NUCLEAR MAGNETIC RESONANCE STUDIES OF IMMUNOGLOBULINS

I. STRUCTURE-FUNCTION RELATIONSHIPS IN PHOSPHORYLCHOLINE-BINDING MOUSE MYELOMA ANTIBODIES

II. ^{19}F NMR STUDIES OF TRIFLUOROACETONYLATED IMMUNOGLOBULINS AS A PROBE OF ANTIBODY CONFORMATION

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.....every man should write a book at some time in his life, both for the mental discipline and because it tends to elevate him in popular esteem to the respected status of an "intellectual."

Richard M. Nixon in "Six Crises"

Gonna get my Ph.D.
I'm a teenage lobotomy.

the Ramones

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Chapter 1 ABSTRACT

This chapter presents a general introduction to mouse myeloma proteins, antibody structure, and the nature of antibody-antigen interactions. The contributions of light and heavy chains to antibody binding properties are also discussed and the 3-dimensional structure of the McPC 603 binding site is examined in some detail.

Chapter 2 ABSTRACT

The interaction of phosphorylcholine-binding mouse myeloma protein McPC 603 and the isotopically substituted hapten phosphoryl[methyl-^{13}C]choline has been investigated using ^{13}C and ^{31}P nuclear magnetic resonance spectroscopy. Upon binding to antibody, upfield shifts of 0.7 and 1.5 ppm are observed for the hapten ^{13}C and ^{31}P resonances, respectively, and both spectra are in the "slow" exchange limit. Linewidth analysis indicates some immobilization of the phosphate group but essentially unrestricted methyl group rotation for the bound hapten. Hapten-antibody dissociation rate constants of 10 and 38 s^{-1} are calculated from ^{13}C and ^{31}P NMR spectra, respectively, suggesting the possibility of differential dissociation rates for the two opposing ends of the phosphorylcholine molecule. The NMR data are entirely consistent with the known X-ray structure of the McPC 603 Fab'-phosphorylcholine complex.

Chapter 3 ABSTRACT

The binding site interactions between the phosphorylcholine-binding mouse myeloma proteins TEPC 15, W3207, McPC 603, MOPC 167, and MOPC 511 and the isotopically subsituted hapten phosphoryl-[methyl-^{13}C]choline have been investigated using ^{13}C and ^{31}P nuclear magnetic resonance (NMR) spectroscopy. Each protein exhibits a unique NMR pattern, but extensive similarities in chemical shift parameters upon binding of hapten to immunoglobulin suggest a significant degree of conservation of important hapten-binding site interactions. Moreover, independent binding studies, in conjunction with the NMR data, allow construction of a simple model of the binding sites of these antibodies, analyzed in terms of the relative strength of interaction between hapten and two main subsites. The NMR evidence supports the view that the heavy chains of these proteins dominate in interacting with bound phosphorylcholine.

Chapter 4 ABSTRACT

We have determined the pH dependencies of the binding affinities of the mouse myeloma immunoglobulins M603, W3207, T15 and M167 for the haptens phosphorylcholine (PC) and L-α-glycerophosphorylcholine (GPC). These affinities are generally maximal near neutral pH with the exception of the binding of PC by M167 which is strongest at pH 5.5. These data have helped to clarify the nature and relative importance of the ionic interactions between hapten and antibody.

^{31}P nuclear magnetic resonance (NMR) techniques were used to probe the influence of pH on the microenvironment of the phosphate group of several haptens when these were bound to M603, W3207, T15, M167 and M511. The phosphate subsites of M603, W3207 and T15 are electropositive and also show other similarities; those of M167 and M511 have more electronegative character than PC experiences in solution. The two hydrogen bonds known to be formed between M603 and the phosphate oxygens of PC are also involved in binding GPC and are essentially unaffected by pH in the region 3-9. Studies with the hapten 3-trimethyl-

amino-1-propanolphosphate show that the binding cavity of M167 is substantially wider than those of M603, W3207 and T15.

These results lead to a detailed, molecular model of the pH dependent binding of PC and related haptens to these antibodies; they further indicate the roles of various amino acid residues in defining the differing ligand specificities of these antibodies.

Chapter 5 ABSTRACT

The phosphorylcholine (PC) affinities of several hybrid immunoglobulins, formed from recombination of light and heavy chains from parent molecules with PC specificity, have been determined. The results indicate a highly specific light-heavy chain interaction in these antibodies since high PC binding affinities are obtained only with autologous recombinants or with heterologous recombinants in which both parent molecules have a highly similar light chain. It is proposed that the heavy chain residues of these immunoglobulins determine both the primary PC specificity and the differing fine specificities; the important role of the different light chains is then to stabilize unique heavy chain conformations.

Chapter 6 ABSTRACT

The domain structure of antibodies, as well as the localization of various effector functions to specific antibody regions, are discussed. Evidence, from the literature, for the induction of conformational changes in the antibody molecule as a result of antigen binding is presented and the possible nature of such changes is discussed.

Chapter 7 ABSTRACT

The six interchain disulfide bonds of TEPC 15 were trifluoroacetonylated and ^{19}F NMR studies used to probe the magnetic environments of the trifluoroacetonyl reporter groups. The use of various enzymatic fragments of the intact immunoglobulin enabled the observed fluorine signals to be partially assigned to the known locations of the disulfide bonds on the antibody molecule and the distinct chemical environments seen for the ^{19}F labels are in good agreement with the known 3-dimensional structures of antibodies. No change in environment of the trifluoroacetonyl groups was observed upon binding of TEPC 15 to phosphorylcholine or to phosphorylcholine conjugated to a protein carrier. Similar studies with trifluoroacetonylated MOPC 315 binding to 2,4-dinitrophenyl-sensitized sheep red blood cells likewise resulted in no observable changes in the ^{19}F NMR spectrum.

ABSTRACTS OF THE PROPOSITIONS

Proposition I

A chemical cross-linking experiment designed to examine the structure of the membrane attack complex of complement is proposed.

Proposition II

A study of the functional relationship between the mast cell receptor for IgE and a membrane serine esterase is proposed.

Proposition III

An NMR study of the binding of phosphoryl-choline and related substances to C-reactive protein is proposed.

Proposition IV

A study of the mode of action of the sweet suppressor from Gymnema sylvestre is proposed.

Proposition V

A study designed to investigate the possible significance of a specific rat liver target protein for azocarcinogens is proposed.

PART I

STRUCTURE-FUNCTION RELATIONSHIPS IN PHOSPHORYLCHOLINE-BINDING MOUSE MYELOMA ANTIBODIES

Chapter 1

BACKGROUND

General Antibody Structure

Antibodies are immunoglobulins with known antigen specificity. They are large (150,000-950,000 daltons) glycoproteins constructed on a common framework. The basic unit of all immunoglobulins consists of four polypeptide chains; two light chains (25,000 daltons) and two heavy chains (50,000-70,000 daltons) symmetrically arranged so as to form two antigen-binding sites (Lennox and Cohn, 1967; Edelman and Gall, 1969). These chains are held together primarily by non-covalent forces but a network of disulfide bonds linking them also usually exists. Higher polymers of this basic unit (up to a pentamer) may exist and result from disulfide bonding between monomers and a small accessory protein termed J chain (Frangione and Milstein, 1969). Immunoglobulins may be divided into different classes on the basis of the C-terminal sequence of the heavy chain they contain. Thus, proteins containing γ, μ, α, ε and δ heavy chains comprise the IgG, IgM, IgA, IgE and IgD immunoglobulin classes. Additional classification into subclass has also been made. Two types of light chain (κ and λ) occur throughout the various immunoglobulin classes but in char-

acteristically different ratios within different species. Molecules of different immunoglobulin classes carry out different biological functions and are often characterized by different degrees of polymerization (Natvig and Kunkel, 1973).

All immunoglobulin chains contain internal homology units which consist of a sequence of ∿ 110 amino acids; there are two such homology units in the light chain and four or five in the heavy chain. The sequence of the N-terminal homology unit in each light and heavy chain is highly variable and forms the molecular basis for the specificity of a particular antibody. The sequence of the remainder of the chain is constant within each immunoglobulin class (Smith et al., 1971). There is a large degree of sequence conservation between homology units; in fact present-day antibody sequences are now believed to have arisen by repetitive gene duplication of an ancestral gene coding for one homology unit (Hill et al., 1966; Singer and Doolittle, 1966). The homology between such units extends to the level of 3-dimensional structure; recent X-ray evidence has shown each homology unit to contain a highly conserved "immunoglobulin fold", the main features of which are a 3-stranded and a 4-stranded β-pleated sheet

which run roughly parallel and are fixed with respect to each other by an absolutely conserved disulfide bond (Davies et al., 1975). Two adjacent homology units from different polypeptide chains interact to form a compact globular region termed a domain. X-ray studies have shown that the antibody molecule is Y-shaped and consists of a series of these globular domains connected by stretches of extended chain structure (Sarma et al., 1971; Huber et al., 1976; Silverton et al., 1977). A further discussion of antibody structure is presented in Chapter 6.

The Antibody Binding Site

Antigen binding activity is confined exclusively to the variable domain which is comprised of the N-terminal homology units of the light and heavy chains. Each monomeric antibody therefore is bivalent with respect to antigen binding. X-ray crystallographic studies of the Fab' fragments of a human IgG1 (Amzel et al., 1974; Poljak et al., 1973; Poljak et al., 1974) and a mouse IgA (Padlan et al., 1973; Segal et al., 1974), both with known hapten binding specificity, have played a crucial role in advancing our understanding of the antibody binding site and allow some general

observations to be made. The hapten binding site is located at the extreme tip of the Fab "arm" and consists of a more or less well-defined cleft between the heavy and light chains. This cleft is lined exlusively by hypervariable residues. The size and shape of the binding cleft appears to be strongly dependent on the sequence and especially the length of the individual hypervariable loops and it is readily apparent how changes in amino acid sequence in the hypervariable regions might give rise to different binding specificities (Padlan, 1977). Furthermore, the region lined by hypervariable region residues is sufficiently large so as to allow the potential binding of many small ligands of dissimilar structures. Thus, the tremendous diversity of possible antibody specificities may arise in part from the multiple antigen specificities that a single combining site is capable of creating (Richards et al., 1975). This model also allows the variable participation of the individual hypervariable loops in forming the antigen contacting surface.

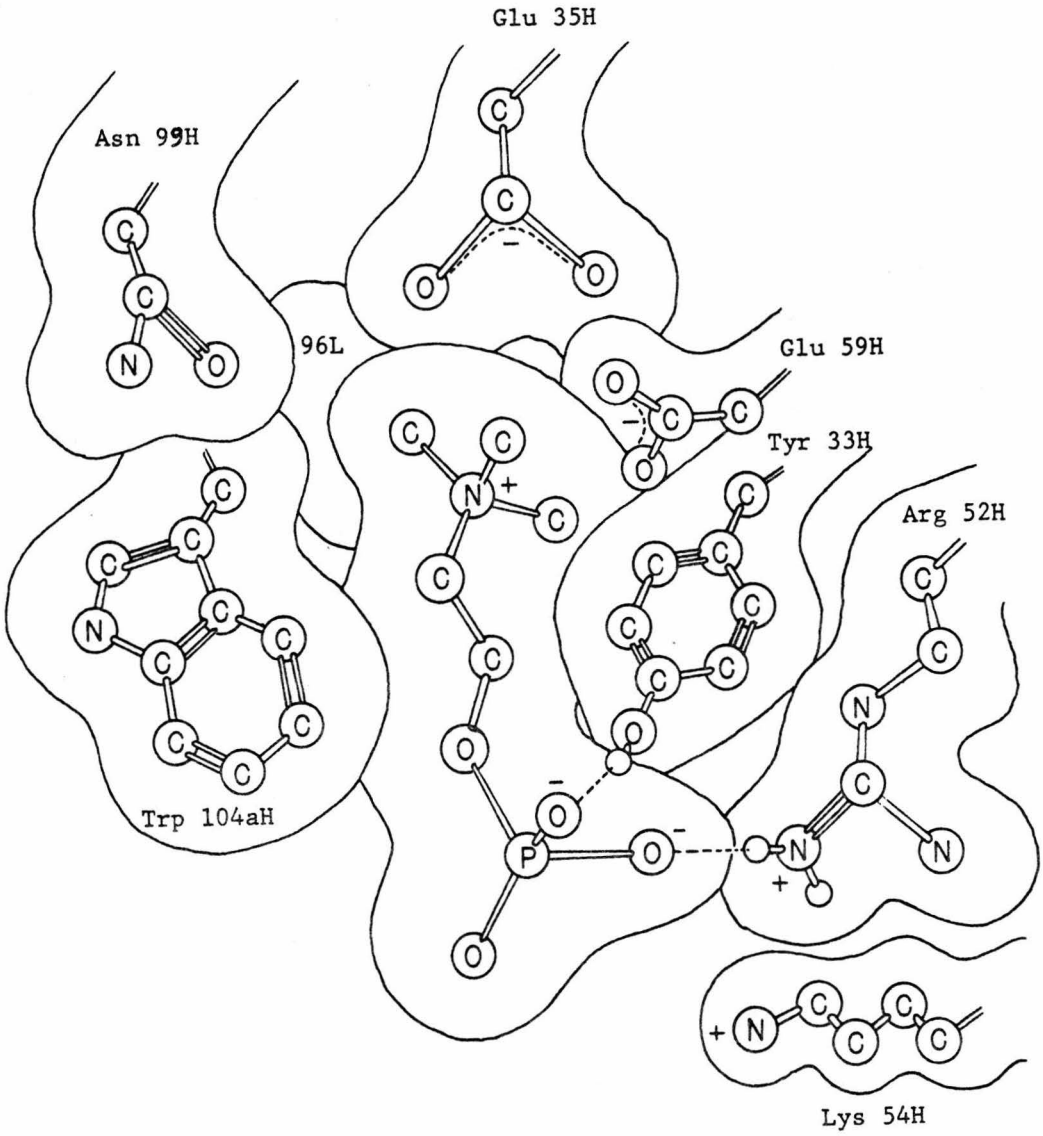
The binding site of IgG New, a human myeloma protein discovered to have a high binding affinity for a γ-hydroxy derivative of vitamin K_1, consists of a shallow depression 6 Å deep and of approximate area 16 x 7 Å (Amzel et al., 1974). Residues from both light and heavy chains contribute to the hapten-contacting surface and, as expected, the binding interaction with this non-polar hapten is predominantly of a hydrophobic nature.

The binding site of the mouse myeloma IgA McPC 603 has been described as a "wedge-shaped cavity" of dimension 12 x 15 x 20 Å (Segal et al., 1974). The differences between proteins McPC 603 and New serve to illustrate how greatly the shape of the combining site can be varied by changes in hypervariable region sequences, even though the binding sites of these proteins occur in identical regions of their respective Fab fragments. McPC 603 binds phosphorylcholine with high affinity and the crystallographic data show that the hapten binds so that the choline portion intrudes into the cavity and the phosphate group binds towards the exterior. In addition, the hapten binds asymmetrically to the walls of the binding cleft such that heavy chain residues contribute the majority of the

binding contacts (Padlan et al., 1976). In fact, only a single light chain residue (96L) appears to contact the phosphorylcholine molecule. Earlier chemical modification experiments had argued for the importance of complementary ionic interactions for this binding interaction (Grossberg et al., 1974) and the X-ray structure confirms this. The hapten quaternary nitrogen is stabilized by the acidic side chains of Glu 35H and Glu 59H whereas the negative phosphate group is stabilized by the ionic influence of Arg 52H and possibly Lys 54H. In addition, there exist hydrogen bonds to the phosphate oxygens from Tyr 33H and Arg 52H and the choline moiety is in extensive Van der Waal's contact with other side chain residues, notably Tyr 33H and Trp 104aH (Padlan et al., 1976). The design of the binding pocket appears to allow the accommodation of the phosphorylcholine determinant even when, as a phosphate diester, it occurs as part of a larger antigenic structure. On the other hand, it seems unlikely that significant structural changes in the choline portion of the hapten can be tolerated because of the close complementary fit of the antibody to this portion of the hapten. The important binding interactions between McPC 603 and phosphorylcholine are shown in Figure 1.

Figure 1

Model of the binding of phosphorylcholine to the combining site of McPC 603. The figure is taken from Capra and Edmundson (1977).


Glu 35H
Asn 99H
96L
Glu 59H
Tyr 33H
Arg 52H
Trp 104aH
Lys 54H

Light-Heavy Chain Interactions

The polypeptide chains of immunoglobulins are held together by disulfide bonds and non-covalent forces (Edelman and Poulik, 1961; Fleischman et al., 1962). After reduction of these interchain disulfide bonds, separation of the light and heavy chains can be achieved by gel filtration in a denaturing solvent. Commonly, 1 M solutions of organic acids (eg. propionic) (Fleischman et al., 1962) or concentrated urea or guanidine-HCl solutions or combinations of these are used to achieve dissolution of quaternary structure (Franek et al., 1965; Marler et al., 1964; Utsumi and Karush, 1964). The stability of the separated chains in neutral buffers is variable. When after chain separation, light chains are dialized against neutral buffers, they stay in solution whereas heavy chains, under the same conditions, generally aggregate and precipitate. Heavy chains can sometimes be induced to remain soluble by maintaining the pH below 5.5 (Stevenson and Dorrington, 1970). Physical studies of the isolated chains show that they retain structures which are ordered yet different from those in the intact immunoglobulin (Björk and Tanford, 1970a, 1970b).

Renaturation of the dissociated immunoglobulin can be effected by combining the separated chains in the

denaturing solution, dializing against water to dilute the denaturant, and subsequent dialysis against neutral buffer (Porter and Weir, 1966). This renaturation can be achieved both under conditions where the interchain disulfides are allowed to reoxidize or where they have been alkylated prior to renaturation.

The antigen binding capabilities of the isolated chains from many immunoglobulin systems have been studied with a view to ascertaining the role that these chains play in creating the binding site. In general, the isolated chains display little or none of the binding activity of the parent molecule (Painter et al., 1972; Forre et al., 1976). This is almost universally true for light chains (Edelman et al., 1963; Fleischman et al., 1963; Hong and Nisonoff, 1966) although a few cases of low affinity binding in these chains have been reported (Yoo et al., 1967; Painter et al., 1972). Substantial binding activity in isolated heavy chains seems to be more prevelant. For example, the heavy and light chains of horse antidiphtheria toxoid antibodies retained 20 and 5% respectively of the binding capacity of the parent antibody (Porter and Weir, 1966) and similar results have been reported for anti-DNP antibodies (Franek et al., 1965). It is clear, however,

that in the large majority of cases studied neither chain is able to bind antigen (hapten) to a significant extent without the presence of its complementary chain.

A more fruitful approach to the study of the role of individual chains has been the study of hybrid antibodies composed of light and heavy chains from different parents. Many studies have shown that the four chain normal immunoglobulin structure is readily regained upon proper renaturation of light and heavy chains from different antibodies of the same (Edelman et al., 1963; Metzger and Mannik, 1964; Hong and Nisonoff, 1966) and even different (Hoessli et al., 1974) species. However specific binding activity is generally regained only with autologous recombinants and this shows the requirements for formation of a functional binding site with the original specificity to be much stricter than those governing renaturation of the normal immunoglobulin structure. Heterologous recombinants of monoclonal myeloma immunoglobulins of similar specificity have been shown to exhibit none of the original binding affinity in the cases of phosphorylcholine (Sher et al., 1971) and DNP-binding (Bridges and Little, 1971) proteins although in a study of galactan-binding myelomas all hetero-

logous recombinants showed high galactan affinity (Manjula et al., 1976). In another study, significant DNP binding activity was obtained in heterologous hybrids formed from DNP-binding antibodies of two different species (Hoessli et al., 1974). However such results are clearly exceptions and one can draw the general conclusion that in the vast majority of cases a particular antigen specificity is dependent on the precise interaction of the parent immunoglobulin chains.

The Nature of Antibody-Antigen Interactions

Before the recent availability of several high resolution X-ray structures of antibody binding sites, considerable effort, utilizing a variety of techniques, was devoted to obtaining insight into the binding site structure and the nature of antibody-antigen interactions. Early studies were concerned with such questions as the degree of antibody specificity, the size of the combining site, and the nature of the amino acids in the binding region. These, as well as some more recent studies, will be briefly reviewed here.

Early studies of Nisonoff and Pressman (1957) had established the extreme specificity of the immune response to the phenylazobenzoate group. These authors

measured the ability of various structurally related compounds to inhibit the precipitin reaction between phenylazobenzoate coupled to a protein carrier and specific rabbit antiserum. Both the negative charge of the carboxylate group and the benzene ring of benzoate were found essential for interaction with antibody. Furthermore, compounds containing both the benzene ring and another negatively charged group (eg. benzene sulfonate, benzene phosphate) were completely ineffective in inhibiting precipitation. Similar studies have been carried out in exhaustive detail on several antibody systems, most notably those with specificity for DNP groups (Eisen and Siskind, 1964). From these sorts of studies several general conclusions have emerged: (i) On immunization with a hapten-protein conjugate, the region farthest from the protein carrier is immunodominant, ie. forms the energetically strongest interaction with the antibody (Schechter, 1970). This is equivalent to saying that the portion of a hapten (antigen) protruding deepest into the binding cavity is bound with the highest degree of specificity. (ii) Antibodies have resolving powers similar to enzymes; errors of as little as 1 Å in hapten dimension can lead to total loss of binding. (iii) The affinity of an antiserum

is directly related to its specificity. (iv) Hydrophobic interactions, coulombic attractions, hydrogen bonds, charge-transfer bonds and Van der Waal's interactions all can contribute to varying degrees in creating a particular antigen specificity (Karush, 1962). (v) It has become a well established principle that antibodies to negatively charged groups usually contain positive complementary residues in their binding sites and vice versa for positively charged haptens (Grossberg and Pressman, 1968; Freedman et al., 1968; Pressman and Siegel, 1953; Grossberg and Pressman, 1960).

Various techniques have been employed in an effort to identify specific binding site residues in immunoglobulins. Affinity labelling was one of the first to implicate hypervariable region residues as forming the antigen binding site (Wofsy et al., 1966; Singer et al., 1967). Residues from both light and heavy chains are labelled in various antibody systems although the heavy chain often contains the majority of the label (Singer et al., 1971). If radioactive reagent is employed, the particular residue(s) labelled can often be identified and this has been used to show that reaction frequently occurs with only a limited number of residues, reflecting, presumably, the highly

specific manner with which the affinity label is initially bound. Large differences in the labelling patterns have been observed between highly similar phosphorylcholine-binding mouse immunoglobulins, showing the great discriminatory powers of the technique (Metzger et al., 1971; Chesebro et al., 1973). An interesting application has been to employ bifunctional reagent to cross-link appropriately-positioned reactive groups. Using this approach, it was possible to cross-link the light and heavy chains of MOPC 315 and to first establish the participation of both chains in the active site (Weinstein et al., 1969). Non-site directed chemical modification may also yield useful information. Highly specific reagents which abolish binding activity were used to infer the presence of arginyl and carboxylate groups in the binding site of the phosphorylcholine-binding protein HOPC 8 (Grossberg et al., 1968). This was later confirmed by X-ray crystallography (Segal et al., 1974). Physical techniques such as solvent perturbation spectroscopy have been used to purportedly show the presence of certain types of aromatic amino acids in the binding site (Callahan et al., 1974) but the information obtained is too indirect to be of much value.

Another approach, especially in view of the interest in the possibility of multispecific binding sites (Richards et al., 1975), has been to attempt to estimate the size of the combining region for large, usually repeating, antigens by measuring the ability of haptens of increasing size to inhibit precipitation. From such studies, it has variously been concluded the the combining site is large enough to accommodate five or six linked carbohydrate residues (Kabat, 1960; Lundblad et al., 1972) or a tri or tetrapeptide (Schechter et al., 1970a, 1970b). This size corresponds approximately to that measured by e.s.r. In this case, Hsia and Piette (1969) examined anti-DNP antibodies using haptens containing a nitroxide radical and DNP ring separated by a spacer group of varying length. The mobility of the spin label, determined from the e.s.r. spectrum, was used to prove the binding site depth. The obtained value of 10-12 Å also agrees well with the value of 12-13 Å estimated by Valentine and Green (1967) from electron microscope studies of anti-DNP antibodies. The summation of these results leads to the conclusion that the very small haptens often determined to bind to monoclonal antibodies do not represent the entire physiological antigenic determinant against which these antibodies are capable of reacting.

Kinetic studies have also played an important part in the characterization of the binding mechanism. Such studies have generally been carried out on hapten-antibody systems using stopped-flow or chemical relaxation methods. In the large majority of cases only a single association rate constant with a typical value of 10^7-10^8 M^{-1} sec^{-1} has been obtained (Froese and Sehon, 1975; Pecht and Lancet, 1976). These numbers approach the theoretically limiting value of 1-2 x 10^9 M^{-1} sec^{-1} (Froese and Sehon, 1965) and strongly suggest that the antibody-hapten encounter is essentially diffusion controlled. However, even for simple antibody-hapten associations, forward rate constants more than three orders of magnitude lower than this limiting value have been observed (Pecht, 1974). Within a given antibody system, the differing binding affinities of various haptens are the results of differences in the dissociation rate constant, as the association rate constants are generally observed to be quite similar (Smith and Skubitz, 1975; Haselkorn et al., 1974). These studies therefore support the notion that variations in the strength of antibody-hapten interactions are principally determined by differences in the activiation energies for dissociation of the complex.

Determination of thermodynamic parameters yields little useful information. Most antibody-hapten systems have both the enthalpy and entropy terms favoring the association (Karush, 1962; Singer, 1965) although examples are known where an unfavorable entropy term dictates that the entire binding reaction be driven by a favorable enthalpy change (Karush, 1957). Attempts have been made to relate changes in ΔH and ΔS on binding to specific types of noncovalent interactions (Karush, 1962), and it appears possible that apolar haptens generally have more favorable entropy terms than polar haptens, but the lack of definitive antibody structural data in these cases generally does not allow meaningful generalizations to be made. It is interesting that although no difference in affinity is usually observed for hapten binding to various sized antibody fragments, a report by Merz et al. (1974) claims that the Fab' and Fv fragments of MOPC 315 bind DNP-lysine with increasingly higher affinity than the intact antibody. An analysis of the experiment suggests that these differences are most likely the result of increasing steric accessibility of hapten to the binding sites of the progressively smaller fragments.

Nuclear magnetic resonance (NMR) studies of several antibody-hapten systems have been carried out. Burgen et al. (1967) examined the binding of tetramethylammonium hapten to rabbit antibodies and concluded from proton relaxation data that the energy barrier to hapten methyl group rotation was directly related to the affinity of the antiserum. Several other studies using antibodies against the haptens o-carboxymethyl-4-methylumbelliferone (Harina et al., 1977) and 2,4-dinitro-4'-(chloromercuri)-diphenylamine (Haugland et al., 1967) have yielded structural information about the binding site. However, all the above studies suffer from the disadvantage of using heterogeneous antibody.

A recent application of NMR has been to attempt to solve the binding site structure of a monoclonal antibody in solution. Dwek and coworkers (Dwek et al., 1975; Dwek et al., 1977; Dwek, 1977) have studied in detail MOPC 315, a mouse myeloma protein specific for 2,4-dinitrophenyl haptens. Their approach has been to employ a wide variety of magnetic resonance techniques (natural abundance proton NMR, ^{31}P NMR, esr, paramagnetic difference spectra etc.) to refine a predicted structure of the protein based on model building studies (Padlan et al., 1976). A final assessment of the accuracy of this

method must await the crystallographic structure determination of MOPC 315. A somewhat different approach to the study of the same protein was taken by Kooistra and Richards (1978). These authors used various trifluoromethyl analogs of dinitrophenyl haptens to probe the antibody binding site by ^{19}F NMR and were able to map certain structural features of the combining site as well as the kinetic constants for several haptens binding to various-sized antibody fragments.

Some further solution studies of antibody-antigen interactions, especially as they relate to possible antibody conformational changes, are described in Chapter 6.

Phosphorylcholine-Binding Mouse Myeloma Immunoglobulins

Plasmacytomas have been extremely helpful for studying individual components of the normally heterogeneous immune response. These tumors appear to be malignant proliferation products of a single plasma cell and can be induced in high frequency by injection of mineral oil into the peritoneal cavity of BALB/c mice. The mechanism of plasmacytoma induction is poorly understood but seems to be dependent on the creation of an abnormal peritoneal environment (substances other than

mineral oil can also be used) and the unique genetic susceptibility of BALB/c mice (Potter, 1972). The tumors grow to a large size in the solid form and can usually be maintained indefinitely by serial transplantation into syngeneic mice (Potter and Fahey, 1960). The tumor cells secrete monoclonal myeloma immunoglobulin which is readily isolated from the serum.

