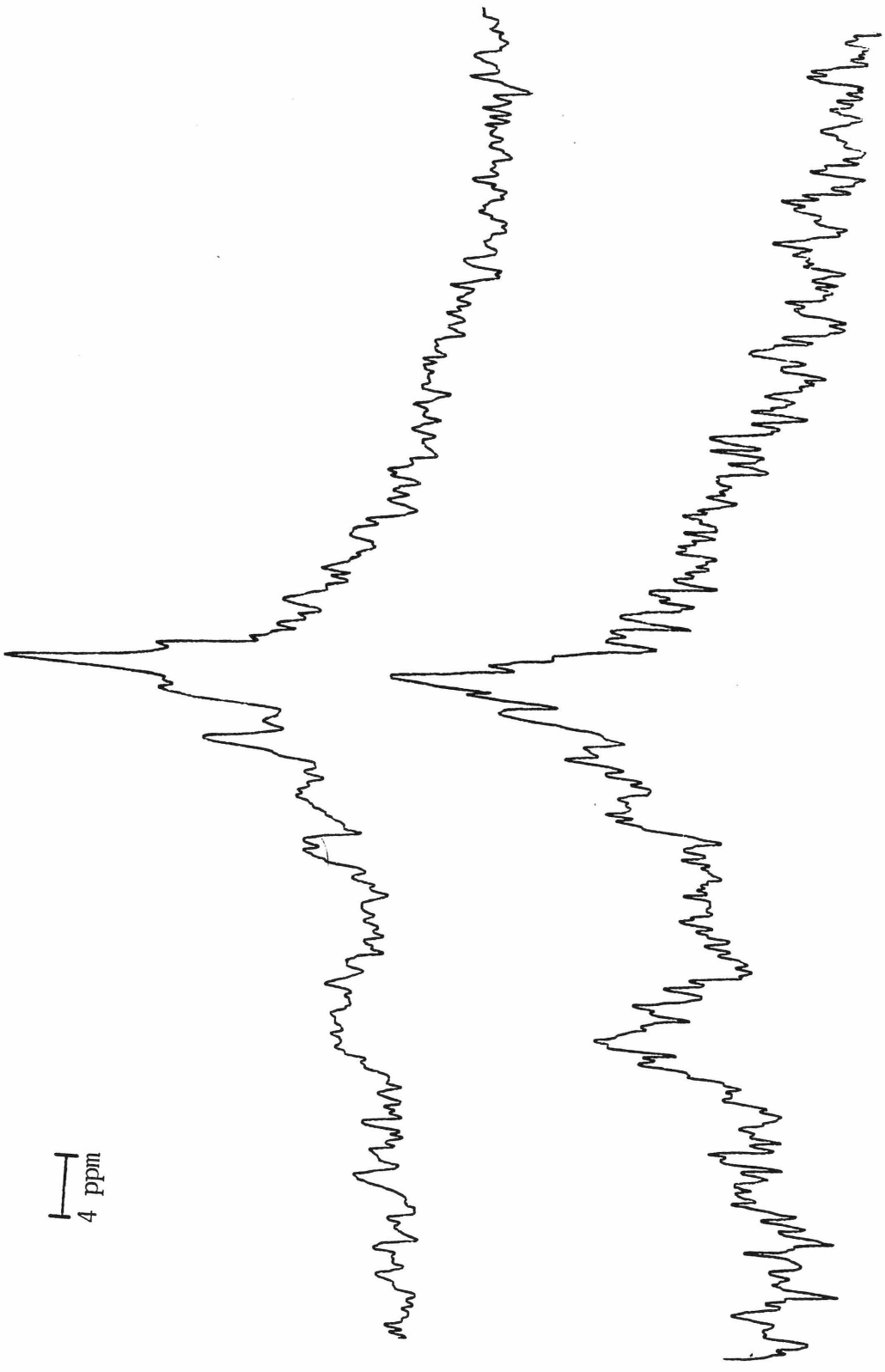

4 ppm

Figure 38

Upper Spectrum: γ-^{13}C-Trp enriched 29.B1 antibody (0.1 mM)
132 K transients at 125 MHz

Lower Spectrum: γ-^{13}C-Trp enriched 29.B1 antibody (0.07 mM)
with hapten (0.08 mM)
108 K transients at 125 MHz
Hapten: DNP-glycine

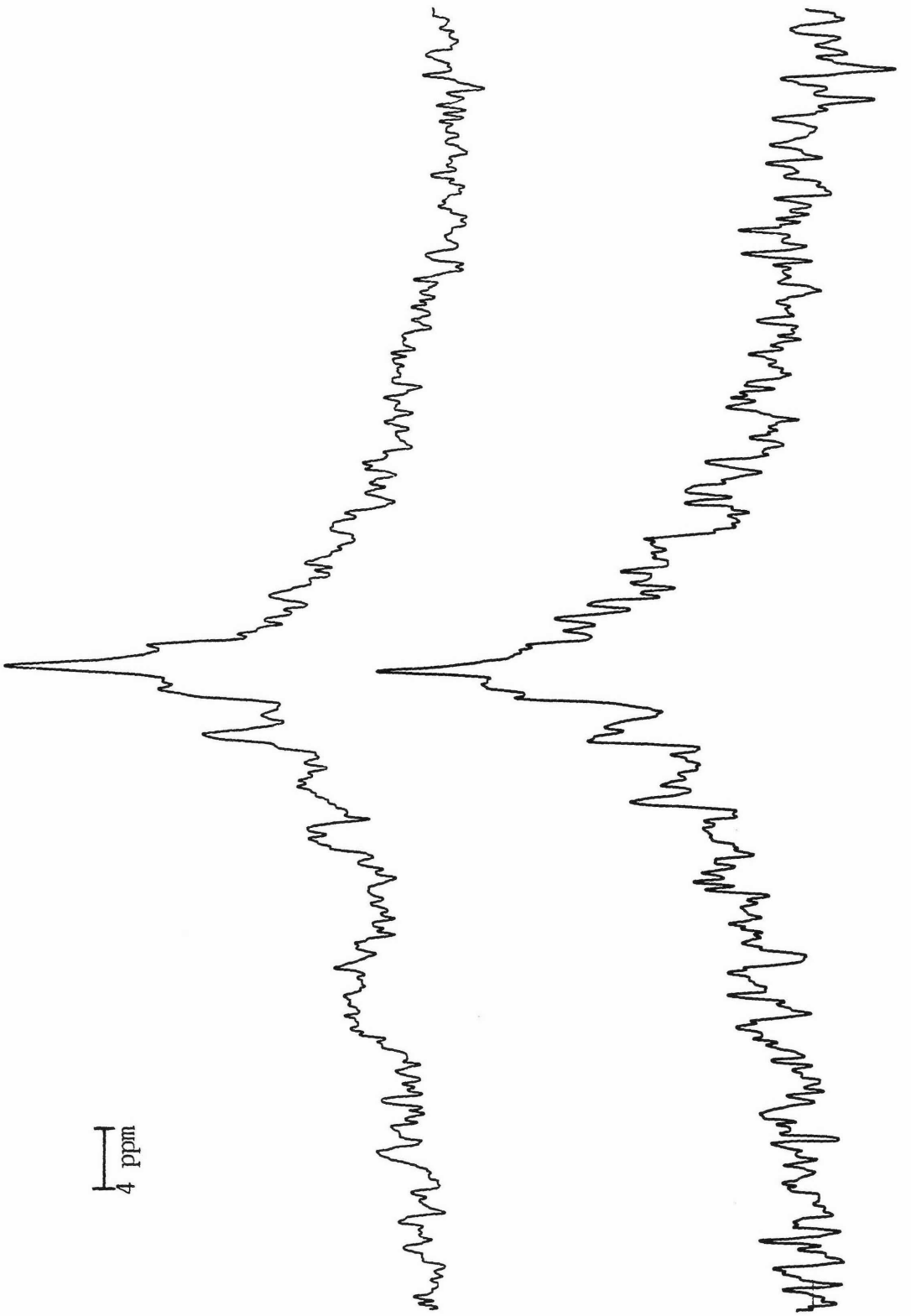

4 ppm

Figure 39

Upper Spectrum: γ-^{13}C-Trp enriched 29.B1 antibody (0.07 mM)
with hapten (0.08 mM)
132 K transients at 125 MHz