Myeloma proteins with antigen binding activity are usually discovered by screening procedures which monitor the ability of the sera to bind radioactive ligands or to precipitate large antigens. Approximately five percent of mouse myeloma proteins exhibit a known antigen affinity. Curiously, most of these proteins are observed to bind either phosphorylcholine, 2,4-dinitrophenyl derivatives or polysaccharides (Potter, 1972). The phosphorylcholine-binding group, of which 11 have now been described (Potter and Lieberman, 1970; Sher et al., 1971), was first observed to precipitate the pneumococcal C polysaccharide; subsequently Leon and Young (1971) demonstrated that the immunodominant group of this antigen is phosphorylcholine. Most mouse myeloma proteins are of the IgA class, which is the major antibody class present in fluids that bathe the mucous membranes of the body. In addition, large numbers of

IgA-containing plasma cells are found associated with the gastro-intestinal and respiratory systems (Tomasi and Grey, 1972). This has led to the rationalization that the high frequency with which certain specificities of IgA myelomas are observed is the result of clonal expansion in the mouse gut due to antigenic stimulation prior to neoplastic transformation. Presumably therefore, the haptens which these immunoglobulins are observed to bind are constituents of, or at least cross react with, the normal bacterial flora in the gut of these mice (Lennox and Cohn, 1967; Potter, 1971).

It is now universally accepted that myeloma proteins with known antigen specificity represent a subset of the normal antibody repertoire of the particular animal and that therefore these proteins represent a conveniently accessible source of monoclonal antibodies for study. Direct evidence for this assertion derives from the observation that, in the case of phosphorylcholine-binding immunoglobulins for example, immunization of BALB/c mice with phosphorylcholine conjugated to a protein carrier or with R36A polysaccharide results in an antibody response largely restricted to the TEPC 15 idiotype (Claflin and Davie, 1974; Cosenza and Köhler, 1972). Thus, the TEPC 15 idiotype represents

the "normal" immune response in these mice to phosphorylcholine-containing antigens. By all measurable criteria the binding of phosphorylcholine and pneumococcal polysaccharide by the mouse myeloma proteins parallels the interactions of haptens and antigens with conventional antibodies.

The in vivo antigens for the phosphorylcholine-binding immunoglobulins are not known but one possibility has been suggested to be (Glaudemans et al., 1977) the species-specific C-techoic acid occurring in the cell walls of all Streptoccocci pneumoniae (Brundish and Baddiley, 1967; Watson and Baddiley, 1974). This antigen is apparently a complex polysaccharide containing phosphorylcholine attached to the 3' position of N-acetylgalactosamine residues via a phosphodiester bond.

Specific properties of the phosphorylcholine-binding myeloma proteins will be discussed in later chapters and only a brief, general outline is given here. Eleven independently induced plasma cell tumors have now been discovered to precipitate the pneumococcus C polysaccharide of which phosphorylcholine is a constituent (Cohn, 1967; Cohn et al., 1969; Potter and Leon, 1968; Potter, 1972). Of these 11, only five are idiotypically distinct (TEPC 15, McPC 603, W3207, MOPC 167 and MOPC 511), the remainder being idiotypically

identical to TEPC 15. The five distinct proteins, while showing moderately high phsophorylcholine affinities (Metzger et al., 1971; Pollet and Edelhoch, 1973; Chesebro and Metzger, 1972) differ amongst each other with respect to binding of phosphorylcholine analogs (Leon and Young, 1971), affinity labelling patterns (Metzger et al., 1971; Chesebro et al., 1973) and changes in various optical parameters upon hapten binding (Morris et al., 1974; Pollet et al., 1974). Immunization with phosphorylcholine results in a predominantly TEPC 15 response in BALB/c mice, whereas immunization of other mouse strains results also in the production of antibodies similar to various of the other idiotypes (Claflin, 1976). This brings to mind such questions as the nature of the functional differences among these immunoglobulins, how these are related to amino acid sequence variations, and the evolutionary advantage of being able to generate different antibodies of apparently similar specificity.

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Chapter 2

MAGNETIC RESONANCE STUDIES OF THE BINDING SITE INTERACTION BETWEEN PHOSPHORYLCHOLINE AND MOUSE MYELOMA IMMUNOGLOBULIN M603

INTRODUCTION

Antibodies, the primary molecules of the immune response, are responsible for the specific recognition of antigen and also for other, biologically important, effector functions which include initiation of the complement cascade, release of histamine from mast cells, and activation of the differentiation of B lymphocytes into antibody-producing plasma cells (Metzger, 1974). X-ray crystallographic techniques have recently revealed the three-dimensional structures of some antibodies and their Fab fragments (Davies et al., 1975; Poljak, 1975) and correlation of antibody structure with various biological functions has attracted interest (Yasmeen et al., 1976; Hurst et al., 1974). A precise knowledge of the molecular details (both structural and dynamic) of the interactions between antigen and antibody is central to a thorough understanding of antibody specificity and the relationship between the structure of an antibody and its function. Various physical techniques have been employed to elucidate such information including circular dichroism (Rockey et al., 1972), chemical modification (Grossberg et al., 1974), and magnetic resonance (Dwek et al., 1976). We have initiated a systematic study of the correlation between antibody

structure and hapten binding properties of a group of myeloma proteins which have specificity for phosphorylcholine and related substances (Potter, 1972). The study of these antibodies offers several advantages: (i) the immunoglobulins can easily be obtained as homogeneous proteins in relatively large (gram) quantities; (ii) extensive amino acid sequence data are available for many of them (Barstad et al., 1974; Hood et al., 1975); (iii) the three-dimensional structure of the Fab' fragment of a typical member of this group, M603, has recently been reported (Segal et al., 1974). This knowledge allows the results of binding experiments to be interpreted in terms of known and inferrable molecular structures.

Magnetic resonance affords a physical technique for studying hapten-antibody interactions which is particularly well suited to phosphorylcholine as one can observe events occurring at either end of the hapten using the signals from ^{31}P (natural abundance) in the phosphoryl group and ^{13}C (enriched) in the trimethylammonium group without significant interference from protein signals. We report here the results of such a study of the interaction between phosphorylcholine and M603. The main goals of this work are (i) to characterize the specific spectral

changes (chemical shift and line width) of the ^{13}C and ^{31}P hapten resonances caused by binding to protein M603; (ii) to rationalize these observations in terms of known binding-site interactions including the dynamics of this hapten-antibody interaction. We hope this particular study can serve as a basis for subsequent studies of the interactions between phosphorylcholine and other phosphrylcholine binding myeloma proteins of somewhat different structure and specificity.

Exchange Rate Theory

The nature of the NMR spectrum observed for a system in which a nucleus is exchanging between two magnetically nonequivalent environments is determined by the ratio of the chemical shift difference between the two environments and the mean lifetime in the two environments (Pople et al., 1959).

(i) The "slow exchange" situation arises when

$$\tau > \sqrt{2}/(2\pi\Delta) \qquad (1)$$

In the case of antibody-hapten association where a nucleus on the hapten is being observed, Δ is the chemical shift difference (in Hz) between the free (F) and antibody-bound (B) states and τ is the mean

lifetime of the hapten in the two environments:

$$\tau = \frac{\tau_F \cdot \tau_B}{\tau_F + \tau_B} \tag{2}$$

A separate signal is seen for the hapten in each of the two environments and the observed linewidth of each signal is related in a simple manner to the intrinsic linewidth and the lifetime in that environment:

$$\pi \Delta\nu_{\frac{1}{2}} = \frac{1}{T_{2_{obs}}} = \frac{1}{T_2} + \frac{1}{\tau} \tag{3}$$

For the antibody-hapten complex, this may be expressed as (Hull et al., 1976)

$$(\pi \Delta\nu_{\frac{1}{2}})_B = \frac{1}{T_{2_B}} + \frac{1}{\tau_B} = \frac{1}{T_{2_B}} + k_{off} \tag{4}$$

and for the free hapten

$$(\pi \Delta\nu_{\frac{1}{2}})_F = \frac{1}{T_{2_F}} + \frac{1}{\tau_F} = \frac{1}{T_{2_F}} + k_{on}[A] \tag{5a}$$

$$= \frac{1}{T_{2_F}} + k_{off} \frac{[AH]}{[H]} \tag{5b}$$

T_{2_B} and T_{2_F} are the intrinsic transverse relaxation times (in the absence of exchange) of the bound and free ligand respectively, $\Delta\nu_{\frac{1}{2}}$ is the observed linewidth

and k_{off} and k_{on} are the dissociation and association rate constants of the antibody-hapten complex.

(ii) As τ decreases the two signals approach each other and finally coalesce at

$$\tau = \sqrt{2}/(2\pi\Delta) \tag{6}$$

This situation is termed "intermediate exchange" and is the most difficult to analyze for relaxation or exchange information.

(iii) "Fast exchange" arises when

$$\tau < \sqrt{2}/(2\pi\Delta) \tag{7}$$

A single absorption is observed which occurs at a chemical shift which is a weighted average of the two environments

$$\delta_{obs} = \rho_F\delta_F + \rho_B\delta_B \tag{8}$$

where δ represents chemical shift and ρ represents mole fraction of hapten. The observed linewidth is the weighted average of the intrinsinc linewidth of the free and bound hapten signals plus an additional term due to the exchange process:

$$(\pi\Delta\nu_{\frac{1}{2}})_{obs} = \frac{1}{T_{2_{obs}}} = \frac{\rho_F}{T_{2_F}} + \frac{\rho_B}{T_{2_B}} + \frac{4\pi^2\rho_F{}^2\rho_B\Delta^2}{k_{off}} \tag{9}$$

MATERIALS AND METHODS

Maintenance of McPC 603 Plasmacytoma

BALB/c mice containing the McPC 603 tumor were obtained from the Salk Institute, La Jolla, California and the McPC 603 tumor line was maintained by serial, subcutaneous transplantation of 1 mm^3 pieces of solid tumor into BALB/c mice. For production of large quantities of immunoglobulin, the tumor was converted to the ascites form in CDF_1 (BALB/c x DBA/2) mice (Cumberland Farms, Tennessee). This was accomplished by mincing the BALB/c solid tumors through a fine mesh metal screen and injecting the resultant cell suspension, diluted with a balanced salt solution, intraperitoneally into female CDF_1 mice. Ascites fluid could be collected from these mice 10-20 days after injection.

On occasion, mice from a variety of different vendors had to be used but no differences in tumor growth were observed.

Preparation of Phosphorylcholine-Sepharose Affinity Column

p-Diazonium phenylphosphorylcholine was synthe-

sized by a procedure similar to that of Bird (1967) and Chesebro and Metzger (1972) as briefly outlined below.

Choline iodide was prepared by the direct combination of methyl iodide and a 10-fold molar excess of dimethylaminoethanol in ether. The reaction mixture was stirred for 24 hours and the precipitated product was washed with ether and dried. The yield was quantitative. Choline iodide (10 mmoles), p-nitrophenylphosphorodichloridate (10 mmoles, Aldrich) and dried quinoline (10 mmoles) were dissolved in a total of 7.5 ml distilled and dried (barium oxide) acetonitrile and stirred in the dark for 5 hours at 0°C. Then 5 ml pyridine and 1 ml water were added and stirring continued for 30 minutes at room temperature. The solvent was removed by flash evaporation and the resultant yellow-green syrup dissolved in water and deionized by passage through an Amberlite MB-3 column. The product, p-nitrophenylphosphorylcholine was obtained from the eluate after lyophilization in 53% yield. It was stored in the dark at room temperature.

p-Nitrophenylphosphorylcholine (1.2 mmole) was

dissolved in 20 ml methanol and quantitatively reduced with H_2 using 80 mg of 5% palladium on charcoal catalyst. After removal of the charcoal by filtration, the solvent was removed by flash evaporation. The unstable amine was immediately dissolved in 8 ml cold 1 N HCl and diazotized with $NaNO_2$ at $\sim 15^{\circ}C$. A small excess of $NaNO_2$ (as monitored with starch-KI paper) was used and after completion of diazotization was destroyed with urea. The p-diazoniumphenylphosphorylcholine was used immediately.

The coupling procedure was based on the general method of Cuatrecasas (1970). To 200 ml carefully washed Sepharose 4B was added 200 ml H_2O and 40 g finely crushed CNBr. Alternatively, the CNBr was dissolved in a small volume of dioxane. The pH was kept at 10.5-11 by the addition of 8 N NaOH and the temperature was kept at $20^{\circ}C$. Activation of the gel, as monitored by cessation of the pH decrease, was complete after 20-30 minutes. The activated gel was washed rapidly with large amounts of ice-cold borate-buffered saline (pH 8). The washed gel was then stirred with 2 g glycyltyrosine in the same buffer for 24 hours at $4^{\circ}C$. After again washing the gel to

remove excess ligand, it was stirred in the presence of 0.6 mmole p-diazoniumphenylphosphorylcholine in borate-buffered saline for 10 hours at room temperature. The intensely orange-colored gel was then extensively washed with buffer. The product consists of phenylphosphorylcholine coupled, via a diazo linkage, to the aromatic ring of the spacer group Gly-Tyr. The gel was routinely stored in 1 N acetic acid to prevent base catalyzed hydrolysis and was equilibrated with buffer just prior to use. Such an affinity gel has remained stable for 4 years.

Purification of McPC 603 Protein

Ascites was obtained from tumor-swollen mice in typical yields of 3-5 ml per mouse per tapping. Each mouse could be tapped 3-6 times before expiring. Mice that had been primed with an intraperitoneal injection of mineral oil (0.5 ml) one month prior to tumor injection (Potter, 1972) gave higher yields of immunoglobulin and were therefore routinely used. From such mice approximately 2-6 mg immunoglobulin per ml ascites could be obtained, resulting in a total of 10-180 mg pure immunoglobulin obtainable from each

mouse. Ascites was either used fresh or stored frozen after centrifugation to remove cellular material.

The ascites was filtered through glass wool to remove gelatinous and oily material and to it was added a 10% volume of 2 M Tris, pH 8.6 buffer. The solution was reduced with 0.01 M dithiothreitol for 1 hour at room temperature, the pH lowered to 8 by the addition of an appropriate volume of 2 M Tris, pH 7.3 buffer, and then alkylated with a 10% excess (0.022 M) iodoacetamide for 1 hour at 0°C. The protein solution was then dialized against a buffer consisting of 0.02 M borate, 0.16 M NaCl, 1mM EDTA (BBS) to remove unreacted reagents and subsequently passed down the phosphorylcholine-Sepharose affinity column equilibrated with the same buffer. After the effluent had reached background absorbance at 280 nm, the McPC 603 protein was immunospecifically eluted with 10 mM phosphorylcholine in BBS. The protein solution was then typically concentrated and exhaustively dialyzed to remove bound hapten.

Fab' Fragments

McPC 603 Fab' fragments, for use in NMR experiments, were prepared as described for MOPC 315 (Inbar et al., 1971). To purified McPC 603 at a concentration of 10 mg/ml in a 0.05 M sodium acetate, 0.05 M NaCl, pH 4.7 buffer was added 1% (w/w) 2X recrystallized pepsin (Sigma, 4100 units/mg). The mixture was incubated for 6 hours at 37°C after which large amounts of precipitated protein (Fc peptides) were seen. On raising the pH this protein redissolved. The entire digestion mixture was then run down the phosphorylcholine affinity column equilibrated with BBS and protein retaining hapten binding activity was eluted with 1 mM phosphorylcholine. SDS polyacrylamide gels (Fairbanks et al., 1971) showed only 2 bands of apparent MW 25,000 and 34,000 attributed to the light chain and Fd fragment respectively. This protein therefore consisted of essentially pure Fab' fragment and was used without further purification.

Synthesis of Phosphoryl[methyl-^{13}C]choline

[methyl-^{13}C]Iodide (90.8 atom % ^{13}C) was obtained from Thompson Packard Inc. [methyl-^{13}C]-

Choline iodide was first prepared by the direct combination of dimethylaminoethanol and [methyl-^{13}C]iodide (Chesebro and Metzger, 1972).

The phosphorylation of the [methyl-^{13}C]choline iodide was similar to the procedure of Baer (1947, 1952). Carefully dried [methyl-^{13}C]choline (7 mmoles), diphenylchlorophosphate (8 mmoles), 10 ml of 5 mm glass beads and 10 ml dried pyridine were vigorously stirred in a closed vessel for 2 days at room temperature. The residue was dissolved in 20 ml H_2O, filtered, and the solvent removed under reduced pressure. The remaining syrup was dissolved in 50 ml H_2O and refluxed in the presence of 17 g $Ba(OH)_2 \cdot 8H_2O$ and 0.1 ml 1-octanol for 1¼ hours to hydrolize the product. The hot solution was neutralized with carbon dioxide and filtered. After extraction of the filtrate with 50 ml ether the water was removed <u>in vacuo</u> at a temperature of <50°C. The residue was dissolved in 15 ml H_2O, an equal volume of ethanol was added, and the resultant precipitate (containing most of the impurities) was discarded. The barium salt of phosphoryl[methyl-^{13}C]-choline iodide precipitated overnight after raising the ethanol concentration to 80%. The crystals were

washed with absolute ethanol and ether and then dried by heating under vacuum at 100° over P_2O_5. The yield was approximately 60%. The purity of the product was verified by ^1H and ^{13}C NMR spectroscopy and thin-layer chromatography (see Figures 2 and 3). It stained positive for phosphate esters (Dittmer and Lester, 1964).

Hapten Binding Assays

Phosphoryl[methyl-^{14}C]choline was obtained from New England Nuclear. Equilibrium dialysis was performed in Lucite cells with 2-ml compartments using reduced and alkylated monomeric IgA at concentrations of 1-2 mg/ml. Protein concentration was determined by absorbance at 280 nm using $\varepsilon_1^{0.1\%}_{cm} = 1.36$. This value was determined experimentally for TEPC 15 by direct absorbance measurements of weighed, lyophilized protein samples and is in good agreement with published values of 1.34 (Chesebro and Metzger, 1972) and 1.40 (Pollet and Edelhoch, 1973). A molecular weight of 150,000 per IgA monomer was used in calculations. The cells were then agitated for 24 hours at a constant temperature (either at 4°C (cold room) or at 30°C (constant temperature bath)) as the binding affinity is strongly

Figure 2

^{13}C NMR spectrum of synthetic phosphoryl[methyl-^{13}C]choline in borate-buffered saline (pH 8).

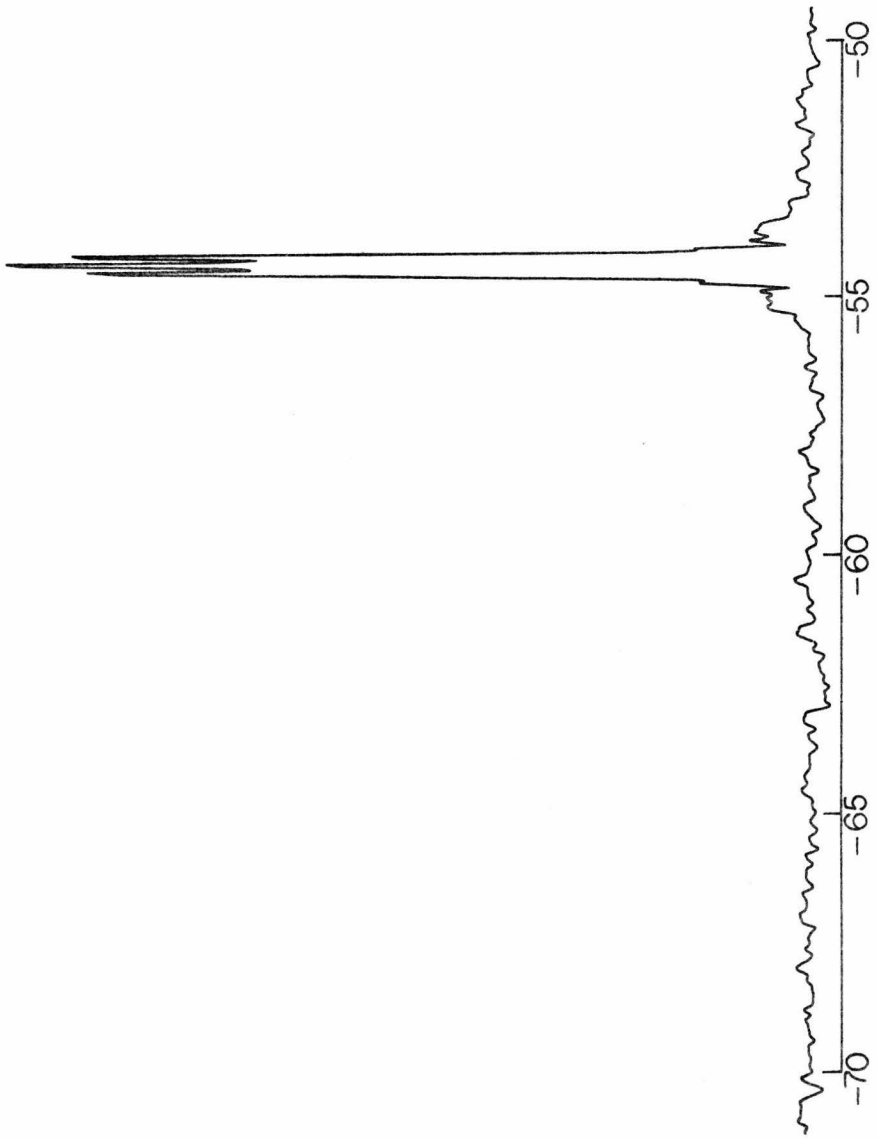
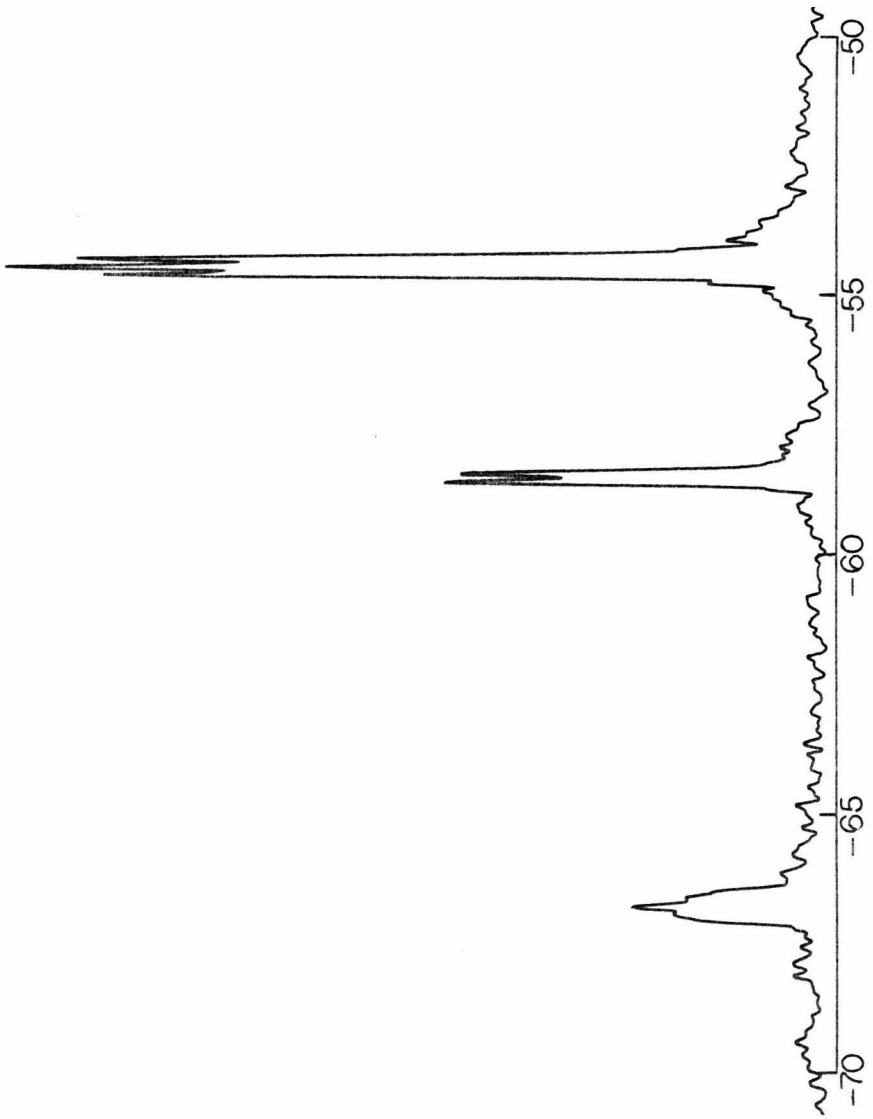

-50
-55
-60
-65
-70
ppm from tetramethylsilane

Figure 3

^{13}C NMR spectrum of commercial phosphoryl-choline (Sigma) in borate-buffered saline (pH 8).


-50
-55
-60
-65
-70
ppm from tetramethylsilane

temperature dependent. 1 ml solution was withdrawn from each side of each dialysis cell and dissolved in 10 ml Aquasol scintillation cocktail (New England Nuclear). Samples contained from 50-20,000 cpm and were counted for 20 minutes so that the counting error ranged from 5% to 0.3%.

The data were plotted as r/c vs. r where c is the unbound hapten concentration and r is the antibody-bound hapten concentration divided by the antibody concentration (Scatchard, 1949). A least-squares fit was used to obtain the best straight line through the data points.

NMR Sample Preparation

After affinity purification, protein solutions were exhaustively dialized to remove bound hapten. They were then concentrated to ultrafiltration in an Amicon cell using a PM-10 membrane. NMR samples consisted of 2-3 ml of 3-4 mM McPC 603 Fab' or reduced and alkylated 7S monomer prepared in this manner. Protein concentration was calculated using $\varepsilon_{1\ cm}^{0.1\%} = 1.36$ and a molecular weight of 55,000 for the Fab' fragments. Small aliquots of a concentrated (0.2 M) phosphorylcholine stock solution were added to the protein sample to achieve the desired hapten concentration. To prevent the formation of small

amounts of turbidity which were occasionally observed after 24 hours, sodium azide to 0.02% was added to NMR samples.

Protein samples used for NMR xperiments could be reused after repurification. The concentrated NMR samples were diluted with BBS, dialized to remove bound hapten, and repurified by affinity chromatography. They were then extensively dialyzed to remove bound phosphorylcholine and reconcentrated.

NMR Experiments

NMR spectra were obtained on a Varian XL-100-15 spectrometer interfaced with a Varian 620i computer and operating in the Fourier transform mode. Both ^{13}C spectra (obtained at 25.2 MHz) and ^{31}P spectra (40.5 MHz) were proton noise decoupled and obtained at the normal probe temperature of 30 ± 2°C. A deuterium field-frequency lock was provided by means of a 5 mm capillary insert containing D_2O which was placed inside the 12 mm sample tube. The data were accumulated using a 90° pulse and an acquisition time of 0.3-0.4 seconds. A sweep width of 1000 Hz was commonly used. Generally at least 100,000 transients were accumulated per spectrum.

RESULTS

Hapten Binding Constants

Scatchard plots of binding of phosphorylcholine to reduced, alkylated McPC 603 at two different temperatures are shown in Figures 4 and 5. The data are linear and extrapolate to 1.9 binding sites per IgA monomer. Mild reduction and alkylation of immunoglobulins has been previously demonstrated to have no effect on their binding properties (Sher and Tarikas, 1971). For McPC 603 and phosphorylcholine this technique gives association constants of 8.2×10^5 (at 4^oC) and 1.0×10^5 M^{-1} (at 30^oC). These constants are in good agreement with those reported previously (Rudikoff et al, 1972; Metzger et al, 1971; Potter, 1972) under somewhat ill-defined experimental conditions. For interpretation of the NMR results, however, we wished to have association constants obtained under conditions identical with respect to buffer composition and temperature to those employed in the NMR measurements. Although the protein concentrations were two orders of magnitude higher in the NMR experiments than in the equilibrium dialysis measurements, previous work has shown the hapten-antibody association constants

Figure 4

Scatchard plot of phosphorylcholine binding to McPC 603 at 4°C. Data were obtained by equilibrium dialysis in a 0.02 M borate, 0.16 M NaCl, 1 mM EDTA buffer.

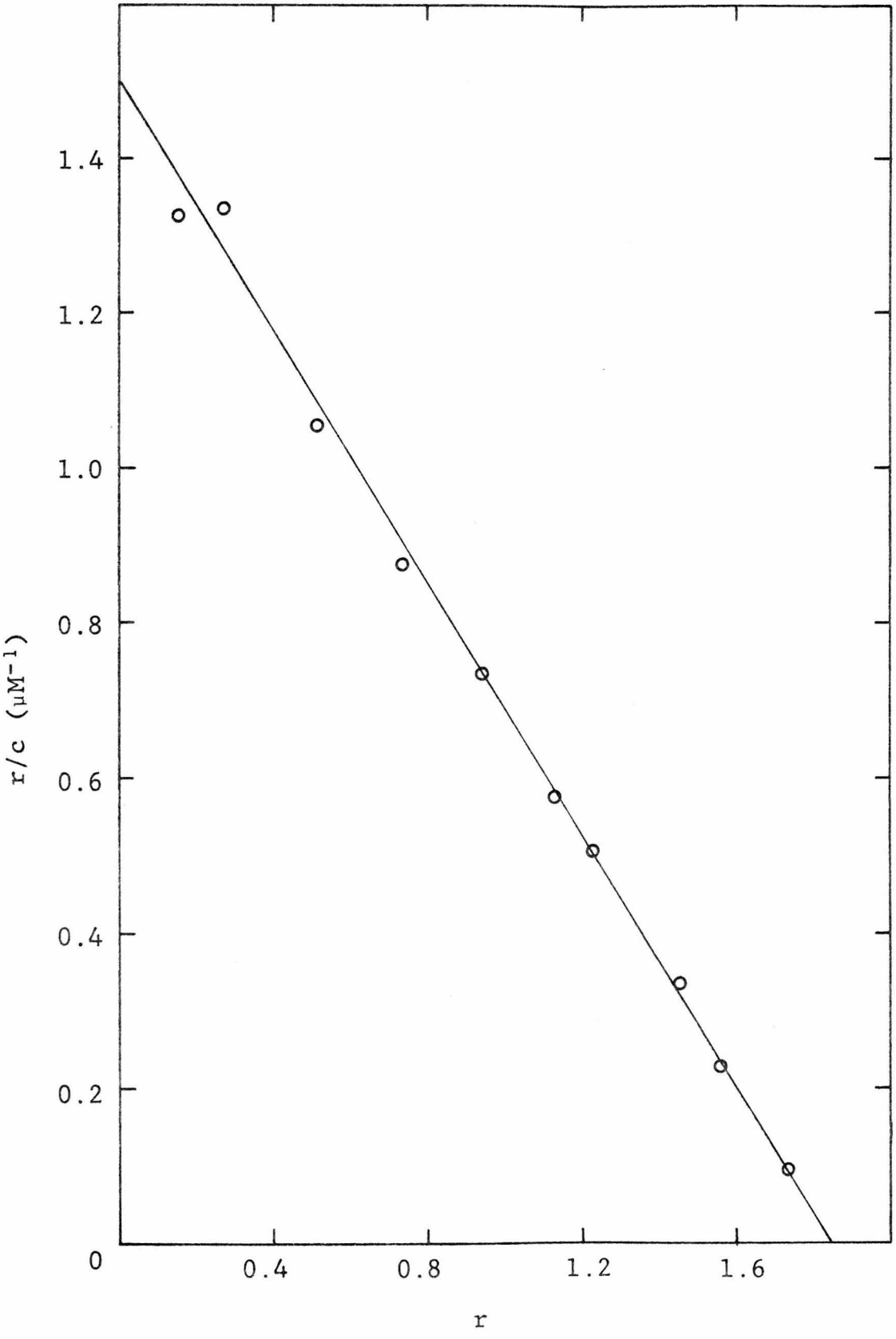
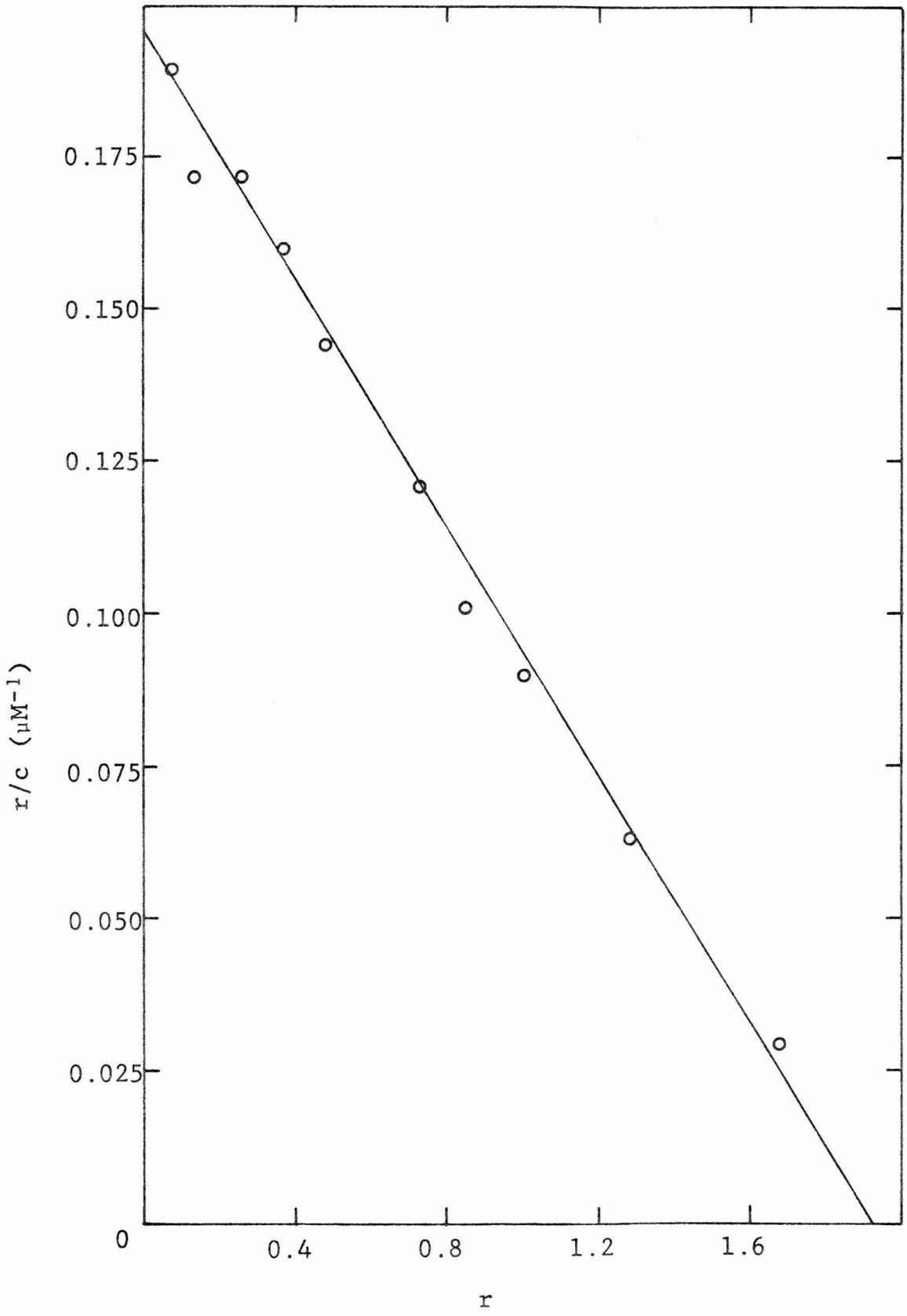

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
r/c (μM⁻¹)
0.4
0.8
1.2
1.6
r

Figure 5

Scatchard plot of phosphorylcholine binding to McPC 603 at 30°C.


0.175
0.150
0.125
0.100
0.075
0.050
0.025
0
r/c (μM⁻¹)
0.4
0.8
1.2
1.6
r

of phosphorylcholine-specific myeloma proteins to be independent of immunoglobulin concentration (Sher and Tarikas, 1971).

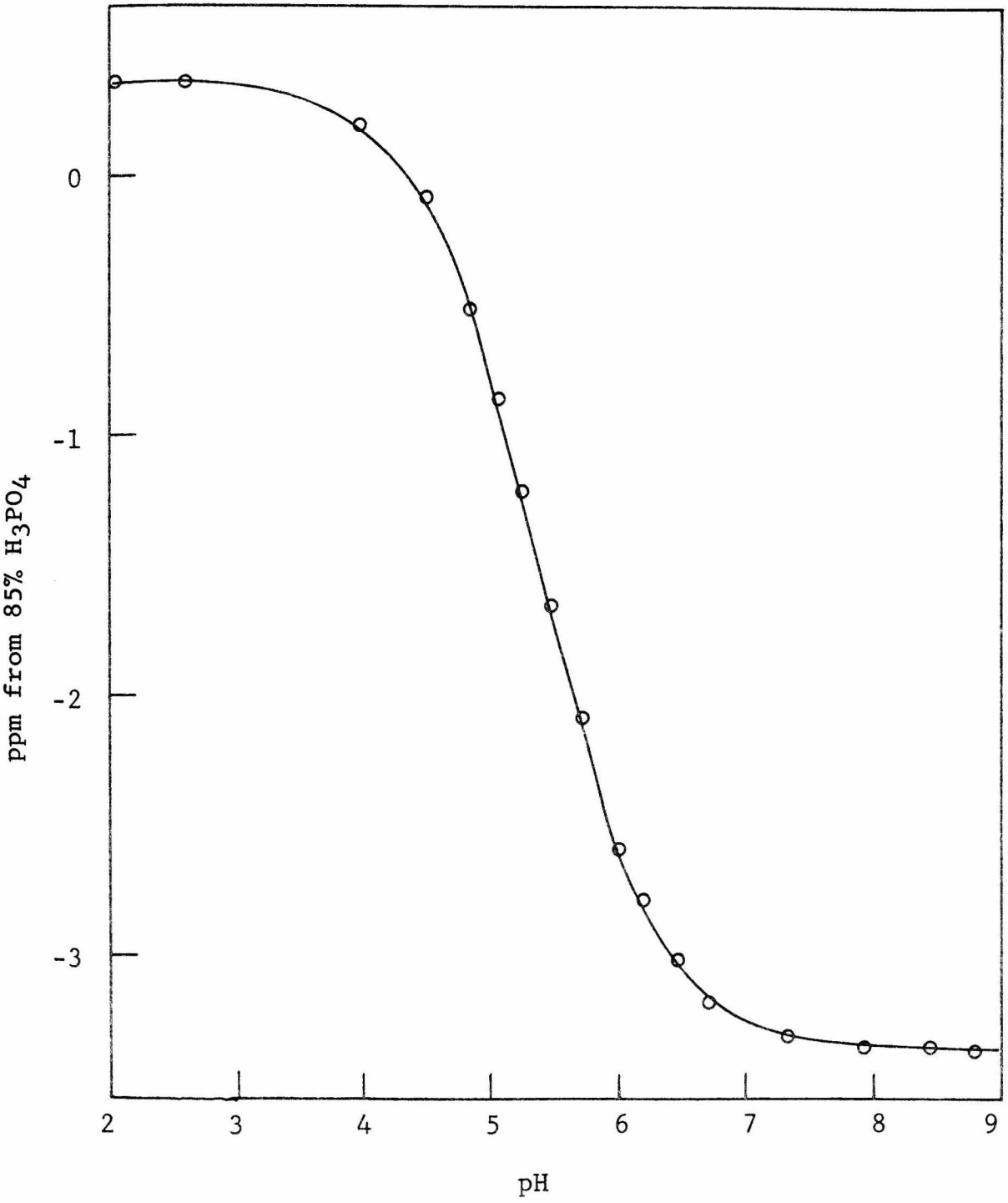
NMR Results

Figure 2 shows the ^{13}C spectrum of the isotopically enriched synthetic hapten phosphoryl[methyl-^{13}C]-choline. The proton-noise decoupled spectrum consists of a 1:1:1 triplet ($J_{^{13}C-^{14}N}$ = 4 Hz) (methyl carbon atoms) which occurs at -54.3 ppm from tetramethylsilane. For comparison purposes, the natural abundance ^{13}C NMR spectrum of commercial phosphorylcholine (Sigma) is shown in Figure 3. The methyl group signal of this latter compound coincides exactly with the spectrum of the isotopically enriched hapten.

^{31}P magnetic resonance may be used to monitor the state of ionization of phosphate esters (Crutchfield et al., 1967; Moon and Richards, 1973) and our results in this case show that monoprotonation occurs with a pK of 5.3 ± 0.1 and results in an upfield shift of 3.8 ppm (Figure 6). At pH > 7 phosphorylcholine exists almost exclusively as the dianion, the ionization state of the hapten which is recognized by the antibody under physiological conditions.

Figure 6

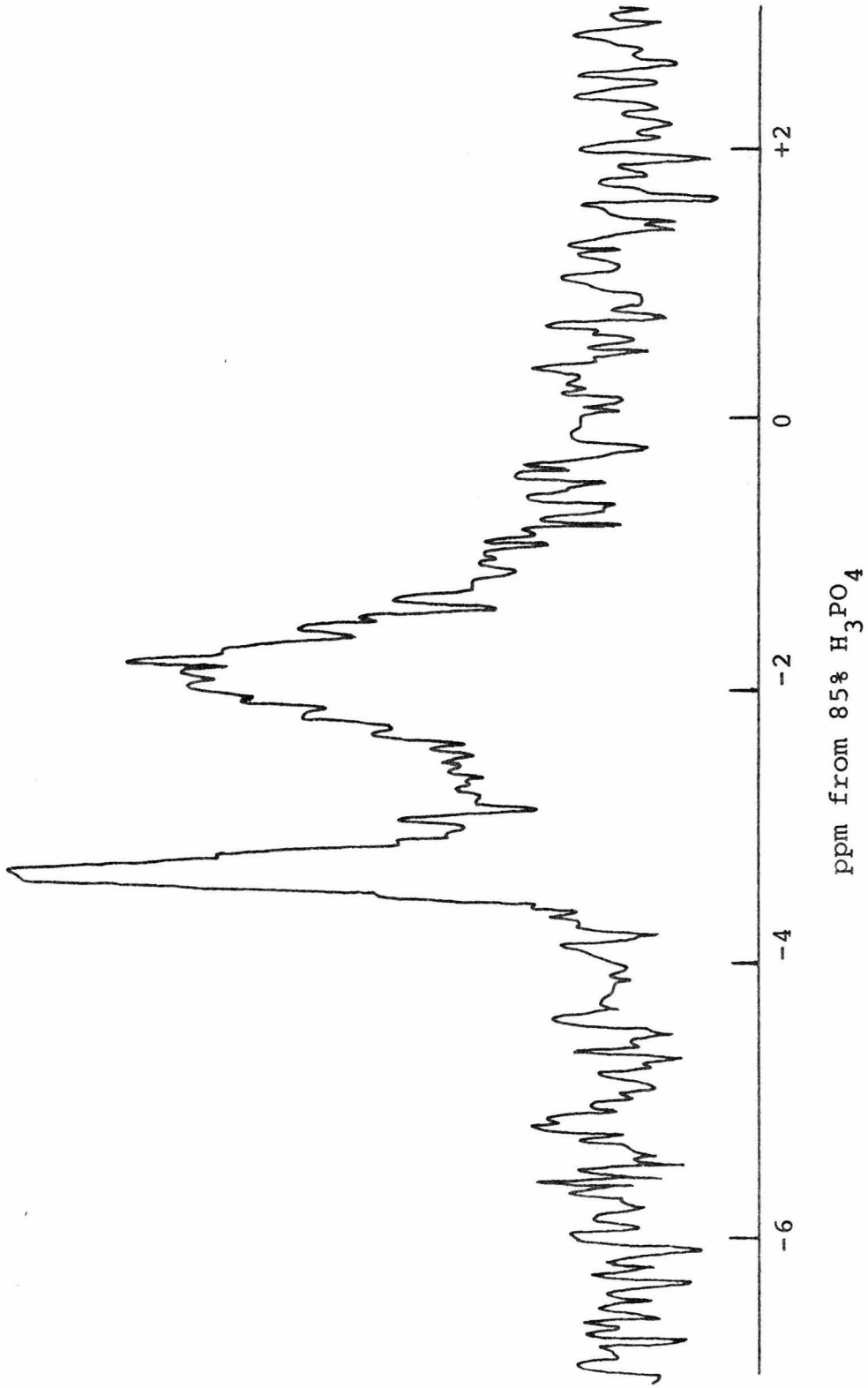
^{31}P NMR titration curve of phosphorylcholine.


0
-1
-2
-3
ppm from 85% H_3PO_4
2
3
4
5
6
7
8
9
pH

At pH 8.0 in the absence of immunoglobulin, the ^{31}P NMR spectrum of phosphorylcholine is a single sharp line ($\Delta\nu_{\frac{1}{2}}$ = 3 Hz) which occurs at -3.36 ppm from an external reference of 85% H_3PO_4. Figure 7 shows a typical ^{31}P spectrum for solutions containing an excess of phosphorylcholine over McPC 603 Fab'. Two separate signals are observed, one for the hapten free in solution and one for the hapten bound to antibody. Binding of hapten to antibody leads to an upfield shift of 1.5 ppm and is accompanied by appreciable line broadening. That both the observed change in chemical shift as well as line-broadening effects were due to specific interactions of the hapten with the immunoglobulin was verified by control experiments with non-binding proteins such as bovine serum albumin in which case neither line width nor chemical shift was significantly affected. Even at high protein concentrations, nonspecific binding of phosphorylcholine to regions of McPC 603 Fab' other than the antigen binding site is considered unlikely since crystals of the protein soaked with phosphorylcholine have been shown to exhibit only one binding site per Fab' monomer (Rudikoff et al., 1972). In addition, no evidence for the presence of additional, weaker hapten binding sites has been observed in the

Figure 7

^{31}P NMR spectrum of 3.7 mM McPC 603 Fab' and 4.7 mM phosphorylcholine at pH 8.0. An acquisition time of 0.4 sec and a pulse width of 105 μsec were used. The spectrum represents the accumulation of 113,000 transients.


+2
0
-2
-4
-6
ppm from 85% H_3PO_4

present work. Fab' fragments were generally used for NMR experiments primarily because their use results in a lower sample viscosity and allows one to obtain higher effective binding site concentrations. When the complete McPC 603 protein was used (reduced and alkylated) spectra were obtained which were identical to those resulting from the use of Fab' fragments.

Binding of phosphoryl[methyl-^{13}C]choline to McPC 603 Fab' results in a small upfield shift (0.7 ppm) of the ^{13}C NMR signal with little concomitant line broadening as shown in Figure 8. Again, two separate peaks are observed indicating that the hapten is in "slow exchange" on the NMR time scale. On binding of hapten to antibody the ^{13}C-^{14}N splitting is lost.

DISCUSSION

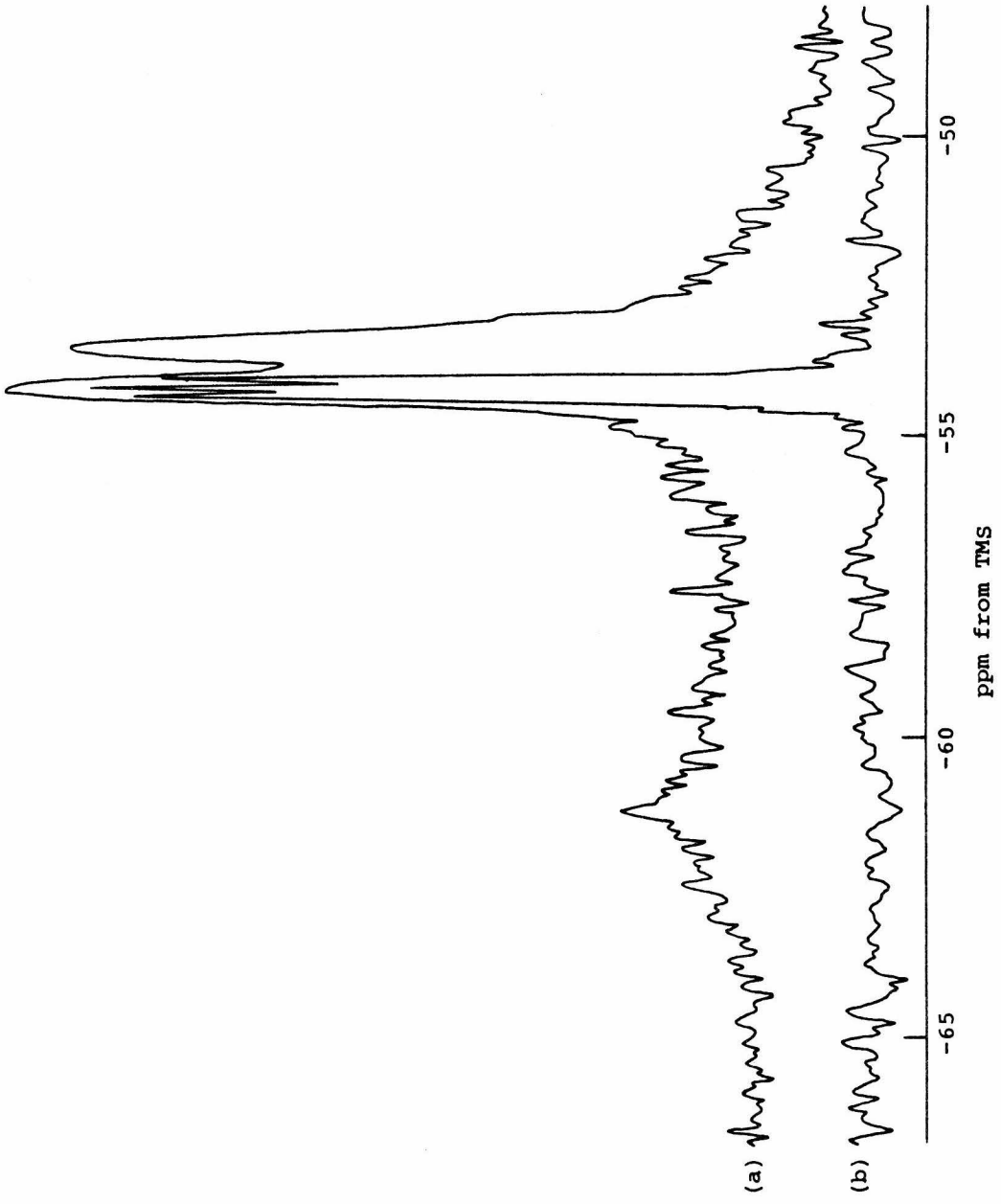
General

The 3-dimensional structure of McPC 603 Fab' has been determined to 3.1 Å resolution (Segal et al., 1974) and provides a structural basis for subsequent discussions. In this study, the phosphorylcholine molecule was shown to bind in a wedge-shaped cavity lined exclusively by residues from the hypervariable regions of the heavy (H) and light (L) chains. The

Figure 8

(a) ^{13}C NMR spectrum of a two-fold excess of phosphoryl[methyl-^{13}C]choline over McPC 603 Fab' at pH 8.0. Fab' concentration was 3.2 mM. An acquisition time of 0.3 sec and a pulse width of 35 μsec were used and 227,000 transients were collected. The spectral baseline is formed by the natural abundance ^{13}C spectrum of the protein.

(b) ^{13}C NMR spectrum of phosphoryl[methyl-^{13}C]choline at pH 8.0.


(a)
(b)
-50
-55
-60
-65
ppm from TMS

choline end of the hapten binds in the interior of this cavity and is in close Van der Waal's contact with mainly hydrophobic protein side chains, whereas the phosphate group binds more toward the exterior of the cavity. Specific hydrogen bonds are formed between the phosphate oxygens and Tyr 33H and Arg 52H; further interaction of an ionic nature may arise from the nearby Lys 54H. Two carboxylate anions (Glu 35H and Glu 59H) (Padlan et al., 1976) interact ionically with the positively-charged quaternary nitrogen of the hapten. Thus, hydrogen bonds and ionic and hydrophobic interactions play specific roles in stabilizing the antibody-hapten complex.

The potential usefulness of phosphorylcholine arises from the possibility of observing chemical shifts as well as relaxation phenomena (T_1 and T_2) independently for both ends of the hapten. Knowledge of chemical shifts for the bound hapten can reveal information about the local environment of the nucleus being observed. Analysis of spin-lattice (T_1) and spin-spin (T_2) relaxation rates may shed light on dynamic processes such as rates for association and dissociation and the mobilities of different regions of the hapten molecule when bound to antibody.

Chemical Shift and Environment

The chemical shift of the ^{31}P nucleus is dominated by the paramagnetic term (Emsley et al., 1966). The paramagnetic term depends on the magnetic moment of the orbiting electrons and is therefore strongly dependent on the symmetry of the electron cloud around the ^{31}P nucleus. Because of the large change in this symmetry upon ionization of the phosphate group, it is possible to sensitively monitor the ionization state of phosphate esters by ^{31}P NMR (Cohn and Hughes, Jr., 1960) and an upfield shift of 3.5-4.5 ppm is commonly observed for the addition of one proton (Crutchfield et al., 1967; Lee and Chan, 1971; Gorenstein and Myrwicz, 1973; Moon and Richards, 1973) which agrees well with the shift of 3.8 ppm upfield on monoprotonation of phosphorylcholine. On binding to McPC 603, the ^{31}P hapten resonance moves upfield by 1.5 ppm which we suggest is due to partial protonation of the phosphate group by the formation, between amino acid residues in the immunoglobulin and the hapten, of hydrogen bonds (from Tyr 33H and Arg 52H) that partially neutralize the negative charge on the phosphate.

This interpretation parallels, for example, that of Gorenstein and Myrwicz (1973) for the binding of

cytidine 3'-monophosphate to ribonuclease A in which the ^{31}P signal experienced an upfield chemical shift of 0.3 ppm which was interpreted to be the result of partial neutralization of the negative charge on the phosphate as a consequence of interaction with positive enzyme residues. Similar conclusions had previously been reached from proton NMR studies (Meadows and Jardetzky, 1968; Meadows et al., 1969) as well as from X-ray diffraction studies of the complex between ribonuclease A and cytidine 3'-monophosphate (Richards and Wyckoff, 1971).

Many factors (Stothers, 1972) could account for the observed ^{13}C shift on binding of 0.7 ppm upfield. One possibility is that the effective charge on the carbon nucleus is reduced when the trimethylammonium group interacts with negatively charged residues in the hapten binding pocket of the immunoglobulin (for example Glu 35H and Glu 59H). This would result in increased shielding of the carbon nucleus and an upfield shift.

However, the experimental observation that the chemical shift of the ^{13}C signal of bound hapten is pH-independent in the region 3-8 (as is the signal of the free hapten) casts some doubt on this interpretation since the antibody carboxylate groups stabilizing the

quaternary nitrogen of the hapten might be expected to titrate (to at least some extent) in this pH range. Examples (such as pepsin) where a carboxyl group titrates with a pK < 3 are, however, also known (Hartsuck and Tang, 1972). Protonation of these carboxylates should significantly decrease the chemical shift on binding, which is not what is observed. Therefore, we favor the explanation that the ^{13}C shift on binding is largely due to the unusually hydrophobic nature of the binding site environment for the hapten methyl groups (see Chapter 3).

It is also conceivable that the hapten may have a substantially different conformation when bound to antibody than when free in solution, which provides another potential contribution to changes in chemical shift of hapten on binding. It can be seen using molecular models that the "eclipsed" conformation would bring the nitrogen and phosphorous atoms into such close spatial proximity that severe steric crowding would result. In solution, therefore, phosphorylcholine probably exists predominantly in a substantially "trans" conformation even though such a conformation would minimize favorable intramolecular ionic interactions between cationic nitrogen

and anionic oxygen atoms. Phosphorylcholine when bound to McPC 603 has been revealed by diffraction studies of crystals (Segal et al., 1974) to have a conformation possibly slightly more eclipsed than one might expect for phosphorylcholine free in solution. Such a change in conformation might cause a small downfield shift in the ^{31}P resonance on binding due to a linear diamagnetic field effect arising from the closer proximity of the quaternary nitrogen. For similar reasons, it might be expected to shift the ^{13}C resonance upfield on binding, as observed. However, since we observe an upfield shift for the ^{31}P signal on binding, such an effect, if present, is nevertheless dominated by the shift contribution we have attributed to charge neutralization by partial protonation of, and hydrogen bond formation to, the phosphate group by antibody residues in the binding pocket.

Though effects other than charge neutralization may contribute to both the observed ^{31}P and ^{13}C shifts, the importance of interactions between charges on the hapten and on the immunoglobulin has been previously demonstrated not only by X-ray diffraction studies but also by binding studies. For example, the inability of the phosphorylcholine analogues phosphorylethanolamine and choline to compete effectively for hapten binding to

McPC 603 (Leon and Young, 1971) provides additional evidence for the importance of both the quaternary nitrogen and the negative phosphate group to the hapten-antibody interaction.

Relaxation-Dynamic Behaviour

The observation of two distinct peaks in both the ^{13}C and ^{31}P NMR spectra which correspond to phosphoryl-choline free in solution and bound to antibody implies that the hapten exchanges between these two environments at a rate which is slow on the NMR time scale. Nevertheless, the situation is not one in which the mean lifetime of the hapten-antibody complex is so long that the observed spectrum is not to some degree influences by the exchange process. Broadening of the linewidths results from this exchange and the magnitude of this broadening allows one to estimate τ_B. This procedure is simplified in spectra where $\rho_A = \rho_B$ and $\tau_F = \tau_B$ so that $\tau_B = 2\tau$ (from equation 2).