Lower Spectrum: γ-^{13}C-Trp enriched 29.B1 antibody (0.06 mM)
with hapten (0.07 mM) after 3 hour papain digest
93 K transients at 125 MHz
Hapten: DNP-glycine

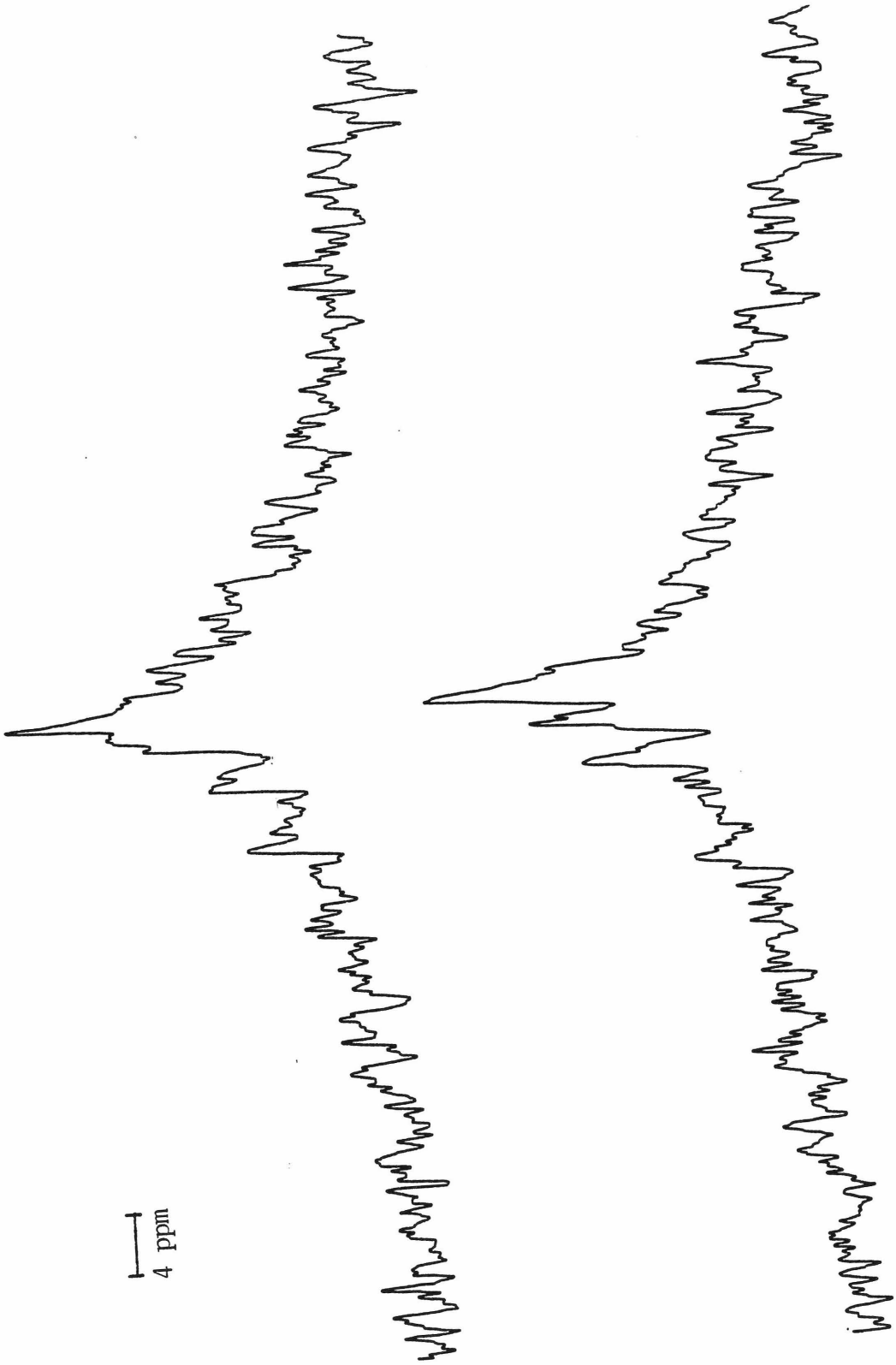

4 ppm

Figure 40

Upper Spectrum: γ-^{13}C-Trp enriched 29.B1 antibody (0.06 mM) with DNP_1-BSA (0.07 mM)
167 K transients at 125 MHz

Lower Spectrum: γ-^{13}C-Trp enriched 29.B1 antibody (0.06 mM) with DNP_1-BSA (0.07 mM) in the presence of 60 mM 2-mercaptoethanol
70 K transients at 125 MHz

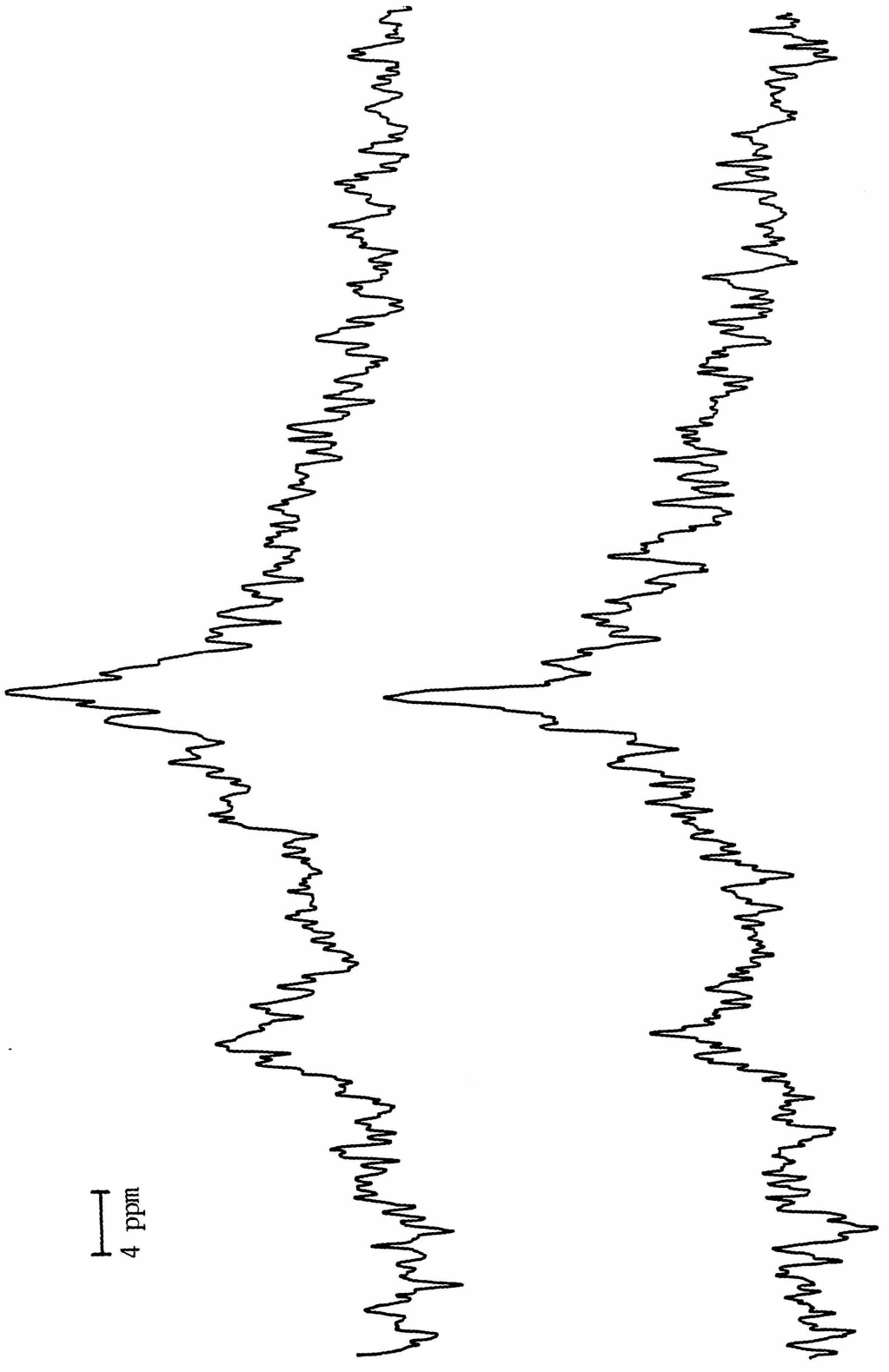

4 ppm

Figure 41

Upper Spectrum: γ-^{13}C-Trp enriched 29.B1 antibody (0.1 mM)
400 K transients at 50 MHz

Lower Spectrum: γ-^{13}C-Trp enriched 29.B1 antibody (0.06 mM)
with DNP_1-BSA (0.07 mM)
460 K transients at 50 MHz

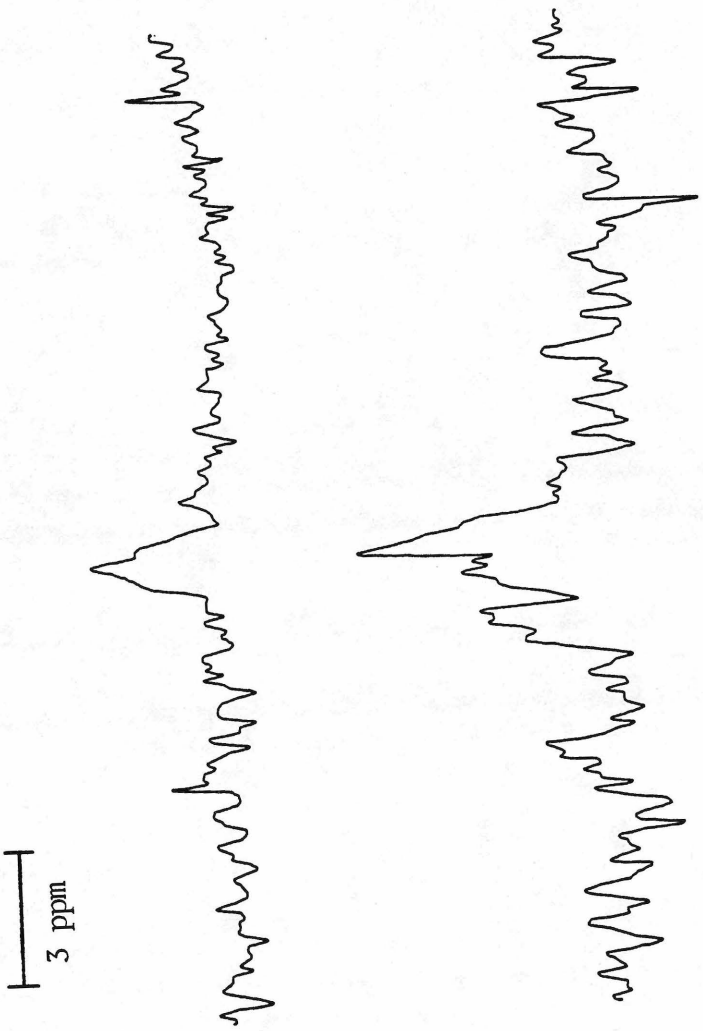
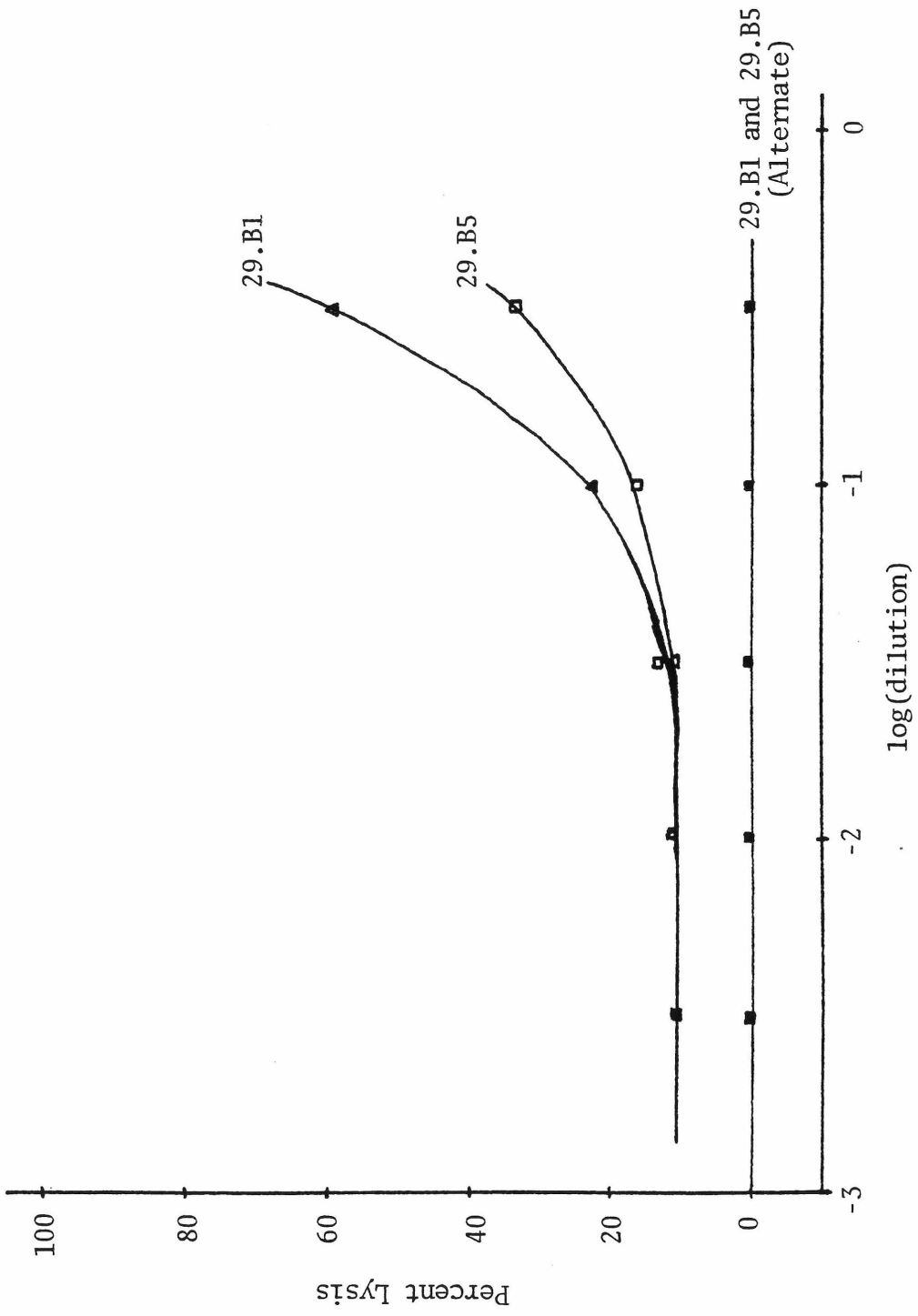

3 ppm

Figure 42

Direct lysis complement assay results with 29.B1 (IgG_{2a}) and 29.B5 (IgG_1) antibodies bound to DNP labeled sheep red blood cells. Alternate pathway assay.


Percent Lysis
100
80
60
40
20
0
-3
-2
-1
0
log(dilution)
29.B1
29.B5
29.B1 and 29.B5
(Altemate)

Discussion

A process of alternate animal and cell culture growth (Periman, 1971) of the MOPC460 tumor line has established a cell culture line of MOPC460. The cells grown in suspension in 10% fetal calf serum supplemented RPMI1640 or DMEM with a doubling time of about 16 hours. The IgA cell product is secreted into the medium as the cells grow and reaches a maximum concentration ($\sim$ 20 micrograms per milliliter) at a confluent density of 10^6 cells per milliliter. The cells grow and produce ascites if injected into mineral oil-primed CDF_1 mice. The cells produce a solid tumor when injected subcutaneously into BALB/c mice.

Similar attempts to establish cell culture lines of MOPC315 and UPC10 (which secretes a levan-specific IgG_{2a}) were unsuccessful after five passages. Therefore an alternate procedure was attempted: somatic cell fusion (Köhler and Milstein, 1975) of the protein-secreting tumor cells with a nonsecreting cell line (NSP2) commonly used in the production of hybridomas. The standard fusion protocol with myeloma tumor cells substituting for spleen cells produced viable protein-secreting cell lines. After several subclonings the lines stabilized as secreting cells (assayed by a solid phase radioimmunoassay).