The spectra of both Figures 7 and 8 manifest some exchange broadening allowing lifetimes for both the phosphoryl and trimethylammonium ends of the bound hapten to be determined. Apparent inequalities between

these two values may reflect the possibility for dissociation of one end of the hapten molecule from its binding pocket on the antibody to an essentially solution-like environment while the other end remains bound. Values of $k_{off} = \tau_B^{-1}$ estimated in this way can be used in conjunction with the independently determined equilibrium association constant of 10^5 M^{-1} (at 30°C) to obtain values for $k_{on} = K_a \cdot k_{off}$, the rate constant for hapten-antibody association. From analysis of the ^{31}P and ^{13}C spectra, k_{off} rates of 38 ± 15 (^{31}P) and 10 ± 4 sec^{-1} (^{13}C) were determined. This allows one to calculate an approximate value for k_{on} of 1-4 x 10^6 M^{-1} sec^{-1}. The observed rate constants fall within the range of values measured independently for a variety of hapten-antibody systems (Haselkorn et al., 1974; Pecht, 1974). In the work of Haselkorn et al. (1974) large differences in k_{on} were observed for haptens with only slightly differing structures, leading these authors to conclude that antibody-hapten association may be more complex than a simple diffusion-controlled encounter. In fact, association rate constants more than three orders of magnitude lower than the theoretically limiting value of 1-2 x 10^9 M^{-1} sec^{-1} (Froese and Sehon, 1965) have been observed for simple

antibody-hapten associations (Pecht, 1974). These discrepancies between observed and diffusion-controlled rates have been attributed to effects ranging from steric and electrostatic repulsion (Day et al., 1963; Pecht et al., 1972) to a two-step encounter mechanism (Haselkorn et al., 1974).

The absence of splitting of the ^{13}C resonance by coupling to the ^{14}N nucleus ($J_{^{14}N-^{13}C}$ = 4 Hz) for the hapten bound to antibody requires that the dissociation rate for the antibody-hapten complex (A·H) be fast compared with the coupling constant, that is $k_{off} > 4$ sec^{-1}. Similarly, the absence of splitting of the ^{13}C resonance for the free hapten which is in equilibrium with bound hapten requires that the association rate of antibody (A) and hapten (H) be faster than the coupling constant, that is, $k_{on}[A] > 4$ sec^{-1}. As $K_a = k_{on}/k_{off} = [A\cdot H]/[A][H]$, whence $k_{on}[A] = k_{off}([A\cdot H]/[H])$, equal concentrations of free hapten and bound hapten (as in Figure 8) will lead to $k_{on}[A] = k_{off}$. Line width measurements show that $k_{off} \sim 10$ sec^{-1} (which is indeed larger than $J_{^{14}N-^{13}C}$ = 4 sec^{-1}) which accounts for the absence of splitting in both the free and bound hapten signals in Figure 8.

The value of k_{off} for the ^{31}P nucleus exceeds that of the ^{13}C nucleus by a factor of almost four. While this is not a large difference and is subject to some uncertainty, it may reflect real differences in the microscopic dissociation rates of different regions of the hapten, suggesting that the phosphoryl end may move between its binding pocket and a solution-like environment faster than does the quaternary ammonium end of the hapten. This view is consistent with the known X-ray structure of McPC 603 (Segal et al., 1974) in which the trimethylammonium end of the bound phosphorylcholine lies deep within, and in intimate contact with, the binding cleft while the phosphate end lies nearer to the entrance of the cleft. This could well allow dissociation of the hapten phosphoryl group to a solution-like environment without appreciable disruption of binding site interactions to the trimethylammonium region. On the other hand, dissociation of the trimethylammonium region prior to phosphate group dissociation is rendered impossible by the topology of the binding site. The dissociation rate constant of the ^{13}C signal of the methyl group would therefore reflect the macroscopic dissociation rate constant of the entire hapten.

A similar situation has been observed recently for the binding of oxytocin to bovine neurophysins I and II (Blumenstein and Hruby, 1977). These authors specifically enriched this peptide hormone with ^{13}C at two positions, one in the N-terminal region and one near the C-terminus. The ^{13}C NMR spectrum showed the N-terminal signal to be in slow exchange with $k_{off} < 15\ sec^{-1}$ and the C-terminal signal to be in fast exchange with $k_{off} > 1000\ sec^{-1}$. The off rate of the N-terminal region of oxytocin, being lower, was interpreted as being equivalant to the macroscopic dissociation rate of the entire hormone from the protein whereas the fast off rate at the C-terminus apparently is due to the additional contribution of other microscopic exchange processes.

Equations 3-5 allow one to estimate not only the rates for hapten-antibody association and dissociation but also T_2 for the bound and free hapten. At equal concentrations of free and bound hapten, the line widths of the free and bound ^{13}C signals are nearly equal, leading to the conclusion that T_2 for this nucleus is essentially the same in the bound and free form. This most probably occurs because the major dipolar relaxation

process depends on the free rotation of the methyl group about the N-C bond; this rotation is apparently not restricted when hapten is bound to antibody. In spite of the close Van der Waal's contact between the hapten methyl groups and antibody side chains, the rotating methyl groups do not occupy a larger volume than is seen in the static X-ray model. On steric grounds therefore, an energy barrier to methyl group rotation is not likely. It is interesting to compare these results to the proton NMR studies of the binding of tetramethylammonium to rabbit antibodies carried out by Burgen et al. (1967). These authors observed a direct relationship between the free energy of complex formation and the energy barrier to methyl group rotation (manifested in an increased nuclear relaxation rate). In contrast to this example, the binding interaction between phosphorylcholine and McPC 603 does not depend on the rotational immobilization of the hapten methyl groups.

The greatly broadened ^{31}P signal of the bound hapten (Figure 7) implies different values of T_2 for the free ($\sim$0.1 sec) and antibody-bound ($\sim$0.01-0.02 sec) phosphate group. The shorter value of T_2 for the bound hapten indicates that the phosphate region of the hapten is significantly less mobile when bound to antibody than

when free in solution. Such immobilization can be visualized as arising from the directional properties of the two hydrogen bonds from Glu 35H and Glu 59H to the phosphate group.

Conclusions

The concurrent use of ^{13}C and ^{31}P magnetic resonance allows one to probe the environments of the two ends of the phosphorylcholine molecule as it binds to a homogeneous, specific antibody. The evidence supports a picture of the hapten in the McPC 603 binding pocket in which the trimethylammonium region is stabilized by ionic interactions with anionic side chains which neutralize to some degree the positive charge of the ammonium nitrogen. Although the choline end is in tight Van der Waal's contact with the binding pocket, the methyl groups are free to rotate about the C-N bond. The phosphate oxygens of the hapten are partially protonated due to hydrogen bonds formed with amino acid side chains within the binding pocket and the phosphate groups is significantly immobilized. This view of the bound complex is entirely consistent with the known X-ray structure.

Studies of the dynamics of exchange indicate that the phosphate end of the bound hapten may exchange with a solution-like environment more rapidly than does the trimethylammonium region.

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Chapter 3

A COMPARATIVE STUDY OF PHOSPHORYLCHOLINE-BINDING MOUSE ANTIBODIES

INTRODUCTION

A number of myeloma proteins produced by plasmacytomas of BALB/c mice have been found to combine specifically with phosphorylcholine (PC)[1] (Potter and Lieberman, 1970; Leon and Young, 1970; Potter, 1972) and sequence analysis of the heavy chains of several of these proteins (Hood et al., 1975, 1976) has revealed a degree of homology greater than 85%. In fact, this high degree of heavy chain homology appears also to extend to a human IgM Waldenström with binding activity toward PC (Riesen et al., 1975, 1976). Though still incomplete, the sequence data of the light chains indicate that they belong to at least three different κ subclasses and therefore bear little homology to each other (Barstad et al., 1974; Claflin et al. 1975; Barstad, 1975). Though these antibodies have similar affinities for PC, they exhibit differing affinities for its structural analogues (Leon and Young, 1971). Because of the extensive homology among the

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[1]Abbreviation used is: PC, phosphorylcholine.

heavy chains of these proteins and the correlation between the subspecificity of an individual immunoglobulin and the subgroup of its light chain, it has been suggested that the heavy chain may well be the principal determinant of the specificity for PC while the light chain "fine tunes" the subspecificity (Barstad, 1975).

All antibodies studied to date show a high degree of conservation of their tertiary and quaternary structures. Thus, one can construct plausible 3-dimensional binding sites using sequence data together with structural information obtained by X-ray diffraction studies of other, related immunoglobulines (Padlan et al 1976a). The 3-dimensional structure of the Fab' fragment of McPC 603, a typical mouse myeloma protein that binds PC, as been determined to 3.1 Å resolution (Segal et al., 1974). This information, together with the existence of a well-characterized group of similar immunoglobulins, has afforded the opportunity to study the molecular details of structure-function relationships and, in particular, to assess the importance of individual amino acid residues to the interaction between antibodies and antigens (Padlan et al., 1976b).

Such studies should help to reveal the molecular source of the exquisite discriminatory powers exhibited by the immune response.

The use of ^{31}P and ^{13}C magnetic resonance techniques to study the binding of PC to McPC 603 was previously described (Goetze and Richards, 1977) and this type of study is now extended to four other myeloma immunoglobulins that also bind PC. In this study the emphasis is on correlating differences in interaction between antibody and hapten with changes of amino acids in the subregions of the binding sites of these antibodies.

MATERIALS AND METHODS

Materials

Plasmacytomas TEPC 15 (abbreviated T15), McPC 603 (M603) and W3207 were obtained from the Salk Institute, La Jolla, California; MOPC 167 (M167) and MOPC 511 (M511) through the courtesy of Dr. Lee Hood. [methyl-^{14}C]Choline chloride was purchased from New England Nuclear and L-α-glycerophosphorylcholine (95%) from Sigma. The latter was used without further purifi-

cation. Phosphonocholine, obtained as the betaine salt, was a gift from Dr. Lee Hood.

Hapten Binding Assays

The binding affinities of the various immunoglobulins for PC and choline were measured directly by equilibrium dialysis as described previously (Goetze and Richards, 1977).

Binding affinities for the haptens L-α-glycero-phosphorylcholine and phosphonocholine were determined indirectly by inhibition of phosphoryl[methyl-^{14}C]-choline binding as follows. 2 ml of a 1-3 mg/ml protein solution were placed on one side of a series of equilibrium dialysis chambers. The opposing chambers contained stoichiometric concentrations of labelled PC as well as increasing concentrations of the unlabelled ligand under study. A control chamber contained a stoichiometric concentration of labelled PC plus an equal concentration of unlabelled PC. After equilibration and counting, an inhibition curve of protein-bound cpm vs. ligand concentration was drawn and the concentration of ligand which resulted in the same protein-bound cpm as the control was determined. The relative affinity of antibody for the ligand was taken as the

ratio of unlabelled PC concentration in the control to the ligand concentration previously determined. An absolute affinity for the ligand was then computed using the known PC affinity.

Amino Acid Numbering

The numbering system of heavy chain amino acids is that of Rudikoff and Potter (1974) for M603.

General

All other experimental procedures were performed in analogy to those described previously for M603 (Goetze and Richards, 1977).

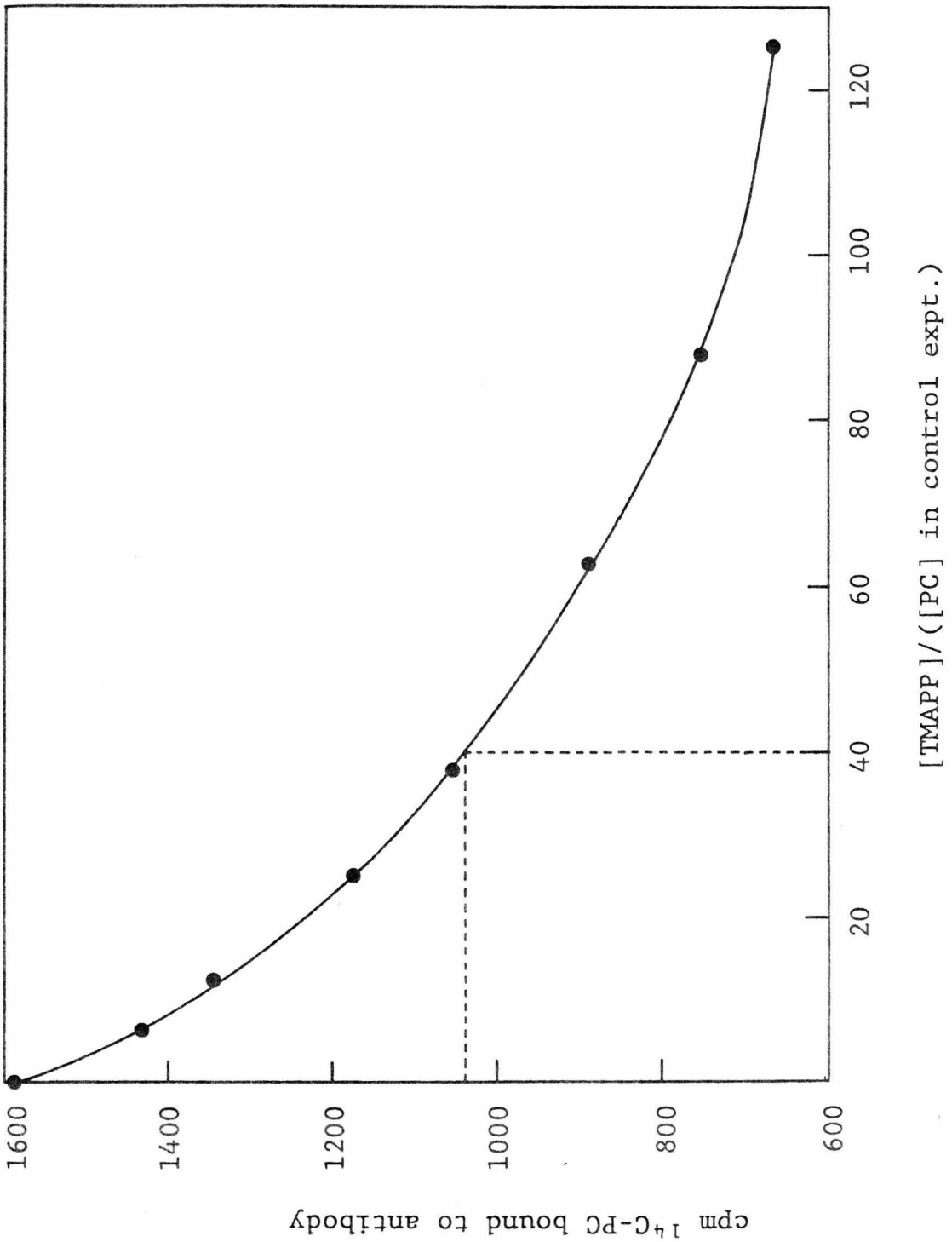
RESULTS

Scatchard plots of binding of PC to all five proteins were linear and extrapolated to close to the expected two binding sites per IgA monomer. A typical inhibition curve, used to obtain the affinity of unlabelled ligands relative to that of PC, is shown in Figure 9.

The data obtained by equilibrium dialysis on the thermodynamics of binding of four haptens to the five

Figure 9

Inhibition curve of the binding of TMAPP to T15. The experiment was carried out in borate-buffered saline (pH 8) at 4°C. The dotted line represents the control sample (see text) and shows that in this case $K_a(PC)/K_a(TMAPP) = 40$.


cpm ^{14}C-PC bound to antibody
1600
1400
1200
1000
800
600
20
40
60
80
100
120
[TMAPP]/([PC] in control expt.)

myeloma proteins, together with pertinent background information, are summarized in Table I. Our binding constants (K_as) for PC agree reasonably well with various published results (Potter, 1972; Metzger et al., 1971; Chesebro and Metzger, 1972; Pollet and Edelhoch, 1973). The binding constant of W3207 for PC is larger than that of any other myeloma protein studied to date. As previously suggested (Potter and Lieberman, 1970), M511 shows a relatively high affinity for choline.

Table II summarizes ^{31}P and ^{13}C nuclear magnetic resonance (NMR) results of interactions between the various proteins and phosphoryl[methyl-^{13}C]choline. Binding of hapten to antibody generally causes a 1.2-1.3 ppm upfield shift of the ^{13}C methyl resonance relative to its position when free in solution; M603 provides the exception with a smaller upfield shift of 0.7 ppm. The ^{31}P resonance moves 1.5 ppm upfield on binding for all the proteins except M167, for which the chemical shift for the bound form is the same as for the hapten free in solution.

Because the value of the dissociation rate constant of the antibody-hapten complex, k_{off}, is similar to the

Table 1. Properties of PC-specific myeloma proteins

Property	Myeloma Protein				
	T15	W3207	M603	M167	M511
Idiotype[1]	A	?(≠A)	B	C	D
Light chain subgroup[2]	I	II	II	III	III
Number of heavy chain differences from T15[3]	-	7	5	13	5
PC $K_a \times 10^{-5}$, M^{-1}					
4°	17.5	30.4	8.2	7.4	0.43
30°	3.5	7.0	1.0	1.2	0.15
Subspecificity[4]					
Choline	550	600	870	6	3
L-α-glycerophosphorylcholine	4	4	5	(0.8)	0.7
Phosphonocholine	(95)	15	(11)	(33)	-

[1]From Potter and Lieberman (1970) and Sher et al. (1971).

[2]From Barstad et al. (1974), Claflin et al. (1975) and Hood et al. (1976).

[3]From Hood et al. (1976).

[4]Represented as K_a(PC)/K_a(inhibitor). Data were determined by direct equilibrium dialysis (choline) or inhibition of PC binding (L-α-glycerophosphorylcholine and phosphonocholine) at 4°C. The numbers in brackets are taken from Leon and Young (1971).

Table II. PC chemical shifts and kinetic rate constants obtained with PC-specific myeloma proteins

Property	Myeloma protein				
	T15	W3207	M603	M167	M511
^{13}C shift on binding, ppm	1.3	1.2	0.7	1.3	1.2
^{31}P shift on binding, ppm	1.5	1.5	1.5	0	1.5
k_{off} from ^{13}C spectra, sec^{-1}	107	34	10	53	195
k_{off} from ^{31}P spectra, sec^{-1}	63	47	38	-	189
$k_{on} \times 10^{-6}$ from ^{13}C spectra, M^{-1} sec^{-1}	37	24	0.94	6.4	2.9
$k_{on} \times 10^{-6}$ from ^{31}P spectra, M^{-1} sec^{-1}	22	33	3.8	-	2.8

change in chemical shift occasioned by binding of hapten to antibody, exchange-broadened resonances were observed in all cases but with differences characteristic of each protein. For example, binding of excess PC to W3207, M603 and M167 results in separate peaks corresponding to free and bound hapten for both the ^{13}C and ^{31}P signals whereas binding to M511 results in a single resonance corresponding to the weighted average of the two environments. Binding of PC to T15 gives two separate ^{31}P signals but only a single greatly broadened ^{13}C resonance. Table II also collects the values of k_{off}, estimated from the observed change in chemical shift on binding and the extent of exchange broadening (see Chapter 2), as well as the calculated values of the rate constants for association ($k_{on} = K_a \cdot k_{off}$). Except for M603, the rate constants from the ^{13}C and ^{31}P observations are in good agreement in that they differ by less than a factor of two.

DISCUSSION

Subsite Interactions Among Phosphorylcholine-Binding Myeloma Proteins

Particular regions of a ligand-binding site on a protein, responsible for a specific type of interaction (e.g. electrostatic, hydrogen bonding or hydrophobic) with a portion of the bound ligand have been termed interaction subsites; this concept was formulated by Haselkorn *et al*. (1974) as a result of kinetic experiments on a myeloma protein that binds dinitrophenyl derivatives. In this model, the total binding energy represents a direct summation of the contributions from each individual subsite-ligand interaction. This concept serves as a convenient basis for our discussion of the binding of PC and its analogues to myeloma proteins with specificity for PC.

For binding of PC one might expect three types of subsites to contribute substantially to hapten binding; a negatively charged subsite for interaction with the quaternary nitrogen; a positive, possibly hydrogen-bond-donating subsite for interaction with the dianionic phosphate; and a hydrophobic subsite for interaction with

the two central methylene groups of PC. Specific interactions, completely consistent with these expectations, have been observed in the 3-dimensional structure of the binding site of M603 which was determined by X-ray diffraction (Segal et al., 1974).

For consideration of the phosphate-binding subsite, proteins T15, W3207 and M603 are useful. These all cause identical hapten ^{31}P shifts and show similar k_{off} rates calculated from their ^{31}P spectra, suggesting similar subsite interactions with the phosphate group. Their affinities for choline are lower by a factor of 600-900 than that for PC, as would be anticipated if interactions with the dianionic phosphate group are important for binding. Also, their affinities for L-α-glycerophosphorylcholine are lower by a factor of 4-10 compared to PC suggesting steric interference and/or loss of electrostatic binding interaction when the larger, monoanionic L-α-glycerophosphate group binds in the phosphate pocket. These results indicate that, for these three proteins, the phosphate subsites are probably very similar; their different absolute affinities for each of PC, choline and L-α-glycerophosphorylcholine arise from differing interactions with the trimethyl-

ammonium region of these ligands. The ^{13}C NMR results support this view and imply that M603 interacts differently than do T15 and W3207 with the methyl groups of the hapten. In addition, different k_{off} rates were calculated from the ^{13}C spectra of labeled hapten interacting with these three proteins. Although M603 binds PC less strongly than do T15 and W3207, M603 nevertheless has a lower value of k_{off}, which requires that it have a substantially lower association rate constant than T15 and W3207.

Comparative affinities do not, however, always correlate well with the apparent subsite interactions. For example, on the basis of these studies at pH 8, M167 is unique among the proteins in causing no shift of the ^{31}P hapten resonance on binding, suggesting a different subsite for binding the phosphate group. It will be shown in Chapter 4 that the phosphate subsite of M511 nevertheless bears a strong homology to that of M167; a similarity not apparent from their dissimilar ^{31}P shifts at pH 8. The choline subsites of M167 and M511 however appear to be very similar to the corresponding subsites of T15 and W3207. Therefore, it is surprising that the dissociation rate constant of the M511-PC complex is an order of magnitude higher

than that of the other proteins which is reflected in a decreased binding constant. This unexpectedly low affinity cannot, by these criteria, be traced to noticeably different interactions between antibody and trimethylammonium or phosphate groups, but appears due to a generally poorer complementarity between hapten and binding site.

Table III summarizes the postulated subsite interactions deducible from a consideration of both the NMR and the binding data. To a first approximation, the affinities of these proteins for PC agree with the total strength of the binding interaction predicted from this simple model. It appears possible, therefore, that the affinities for PC and related haptens can be modulated by structural changes in relatively independent subregions of the binding pockets of these antibodies, which are known to exhibit diverse specificities for a variety of bacterial antigens (Potter and Leon, 1968; Potter, 1972), a diversity which likely arises from these differences in subsites.

Comparison of the dynamic aspects of hapten binding by these five proteins shows that differences in affinity result not only from differing dissociation

Table III. Postulated strength of subsite interactions of phosphorylcholine-specific myeloma proteins

	Myeloma protein				
	T15	W3207	M603	M167	M511
Choline subsite	++	+++	++	+++	+
Phosphate subsite	+++	+++	++	+	+
Total	5+	6+	4+	4+	2+
$K_a \times 10^{-5}$, M^{-1} (30°)	3.5	7.0	1.0	1.2	0.15

+, weak; ++, moderate, +++, strong

constants reflecting, probably, differences in complementarity between antibody and hapten, but also from differing association rate constants. The calculated association rate constants are typical of those previously observed for other antibody-hapten systems (Sehon, 1963; Kelly et al., 1971; Pecht, 1974; Haselkorn et al., 1974). In particular, the k_{on} value of 4.6×10^7 M^{-1} sec^{-1} calculated by Pecht (1974) for HOPC 8 agrees with our estimate of 2 to 4×10^7 M^{-1} sec^{-1} for T15, a protein idiotypically identical to HOPC 8. However, the association constants for M603, M167 and M511 are lower by an order of magnitude than those of T15 and W3207. This could be explained if; (a) T15 and W3207, for simple topological reasons, allow more ready access of hapten into the binding cleft than do the other proteins, or (b) binding of hapten to M603, M167 and M511 is accompanied by a protein rearrangement which has a slower time constant than the diffusion-controlled encounter of antibody and hapten. Because little evidence for anything but a simple diffusion controlled antibody-hapten association step is commonly observed in other systems (Froese and Sehon, 1975; Pecht and Lancet, 1976), the former

explanation is considered more likely in this case. This would suggest that there is a significant structural difference between the binding sites of T15 and W3207 on the one hand and those of M603, M167 and M511 on the other. No molecular explanation for such a difference can be given at this time.

Relationship Between Differences in Binding Site Interactions and 3-Dimensional Structure

The similarity of both the ^{13}C and ^{31}P hapten chemical shifts upon binding (at pH 8) to most of these proteins argues for a high degree of similarity in the important electrostatic interactions between hapten and binding site. By this criterion of comparative chemical shifts, however, M167 shows an unusual phosphate-binding subsite and M603 a unique choline subsite; the possible molecular origins for these anomalies will now be investigated.

The heavy chain appears to play the dominant role in the intaraction between these antibodies and hapten (Barstad et al., 1974). This is supported by the high degree of conservation of heavy chain sequence among the PC-binding immunoglobulins and also by the presence of

three widely differing light chain sequences among the five proteins. Such a view is confirmed by the 3-dimensional structure of the M603-PC complex, in which the phosphate binding subsite is found to be formed exclusively by heavy chain residues. The following amino acids are in direct contact with PC: Tyr 33H, Glu 35H, Arg 52H, Glu 59H, Trp 104aH and an unidentified residue at position 96 of the light chain (Segal et al., 1974; Padlan et al., 1976b). The first five of these residues, as well as the ionically important Lys 54H, are, with a single exception, conserved among all the PC-binding mouse myeloma antibodies.

We have previously interpreted the ^{31}P chemical shift on binding of PC (+1.5 ppm) as being caused largely by hydrogen bonding between specific amino acid residues and the phosphate group (Goetze and Richards, 1977). Binding of PC to M167 at pH 8 results in no such shift. Why? A trivial answer would be to suggest that the lack of ^{31}P shift on binding is due to the absence of a specific interaction between M167 and the phosphate. This explanation, however, is not acceptable for the following reasons: (i) M167 binds PC with a higher affinity than choline; (ii) the linewidth of the bound

^{31}P signal ($\Delta\nu_{\frac{1}{2}}$ = 24 Hz) is broadened to an extent similar to that observed upon binding of PC to the other immunoglobulins suggesting restricted mobility of the phosphate group; (iii) a study of the pH-dependence of the ^{31}P chemical shift on binding (Chapter 4) clearly shows the absence of any shift at pH 8 to be coincidental.

Examination of the M603 X-ray structure reveals that heavy chain hypervariable region 2 (residues 50-65) forms a crucial portion of the phosphate binding subsite. Relative to T15, M167 has 3 amino acid substitutions in this second hypervariable region (Asn 54→Ser, Asn 56→His, and Thr 58a→Arg). Any one of these, especially the positively charged Arg 58a, may, either directly or indirectly, alter the local magnetic environment so as to perturb the ^{31}P chemical shift. In addition, M167 (and M511) has an extra residue, Asp 100aH, located in the vicinity of the phosphate (Padlan et al., 1976b) which may also influence the ^{31}P shift. A more detailed study of this problem is presented in Chapter 4.

The anomalous ^{13}C shift observed on binding of PC to M603 is difficult to rationalize because the light chain contributes to formation of the choline subsite and sequence data for the light chains are still incom-

plete. However, the other 4 proteins have, relative to M603, an extra residue in the third hypervariable region of the heavy chain (residue 103aH) (Padlan et al., 1976b). This hypervariable loop is in extensive contact with the choline portion of the bound hapten. It has been suggested that amino acid insertions or deletions in the hypervariable loops are generally capable of introducing larger structural changes (and hence, specificity changes) than amino acid substitutions (Padlan et al., 1976a; Padlan, 1977; Kabat et al., 1977) and this deletion in a crucial region of the M603 binding site might explain the unique ^{13}C shift.

It is informative to compare the nature of the amino acid residues contributing to formation of each of the two main subsites. The X-ray structural data of M603 reveals (Segal et al., 1974; Padlan et al., 1976b) that, to a first approximation, the choline binding subsite is lined with residues mainly from the third hypervariable region of the heavy chain (H3) and to a much lesser extent the first (L1) and third (L3) hypervariable regions of the light chain. The H3 loop of M603 has 7 out of 12 residues with significant hydrophobic character (3 Tyr, 2 Trp, 1 Phe, 1 Val for

a total hydrophobic percentage of 58%) (Rudikoff and Potter, 1974, 1976). Therefore the choline subsite is extensively hydrophobic and this may be a contributing factor to the ^{13}C chemical shift of hapten on binding. Since the other four immunoglobulins have an additional residue with hydrophobic character (Tyr) in H3, the smaller ^{13}C shift for M603 may result from such a difference. The independence of the M603 ^{13}C shift to pH in the region 3-8 also argues against the origin of this shift being due to the ionic influence of Glu 35H and Glu 59H.