Two hybridoma cell lines that secrete IgG protein specific for DNP were obtained from Dr. Leonard Herzenberg's laboratory and grown in large scale culture. One line known as 29.B5, an IgG_1 secretor, grows relatively slowly with a doubling time of nearly 24 hours but yields a large amount of protein (about 40 millgrams per liter at 10^6 cells per milliliter). The other line, 29.B1, an IgG_{2a} secretor,

grows more rapidly with a doubling time of 16 hours but yields less protein (about 20milligrams per liter at 10^6 cells per milliliter). Both lines can also be grown as solid or ascites tumors in SJL x BALB/c F_1 hybrid mice.

All of the ^{13}C enriched antibody was isolated by affinity chromatography using a DNP-sepharose support. Isolation of IgA protein was relatively time consuming as a reduction and alkylation step prior to chromatography required considerable concentration of the medium. The IgG proteins were extremely easy to isolate simply by flowing the spent medium through the column. It was relatively difficult to remove hapten used to elute these proteins from the affinity column because of their high binding constants. This difficulty was overcome by addition of a small molar excess of sodium dithionite to the protein solution which reduced the DNP group and weakened binding sufficiently to permit removal by dialysis.

All of the ^{13}C spectra were accumulated with a very small flip angle because the T_2 for the tryptophan resonance at high fields is relatively short (a relatively broad line) while the T_1 is very long (on the order of 20 seconds in these proteins). Thus to avoid saturation while accumulating a large number of transients a 12° pulse was employed with a one-half second acquisition time. The increased linewidth at high field was expected from previous work (Oldfield et al., 1975) and the long T_1 is quite reasonable for a protein of 150 thousand dalton molecular weight (the T_1 reflects a correlation time of 150 nanoseconds). This compares with a correlation time of 72

nanoseconds for alkaline phosphate at a molecular weight of 80 thousand dalton (Hull and Sykes, 1975), 40 nanoseconds for hemoglobin at 62 thousand dalton and 12 nanoseconds for myoglobin at 17 thousand dalton (Gilman, 1979). Quite respectable signal to noise ratios were obtained from 0.1 millimolar antibody solutions in about 12 hours. One set of spectra obtained at lower field (50 MHz) yielded somewhat sharper lines, but the signal to noise ratio was not as good so this was not employed for any further work. Improvements in sensitivity at this lower field would make this an optimum observation frequency probably resolving most of the resonances in the envelope found at 125 MHz.

The spectra are in accord with previous results that indicated that the gamma carbon of tryptophan was extremely sensitive to environment as resonances are observed over a 10 part per million range. The tryptophan region is clearly an envelope at 125 MHz with a number of overlapping signals. This isn't particularly surprising considering there are 10 to 15 nonequivalent tryptophan residues per immunoglobulin. A comparison of the three classes studied shows that the IgA tryptophan envelope is quite different in shape from the spectra of the two IgG classes observed. There are also differences between the two IgG classes but less than between either and the IgA. This is expected since the Fc regions of the IgG subclasses are more related than either to an IgA Fc region. Also, the IgA was reduced and alkylated in the isolation procedure which could certainly have

an effect on the conformation of the Fc domains (Seegan et al., 1979).

There is a marked difference between the three antibodies when spectra are obtained with antigen bound. Slight differences are observed in the tryptophan envelope when 29.B5 binds monosubstituted DNP-BSA, but large differences are seen with M460 and 29.B1. One might expect one tryptophan in the binding site to be perturbed in all DNP-specific antibodies as a charge transfer interaction between nitrophenyl hapten and a binding site tryptophan is a common feature of these antibodies (Little and Eisen, 1967). However, as 29.B5 certainly has this interaction (and the highest binding constant) clearly the charge transfer interaction alone does not cause major changes in the tryptophan resonance envelope. A nonspecific effect, due to the longer correlation time of the larger antigen-antibody complex (150 thousand dalton increases to 280 thousand dalton) might change the relaxation times of the various resonances enough to alter their intensities. That is, even with a 12° flip angle, a pulse every half-second might saturate some resonances and thereby reduce their intensities. Again, 29.B5 acts as a control and aside from some broadening no real change in intensity is seen. Thus the use of a 12° pulse avoids saturating the resonances at the correlation times both of the antibody and of the complexes.

A further control is the spectrum of hapten bound to 29.B1 which is not very different from the spectrum of antibody alone and is quite distinct from the spectrum of the antibody-antigen complex. This is not surprising as previous physical studies using circularly polarized

luminescence (Jaton et al., 1975) found that a ceratin minimum size antigen was necessary to induce conformation changes in the Fc. Prior studies using circular dichroism (Holowka et al., 1972) had found changes on binding small hapten presumably due to changes nearby the binding site. This correlates with the observation that even in the "preaggregated" antibody IgM hapten alone will not trigger complement fixation (Brown and Koshland, 1975).

An experimental attempt to correlate the observed NMR differences with complement fixation was carried out by reducing the antibody in the presence of antigen. This is known to considerably decrease the ability of the complex to fix complement (Press, 1975) and again large changes are seen in the tryptophan envelope with most resonances moving toward the free solution resonance position. This is closer to the shape of the envelope of the antibody alone which has many resonances in a central envelope. When antigen is bound, on the other hand, there are more resonances downfield of the central peak.

Finally, it is well-known that papain digest of IgG which cleaves the Fab fragment from the Fc region leaves the Fab relatively unaffected while altering the conformation of the Fc considerably (Cathou et al., 1968). Such a digest of 29.B1 produces large changes in the tryptophan envelope and according to the above-mentioned observation is most likely due to Fc tryptophan resonances. Interestingly, one obvious result is the increase in the number and intensity of resonances downfield of the central envelope. It is known that cross-linked Fc fragments fix complement quite efficiently (Isenman et al.,

1975) so one might speculate that removal of the Fab allows the Fc to assume a relaxed complement triggering conformation characterized by certain downfield resonances.

One puzzling feature of this study is that while the results with 29.B1, an IgG_{2a}, are consistent with conformational change as the trigger of complement fixation, the other two antibodies behave somewhat anomalously. First, 29.B5 (an IgG_1) commonly considered to be of a non-fixing class was found to fix complement in the standard complement depletion assay (Chapter 8) and also to lyse DNP-labeled erythrocytes (Chapter 9). There is a report that some IgG_1 antibody in mouse can fix complement by the classical pathway (Ey et al., 1979), but the finding is still somewhat controversial. An attempt was made to determine if the lysis observed was by alternate pathway (which skips the interaction of Fc with C1 completely) and no lysis was observed. So it is still unclear exactly how 29.B5 is activating complement. It is certainly less efficient than the IgG_{2a} so possibly the Fc interaction with C1 is not really "classical". Precise individual component complement assays might help to resolve this problem.

The other anomaly is that M460 (a reduced and alkylated IgA) shows considerable change in its tryptophan envelope on binding antigen. This result was not a complete surprise as kinetic studies (Lancet and Pecht, 1976) showed two distinct rate constants for the binding of hapten to M460. This was interpreted as reflecting a conformational change in the Fab which altered the affinity constant of

the antibody on binding hapten. It has been reported that IgA antibodies can trigger the alternate pathway through their Fab domains so this result may relate to alternate pathway triggering.

Certainly considerable work remains to be done with this system before it can be definitively stated that the changes seen in the tryptophan envelope on binding antigen reflect changes in the Fc region correlated with complement fixation. Enzymatic fragmentation studies may help to assign Fab and Fc tryptophan resonances. Of course the work can be extended to the IgM class and to other IgG antibodies. In particular, the results with the non-fixing IgG_3 class would be informative. In any event, the biosynthetic incorporation of this enriched tryptophan coupled with hybridoma antibodies provides a very powerful tool for the further investigation of effector function triggering at the molecular level.

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PROPOSITIONS

PROPOSITION I

Hemoglobin, a protein comprising 90% of an erythrocyte, provides the means whereby oxygen is transported from the lungs to the body's tissues and facilitates the return of carbon dioxide from the tissues to the lungs (Lehman and Huntsman, 1974). The protein is composed of two pairs of polypeptide chain termed the alpha and beta chains, each of which contains the oxygen-binding prosthetic group, heme. A simpler protein, myoglobin, which is similar to a single beta subunit, is found in muscle tissue (Lehman and Huntsman, 1974). The major functional difference between the two proteins is a property of hemoglobin called cooperativity (Antonini and Brunori, 1971) made possible by its multisubunit structure.