The H1 loop, whose sequence is constant among all PC-binding immunoglobulins, mainly forms one "side" of the cavity, i.e. contributes residues in close contact with the methylene groups of bound hapten. The phosphate subsite is lined almost exclusively by H2 residues. A sequence analysis of H2 shows a preponderance of hydrophilic residues with only 2 out of 16 residues (12.5%) being hydrophobic. The phosphate subsite therefore, as might be expected from its relatively high degree of exposure of solvent and the charged nature of the group it binds, is constructed almost exclusively of polar side chains.

One would like to assess the relative contributions of hydrophobic and coulombic forces in determining the binding energy for the trimethylammonium portion of PC. An ideal way to answer this question would be to quantitate the binding of the PC analogue 3,3-dimethyl-1-butanol phosphate to these antibodies but preliminary attempts to synthesize this compound have not been successful. The inability of phosphorylethanolamine to compete for binding (Leon and Young, 1971) is not proof for a positive charge requirement on the hapten since the lack of the bulky methyl groups might also account for the loss of binding ability. Karush (1962) has argued that ionic interactions cannot dominate the energetics of an antibody-hapten complex since in most cases large variations in ionic strength have little effect on the binding affinity. In fact, exactly this argument was used to propose that electrostatic interactions contribute little to the binding energy between T15 and PC (Pollet and Edelhoch, 1973). Since thermodynamic considerations predict that hydrophobic bonding will generally contribute most to the free energy of binding (Karush, 1962), the complementary electrostatic interactions on binding, though

important for conferring specificity, may not be dominant contributors to the binding energy.

Comparison of Phosphorylcholine-Binding Sites

We may now summarize our understanding of the individual binding sites of these proteins. W3207, which has the highest affinity for PC, forms strong interactions with both ends of the hapten. T15 has differences from W3207 in the third hypervariable region of its heavy chain which, by analogy to the X-ray structure of M603, would be expected to influence mainly the subsite for choline. Accordingly, T15 exhibits slightly weaker interactions in this subsite, but retains the strong phosphate subsite interactions of W3207; the net result is a 2-fold decrease in the affinity of T15 for PC. M603 retains the W3207-type light chain but has significant differences throughout the heavy chain, causing a substantial alteration in the choline subsite; the overall affinity of M603 for PC is reduced by a factor of 4-6 relative to that of W3207. M167 has a heavy chain with a wider diversity from the W3207 prototype than that of any of the other proteins. M167 also has a light chain of a different

subgroup. These changes give rise to a greatly altered phosphate subsite, where decreased specificity is attested by the high relative affinity for choline and L-α-glycerophosphorylcholine. M511 has a light chain of the M167 type and only a moderate number of amino acid differences in its heavy chain relative to W3207; we have no explanation for the greatly decreased affinity of M511 for PC.

Conclusions

The generally similar hapten-binding site interactions observed for this group of five idiotypically distinct mouse myeloma proteins demonstrate a general conservation of at least the important electrostatic interactions in the binding sites of this family of immunoglobulins. This conservation is, however, not absolute, and deviations are observed in subregions of the binding pockets of M167 and M603. The dynamics of the hapten-antibody interactions, as monitored by the exchange rates of the ^{13}C and ^{31}P nuclei of the PC hapten, correlate with the binding affinities of these proteins for various haptens. The concept of subsite interactions has proved especially fruitful in understanding these effects.

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CHAPTER 4

THE MOLECULAR BASIS FOR SUBSPECIFICITY DIFFERENCES AMONG PHOSPHORYLCHOLINE-BINDING MOUSE ANTIBODIES

INTRODUCTION

A group of mouse myeloma immunoglobulins which specifically bind phosphorylcholine (PC)[1] (Potter, 1972; Rudikoff et al., 1972; Potter and Lieberman, 1970) serves as a convenient system for studying structure-function relationships between similar, homogeneous antibodies. In this group, the primary structures of the heavy chains and of portions of the light chains from six different proteins are now known (Hood et al., 1975, 1976). The three-dimensional structure of the Fab' fragment of one one these proteins, M603, as well as its complex with PC (Segal et al., 1974), has allowed direct visualization of the interactions between hapten and specific residues in the antigen combining site of the immunoglobulin which define the antibody specificity.

The high degree of sequence homology among the heavy chain residues of immunoglobulins specific

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[1]Abbreviations used are: PC, phosphorylcholine; GPC, L-α-glycerophosphorylcholine; NPPC, p-nitrophenylphosphorylcholine; TMAPP, 3-trimethylamino-1-propanolphosphate; NMR, nuclear magnetic resonance.

for phosphorylcholine in both mouse and man (Padlan et al., 1976; Riesen et al., 1976) and the observation that most of the residues which interact with hapten in M603 are located in the heavy chain have allowed some rationalization of the molecular origins of the varying binding properties of these proteins (Padlan et al., 1976; Goetze and Richards, 1977a). These facts also suggest that a single general structure may define PC specificity in several immunoglobulins and this work is an attempt to understand how, or if, this structure can serve as an example of the ability of the immune response to create a binding site optimally complimentary to a class of antigenic determinants. We have, therefore, examined the detailed molecular interactions responsible for the high affinity of these proteins for PC and related ligands and how these affinities are modulated by changes in a small number of crucial amino acids within this group of immunoglobulins. Such information may yield insight into the question of how immunoglobulins with subtly differing binding specificities can be created with a minimum number of changes in amino acids and thereby, help us to under-

stand the origins of the broad diversity and exquisite specificity of the immune response.

In this work we have used ^{31}P NMR to probe the molecular details of the environment of the phosphate group of several haptens when bound to five idiotypically distinct PC-binding myeloma proteins (M603, W3207, T15, M167 and M511). Several factors proved fortuitous for these experiments: (i) the phosphate binding sites in these proteins appear (by analogy to M603) to be formed exclusively by residues from the heavy chains whose complete sequences are known; (ii) the various known subspecificities of these proteins for ligands differing structurally at the phosphoryl end (Leon and Young, 1971) most likely result from differences in the phosphate binding subsites; (iii) ^{31}P NMR is an especially useful technique for not only can one use ^{31}P chemical shift information to probe the microenvironment of the phosphorous but one can also study such chemical shift behaviour as a function of pH and thereby learn about the electrostatic nature of groups in the phosphorous microenvironment by the way they perturb the pK of the phosphate group of bound haptens.

Recent studies have shown that a large portion

of the binding energy for the interaction between haptens and PC-specific antibodies arises from ionic attractions between groups of opposite charge (Grossberg et al., 1974; Krausz et al., 1976). However, little information is available on the pH dependence of the binding affinities to allow one to assess the relative importance of the various charged residues of the protein to the total binding interaction. Though some data on T15 are available, notably the observation that the PC affinity of T15 drops sharply below pH 5 (Pollet and Edelhoch, 1973), one has not been able to ascribe this effect unambiguously to protonation of the phosphate group of the hapten or to protonation of carboxylate groups on the protein which are known to interact strongly with bound hapten (Grossberg et al., 1974). In order to resolve such ambiguities we have studied the pH dependence of the binding affinities of these proteins both for PC and for L-α-glycerophosphorylcholine (GPC).

MATERIALS AND METHODS

Haptens

Phosphorylcholine (PC) and L-α-glycerophosphorylcholine (GPC) were purchased from Sigma and phosphoryl-[methyl-^{14}C]choline from New England Nuclear. p-Nitrophenylphosphorylcholine (NPPC) was synthesized as previously described (Chesebro and Metzger, 1972). 3-Trimethylamino-1-propanolphosphate (TMAPP) was synthesized in complete analogy to PC (Chesebro and Metzger, 1972; Baer, 1952) except that 3-dimethylamino-1-propanol (Aldrich) was substituted for dimethylaminoethanol.

Protein Purification

Ascites fluid was mildly reduced, alkylated and filtered through a Sepharose-PC column (Chesebro and Metzger, 1972). PC-specific protein was immunospecifically eluted by washing the column with 1 mM PC in borate-buffered saline, pH 8.

Hapten Binding Affinities

Affinities for PC at varying pH were determined by equilibrium dialysis as previously described (Goetze and Richards, 1977b). Experiments were carried out in borate-cacodylate buffered saline (0.02 M borate, 0.02 M cacodylate, 0.13 M NaCl, 1 mM EDTA, 0.02% NaN_3) and care was taken to pre-equilibrate all samples and stock solutions to the desired pH. All binding affinities were measured at $5^o \pm 1^oC$.

Affinities for all other haptens were determined indirectly by inhibition of labelled PC binding (Karush, 1956; Michaelides and Eisen, 1974).

NMR Experiments

A Varian XL-100-15 spectrometer interfaced with a Varian 620i computer was used. Spectra were obtained at 40.5 MHz at a probe temperature of $25^o \pm 1^oC$ and were proton-noise decoupled. A capillary insert containing D_2O provided the field-frequency lock. A 90^o pulse with an acquisition time of 0.4 sec and a sweep width of 1000 Hz were used to accumulate the data.

NMR Titrations

NMR samples consisted of immunospecifically-purified Fab' fragments (Inbar et al., 1971) which had been extensively dialyzed to remove bound hapten. Protein solutions were concentrated to 3-5 mM by ultrafiltration in an Amicon apparatus and the desired hapten concentration was achieved by the addition of a small volume of a concentrated stock solution.

Titration experiments were started at the high pH extreme by having previously dialyzed the protein sample against borate-cacodylate buffered saline of the appropriate pH. Samples were titrated towards low pH by the addition of 5 N HCl. With care, it was possible to avoid protein precipitation above pH 3. The pH of each sample was measured immediately after each NMR spectrum was obtained.

Amino Acid Numbering

The numbering system of heavy chain amino acids is that for M603 (Rudikoff and Potter, 1974).

RESULTS

pH Dependence of Binding Constants for PC and GPC

The binding affinities of M603, W3207, T15 and M167 for the haptens PC and GPC at 5°C are shown in Figures 10 to 13. Affinities were measured over the pH range 4-9 and in several cases were extended somewhat beyond these limits.

The curves for M603 and W3207 are qualitatively similar. Affinities for both PC and GPC reach a maximum at pH 7.0-7.5 with those for W3207 being $\sim$ 7-fold higher on an absolute basis. Raising or lowering the pH from neutrality causes the affinities to decrease appreciably but the amount of this decrease as the pH is lowered is significantly different for M603 and W3207. For example, the affinity for PC of M603 drops 47-fold from pH 7.3 to pH 4 whereas that of W3207 drops 12-fold. Both proteins bind PC with a higher affinity than GPC over the entire pH range studied although these affinities converge near pH 3.

The binding data for T15 exhibit a substantial amount of scatter at pH 7-10 for unknown reasons. The binding curves are similar to those of M603 and

Figure 10

The pH dependence of the binding constants of M603 for PC (●) and GPC (▲). The experiments were performed by equilibrium dialysis at 5°C in borate-cacodylate buffer except for those at pH 8 which were obtained in borate-buffered saline. Each data point represents the result of a Scatchard plot of 10 points (PC) or of an inhibition curve (see text) of a similar number of points (GPC). The binding constants were estimated to be accurate to ± 10% for PC and ± 15% for GPC

The PC affinity of M603 at pH 8 is 5.4×10^5 M^{-1} or 34% lower than the value reported previously (Goetze and Richards, 1977a). The previous value had been obtained using protein from the M603 tumor line obtained from a different source than that used in the current experiments.

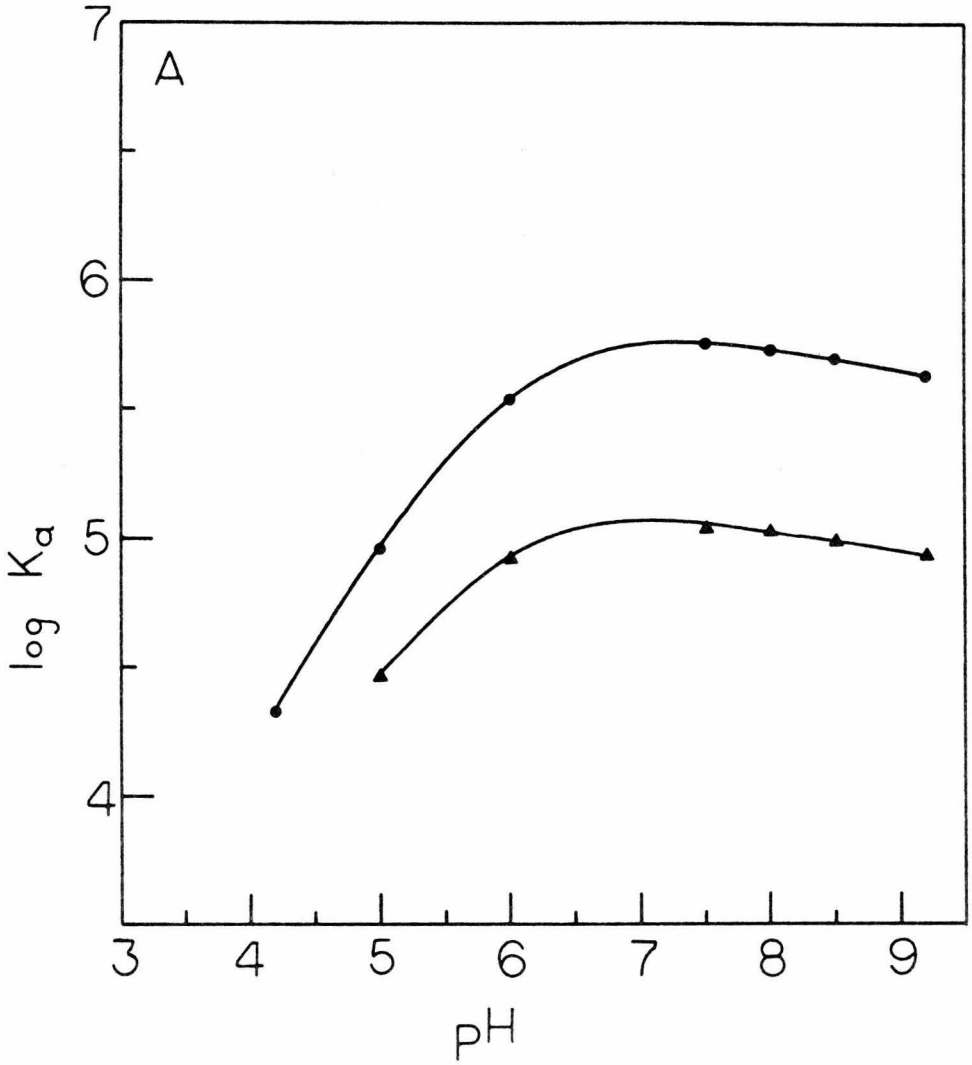

A
7
6
5
4
log K_a
3
4
5
6
7
8
9
pH

Figure 11

The pH dependence of the binding constants of W3207 for PC (●) and GPC (▲). For further details, see Figure 10.

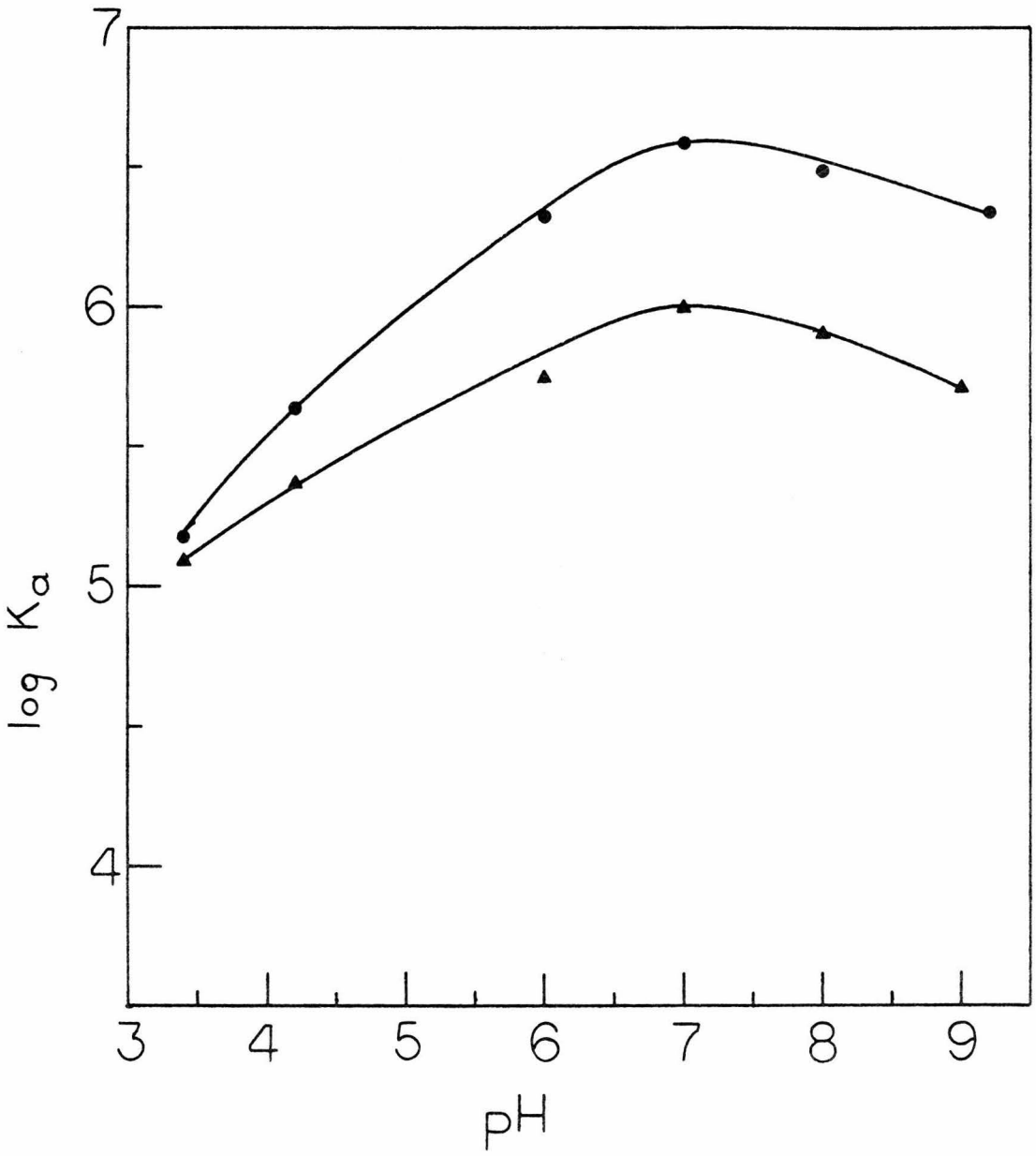

7
6
5
4
log K_a
3
4
5
6
7
8
9
pH

Figure 12

The pH dependence of the binding constants of T15 for PC (●) and GPC (▲). For further details, see Figure 10.

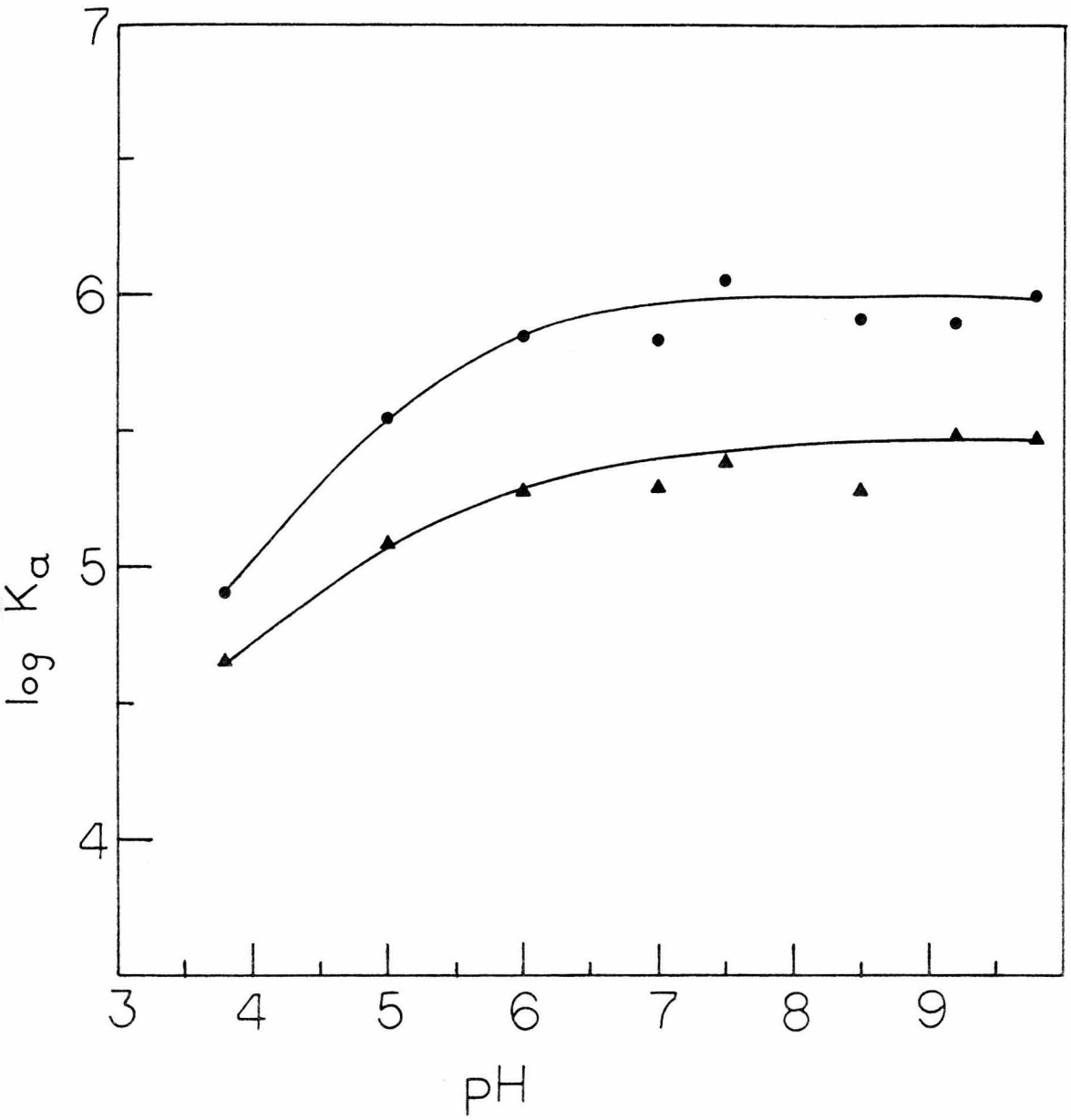
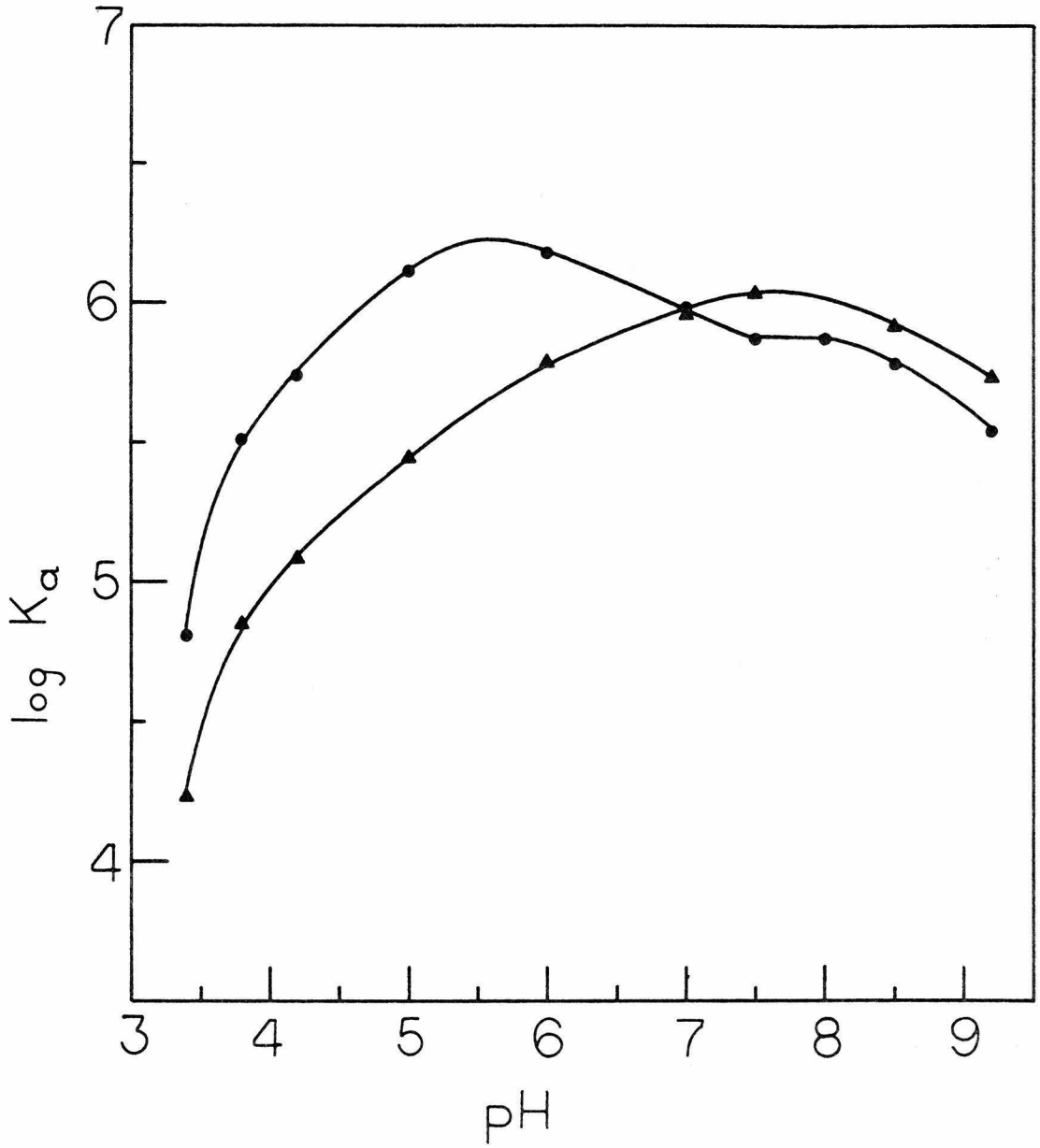

7
6
5
4
log Ka
3
4
5
6
7
8
9
pH

Figure 13

The pH dependence of the binding constants of M167 for PC (●) and GPC (▲). For further details, see Figure 10.


7
6
5
4
log K_a
3
4
5
6
7
8
9
pH

W3207 at pH < 7 but are unique in that the affinity for both haptens is pH-independent in the range 7-10.

The pH dependence of GPC binding to M167 is similar to that for GPC binding to M603 and W3207. In contrast, the pH at which the affinity of M167 for PC is maximum is lowered to $\sim$ 5.5 and one observes two distinct ionizations as the pH is raised above 5.5. Because of this, the affinity of M167 is actually greater for GPC than for PC in the region pH > 7.

Table IV lists the affinities at pH 8 of these four proteins as well as M511 for the haptens GPC, NPPC and TMAPP relative to their affinities for PC. Again, one notes the similarity between M603 and W3207; all three haptens bind to either protein with a similar affinity.

NMR Titrations

At neutral pH, the ^{31}P signal of PC (Goetze and Richards, 1977a, 1977b) and also that of the other haptens, obeys the condition of slow exchange on the NMR time scale, $k_{off} < \frac{2\pi\Delta_{AB}}{\sqrt{2}}$ (Pople et al., 1959) (where k_{off} is the dissociation rate constant and Δ_{AB} is the chemical shift difference between free and

Table IV

Subspecificities of PC-Binding Myeloma Proteins[1]

Hapten	W3207	M603	T15	M167	M511
GPC	4	5	4.3	0.8[2]	0.7
NPPC	5.6	6.8	-	1.5	-
TMAPP	18	13	40	2	1.5

[1]Represented as K_a(PC)/K_a(inhibitor). Binding experiments were performed by equilibrium dialysis in borate-buffered saline at 5°C.

[2]From Leon and Young (1971).

bound hapten) so that one observes separate peaks for the hapten free in solution and bound to antibody.

However, binding affinities and hence the dissociation rate constants, vary with pH as do the differences in chemical shift of the various haptens (Δ_{AB}) when free in solution as compared to bound to the antibody. Accordingly, the NMR spectra vary from the slow exchange limit as a function of pH. In order to minimize interference from free hapten signal under conditions of slow exchange, especially in regions where the positions of the signal for bound and free hapten were very close, and also to maximize the contribution to the observed chemical shift from the bound hapten under condtions of fast exchange, the NMR experiments were generally carried out under conditions where antibody was in excess; in this way almost all hapten present was bound to antibody. The line width of the observed peak for bound hapten ($\Delta\nu_{\frac{1}{2}}$ = 20-30 Hz) generally remained constant as the pH was lowered to pH $\sim$ 4 indicating that conditions of fast exchange had not yet been reached. (This statement is not true for M511, which will be discussed separately later.) With M603, which has a lower affinity for hapten

(and therefore a higher k_{off}), experiments involving hapten binding approach the fast exchange limit at slightly higher pH values.

These observations were confirmed by carrying out some titration experiments under conditions of excess hapten. When exchange was slow, separate signals were observed for free and bound hapten; the position of the signal for bound hapten was identical to that previously observed when antibody was in excess.

The binding affinities for haptens generally decrease as the pH is raised above neutrality; this decrease is, however, sufficiently small so that all spectra continued to reflect slow exchange in this region.

Below pH $\sim$ 4, the observed signal often narrowed appreciably indicating fast exchange. The NMR titrations were carried out at 25°C and the binding affinities are not known accurately at this temperature. As a result, precise determination of the chemical shift for the bound hapten was not possible. However, by assuming that the affinities at 25°C are about 25% of those at 5°C (Goetze and Richards, 1977a) one could

establish an approximate position for the chemical shift of bound hapten although these data (below pH 4) are considerably less accurate than those obtained under conditions of slow exchange.

NMR studies of M511 with the haptens PC and TMAPP present a unique situation. A careful analysis showed that these haptens are in fast exchange, with reasonably narrow linewidths, both at high pH (pH $\geq$ 8) and at low pH (pH $\leq$ 5). In the intermediate pH range however, a greatly broadened resonance is observed. This is due to exchange broadening as the increased chemical shift difference between free and bound hapten as well as the higher binding affinity (by analogy to M167) in this pH range lead to a slower exchange rate. The effect of this broadening on the NMR spectra is illustrated in Figure 14 and can be compared with the relatively constant linewidth of the ^{31}P resonance in cases where exchange broadening does not play such a variable role (Figure 15 and 16).

Figure 17 illustrates the titration behaviour of the ^{31}P resonance of PC both free and bound to M603 and W3207. Upon addition of a single proton to the free, dianionic hapten, the ^{31}P signal moves upfield

Figure 14

^{31}P NMR spectra of 3.70 mM TMAPP plus 4.49 mM M511 Fab' at different pH values: (A) pH 8.86 (B) pH 7.73 (C) pH 7.13 (D) pH 5.99 (E) pH 5.21. Data were collected with an acquisition time of 0.4 sec at a probe temperature of 25 ± 1°C. The sharp resonance is due to a small amount of P_i which increased with time (the spectra were taken in the order A, B, D, E, C).

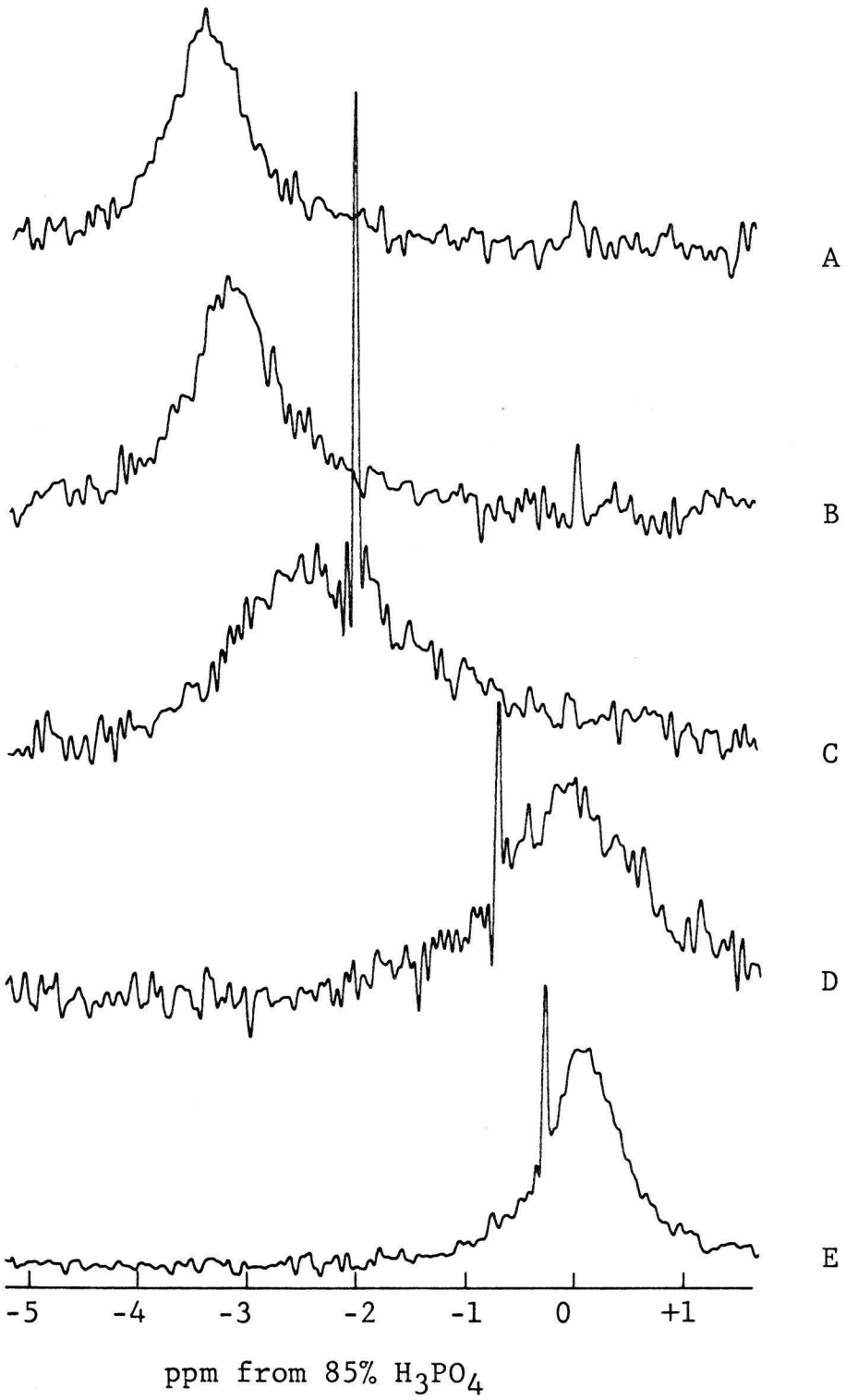

A
B
C
D
E
-5
-4
-3
-2
-1
0
+1
ppm from 85% H_3PO_4

Figure 15

^{31}P NMR spectra of 3.15 mM PC plus 4.12 mM T15 Fab' at different pH values: (A) pH 5.82 (B) pH 5.19 (C) pH 4.70 (D) pH 3.50.

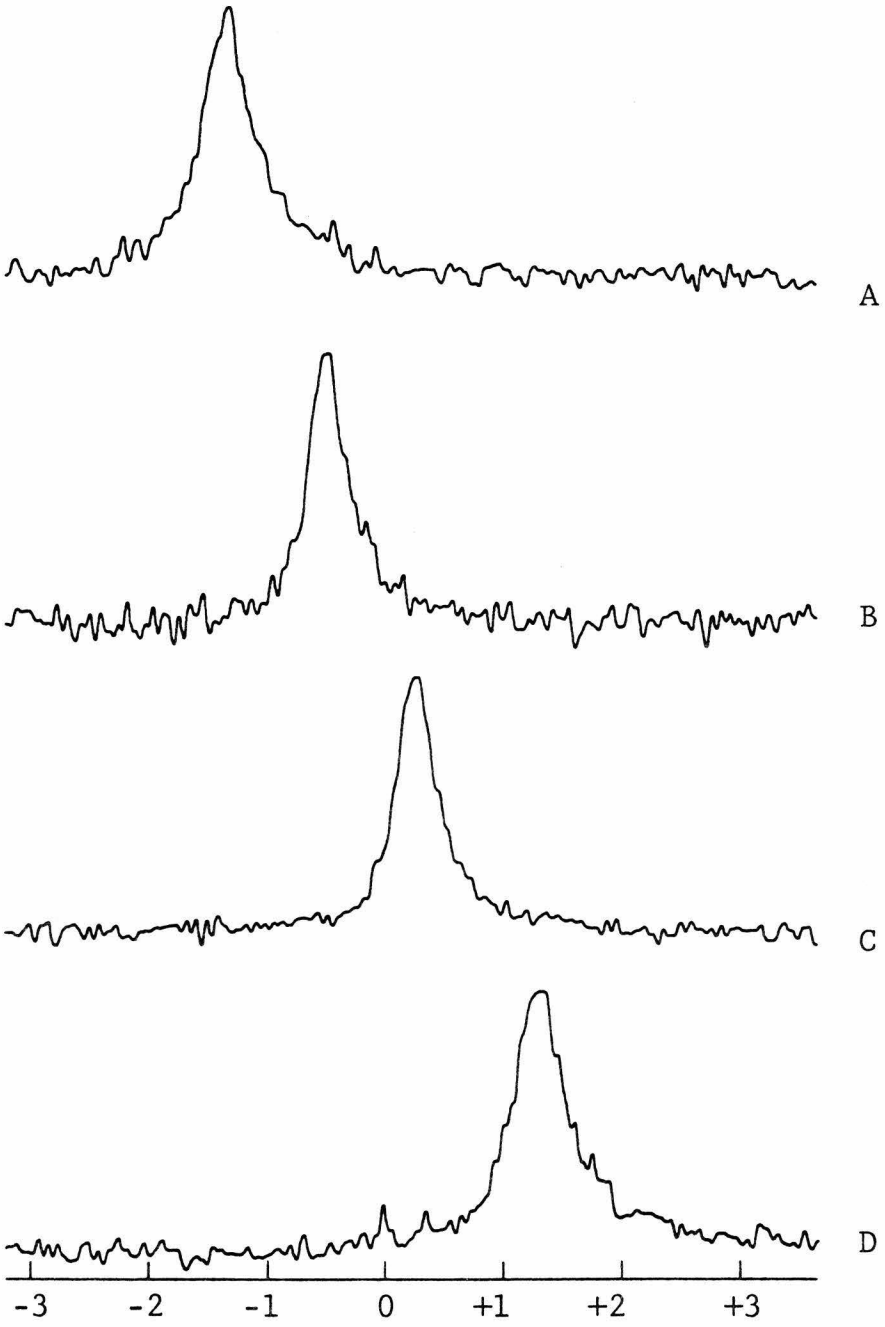

A
B
C
D
-3
-2
-1
0
+1
+2
+3
ppm from 85% H_3PO_4

Figure 16

^{31}P NMR spectra of 3.10 mM TMAPP plus 3.68 mM T15 Fab' at different pH values: (A) pH 8.69 (B) pH 6.36 (C) pH 5.60 (D) pH 4.35. The large signal represents protein-bound TMAPP whereas the small, broader signal is due to residual protein-bound PC.

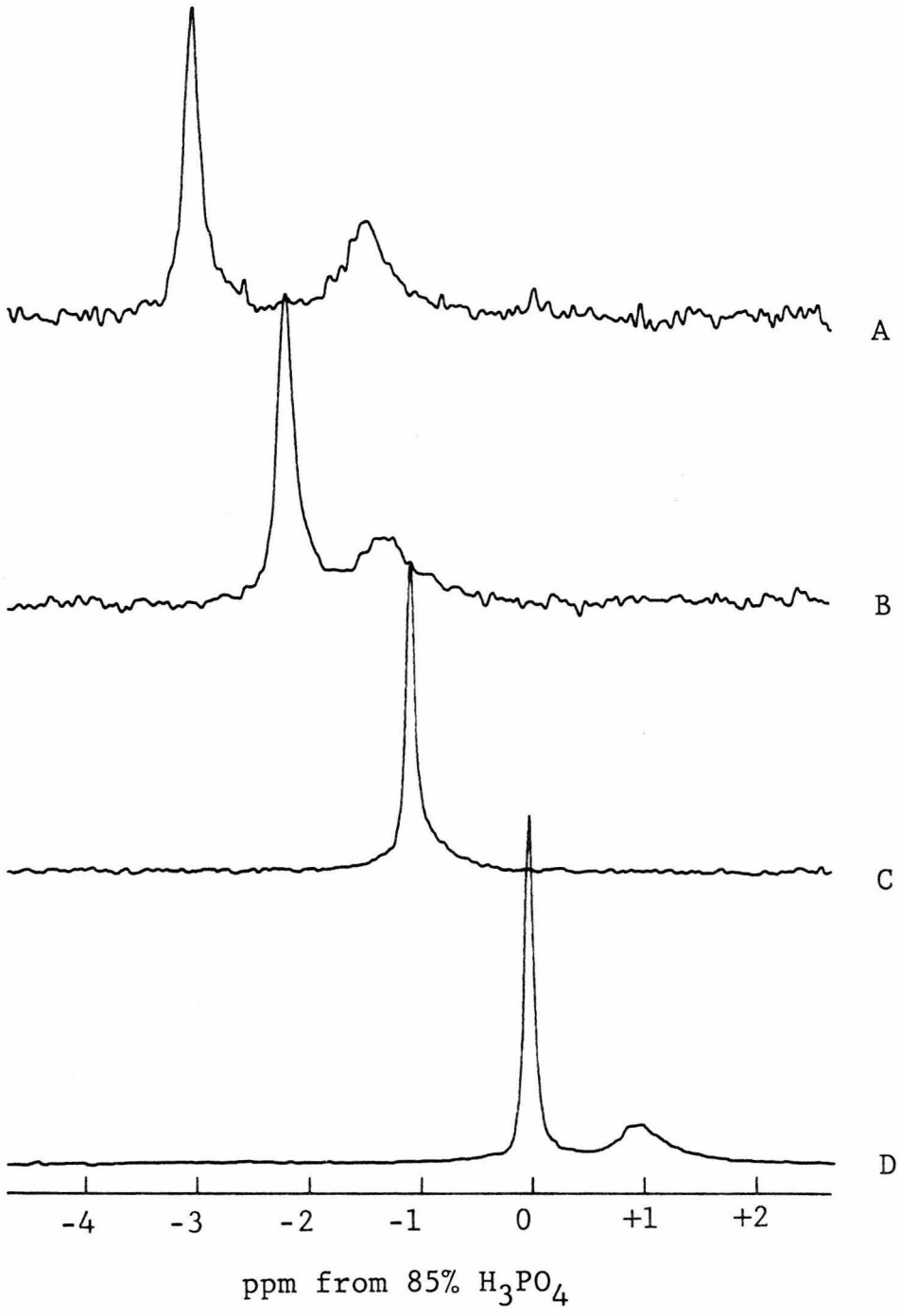
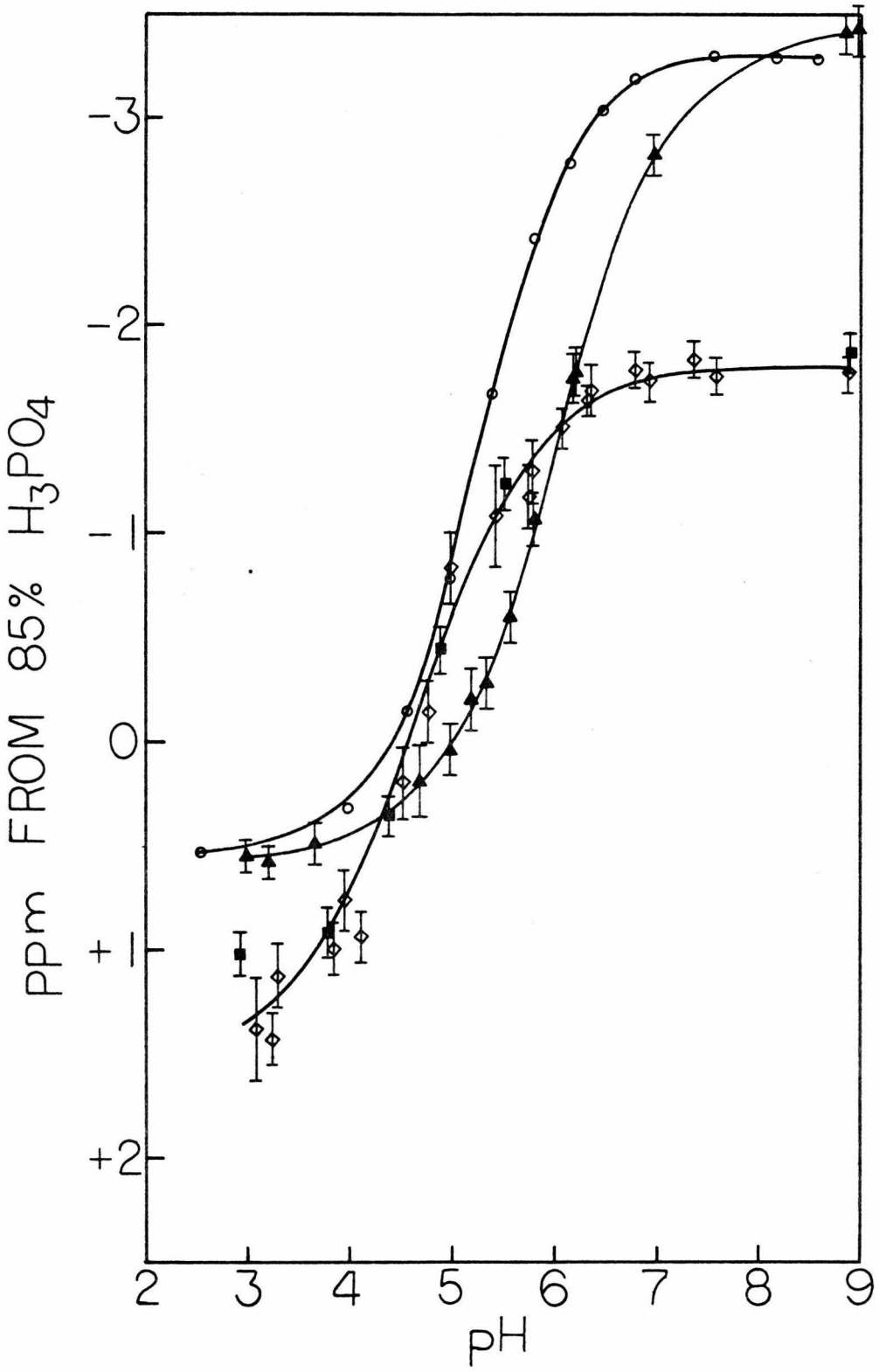

A
B
C
D
-4
-3
-2
-1
0
+1
+2
ppm from 85% H_3PO_4

Figure 17

^{31}P NMR titration curves of PC bound to M603 (■), W3207 (◇), M167 (▲) and free in solution (○). Upfield shifts correspond to an increase in offset (ppm). Spectra of bound hapten were obtained under conditions of protein excess. Offsets of the free hapten were measured in separate experiments using protein-free samples. All NMR titrations were performed in borate-cacodylate buffered saline at 25°C. Errors were estimated from the quality of the spectra and represent the maximum uncertainty in peak position.


PPm FROM 85% H_3PO_4
-3
-2
-1
0
+1
+2
2
3
4
5
6
7
8
9
pH

3.9 ppm and titrates with a pK_2 of 5.3 ± 0.1. Titration of the hapten bound either to M603 or W3207 occurs with a decreased pK of 4.7 ± 0.2. Within experimental error, PC bound either to M603 or W3207 experiences the same chemical shift over the range pH 4-9, although a small difference may exist at the lower pH limit. The total shift caused by protonation of PC bound to W3207 is ∿ 3.5 ppm which is about 0.4 ppm less than for protonation of the free hapten. At pH > 7.5 the position of the ^{31}P signal of PC bound to W3207 is shifted upfield by 1.5 ppm relative to the signal for the free hapten; at pH < 2 the analogous difference extrapolates to 1.0-1.3 ppm upfield.

After completion of the work described in this thesis, Gettins et al. (1977) published a report also describing ^{31}P NMR studies of PC binding to M603. Although differing somewhat in experimental detail, their results with respect to chemical shift and pK changes as well as exchange rates are in essential agreement with those described in this report.

The ^{31}P titration curve of PC bound to T15, which is shown in Figure 18, is identical in all respects to those obtained for M603 and W3207 (Figure 17).

Figure 18

^{31}P NMR titration curves of PC bound to T15 (▽), M511 (●) and free in solution (○). For further details, see Figure 17.

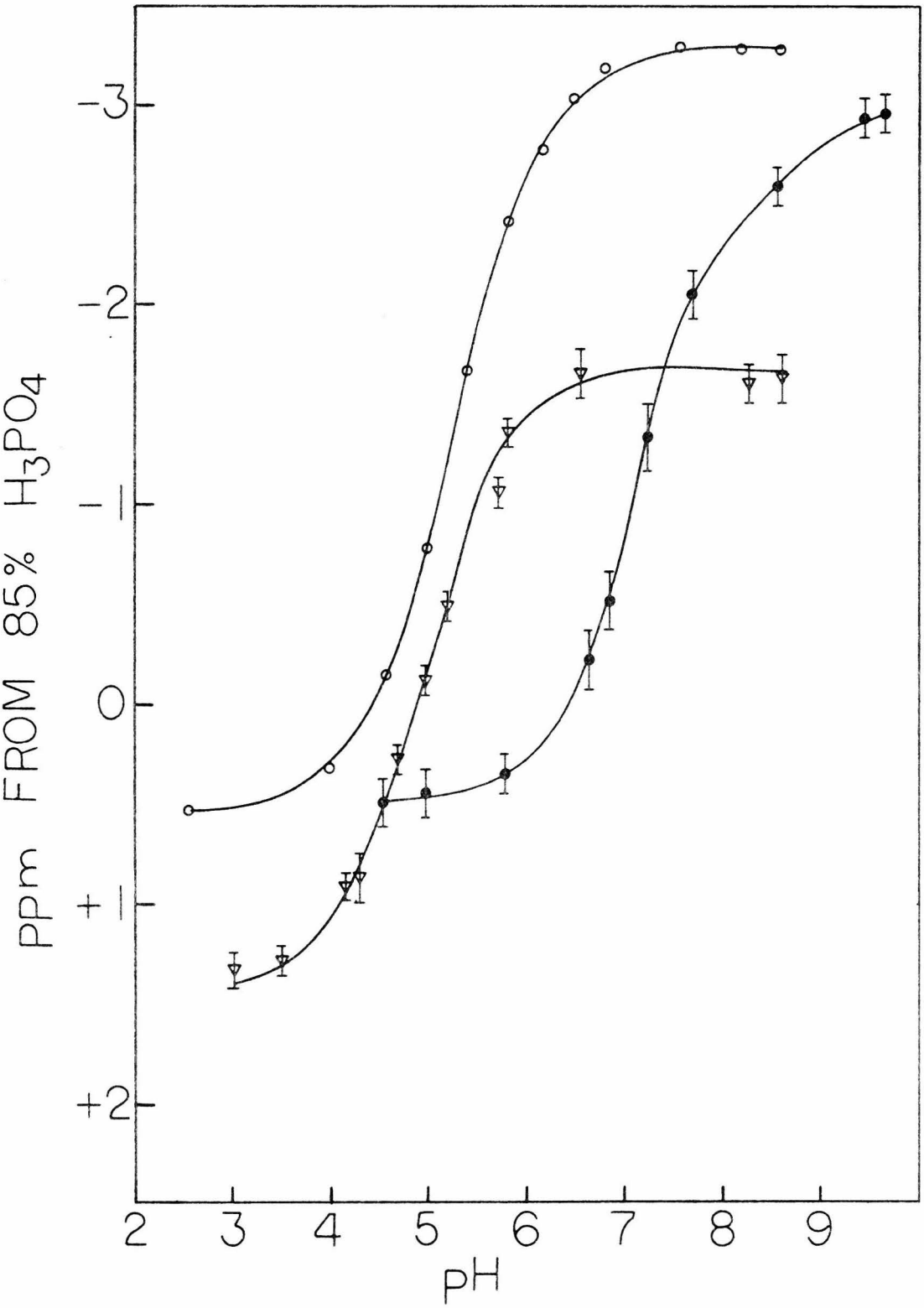
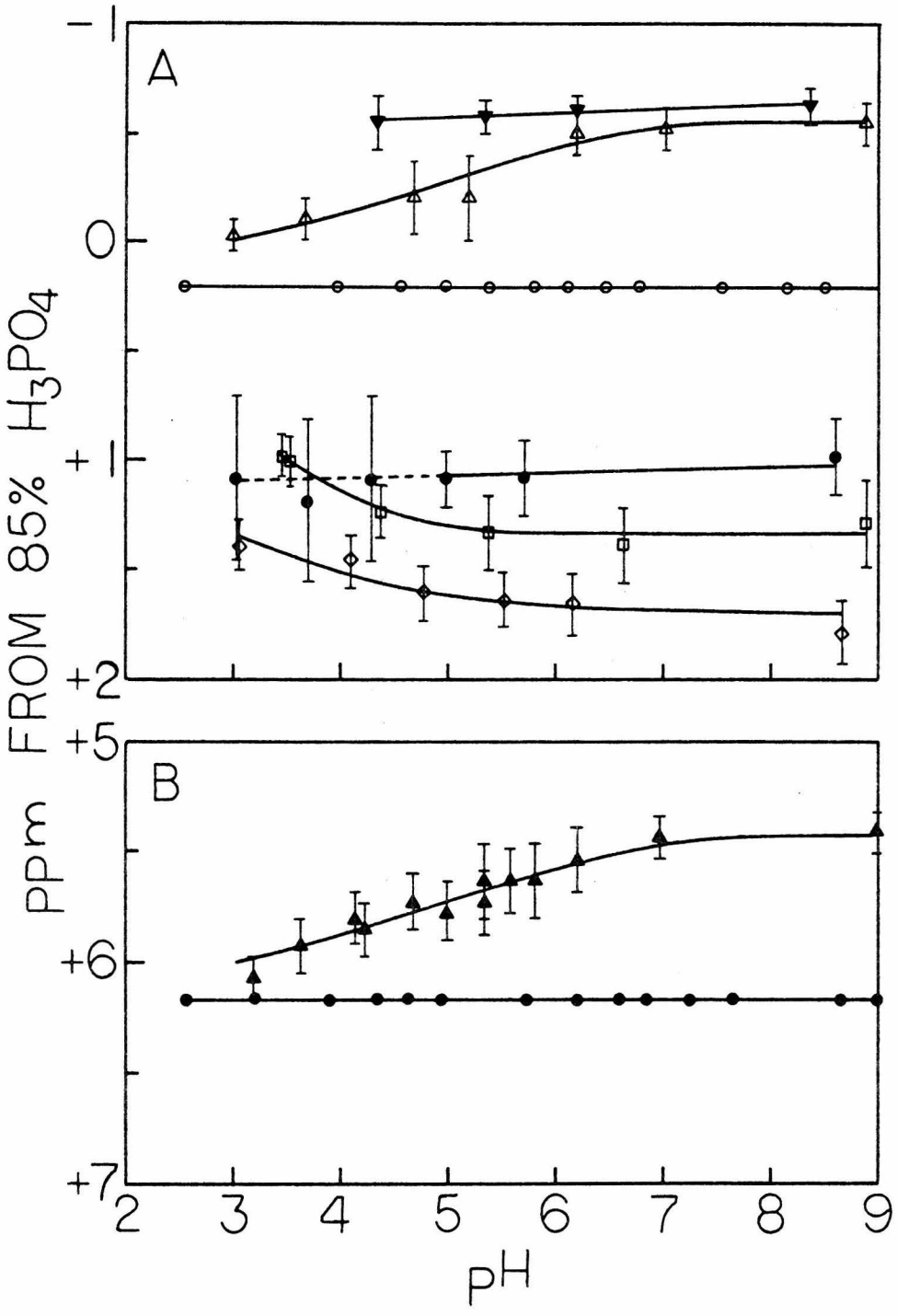

ppm FROM 85% H_3PO_4
-3
-2
-1
0
+1
+2
2
3
4
5
6
7
8
9
pH

Figure 17 shows the titration results for PC bound to M167. In this case, the bound PC titrates with an increased pK of 6.0 ± 0.1 and shows only minimal changes in chemical shift on binding at the two pH extremes. The titration curve of PC bound to M511 is very similar (Figure 18) except that in this case the pK of the bound hapten is increased to 7.3 ± 0.2.