The cooperative binding of oxygen is most easily visualized if one compares the saturation of myoglobin and hemoglobin at various oxygen partial pressures on the same graph. On such a graph the myoglobin binding curve is hyperbolic in shape while that for hemoglobin is sigmoidal. At relatively high oxygen partial pressure, as is found in the lungs, both proteins have similar high affinities for oxygen. However, at relatively low oxygen partial pressure, as is found in the tissues, hemoglobin can release considerably more oxygen than myoglobin. Thus the loss of one subunit's oxygen lowers the affinity of the other subunits for their oxygen and this continues for the third and fourth oxygen. This property permits hemoglobin to transport considerably more oxygen than a similar quantity of myoglobin.

Fine control of the transport of oxygen is mediated by molecules that shift or change the shape of the sigmoidal binding curve. Both protons and the effector molecule, diphosphoglyceric acid (DPG), carry

out such control (Wyman, 1964; Benesch et al., 1968). Such effector molecules can be used to investigate the phenomenon of cooperativity in hemoglobin.

A theory has been formulated to explain the manner in which information on the ligation of one subunit might be communicated to the other subunits. This theory, describing allostery in multisubunit enzymes, by Monod, Wyman, and Changeux (Monod et al., 1965) has been applied to explain oxygen binding in hemoglobin (Perutz, 1970). The theory postulates that at least two structures exist for hemoglobin: a relaxed state with maximal oxygen affinity and a tensed state with reduced affinity. The fully oxygenated structure is the R state and the fully deoxygenated structure is the T state. The loss of oxygen from a subunit permits it to shift to the T state and this in turn influences the other subunits to shift to this state, lowering their oxygen affinity.

The means of communicating the information between subunits is the formation or breaking of salt bridges (Braunitzer et al., 1964) between the subunits. The sequences of both alpha and beta chains are known (Popp, 1967; Popp and Bailiff, 1973) and X-ray structures for both oxygenated (Perutz et al., 1968) and deoxygenated hemoglobin (Muirhead and Greer, 1970) have been determined. This information has established the interchain contacts and has permitted the description of the salt bridge interactions. The importance of salt bridges in the T to R transition also helped explain the activity of DPG. The T structure with more salt bridges has a pocket between the beta chains lined with positively charged residues. Binding DPG in this pocket forms another set of salt bridges and stabilizes the T structure. Thus, addition of DPG to oxygenated hemoglobin shifts it to the T state and favors release of oxygen

(Perutz et al., 1976).

The precise mechanism whereby the presence of oxygen at one heme can cause changes in the salt bridges has been discovered by very precise comparison of X-ray structures of oxygenated and deoxygenated hemoglobin (Fermi, 1975). The iron in deoxygenated hemoglobin is slightly out of the plane of the prophyric ring, and on oxygenation moves into the plane. The proximal histidine liganded to the iron is also moved, as is the F helix of which it is a part. The movement of this length of alpha helical chain acts as a sort of lever arm to break the T state salt bridges. Very precise definition of the changes that take place on ligation has been described by Baldwin and Chothia (Baldwin and Chothia 1979) who state that movement of the F helix shifts the β porphyrin, removing it from a region hindering binding of oxygen. Thus binding of oxygen to one subunit induces strain which tends to favor the oxygenated quaternary structure and this oxygenated structure releases the strain by repositioning the F helix and the β heme.

These studies have all been carried out with crystals of oxygenated or deoxygenated hemoglobin. Thus there is still relatively little known about the intermediate states that hemoglobin passes through on going from deoxygenated to fully saturated (Koshland et al., 1966). For example, does ligation of one subunit shift all of the others to the R state so that all rapidly saturate, or are partially saturated R state/T state intermediates present? This question has been approached by Huestis and Raftery (Huestis and Raftery, 1972, 1973, 1975) and later by Adler and Richards (Adler, 1980) by attaching a fluorine reporter group to a cysteine on the β chains. The modified hemoglobin could then be studied by fluorine NMR as the hemoglobin was oxygenated. Small

resonances indicative of possible intermediate states have been observed in such studies, but except in the presence of certain effectors the signals are difficult to identify unambiguously.

Another nucleus commonly observed by NMR is ^{13}C which is very sensitive to conformation (Stothers, 1972). The major disadvantage compared to fluorine is the poor sensitivity of the ^{13}C resonance and the low natural abundance of this isotope (Dwek, 1973). Also observation of the ^{13}C spectrum of hemoglobin would be hopelessly complex compared to the single fluorine resonance. The problems of background signals and low abundance can be overcome if a carbon atom of a single type of amino acid residue is selectively enriched (Hunkapiller et al., 1973). The lower natural sensitivity of carbon may be compensated for by observing at higher field and for somewhat longer times (Pople et al., 1959). The major problem is how one might enrich a specific residue of hemoglobin.

Fortunately, a unique system exists which makes the incorporation of specifically labelled amino acid residues into hemoglobin possible. This is the Friend erythroleukemia cell line (Patuleia and Friend, 1967) which, under normal conditions, is a proliferating preerythroid murine cell line. Upon addition of 1 to 2% of dimethylsulfoxide to the medium a series of differentiation events are induced in the cells, leading to accumulation of hemoglobin within the cells (Friend et al., 1971). The hemoglobin so produced is readily isolable by lysis of the cells and chromatography of the lysate on ion exchange cellulose. This inducible differentiation has been extensively studied both by protein biochemists (Boyer et al., 1972) and by molecular biologists (Ross and Sautner, 1976).

Friend cells grown and induced in ^{13}C labelled medium will

incorporate this labelled residue into the hemoglobin produced. Ideally, the residue selected for enrichment should not occur too frequently and should be in "interesting" parts of the protein. The gamma carbon of both tryptophan and tyrosine meet these requirements (Oldfield and Allerhand, 1975) and in addition are extremely sensitive to conformation. Tyrosine in particular is in a part of the molecule exquisitely sensitive to change in tertiary and quaternary structure. A technical point that makes these carbons easier to observe is that since they have no directly bonded hydrogen atoms the resonances are extremely sharp. Also the sample need not be proton decoupled so carbons in the rest of the hemoglobin, most of which have directly bonded hydrogen atoms, will be coupled and less likely to be observable.

The investigation of hemoglobin conformation using ^{13}C-enriched hemoglobin should be relatively simple once the labelled protein is isolated. Spectra would be accumulated at various stages of oxygen ligation with the degree of oxygen saturation followed by standard spectrophotometric techniques (Benesch et al., 1965). The use of effector molecules such as diphosphoglycerate or inositol hexaphosphate could be employed to shift the T to R equilibrium of hemoglobin at various stages of ligation. Several methods could be employed to determine which residue was responsible for each signal. Chain separation and recombination with unlabelled heterogamous chain would yield hybrids with only one type of chain labelled (Ogawa and Shulman, 1972). Further localization would probably require proteolytic fragmentation of the separated chains. One particularly useful enzymatic digest would be to employ carboxypeptidase (Kilmartin et al., 1974) which removes the C terminal tyrosine, a residue that should be extremely sensitive to ligation. Each mouse alpha chain

has one tryptophan and three tyrosine residues, while each beta chain has two tryptophan and three tyrosine residues so the resulting spectra would not be very complex.

Thus the system described would provide a sensitive, nonperturbing method for the study of hemoglobin conformation. Such studies might include detection of preferential ligation (alpha or beta), of intermediates, of the T or R state, and of the results of effector perturbation. As there are labelled residues in each chain, several sites on the hemoglobin tetramer would be monitored simultaneously, increasing the amount of information derived.

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PROPOSITION II

A major unresolved problem in immunology is the mechanism whereby the immune response is regulated. Jerne, in his network hypothesis (Jerne, 1974), proposed a theoretical framework to account for a great deal of data by a series of idiotype-antiidiotype interactions. Idiotypes are antigenic determinants on the variable region of an antibody and antiidiotypes are antibodies that bind to these determinants. Recent work has implicated in this network a host of cellular interactions including T helper cells (Rajewsky et al., 1969; Woodland and Cantor, 1978), T suppressor cells (Ju et al., 1978), B memory cells (Herzenberg et al., 1980a), and macrophages (Calderon et al., 1975). Highly specific antisera including monoclonal antibodies have permitted studies of allotype regulation (Herzenberg et al., 1976) and idiotype-antiidiotype feedback interaction (Urbain et al., 1977). However, outside of these rather restricted cases the regulation of the normal heterogenous immune response has not been closely examined. The general view has been that carrier specific T helper and suppressor cells provide the principal control step in the response to a hapten-carrier conjugate (Abbas et al., 1977).

Work on the regulation of memory B cell development and affinity maturation in the Herzenberg laboratory has demonstrated a novel form of control: carrier-dependent hapten-specific suppression (Herzenberg et al., 1980b). Simply stated, it was found that if an animal was first primed with carrier alone and then immunized later with hapten-carrier conjugate, then the IgG response was depressed and of lower affinity compared to the response in noncarrier-primed controls. A further boost

with hapten-carrier conjugate fails to increase the response which remains about two orders of magnitude lower in amount and affinity compared to controls. The suppression is clearly hapten-specific: 1) a normal response is mounted to carrier protein; and 2) a booster immunization with hapten conjugated to a different carrier is still suppressed. The suppression is carrier dependent; for, if the initial priming is done with a carrier different from the first hapten-carrier immunization, then suppression is not established. The system has been studied with monoclonal anti-allotypic and antiidiotypic reagents in order to determine the degree of control exerted on the various IgG subclasses (Herzenberg, 1981). Many of the precise details of the suppression system, and the molecular mechanism which mediates it, are yet to be elucidated.

The system on which most of the work has been carried out to date is the response to 2,4-dinitrophenyl keyhole limpet hemocyanin (DNP-KLH). A major tool in the investigation was a solid phase radioimmunoassay (Oi and Herzenberg, 1979). Dinitrophenylated antigen is applied to the wells of a microtiter plate to which it binds noncovalently. Next, the test serum sample is applied and any DNP-specific antibody binds to the wells. This bound antibody may then be analyzed by a variety of reagents. For example, ^{125}I-labeled anti-allotypic antisera will permit determination of the relative amounts of various allotypes present. The levels of two well-known DNP-idiotypes (M315 and M460) (LeGuern et al., 1979; Lynch et al., 1979) can be monitored by coating the plate first with antiidiotypic antisera followed by application of the test serum, and then detecting the bound antibody with the above-mentioned ^{125}I-labeled antisera.

The procedure described above permitted the observation of

independent suppression of various classes of IgG, of differential suppression depending on allotype, and of the relative amount of suppression of the two idiotypes mentioned above. A major problem with the use of the M315 and M460 idiotype specific reagents is that both of these idiotypes are low affinity antibody idiotypes (on the order of 10^4 to 10^6 m^{-1}) and as such are not a major part of the suppressed secondary response. Thus, an important tool in the further investigation of this system would be monoclonal antibodies with high binding constants for DNP. Monoclonal antiidiotypic antibodies could be raised against such high affinity antibodies and these anti-high affinity idiotype antibodies could be used in an investigation of the suppression of the high affinity response so as to more precisely define the hapten-specific suppression system. One might discover whether different idiotypes are separately suppressed or possibly have different rates of escape from suppression. This might explain the existence of "dominant idiotypes" (Kuettner et al., 1972) as, for example, the cross reactive idiotype of anti-arsonate antibodies in A/J strain mice.

A comparison of the regulation of the response to two structurally similar haptens such as DNP and TNP would also prove instructive. Klinman (Klinman et al., 1973) has found that responses to these two determinants are controlled separately, and so one might expect hapten-specific suppression to follow this pattern. A very useful assay (Herzenberg et al., 1980a) that measures affinity by differential binding in unfractionated serum could be applied to the study of this system. The amount of antibody bound to highly substituted and less highly substituted hapten on carrier is measured. The ratio of these quantities is related logarithmically to the association constant of the immunoglobulin. This

would permit discrimination between primarily DNP-specific and primarily TNP-specific antibody populations in the suppression system outlined above.

Finally, this relatively well-defined system can be employed in an attempt to determine the molecules that mediate the suppression. Such molecules would likely include cell surface proteins (Vitteta and Uhr, 1977) and possibly soluble factors (Granger et al., 1975) with specificity for the suppressed immunoglobulin or immunoglobulin receptor. Certainly monoclonal antiidiotypic antibodies would play a major role in this portion of the study, both in detection and probably in isolation of the suppressor species. This investigation would require large amounts of relatively homogenous cell populations (for example, T suppressor cells) and such populations might be obtained by use of the fluorescence activated cell sorter (Loken and Herzenberg, 1975).

In summary, the hapten-specific suppression system is very useful in the study of immune regulation. An investigation of the control of high affinity idiotypes in this system might help to explain the existence of "dominant idiotypes" in the immune response. A comparison of the control of the anti-DNP and anti-TNP responses might give some clue as to the diversity of the immune response, for if the response to these two very similar antigenic determinants are separately controlled then cross reactivity (Richards and Konigsberg, 1973) plays a less important role physiologically in generating diversity than is currently believed. Finally, isolation and characterization of the molecule(s) responsible for this suppression may be more readily accomplished in this well defined system than in others in which the details of the response are less well known.

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PROPOSITION III

There is a variety of so-called "secondary functions" in the immune system (Winkelhake, 1978) for which the binding of antigen by antibody serves as an initiating event. These functions include the triggering of the complement cascade (Porter and Reid, 1980), initiation of degranulation of basophils or mast cells (Segal et al., 1977), phagocytosis by macrophages (Diamond et al., 1978), and triggering of differentiation of B lymphocytes (Dickler, 1976). The initiating event, the binding of antigen by the F_{ab} arm of an antibody, is coupled to these effector functions by the F_c domains (Spiegelberg, 1974), principally the C_H2 and C_H3. The precise molecular mechanism of this coupling is currently not well understood and so a more precise definition of this process would have considerable importance.

Of the various secondary functions listed above, certainly the most studied is the complement pathway (Müller-Eberhard, 1969). Complement consists of a group of nine proteins in which early events in the pathway produce enzymes that amplify the response which includes a number of events (Feuron and Austen, 1976) such as cellular lysis, virus inactivation, and production of chemotactic and anaphylactic factors. It is the interaction of the first complement component through the protein called C_{1q} with the C_H2 domain of IgG that triggers the pathway (Reid and Porter, 1975).

One theory, the associative (Metzger, 1974), states that antigen serves to crosslink IgG and through this crosslinking produces a group of closely associated F_c regions. The binding interaction between individual F_c regions and C_{1q} is assumed to be low so that aggregation serves

to augment binding to the polyvalent C_{1q} by avidity. This binding is said to trigger the pathway. An alternate theory, the distortive (Hoffman, 1976), states that antigen produces a conformational change in antibody when it is bound, which is conveyed to the C_H2 domain in a manner analogous to allostery in enzymes (Monod et al., 1965). This change may increase the binding constant between F_c and C_{1q} or it may simply produce a complementary change in C_{1q} which is in turn relayed to the other proteins of the C_1 complex to trigger the cascade.

Considerable evidence has accumulated to support the associative model, at least for IgG. It has been found that IgG must be crosslinked into at least dimers in order to initiate fixation (Jaton et al., 1976). Also, chemically crosslinked (Ishizaka and Ishizaka, 1960) or heat aggregated (Ishizaka and Ishizaka, 1959) IgG will fix complement in the absence of antigen. On the other hand, certain studies show that relatively mild treatment of IgG can reduce fixation while still allowing aggregation. The best known procedure of this type is the use of reducing agents such as dithiothreitol or 2-mercaptoethanol to remove the inter-heavy chain disulfide bond which diminishes complement fixation considerably (Press, 1975). Also, reagents that specifically modify tryptophan lead to large decreases in the ability of such modified IgG to fix complement (Alan and Isliker, 1974). It appears that modification of a tryptophan in the C_H2 domain is sufficient to produce this effect.

A great deal of effort has been expended in an attempt to demonstrate conformational changes in antibody after the binding of antigen. Differences in susceptibility to proteolysis, depending on whether antigen is bound, have been reported (Ashman and Metzger, 1971) as has the converse (Wright et al., 1978). Work with circularly polarized

luminescence (Jaton et al., 1975) has shown differences localized to the F_c region that occurs on binding fragments of antigen to rabbit antibody. Small fragments did not produce the changes even though they bound quite well. Unfortunately, much larger fragments were required to fix complement (Jaton et al., 1976). Many reports exist that suggest changes in a variety of physical properties of immunoglobulin (sometimes localized to the F_c) but only rarely is an attempt made to correlate this observed behavior with the triggering of effector function.