Figure 19A illustrates the pH dependence of the ^{31}P resonance of the hapten GPC bound to all five proteins. The signal for GPC bound to M603, W3207 and T15 occurs upfield of the position of the free hapten which, itself, does not titrate in the region pH 2-9 (pK_a < 1.0). However, the amount of upfield shift at neutral pH is different for each protein and occurs in the order W3207 = 1.5 ppm > M603 = 1.2 ppm > T15 = 0.9 ppm. In addition, the signal of GPC bound to M603 and W3207 progressively shifts downfield upon lowering the pH below 6 whereas that of GPC bound to T15 appears not to shift although in this case the experimental uncertainty in the peak position at low pH is rather high. The ^{31}P signal of GPC experiences an 0.8 ppm downfield shift upon binding to M511 and M167 at pH > 7 and, in the case of M167, this shift

Figure 19

pH-dependence of the ^{31}P resonance of the diester haptens GPC and NPPC. (A) pH-dependence of GPC free in solution (○) and bound to M603 (□), W3207 (◇), T15 (●), M167 (△), M511 (▼). (B) pH-dependence of NPPC free in solution (●) and bound to M167 (▲). For further details, see Figure 17.


A
B
−1
0
+1
+2
+5
+6
+7
PPm FROM 85% H_3PO_4
2
3
4
5
6
7
8
9
pH

decreases upon lowering the pH.

The titration behaviour of the hapten NPPC, both free in solution and bound to M167 is shown in Figure 19B. For either GPC or NPPC, the difference in chemical shift for the hapten when free in solution and when bound to M167 is identical at any given pH over the entire pH range observed.

The ^{31}P signal of TMAPP titrates with a pK of 5.6 ± 0.1 and a total chemical shift of 3.6 ppm upfield on addition of a single proton. Figure 20 shows that binding of TMAPP to M603 or to W3207 causes only a slight perturbation in the magnetic environment of the ^{31}P nucleus of this hapten. The titration behaviour of TMAPP bound to T15 is similar except that at pH > 7 the ^{31}P resonance occurs slightly further upfield (Figure 21). For all three antibodies the pK of bound TMAPP is raised slightly from its value in solution.

Figure 20 also shows the pH dependence of TMAPP bound to M167. In this case the pK of the bound hapten is raised to 6.3 ± 0.1 and the changes in chemical shift on binding virtually parallel the behaviour of PC bound to M167 over the entire pH range.

Figure 20

^{31}P NMR titration curves of TMAPP bound to M603 (■), W3207 (◇), M167 (▲) and free in solution (○). For further details, see Figure 17.

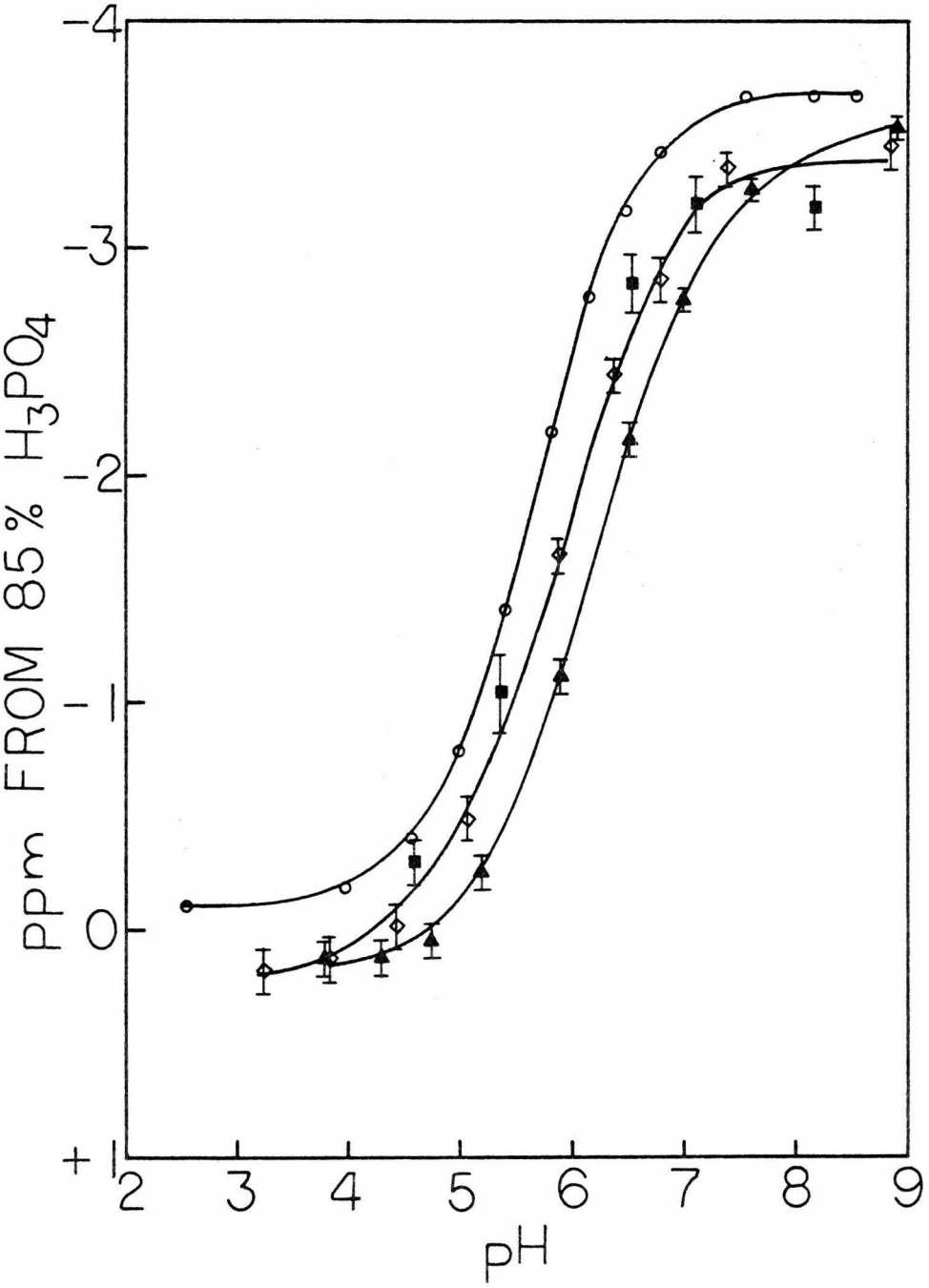
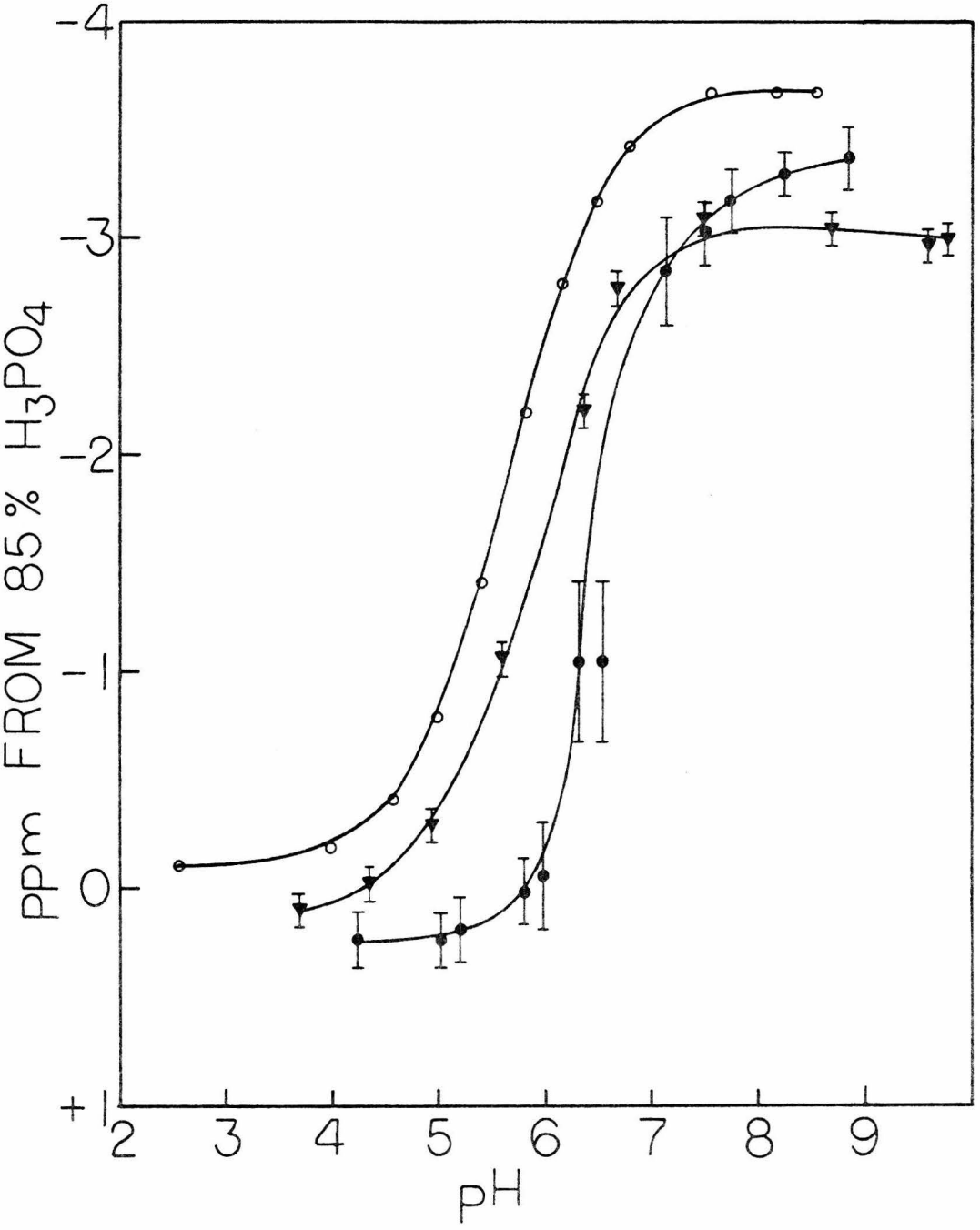

-4
-3
-2
-1
0
+1
PPm FROM 85% H_3PO_4
2
3
4
5
6
7
8
9
pH

Figure 21

^{31}P NMR titration curves of TMAPP bound to T15 (▼), M511 (●) and free in solution (○). As explained in the text, the curve for M511-bound TMAPP likely does not represent the true positions of the bound hapten. For further details, see Figure 17.


-4
-3
-2
-1
0
+1
ppm FROM 85% H_3PO_4
2
3
4
5
6
7
8
9
pH

The titration curve of TMAPP bound to M511 (Figure 21) is steeper than that of the free hapten. A likely explanation is that the data points at high pH do not represent the true position of the bound peaks, because the hapten is in fast exchange and the binding affinity is likely to be low enough so that a significant portion of the hapten is not bound to antibody. A detailed analysis is not possible because the affinity of M511 for TMAPP is not known as a function of pH. Thus, although the titration curve of TMAPP fully bound to M511 cannot be ascertained from the data, it is conceivable that its pK is increased by 2 units, as was observed upon binding of PC to M511. Both M167 and M511 might therefore perturb the environment of the phosphate group of the haptens PC and TMAPP to a similar degree.

Table V summarizes the pK changes of PC and TMAPP upon binding to the various immunoglobulins.

Table V

pKs of free and antibody-bound haptens

	PC	TMAPP
pK	5.3 ± 0.1	5.6 ± 0.1
pK when bound to W3207, M603, T15	4.7 ± 0.2	5.9 ± 0.1
ΔpK	-0.6	+0.3
pK when bound to M167	6.0 ± 0.1	6.3 ± 0.1
ΔpK	+0.7	+0.7
pK when bound to M511	7.3 ± 0.2	6.6[a]
ΔpK	+2.0	+1.0

[a]This value is likely to be inaccurate. See discussion in Results.

DISCUSSION

pH Dependence of Binding Constants

The data in Figures 10 to 13 illustrate that, though individual differences in the affinities of M603, W3207, T15 and M167 for PC and GPC do exist, there are strong similarities among M603, W3207 and T15. Accordingly, we shall first examine possible origins of the similar behaviour of these three immunoglobulins and then discuss explanations for the affinities of M167.

W3207 has a higher affinity for PC than for GPC over the entire pH range although the affinities for the haptens converge with decreasing pH. At pH 7, the phosphate group of PC exists as a dianion and acquires a proton with a pK of 5.3 ± 0.1. In contrast, the phosphate region of GPC remains a monoanion. Thus the larger affinity of W3207 for PC than for GPC most likely arises from an additional favorable ionic interaction between the protein and the dianionic phosphate of PC; this advantage is lost after protonation of PC below pH 5.3 where the affinities for the two haptens converge. One can even explain the differences from this source on a semiquantitative

basis. The ratio of the affinity for PC to GPC at pH 7.3 is 3.9. If one assumes that this represents the maximum stabilization due to interaction of the protein with a dianionic, as contrasted to a mono-anionic, phosphate, then the ratio of the affinity for PC relative to that for GPC at any pH correlates with the degree of ionization of PC bound to W3207 as monitored by ^{31}P NMR (Figure 17). A similar situation is observed in the cases of M603 and T15.

Since the NMR evidence (to be discussed later) shows that Arg 52H interacts similarly with both PC and GPC, the larger affinity for PC probably reflects the influence of Lys 54H, the only other cationic residue in proximity to the phosphate. The fact that Lys 54H is close to the outside of the binding cavity (Padlan et al., 1976), and has been shown by chemical modification experiments not to be in direct contact with PC (Grossberg et al., 1974), readily explains the relatively small effect that Lys 54H exerts on the phosphate group of bound hapten.

Since GPC does not itself change its state of ionization between pH 2-10, the dramatic decrease in the absolute affinity of W3207, M603 and T15 for GPC as the pH is lowered from 7.3 to 3.4 must reflect

ionization changes in the protein, most likely residues with carboxylic acid groups such as Glu 35H and Glu 59H which interact with the positively-charged quaternary nitrogen of the hapten (Padlan et al., 1976; Segal et al., 1974). The crucial importance of such an interaction has been verified by chemical modification experiments on a related protein, H8 (Grossberg et al., 1974), and by the observed inability of any compound lacking the quaternary nitrogen to bind effectively to these proteins (Leon and Young, 1971; Krausz et al., 1976). The decrease in absolute affinity for PC as the pH is lowered reflects both protonation of the protein carboxylates and monoprotonation of the phosphate group of the hapten. Quantitatively, protonation of the phosphate group of PC accounts for $\sim$ 35% of the decrease in affinity of W3207 observed on lowering the pH from 7.3 to 3.0.

The decrease in affinity for both PC and GPC with decreasing pH is considerably steeper for M603 than for W3207 and T15 suggesting significant differences in the interactions between these proteins and the trimethylammonium region of bound hapten. In

agreement with this observation, differing environments for the trimethylammonium group of bound phosphoryl[methyl-^{13}C]choline have been observed by ^{13}C NMR (Goetze and Richards, 1977a). As has been previously suggested (Padlan et al., 1976), the substitution Asp 99H→Asn, found only in M603, may impart unique characteristics to the choline subsite of this protein and thereby explain its lowered affinities relative to W3207 and T15 for PC and GPC.

For three of the proteins studied (W3207, M603 and M167) a decrease in the affinity for PC and GPC on raising the pH above 7.3 is observed. In each case, the ratio of the affinity for PC relative to that for GPC remains constant in this pH region, indicating that both haptens are equally affected. We shall subsequently discuss the hydrogen bonds between antibody and hapten; as the NMR evidence suggests that they are not altered in this pH range, we cannot ascribe this decrease in affinity to ionization of Arg 52H which is the cationic residue in closest proximity to bound PC. Possibly, conformational changes in the binding pocket result from changes in the state of ionization of more distant groups which occur in this pH range.

T15 is unique among these antibodies in that its affinity for PC and GPC is pH independent in the range 7-10. This behaviour is in agreement with a previous study on binding by T15 using fluorescence techniques (Pollet and Edelhoch, 1973). Since the important binding interactions between antibody and hapten are likely to be highly similar among T15, M603 and W3207, this further suggests that the anomalous pH independence of T15 in this case likely reflects more distant changes in the binding site structure.

Padlan et al. (1976) have suggested that the presence of Asp 100aH in M167 and M511 may account for the unique binding specificities of these two proteins. This residue lies between the positive nitrogen and negative phosphate of the hapten and may interact with both. Such an interaction would enhance binding of choline but simultaneously depress binding of PC and such effects have been observed (Goetze and Richards, 1977a; Leon and Young, 1971). The presence of Asp 100aH can also explain our binding data for M167 (Figure 13). The increase in PC affinity from pH 3.4 to 5.5 is due to ionization of Glu 35H, Glu 59H and Asp 100aH; all three of these residues, when ionized, help to stabilize the positive charge on the

quaternary nitrogen of the hapten. The 2.3-fold drop in affinity from pH 5.5 to 7.5 results from the ionization of the phosphate of PC which occurs with a pK of 6.0 when this hapten is bound to M167 (Figure 13). The additional negative charge on the phosphate is repelled by the negative charge on Asp 100aH and the affinity for PC is accordingly reduced. The further decrease in affinity at pH > 8 is probably caused by the ionization of another, presently unknown residue(s).

The results for binding of GPC to M167, though qualitatively similar to those observed for binding to M603, W3207, and T15 also agree with this model. Since GPC does not itself ionize in the pH range in question, any repulsion between the monoanionic phosphate and Asp 100aH should depend only on the state of ionization of Asp 100aH. This effect is therefore included in the ascending line of the curve from pH 3.4 to 7.3 and is not observed as a separate titration. Further support for this argument comes from the observation that M511, which also contains Asp 100aH, similarly binds GPC with a higher affinity than PC at pH 8 (Goetze and Richards,

1977a).

The low binding affinity of M511 for PC prevents an accurate determination of the pH-dependent binding behaviour in this case. However, the similarities between M511 and M167 in the environments of bound PC as determined by NMR (to be discussed later), the observation by NMR that the affinities of M511 for PC and TMAPP appear to be maximal below neutral pH, and the observation that M511 binds GPC with a higher affinity than PC at pH 8 all suggest that M511 would show a similar dependence of binding affinity on pH as does M167.

M167 shows another difference from M603, W3207 and T15 in that, with M167, GPC and monoprotonated PC do not bind with equal affinity. At pH 3.4, where bound PC is essentially monoprotonated as determined by ^{31}P NMR (Figure 17), the affinity of M167 for PC is 3.8 times greater than that for GPC. This ratio varies only slightly from pH 3.8 to 5.5. Accordingly, some unfavorable steric interactions are likely to exist between residues in the binding pocket of M167 and the glycerol group of GPC.

NMR Titrations of PC and GPC Bound to M603, W3207 and T15

At pH $\geq$ 7.5, the ^{31}P resonance of PC bound to M603, W3207 and T15 occurs 1.5 ppm upfield of that of the free hapten (Figure 17). We have attributed this shift to the formation of two specific hydrogen bonds from Tyr 33H and Arg 52H of the antibody to the phosphate oxygens of bound hapten (Goetze and Richards, 1977b). This chemical shift change experienced by hapten on binding remains constant in the region pH 7.5-9 indicating that Arg 52H does not ionize in this range and therefore cannot be the cause of the observed decrease in binding affinity at the high pH limit observed with M603 and W3207. The similarities in the titration curves of PC bound to these three proteins suggests that the specific interactions between the phosphate region of the hapten and the phosphate subsites of W3207 and T15 are essentially identical to those observed in the M603-PC complex for which the 3-dimensional structure is known (Segal et al. 1974).

The pK of PC is lowered from 5.3 in solution to 4.7 when bound to each of these immunoglobulins

(M603, W3207, T15). This decrease results from the ionic influence of the positive residues which are located in the phosphate subsite — Arg 52H which forms a hydrogen bond with bound hapten and, to a lesser extent, the more distant Lys 54H. This observed decrease in pK is of only moderate size when compared to that observed in analogous biological systems. For example, the pK of the ^{31}P resonance of the 2'-phosphate of $NADP^+$ and NADPH is lowered by more than 3 units upon complexing with dihydrofolate reductase (Feeney et al., 1975).

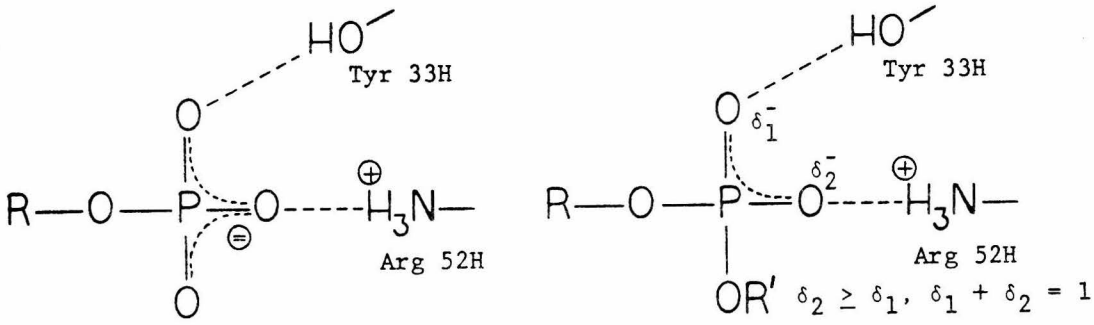
The chemical shift changes upon binding of GPC to these three immunoglobulins at pH $\geq$ 7.5 (Figure 19A), although unique for each protein, are roughly comparable to those observed upon PC binding (Figures 17 and 18). The simplest explanation is that the hydrogen bonds which exist between antibody and bound PC also exist between antibody and bound GPC. Because of the topography of the binding site, this implies that the choline portion of both haptens likewise binds in an identical manner and, furthermore, that, for bound GPC, the phosphate oxygen atom which is seen to be uncoordinated to any

protein residue in the crystal structure of the M603-PC complex (Segal et al., 1974) is esterified.

As the pH is lowered below 6, the ^{31}P resonance of GPC bound to M603 and W3207 shifts slightly downfield. Because of the pH at which this occurs, it is unlikely that this shift is caused by titration of groups directly involved in hydrogen bonding to hapten but may instead reflect a small conformational rearrangement within the binding pocket. Any such change, even if very small, could influence the ^{31}P chemical shift which is extremely sensitive to changes in the O-P-O angle (Blackburn et al., 1971; Gorenstein and Kar, 1975). The somewhat anomalous shift for GPC bound to T15 at high pH might similarly arise from such a distortion. This explanation seems preferable to one which would invoke fundamentally different interactions between each immunoglobulin and the phosphate group of GPC since the thermodynamic binding affinities and the conservation of residues which contact hapten argue for a high degree of similarity in the interactions of these antibodies with similar haptens (Padlan et al., 1976).

The observation that the chemical shifts upon

binding of monoanionic PC and of dianionic PC are similar and that GPC and monoanionic PC bind with equal affinity to M603, W3207 and T15 argues that, at low pH, both these haptens are involved in identical hydrogen bonding interactions. The postulated charge distribution of the phosphate group of these two haptens in the binding pocket of these three antibodies is schematically illustrated below:



PC at pH $\geq$ 7 PC at pH $\leq$ 3 or GPC

$R = (CH_3)_3\overset{+}{N}CH_2CH_2-$

R' = H for PC, $-CH_2CHOHCH_2OH$ for GPC

In summary, when dianionic PC binds to M603, W3207 or T15 at neutral pH, it experiences a ^{31}P upfield shift of 1.5 ppm due to formation of hydrogen bonds with Tyr 33H and Arg 52H. Similar hydrogen bonds may exist when GPC or monoanionic PC (at low pH) bind. The pK for the second ionization of PC bound to antibody is decreased by 0.6 units from that of PC in solution due to the electrostatic complementarity between the negative phosphate group of the hapten and its positively-charged binding subsites on the proteins.

NMR Titrations of PC, GPC and NPPC Bound to M167 and M511

Figure 17 shows that PC when bound to M167 has its second pK_a raised to 6.0 from 5.3 when in solution. This stands in sharp contrast to binding of PC to M603, W3207 and T15 which lowers the second pK_a to 4.7. This unprecedented result with M167 suggests that the phosphate subsite has an electrostatic character more negative than that which PC experiences in solution which is likely caused by the presence of Asp 100aH, the only apparent, anionic group in close proximity to the phosphate (Padlan et al., 1976). In

analogy to the known structure of M603, one would expect that Arg 52H and, to a lesser degree, Lys 54H and Arg 58aH might all be positioned close enough to the phosphate group of the hapten to perturb its ionization. The fact that the presence of a single anionic residue, Asp 100aH, can apparently outweigh the possible influence of several cationic groups on the phosphate ionization, leads us to suggest that the architecture of the phosphate subsite of M167 is appreciably different from those of M603, W3207 and T15. Indeed, M167 has several potentially important substitutions in this region relative to M603 (for example, Asp 56H→His, Thr 58aH→Arg, Trp 104aH→Gly) and these could so alter the structure of the phosphate subsite that Arg 52H, Lys 54H and Arg 58aH are moved sufficiently far from the bound phosphate that the hydrogen bonding and electrostatic interactions characteristic of the M603-PC complex are not possible with M167. However, in spite of the negative character of the phosphate subsite of M167, as evidenced by the raised pK of bound PC and likely due to the presence of Asp 100aH, a <u>net</u> favorable thermodynamic interaction between protein and the phosphate group of the hapten

must exist because, at pH 8, M167 exhibits an affinity for PC that is six times that for choline (Goetze and Richards, 1977a). Thus the situation may be one in which the favorable energetic consequences of a hydrogen bond between Tyr 33H and hapten outweigh the repulsive interactions between the negative phosphate and a negative binding subsite which has been created by the intrusion of Asp 100aH into, and the ejection of Arg 52H from, the immediate phosphate vicinity.

The titration curve of PC bound to M511 (Figure 18) shows that the pK of bound hapten is now raised to 7.3. Thus, this antibody creates a phosphate subsite even more electronegative than that of M167. An inspection of the heavy chain amino acid sequence of M511 reveals that this antibody is unique in having an Asp residue at position 53H. Thus, M511 has an anionic side chain between the important Arg 52H and Lys 54H and, since M511 also contains Asp 100aH, this antibody may contain two Asp residues projecting into the phosphate subsite. The net effect is to create a highly electronegative environment. Nevertheless, M511 shows a small net

favorable thermodynamic interaction with the hapten phosphate group since binding affinity for PC is still 3 times higher than for choline at pH 8 (Chapter 3).

In view of the correlation among these antibodies between the electrostatic character of the phosphate subsite (as determined by NMR) and their binding affinities for choline and GPC relative to PC, varying the electrostatic character of this region of the binding pocket appears to be a highly effective method of achieving a different spectrum of antigen specificities. For example, M167 and M511 are expected to exhibit high affinity for antigens containing the choline determinant whereas M603, W3207 and T15 would be effective only against those containing PC determinants. Furthermore, these distinctions in fine specificity can result from as small a structural change as a single amino acid substitution.

The absence of an appreciable ^{31}P chemical shift change on binding of dianionic and monoanionic PC is common to M167 and M511. This is likely due to a weakening of the hydrogen bonds to the phosphate group as well as possibly the ionic influence of Asp 100aH.

In spite of the large difference in the absolute chemical shift between the signals of the free haptens GPC and NPPC, both haptens experience changes in chemical shift on binding to M167 which are identical at any given pH over the entire pH range studied (Figure 19). This implies that the binding site of M167 perturbs the phosphorous environment of both haptens to a very similar degree and that the phosphate group of both haptens has essentially identical interactions with binding site residues. The specific antibody interaction with the PC portion is thus not significantly affected by such diverse substituents attached to the phosphate as glycerol or p-nitrophenyl. Moreover, as shown in Table IV, each of W3207, M603 and M167 exhibits similar affinities for both GPC and NPPC which suggests that there is little, if any, specific interaction between these antibodies and the glycerol or p-nitrophenyl substituents.

However, with M167 and M511 the phosphate environment of PC differs appreciably from that for the diester haptens (GPC and NPPC) since these two classes of haptens experience significantly different changes in the chemical shifts of their ^{31}P resonances on binding. Possibly Asp 100aH (and also Asp 53H in

the case of M511) affects the phosphate portion of these two groups of haptens differently; or there may be a non-specific steric crowding of the glycerol and p-nitrophenyl substituents of GPC and NPPC. The present evidence does not discriminate between these, or other possibilities. However, affinity labelling experiments using the hapten, p-diazoniumphenyl-phosphorylcholine (Chesebro et al., 1973) have shown extensive differences in the labelling pattern between many of the PC-binding myeloma proteins; these differences suggest that the outer edges of the binding pockets, with which the diazonium group presumably react, may have appreciably different structures. Thus, whereas M603, W3207 and T15 can freely accommodate the bulky glycerol and p-nitrophenyl substituents, M167 and M511, for steric reasons, cannot Such repulsive interactions have been previously discussed to account for the pH dependence of the binding affinities of M167. With decreasing pH, there is a decrease in the downfield shift of GPC and NPPC; this suggests that there may be a reduction in this non-specific repulsion with decreasing pH.