Probably the best evidence correlating conformational change with complement fixation comes from work by Koshland (Brown and Koshland, 1975) with an anti-lactose IgM raised in rabbit. In this system a monosubstituted antigen (lactose conjugated to ribonuclease) triggered complement fixation. It appeared that the binding of one antigen to an IgM pentamer was sufficient to initiate fixation. In a later report (Brown and Koshland, 1977) it was found that the exposure of J chain varied, depending on whether monovalent antigen was bound. This suggests a conformational change is produced by occupation of the binding site by antigen with no crosslinking necessary. A minimum size antigen was necessary as hapten alone did not trigger fixation and hapten could be used to inhibit complement fixation by disrupting the antibody-antigen complexes.

The study with IgM has been criticized on a number of points (Metzger, 1978), but it is supported by work in this thesis (Chapter 8) with a myeloma system using hybridoma IgG controls, which answers most of the objections raised against Koshland's work. Further work (Chapter 9) with a complement-fixing DNP-specific hybridoma antibody indicated that conformational changes in tryptophan were taking place when monosubsti-

tuted DNP antigen was bound, but under these conditions no complement was fixed. This suggests that both crosslinking and conformational change are necessary for effector function triggering. As the IgM is already "crosslinked" it can fix complement with monovalent antigen while the isolated IgG molecules require some sort of crosslinking.

A major objection to this concept is the observation that aggregated IgG, with no antigen present, is capable of fixing complement. One reply to this objection is that the physical (heat, denaturants) and chemical methods may be severe enough to produce irreversible changes in the structure that mimic the conformational change induced by antigen. A relatively mild chemical crosslinking procedure has been employed by Jaton (Wright et al., 1980) to produce dimers and higher aggregates of IgG which do fix complement, but not as efficiently as antibody-antigen aggregates.

This brings up the important point of background or spontaneous fixation observed in complement assays. It is well known that even after IgM is carefully deaggregated to give exclusively 19S pentamers, it can still fix complement without antigen (Augener et al., 1971) if relatively high concentrations of the IgM are employed. The same effect, but to a lesser extent, is seen with IgG (Strader, 1980). Thus, the studies with aggregated IgG may simply have increased this background fixation in a manner similar to the increase in IgM compared to IgG.

This suggests a very interesting experiment to test the distortive model and simultaneously remove an objection raised by those who support the associative model. A relatively mild procedure can be utilized to produce defined aggregates (dimer, trimer, etc.) of a hybridoma IgG with a defined specificity (such as DNP). Monovalent antigen can be checked

for monovalency by a complement assay with monomeric IgG. Then a complement assay can be employed to observe the level of fixation by crosslinked IgG as monovalent antigen is bound. If the results are similar to those found with IgM, then this strongly supports the combined distortive-associative model for effector triggering, at least in the case of complement.

Experimentally, the procedures are quite straightforward. A previous study with chemically crosslinked IgG gives the technique for crosslinking and describes separation of the resulting aggregates (Wright et al., 1980). Such work would most reasonably be carried out on a hybridoma immunoglobulin with some simple specificity and high binding constant (Oi et al., 1978). A method described in Chapter 8 of this thesis will serve for the production of monovalent antigen. The complement assay would be the standard chromium release assay (Bengali et al., 1980) as described in Chapter 8. Comparisons could be made among the amounts of fixation obtained with antibody-antigen complexes, with crosslinked antibody and with crosslinked antibody-monovalent antigen complexes.

The results of these experiments would be very important to the aggregation-conformational change hypothesis. An increase in fixation by the crosslinked IgG on binding monovalent antigen would strongly support the importance of conformational change in effector triggering. On the other hand, if no change was observed it would imply that the associative model is probably correct for IgG and any conformational changes observed in IgG are simply irrelevant. This would still leave open the question of conformational change in the IgM system, which presumably would differ fundamentally from effector triggering in IgG.

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PROPOSITION IV

One approach in the study of the way antibody structural features generate a particular binding specificity has been to compare several different antibodies that interact with the same hapten. A precise definition of these fine specificities correlated with amino acid sequence data permits one to judge the importance of a particular amino acid residue in the site, if it is present in one and absent in another. This approach has been applied, so far, only to the phosphorylcholine binding myeloma proteins (Padlan et al., 1976b) as this is the only simple hapten for which a number of sequenced antibodies exist. The emergence of the hybridoma technique (Köhler and Milstein, 1975) should allow the extension of this approach to other systems, but another technique might work as well or better. This is the mutation of a well-characterized myeloma cell line followed by fusion and cloning of the resulting variants.

A mutation-fusion approach has much to recommend it in the investigation of structure-function relationships. While an enormous diversity of antibody protein will be generated by fusion requiring considerable sequencing before comparison studies are possible, the variation of an already sequenced and characterized protein would yield a much simpler system. The use of the hybridoma technique is necessary only as a method to isolate single cells from the mutagenized population. A careful adjustment of the relative numbers of mutagenized cells and fusion partner cells per cloning well should yield one growing hybrid per well. The other requirement for this technique to work is a procedure that simply and rapidly permits the screening of many hybrids so as to select out mutated proteins.

A binding assay commonly used in the screening of normal hybridoma proteins (Marier et al., 1979) would permit the elimination of protein mutated to the extent that binding is abolished. After this preliminary screening, then would remain a population of hapten binding proteins most of which would be identical to the parent myeloma protein. One approach to deplete this population would be to treat the cells with anti-idiotype antibody and complement. This would deplete the idiotype positive cells and so leave those antibodies that differ significantly at the binding site from the parent myeloma protein. If several antiidiotype monoclonal antibodies were available then several subpopulations of proteins could be selected.

The resulting cells could be analyzed further by use of a novel affinity binding assay (Herzenberg et al., 1980) that is capable of screening very large numbers of cloning well supernatants. The method involves the measurement of the amount of antibody that binds to a given amount of antigen at two different epitope densities. For example, to study the binding of DNP-specific antibodies the binding to DNP_5-BSA and DNP_{40}-BSA is compared. The ratio of these amounts when plotted against the log of the association constant is linear. The assay may be calibrated with myeloma or hybridoma proteins of known affinity previously measured by equilibrium dialysis (Eisen and Karush, 1949) or fluorescence quenching (Velick et al., 1960).

The combination of these two screening assays coupled with selection against the parent binding site would yield exactly what is required in the study of structure-function relationships: a spectrum of antibodies all of which are variations on a known characterized structure and which vary in binding constant. Sequence studies of the variable region might

provide considerable insight as to which residues are most important in generating a particular specificity. A comparison of the fine specificity of binding a group of related haptens might provide information on the phenomenon of cross-reactivity (Richards and Konigsberg, 1973) and how it is generated. Finally, a more precise study by a technique such as NMR (Wuthrich, 1976) on selected antibody clones would precisely define the differences in interaction produced by specific amino acid substitutions.

A myeloma ideally suited for this procedure is the DNP-specific protein M315, whose binding site and fine specificity have been extensively studied over the past ten years (Padlan et al., 1976). Particular amino acid residues are thought to contribute specific interactions to the binding site (Givol et al., 1971) so such a study would both test these assertions and more precisely define the interaction between the DNP hapten and the binding site. The binding assay is well-established in the screening of DNP-specific hybridomas and the affinity assay was first applied to the study of DNP-specific antibodies, so these procedures are completely worked out in this system. The protein has been studied by NMR (Kooistra and Richards, 1978; Hardy and Richards, 1978) and such studies could be extended to analyze selected variants. Finally, M315 is noted for its multispecificity (Michaelides and Eisen, 1974) as it binds DNP, TNP, dinitronaphthol, menadione, and a number of others. A comparison of the relative affinities of the parent protein for these compounds with selected variants for the same compounds might help to clarify the question of cross-reactivity and multispecificity.

The relationship between structure and function could be studied in areas outside the binding site if suitable assays are possible. For

example, there exists a DNP-specific hybridoma which fixes complement, an effector function known to be associated with the C_H2 domain (Winkelhake, 1978). Mutation of this hybridoma line followed by growth of the hybrid cells in soft agar (Coffino et al., 1972) would provide a method to test for changes in complement fixation. If the growing clones are overlayed with DNP-erythrocytes and complement then clones that don't form lysis plaques lack complement fixing capability (Jerne et al., 1974). The standard binding assay could be used to verify that antibody was still being secreted. Such variant immunoglobulin could be analyzed by enzymatic fragmentation (Putnam, et al., 1962) and SDS gel electrophoresis to determine the nature of the change.

A variety of mutagens have been defined in the study of cells in culture (Friedrich and Coffino, 1977). Among these are ultraviolet light, X-rays, and a host of chemicals. Because of the ease of the screening procedure outlined above, several of these mutagens could be employed in order to select the simplest and most efficient.

It should be noted that the method described might be supplanted by a direct genetic manipulation approach. One would have to clone the genes, coding for the immunoglobulin heavy and light chains, specifically change them and then, somehow, reinsert them into a plasmacytoma cell with the hope that they would be expressed. This is probably not going to work, but another approach making CDNA copies (Taniguchi et al., 1980) of MRNA isolated from the parent myeloma might. The CDNA specifying the heavy and light chains would be inserted into bacteria on an inducible plasmid (Mercereau-Puijalon, 1980). The heavy and light chains presumably could be recombined later. This procedure would require a great deal of work, much more than would be required in the screening

assays of the mutation approach. Thus, until the state of the art of genetic engineering or peptide synthesis improves substantially, the mutation-hybridoma selection procedure described would likely be the best method for the generation of a family of related antibody binding sites. As a bonus the technique might also facilitate investigation of the regions of antibody involved in effector function triggering.

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PROPOSITION V

The nature of transformation in an in vitro culture cell line is still not well understood. By "transformation" one refers to the change of a normal cell type such as a fibroblast from growth that is contact inhibited and persists for only a limited number of generations to growth that is not contact inhibited, and that will continue as long as the cells are kept in culture (Sanford, 1974). Such transformation is quite obvious morphologically and has been studied in some detail in a variety of lines such as liver cell lines (Hagiwara and Kitahara, 1974). Transforming agents include ultraviolet light, certain chemicals and a large number of viruses (Gross, 1970). One important characteristic of the transformed line is its ability to form a tumor in the animal from which it was derived. Thus, the study of transformation in vitro may shed some light on the process of oncogenesis in vivo.

Aside from changes in cellular morphology and the ability to induce a tumor, one other readily observable change that occurs on transformation is the cell's nutritional requirements and metabolism (Dieter et al., 1974). In general, transformed cells usually are culturable under less stringent conditions and evince a lowered requirement for serum. This change in growth characteristics is maintained for the life of the cell line and provides a useful marker of the transformed phenotype. These changes may also provide a clue as to the basic nature of the transformation process.

A very interesting observation is that certain transformation processes are reversible. For example, temperature sensitive transformed lines exist and such systems have been employed in an attempt to elucidate the mechanism of transformation (Dulbecco and Eckhart, 1970).

Another unusual observation is that occasionally a normal transformed line originally isolated from an animal tumor may undergo this reversion to the nontransformed state in vitro. In one case (Paraf et al., 1970) a mouse plasmacytoma line "differentiated" in vitro into two sets of cells: one was fibroblast-shaped and produced tumors in mice, while the other was epitheloid in morphology and did not induce tumors. The two cell populations were extensively characterized and a major difference between them turned out to be the presence of type A virus particles in the fibroblastic cells. Such particles were essentially absent from the epitheloid cells.

Viruses have long been implicated in the oncogenic process and a number of in vitro and in vivo systems exist that demonstrate the transforming ability of certain viruses (Diamandopoulos, 1973). In addition virus particles are often found upon electron micrographic investigation of transformed cells (Dalton et al., 1961). One extensively studied system is the induction of T lymphomas by certain RNA tumor viruses. In particular it has been found that T lymphoma cells have receptors on their surfaces specific for the particular virus that induced them (McGrath and Weissman, 1979). In the case of oncogenic induction by agents other than viruses, it has been found that in many cases such agents activate endogenous RNA tumor virus. The virus multiplies and spreads, eventually binding to a cell with receptor for it. This cell is transformed and so yields a tumor cell population with viral receptor on its surface.

Further study of T lymphoma cells has shown that only cells with viral receptors on their surfaces are capable of autonomous replication. Indeed, if the receptor is blocked by a monoclonal antibody directed

against it, then normal T lymphoma growth is halted (McGrath, 1981). Monoclonal antibodies directed against other surface determinants have no effect on cell growth and excess UV-inactivated virus protects the cell from monoclonal blocking. This result suggests the hypothesis that these T cells are producing their own nitrogen - that is, their own growth factor. It has been suggested that this phenomenon may explain transformation in a number of cases, especially for diploid cell lines. Cells that increase their chromosome number on transformation conceivably could be becoming transformed by such increase, but cells that remain diploid demand another explanation.

Different studies with mouse sarcoma virus transformed cell lines (DeLarco and Todaro, 1978) have shown that these cells release a growth stimulating peptide that binds to epidermal growth factor receptors. Such growth stimulating activity may be assayed by the effect of culture supernatants on normal fibroblast or epithelial cells. Recent work (Todaro et al., 1980) with three human cell lines, a rhabdomyosarcoma, a bronchogenic carcinoma, and a metastatic melanoma demonstrated the release of transforming growth factor by these cells. Again, these protein(s) interact with EGF receptors on fibroblasts and produce profound changes in cellular morphology. In particular, the transformed fibroblast can then grow in soft agar culture. An important point is that removal of the transforming protein leads to reversion to the normal phenotype. Finally, it was found that the tumor cells that grow best in soft agar release the greatest amount of these transforming proteins.

Thus, it appears that in a number of cases the maintenance of the transformed phenotype is due to the production of protein, or factors that act to promote continuous cellular division. That is, the cells are

auto-stimulatory and produce their own mitogens. This is an extremely important concept, for if it is generally true then it may provide a means for controlling the expression of the transformed phenotype in vivo. Therefore, further investigation of other transformed cell types and characterization of the factors involved in cellular growth seems warranted.

A large number of myeloma tumor and cell lines exist in a number of labs. Some arose spontaneously in susceptible mouse strains (Potter, 1972) such as BALB/C while others were induced by various agents such as injections of mineral oil or X-ray irradiation. Viral particles have been observed in many of these tumor cells (Dalton et al., 1961). It is not clear how extensive the transformation mechanism outlined above may be, but it is more likely to be involved in diploid lines rather than polyploid lines. The increase in chromosome number as mentioned previously may serve, somehow, as another mechanism of transformation so that at least some cell lines examined should be diploid. Such a cell line is S194, a myeloma line that secretes a DNP-specific IgA immunoglobulin (Waring, 1975).

One method currently employed in the investigation of cell surface antigens is xenogenic immunization of rats with mouse cells, followed by somatic cell fusion of the rat's spleen cells with a myeloma line that yields cells secreting antibody with a variety of specificities, some directed against cell surface determinants (Springer et al., 1978). The hybridoma clones secreting antibody specific for S194 surface protein can be screened by a solid phase radioimmunoassay (Oi and Herzenberg, 1979). Those antibodies that bind to S194 membrane can then be screened for growth inhibition. If these cells have viral receptors on their surfaces

then it is quite likely that a few fusions would generate at least one antibody against the mitogen receptor. Growth inhibiting antibodies could then be utilized to isolate the protein they bind by conjugating the antibody to a solid support (Barra et al., 1979). Detergent solubilized membrane extracts could then be passed down the column and the membrane protein would be bound by the hybridoma antibody in a manner analogous to the isolation of Ly 2,3 (Ledbetter, 1980). Such an approach would permit isolation of the growth inhibiting surface protein which could then be examined for viral binding. The cell surface protein could also be assayed for peptide binding as in the TGF study.

A similar study could be carried out on a polyploid myeloma line to determine the importance of this self-mitogenic mechanism in nondiploid transformed lines. The isolation and characterization of the receptor and the transforming factors, either in diploid or polyploid lines, should provide very basic information concerning the nature of the transformed state. Additionally, information gained from such a study might provide a means of selectively inhibiting this transformation in vivo and lead to a treatment of spontaneous tumors. As the production of transforming factors is the first event in oncogenesis an assay for such factors (utilizing monoclonal antibodies) would permit a very quantitative measure of transformation. Potentially this sort of system could be incorporated into an in vitro assay, useful in screening substances for oncogenic capability. Such a system might be more predictive of oncogenesis in vivo than the Ames test currently in use (Ames et al., 1973).

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