Binding of TMAPP

The requirements on the binding sites of these immunoglobulins for the optimal relationship between the phosphate and trimethylammonium groups of the hapten were probed by studying the binding of TMAPP in which these groups are separated by three methylene groups rather than by two as in PC itself. Table IV shows that TMAPP binds to M167 and M511 with an affinity nearly equal to that of PC but binds much less well to M603, W3207 and T15.

Figures 20 and 21 show that the ^{31}P chemical shift of TMAPP bound to M603, W3207 and T15 varies only slightly from the chemical shift of the free hapten. Moreover, the titration behaviour of bound TMAPP is completely different from that of bound PC. For example, the pK of TMAPP bound to W3207 is 0.3 units higher than that of the free hapten whereas the pK of PC bound to W3207 is lower by 0.6 units. Thus, the ionic influence of W3207 is quite different on the phosphate of bound TMAPP than it is on the phosphate of bound PC. Moreover, the 1.5 ppm upfield shift attributed to hydrogen bonding between protein and bound PC and GPC is not observed with bound TMAPP. All these observations suggest that the phosphate

group of TMAPP is not able to interact correctly with the residues of the phosphate subsites in W3207, M603 and T15. The structure of the M603-PC complex shows extensive Van der Waal's contacts of the methyl and methylene groups of the hapten with Tyr 33H, Trp 104aH and residue 96 of the light chain (Padlan et al., 1976; Segal et al., 1974). These residues define the width of the binding cavity and discourage accommodation of much structural variation in the complementary region of the hapten (see Figure 1). PC has been shown to bind to M603 with the trimethylammonium portion of the hapten projecting into the binding cavity. Therefore, TMAPP probably also binds to these antibodies in such a fashion as to preserve the important interactions between the positive, quaternary nitrogen and negative, acidic groups on the antibody. Such an orientation of a hapten with three methylene groups forces the phosphate out of the binding pocket past the phosphate binding subsite. This destroys the energetically favorable interactions of the phosphate group with antibody residues and probably accounts for the 13-40 fold drop in affinity for TMAPP relative to PC.

The "looser" binding of the phosphate group of TMAPP compared to that of PC is confirmed by the decreased linewidth of the ^{31}P resonance of TMAPP. Thus, whereas the intrinsic linewidth of the ^{31}P signal of PC bound to M603, W3207 and T15 is $\sim$ 20-30 Hz, that of TMAPP bound to M603 and W3207 is $\sim$ 15 Hz and that of TMAPP bound to T15 is only 5-10 Hz. Thus, there appears to be a subtle difference even between the interaction of T15 as opposed to M603/W3207 with TMAPP and this correlates with the weaker binding of this hapten by T15 (Table IV).

In contrast to the difference between PC and TMAPP interacting with M603, W3207 and T15, there are many parallels between PC and TMAPP binding to M167. The ^{31}P NMR titration curve of TMAPP bound to M167 (Figure 20) bears virtually the same relationship to that of free TMAPP as the titration curve of PC bound to M167 does to that of free PC (Figure 17). In both cases the pK change on binding is + 0.7 units and the lack of an appreciable change in chemical shift on binding at both high and low pH limits observed for TMAPP is very similar to the behaviour of PC. These parallels suggest that, in contrast to M603, W3207 and T15, the binding pocket of M167 is able to accommodate TMAPP so that this larger hapten is able to bind

with its phosphate group located in an essentially normal position within the phosphate subsite of the protein (as is the case also for PC and GPC). This accommodation may be allowed by the substitution Trp 104aH→Gly which creates a wider binding cavity thereby making it possible for the extra methylene group of TMAPP to "buckle out" into this extra space. In this way, the net N-P distance in bound TMAPP can remain close to that for bound PC. Such a distortion has the advantage of retaining the favorable interactions of both the phosphate and trimethylammonium groups of the hapten with the complementary antibody subsites so that the net affinity for TMAPP is only lower by a factor of two than that for PC.

For reasons discussed previously (see Results) it has not been possible to precisely determine the ^{31}P NMR titration curve of TMAPP bound to M511. It is conceivable from the data however that, as is true for M167, the titration curve of TMAPP is perturbed in a similar manner to that of PC. As M511 does not contain the substitution Trp 104aH→Gly, the possible ability of this antibody to interact similarly with the phosphate group of both haptens is not due to a wider binding cavity but quite likely a direct result

of the extreme weakness of the interactions with the phosphate. Thus, the fact that M511 binds PC, TMAPP and choline with nearly identical binding affinities is evidence for an unimportant role for the phosphate group in contributing to the total binding energy and in this case there would therefore be no energetic advantage to attempting to accommodate the phosphate group of TMAPP within the phosphate subsite.

Comparison of the Binding Sites

The data in this report allow a molecular explanation for some of the specificity differences among these antibodies. The arrangement of the important charged residues in the binding cavities of these immunoglobulins creates sites with high affinities for diester PC analogues at physiological pH. In the case of GPC binding, the protonation of carboxylate groups on the antibodies is the sole cause of the large decrease in affinity with decreasing pH. The exact pH-dependence of this decrease varies among the four proteins examined and indicates that the precise arrangement of the anionic residues which interact with the trimethylammonium region of the bound hapten differs among these antibodies. Our studies provide

no evidence that these antibodies specifically recognize an antigenic determinant larger than PC.

Although M603, W3207 and T15 exhibit a 4-5 fold increase in affinity for PC relative to GPC, the presence of Asp 100aH in the binding cavities of M167 and M511 results in a pH-dependent repulsion between these proteins and PC such that, at physiologicl pH, PC which is a dianion, is bound with a lower affinity than GPC, which is a monoanion.

The two specific hydrogen bonds formed between antibody and the phosphate oxygens in the M603-PC complex (Segal et al., 1974) appear to be independent of pH in the range 3-9 for M603, W3207 and T15. These hydrogen bonds may also be intact in the complexes with GPC. These observations give some insight into the manner in which larger, diester haptens are situated in the binding pocket, since the favorable energetics of the two hydrogen bonds apparently dictate that the third remaining phosphate oxygen may be esterified or protonated.

In contrast to the electropositive phosphate subsites of M603, W3207 and T15, those of M167 and M511 have a character more electronegative than that which PC experiences in solution. This indicates that

various amino acid substitutions have altered the local topography of the site such that the ionic influence of Arg 52H is no longer dominant in affecting the phosphate pK.

Studies with TMAPP show that the narrow width of the binding cavities of M603, W3207 and T15 (largely defined by Tyr 33H, Trp 104aH and residue 96L) forces the phosphate group of TMAPP to project past the phosphate binding subsite of these proteins with a significant resultant loss of binding energy and large changes in the NMR parameters of the ^{31}P signal of the bound TMAPP relative to those of bound PC or GPC. In M167, in contrast, the substitution Trp 104aH→ Gly creates a wider binding cavity which can accommodate the extra methylene group of TMAPP so that both ends of the hapten (phosphate and trimethylammonium group) can interact with the protein essentially as they do with PC and GPC.

Implications for Binding of Physiological Antigens

The phosphate subsites of M603, W3207 and T15 appear to have evolved to bind phosphate diesters, especially as relatively little provision has been made for stabilization of a second negative charge on the

phosphate. One sees this, for example, in the case of W3207 where, at neutral pH, loss of one negative charge (when the hapten is changed from PC to GPC) causes only a 4-fold drop in hapten affinity. Loss of the entire phosphate group, however, (choline vs. PC) leads to a 600-fold decrease in binding affinity (Goetze and Richards, 1977a). Such results seem teleologically reasonable for the following reasons: (i) The in vivo antigens for these antibodies in the BALB/c mouse are a variety of bacterial antigens (Potter, 1972; Potter and Leon, 1968) which generally have PC attached to some carbohydrate by a phosphodiester link (Liu and Gotschlich, 1963; Watson and Baddiley, 1974; Brundish and Baddiley, 1968). For example, immunization of BALB/c mice with PC conjugated to proteins (Lee et al., 1974) or with pneumococci R36A (Cosenza and Köhler, 1972a; 1972b) yields largely an antibody response of the T15 idiotype. This also occurs in eight other inbred mouse strains (Claflin, 1976). Thus, protein T15 seems to be a "natural" antibody in these mice against antigens containing PC with a phosphodiester link. (ii) The structure of the M603-PC complex shows that only two of the phosphate oxygens specifical-

ly interact with residues in the protein (Segal et al., 1974). Presumably, interaction with a third phosphate oxygen does not occur because, in physiological antigens, this oxygen is esterified. Only the guanidinium group of Arg 52H seems directly implicated in ionic stabilization of the phosphate group since Lys 54H is not in direct contact with PC in the closely related protein H8 (Grossberg et al., 1974). If the binding site had evolved to be maximally complementary to a dianionic phosphate, one might have expected the phosphate subsite to contain at least two cationic residues.

M167 and M511 represent immunoglobulins that, because of a few key amino acid changes, exhibit high affinity for choline; M167 also retains high PC affinity so that it is capable of cross-reacting with antigens containing both determinants. In addition, various other differences, such as the widened binding cleft of M167 and the unique choline subsite of M603, most likely further affect the differential affinities of these antibodies for various physiological antigens. The preservation of these clonotypes among many mouse strains (Claflin, 1976) argues strongly that these antibodies, because of their subtly differing

interactions with PC type antigens, may more successfully protect their host against a wider range of antigenic challenges than would be possible with a more limited diversity of anti-PC immunoglobulins.

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CHAPTER 5

LIGHT-HEAVY CHAIN INTERACTIONS

INTRODUCTION

It is now well established that the heavy (H)[1] and light (L) chains of immunoglobulins cooperate to form the antigen binding site. However, the degree of participation of the individual chains in determining a particular binding specificity is highly variable. For example, binding studies using various enzymatic fragments of MOPC 315, a mouse myeloma immunoglobulin with high affinity for 2,4-dinitrophenyl (DNP) derivatives, have shown that the V_L dimer binds 2,4-dinitrophenol with nearly the same affinity as does the intact antibody (Gavish et al., 1977). This result localizes the DNP-contacting residues on the light chain. Similarly, it was shown that the 1000-fold higher affinity of MOPC 315 for DNP-lysine over DNP-OH is the result of specific binding of the lysine residue

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[1]Abbreviations used are: H, heavy; L, light; C, constant; V, variable; HV, hypervariable; PC, phosphorylcholine; DNP, dinitrophenyl.

to heavy chain amino acids (Gavish et al., 1977). In contrast, the X-ray structure of M603 has shown that all but one of the hapten-contacting residues are from the H chain (Segal et al., 1974). In other cases, it appears that both chains contribute significantly to formation of a particular specificity (Manjula et al., 1975; Amzel et al., 1974).

L-H chain hybridization experiments have indicated that, in the large majority of cases, only the original L-H chain pairs are capable of exhibiting high antigen affinity of the parent specificity (Bridges and Little, 1971; Klinman, 1971; Edelman et al., 1963; Franek and Nezlin, 1963) although this does not exclude the possibility of heterologous hybrids exhibiting affinities for other types of antigens (haptens). The strict requirement for a specific L-H pairing, even in cases where one chain appears to dominate the binding interaction, suggests that possibly the second chain modulates the conformation of the dominant chain through a synergistic interaction. On the other hand, a variety of experiments indicate that the contributions of L and H chains to the binding interaction are purely additive

(Painter et al, 1972a, 1972b). These conflicting results have motivated the current study.

The phosphorylcholine-binding mouse myeloma immunoglobulins present a useful opportunity to investigate the role of individual chains in formation of the binding specificity. In these proteins, a highly similar H chain is paired with an L chain from any of at least three different κ subclasses (Barstad et al., 1974; Hood et al., 1976). Proteins with L chains from different subclasses exhibit differing affinities for various phosphorylcholine (PC) analogs (Leon and Young, 1971) and this has led Barstad (1975) to suggest that in these proteins the L chain serves to "fine tune" the PC specificity which is created by the H chain. In this chapter, the results of NMR binding studies (Chapters 3 and 4) and hybridization studies of PC-binding immunoglobulins are discussed with respect to the roles of L and H chains in determining antibody specificity.

MATERIALS AND METHODS

Chain Separation

Mouse ascites fluid was reduced and alkylated and PC-binding immunoglobulin immunospecifically isolated as previously described (Goetze and Richards, 1977a). The purified antibody was again reduced and alkylated under identical conditions but at a protein concentration of approximately 5 mg/ml. It was then dialyzed vs. 3 changes of a 100-fold excess of 0.1 M NH_4HCO_3 buffer and lyophilized.

Approximately 500 mg lyophilized antibody was dissolved in 25-40 ml 4.5 M urea (Ultra-Pure, Schwarz-Mann), 1 M propionic acid by gentle stirring in the cold (4^oC) for 1 hour. This solution (always less than 2% of the column volume) was chromatographed down a 5 x 110 cm Sephadex G-100-120 column equilibrated with the same solution. The chromatography was carried out at 4^oC and a flow rate of 30 ml/hr. To minimize any potential irreversible chain denaturation, the eluted light and heavy chains were each pooled and immediately dialyzed vs. 3 changes (12 hours each) of a 20-fold excess of distilled water at 4^oC.

This effectively reduced the concentration of denaturants 8000-fold. If a fourth change of the dialysis solution was attempted, the heavy chains precipitated whereas the light chains remained soluble. The isolated chains were then lyophilized and stored frozen.

Immunoglobulin Renaturation

This renaturation procedure is based on that of Bridges and Little (1971) and was the most successful of several tested. Lyophilized light chain (20 mg), heavy chain (50 mg) and human serum albumin (50 mg, Sigma) were dissolved in 100 ml 4.5 M urea, 1 M propionic acid and gently stirred for 1 hour at 4°C. The solution was then dialized in the cold against 10 litres H_2O for 24 hours and then against 1 litre H_2O for 24 hours (final propionate concentration = 1 mM). This was followed by dialysis against 10 litres 0.01 M Tris, 0.05 M NaCl, pH 8 buffer for 12 hours. Subsequent additions of NaCl to concentrations of 0.10 M and 0.15 M were each followed by a 12 hour dialysis period. A final dialysis against borate-buffered saline was then

carried out prior to affinity chromatography on a Sepharose-PC resin. Protein that was eluted only after washing the column with PC represents renatured, active antibody. In many cases the binding activity was quantitated by equilibrium dialysis as previously described (Goetze and Richards, 1977a).

RESULTS AND DISCUSSION

General

Two criteria were used as assays for functional, renatured molecules. The renatured protein was first run down the PC affinity column. This gives a qualitative indication of whether PC-binding immunoglobulins of any reasonable ($\sim > 10^3\ M^{-1}$) affinity had been formed. In cases where protein was found to bind to the PC column, it was specifically eluted and PC affinity quantitated by equilibrium dialysis. The criterion of adherance to the PC column only gives an indication of PC binding activity but in cases where no such activity was found does not indicate whether immunoglobulins of normal H_2L_2 structure were reformed. However, it is generally accepted

that under the conditions used, good yields of renatured immunoglobulins are obtained (Roholt et al., 1967; Painter et al., 1972b; Manjula et al., 1976). In cases where there was no PC binding activity this indicates that the renatured immunoglobulin did not contain a PC-specific binding site.

Table VI lists the results of hybridization studies carried out. One may make the following observations: (i) In the two cases where autologous recombinations were carried out, affinity for the PC column was restored. In the case of the renatured W3207 molecule, quantitative determination of the PC affinity gave results identical (within experimental error) to those of the parent molecule. (ii) In cases where heterologous recombinations were carried out between two parent molecules having light chains from different subgroups (i.e. T15/M167, T15/W3207, T15/M511) the hybrid antibodies either had unmeasureable binding affinity (failure to adhere to PC affinity column) or had much weaker PC affinity than either parent as measured by equilibrium dialysis. (iii) In two cases where recombinations involving W3207 and M603 were carried out (both antibodies have light

Table VI

PC Affinities of Hybrid Antibodies

H chain	L chain	Ability to bind to PC affinity column	K_a, M^{-1} (4^oC)	% Yield
T15	T15	+		42
W3207	W3207	+	33×10^5	30
M167	T15	-		
T15	M167	+	$< 10^4$	32
W3207	T15	-		
T15	W3207	+	3×10^4	29
W3207	M603	+	22×10^5	29
M603	W3207	+	8.6×10^5	34
T15	M511	-		
M511	T15	-		
T15			17×10^5	
W3207			30.4×10^5	
M603			8.2×10^5	
M167			7.4×10^5	

chains of same κ subclass) binding affinities comparable to those of the parent molecules were obtained. In particular, the affinity of each hybrid was similar to that of the parent donating the heavy chain.

The yield of active protein obtained was similar in heterologous and homologous recombinants of widely differing affinities. This indicates that the requirements for the formation of native structure immunoglobulin are less severe than, and quite different from, the requirements that determine PC activity. This principle has been noted previously (Painter et al., 1972b; Björk and Tanford, 1971). The % yield is defined as weight of active protein (measured by optical absorbance at 280 nm) divided by the total weight of L and H chains initially mixed together. Because the H and L chains were not mixed together in a 1:1 molar ratio, and the criterion for product formed is stricter than in literature reports, the yields of 30-40% obtained here seem reasonable compared to the reported yields of 40-50% (Sher et al., 1971) and 30-70% (Bridges and Little, 1971).

The Molecular Basis for Specific Chain Pairings

The present results point out that high PC affinity is obtained only in cases of autologous hybrids or heterologous hybrids that have at least one highly similar chain in common (here the L chains of M603 and W3207). This is in agreement with previous studies showing the necessity of specific H-L pairing for recovery of PC (Sher et al., 1971, DNP (Bridges and Little, 1971) and other specificities. The fact that both heterologous hybrids of W3207 and M603 show PC affinities similar to the parent antibody donating the H chain implies that the L chains of W3207 and M603 are functionally interchangeable and probably highly similar. In fact, these L chains differ by only 1/31 of the amino terminal residues (a Lys/Arg interchange) (Barstad, 1975) yet these proteins exhibit slightly differing properties (Goetze and Richards, 1977b) related, presumably, to their different heavy chains. The binding data further show that although the combination of W3207 H and M603 L results in an immunoglobulin with slightly lower affinity than W3207, the combination of M603 H and W3207 L has essentially identical affinity to M603.

The efficacy of chain recombination could potentially depend on molecular interactions in several regions of the antibody. First of all, it could depend on C_L-C_H1 complementarity such that a given H chain might optimally pair only with a certain class or subclass of L chain. Secondly, the crucial region of comlementarity might exist in the variable region, either between the framework or hypervariable residues of different chains. Experimental evidence indicates that there is no preferential recombination of a given H chain with specific L chain classes or subclasses (Bigelow et al., 1974; Bunting et al., 1977; Préval and Fougereau, 1976). On the other hand, experiments in which both autologous and heterologous L chains are allowed to compete for association with H chain have shown that in many cases there is a greater tendency for the autologous chains to reassociate (Stevenson and Mole, 1974; Grey and Mannik, 1965) due, therefore, to more favorable V region contacts in the case of autologous partners. Rowe (1976) has shown that in the case of human IgG1, the C region L-H contacts are 10^2-10^4 times more stable than the corresponding V region contacts.

In spite of the relatively small contribution that V region contacts make to the total L-H chain interaction, it can be argued on thermodynamic grounds that small differences in the energies of interaction in the V region may indeed result in a significant degree of preferential recombination (Nisonoff et al., 1975).

A structural basis for the necessity of combining autologous L and H chains if binding activity is to be retained has recently been proposed on the basis of X-ray data analysis (Padlan et al., 1976; Vrana et al., 1977). The authors note that in the mouse the first hypervariable region of the light chain (L1) and the third hypervariable region of the heavy chain (H3) are the most variable of the hypervariable loops with respect to size. This suggests a special function for these regions. An examination of the 3-dimensional structure of M603 (Segal et al., 1974) shows these two loops to be in intimate and extensive contact and it is suggested that amino acid substitutions or deletions/insertions in one or both of these regions would significantly alter the shape of the hapten binding cavity (Padlan et al., 1976). The authors note that in PC-binding immunoglobulins where a highly specific pairing of L and

H chains is necessary for binding activity, L1 and H3 are large and in extensive contact. In contrast, two mouse galactan-binding proteins have L1 7 residues shorter and H3 2 residues shorter than in M603. This might indicate that in these proteins the L-H complementarity is less specific because of the decreased L1-H3 contact. For this reason, heterologous association of L and H chains among this group of proteins should have little modulating effect on the binding site structure and indeed hybrid molecules all show hapten affinities similar to those of the parent antibodies (Manjula et al., 1976). An extreme case is the inulin-binding mouse myeloma protein ABE-47N, where H3 is so short (1 residue) that L1-H3 contacts are absent (Vrana et al., 1977). Another effect of this short H3 region is that the binding site now consists of a shallow groove instead of the cavity seen in M603.

If the L1-H3 interaction is primarily responsible for determining chain complementarity, it is interesting to note that M603 and W3207 differ by only 1 residue in L1 (Lys/Arg) but by 6 residues in H3. It is not easy to see how a strict complementarity between L1 and H3 can exist in view of these many differences.

Furthermore, W3207 and M603 have different choline subsites as monitored by ^{13}C NMR (Goetze and Richards, 1977b) and this is the portion of the binding cavity largely created by L1 and H3. The present evidence therefore indicates that the molecular basis for specific L-H pairing is more complicated than a simple requirement for L1-H3 complementarity.

The only immunoglobulin hybrid arising from parent molecules with different light chain subclasses, and also exhibiting moderate PC affinity (although 60-100 times less than the parent molecules), is the T15H-W3207L hybrid. This result is consistent with the observation that T15 and W3207 have very similar binding sites as determined by NMR (Chapters 3 and 4), T15 being closer in this respect to W3207 than to M603, M167 or M511. This argument fails to explain, however, why the reverse hybrid (W3207H-T15L) does not exhibit significant PC affinity.

The NMR data suggest that, to a first approximation, the various subspecificities in these proteins result from the independent modification of one of the two main subsites through changes in critical heavy chain hypervariable residues. These few, but important, changes apparently require the concomitant presence

of a light chain that is functionally compatible with such an altered heavy chain. The light chain therefore plays an indirect but critical role in determining binding specificity be serving as a precise complementary structure with which the heavy chain can combine to create the binding site. Thus, it appears that the mechanism for generating different fine specificities in PC-binding antibodies is through a small change in the sequence of the highly conserved heavy chain accompanied by selection of a new compatible light chain from a large pool of available structures. This scheme may be of general importance as sequence analysis of human and mouse myeloma proteins and of induced antibodies in rabbits (Margolies et al., 1975), mice (Friedenson et al., 1975) and guinea pigs (Cebra et al., 1974) shows a substantially larger amount of sequence variation among the L chains than among the H chains which suggests that more L than H chains are available for incorporation into antibodies (Poljak et al., 1976). One possible explanation is that somatic mutation is important for the generation of L chain diversity whereas H chain diversity is more dependent on germ-line variability (Mäkelä et al.,

1976).

From the above discussion one can conclude that the ability to form functional hybrid antibodies is a direct function of the binding site similarities between the two parent molecules. The L-H partnership is uniquely stable since in light chain dimers one chain assumes the conformation of a heavy chain (Schiffer et al., 1973). Although the contacts along the L-H interface are now understood in increasingly greater detail (Poljak et al., 1975) the precise nature of the synergistic effects that these chains exert on each other remains to be understood.

The Dominance of the Heavy Chain

The approximately equal participation of L and H chains in forming the binding cleft, the localization of the antigen binding site to a contact area between L and H chains, and the similar number of complementarity-determining loops in both chains argues, a priori, for a similar degree of participation of L and H chains in contacting hapten (antigen). It is an intriguing observation therefore that in many immunoglobulins the heavy chain residues appear to dominate the inter-

action with bound hapten (antigen). Appreciable hapten binding activity in isolated heavy chains is frequently observed but is rare in isolated light chains existing either as monomers or dimers (Painter et al., 1972b; Forre et al., 1976). In both high resolution X-ray structures of hapten bound to specific antibody which are currently available (Segal et al., 1974; Poljak et al., 1974) the heavy chain hypervariable (HV) loops contribute a larger area to the region of the active site than the light chain loops. Poljak et al. (1974) suggest that in the case of IgG New this may be partially due to the greater length of the heavy chain HV loops. In the PC-specific mouse immunoglobulins the H chain dominates the interaction with hapten completely so that differences in binding interactions between these proteins can be accounted for solely on the basis of H chain sequence differences (Chapters 3 and 4).

Kabat et al. (1977) have used a data bank of V region sequences to compute the incidences of the amino acids at various positions of the HV loops of light and heavy chains and from this deduced either

a structural or a contacting or conformation-determining function for amino acids in each position. This study showed that twice as many L chain HV residues as H chain HV residues function as structural elements. This leaves more H chain residues to function as antigen-contacting residues and may provide a molecular foundation for the frequently observed H chain dominance in the interaction with bound ligand. Such an apparent lack of symmetrical participation in antigen binding would have important consequences for the number of possible antigen specificities (to be discussed subsequently).

Consequences of Different Light-Heavy Chain Pairings

An efficient way of generating a large number of different binding site structures is to allow random L-H pairing (Edelman and Gally, 1964). In this way, m H and n L chains may potentially generate up to m x n different binding sites. A recent analysis of the 2 Å resolution model of Fab' New and of sequences of human and murine H and L chains revealed that such a mechanism may be feasible since positions which provide close contacts between V_L and V_H were shown

to be occupied by either constant residues or conservative replacements (Poljak et al., 1975; Poljak, 1975). These interactions are independent of H or L subgroup and even L chain class so that the pairing of a given H chain with any L chain is feasible on structural grounds. It remains to be proven however that all L-H combinations generate molecules with viable antibody affinities. For example, the heterologous hybrids of the PC-binding mouse immunoglobulins generally exhibit no detectable PC affinity but only exhaustive screening tests could determine whether other binding specificities exist. The results of competitive hybridization experiments, in which significant differences in L-H affinities were found even among highly similar κ chains binding to a single γ chain, have been interpreted as indicating that m H chains and n L chains cannot generate the maximum m x n number of antibody molecules. Instead, an upper limit of (m x n)/10 was estimated (Stevenson and Mole, 1974).

Another issue that has direct bearing on this problem is that of the multispecificity of antibody binding sites. It has been suggested that each

antibody site may bind, with varying affinities, a large number of structurally unrelated ligands (Richards et al., 1975) but, because the surface area of the binding site exceeds that of most ligands, this would almost certainly require that a substantial portion of them bind exclusively or predominantly to residues from one chain. If the contribution of L and H chains to the ligand binding energy is additive, different L-H combinations would not alter such specificities and this imposes a limit on the number of new affinities which can be created by chain recombination. It should be noted, however, that the concept of multispecific antibodies is not universally accepted as recent arguments (Johnston and Eisen, 1976; Gavish et al., 1977) have cast doubt on the experimental results which led to this proposal.

Conclusions

The evidence in this study suggests that in the PC-binding mouse myeloma proteins the primary phosphorylcholine specificity, as well as the various fine specificities, are directly determined by the

heavy chain amino acid residues. Thus, a small number of heavy chain amino acid substitutions are sufficient to cause functionally differing antibodies. We have seen in Chapter 4, for example, the important implications of the presence of Asp 100aH for the fine specificities of M167 and M511. Similarly, the unique substitution Trp 104aH→Gly in M167 allows this antibody to bind "longer" PC analogs. We suggest that the correlation between light chain subclass and fine specificity (Barstad, 1975) is due to the fact that a heavy chain of a given fine specificity is conformationally distinct from the heavy chains of other fine specificities; each such conformationally-distinct heavy chain can pair only with the light chain of a suitably complementary subclass. In this view, the role of the light chain in defining the binding specificity is to stabilize a unique heavy chain conformation. The light and heavy chains therefore, rather than contributing additively to the binding energy, appear, in the phosphorylcholine system, to interact synergistically.

The hybridization experiments lend support to this view. The inability of the heterologous hybrids

to generally exhibit appreciable PC affinity may well arise from the fact that in such molecules a given heavy chain is forced to pair with a conformationally incompatible light chain. As discussed previously, this incompatibility extends only to the level of PC specificity and does not prevent the formation of structurally normal immunoglobulin molecules.

The finding that the light chains of W3207 and M603 are functionally interchangeable (Table VI), argues that the heavy chains of these two proteins determine similar fine specificities and therefore have similar light chain requirements. The similarities between W3207 and M603 with respect to binding affinities for various ligands as well as details of their phosphate subsites as determined by ^{31}P NMR (Chapters 3 and 4) lend strong support to this concept. Thus, it appears that even though W3207 and M603 have a moderate number of heavy chain amino acid differences, these differences do not occur in crucial residues and as a result the phosphate subsites of these two proteins are functionally similar. It is noteworthy that the choline subsites of these two proteins differ by ^{13}C NMR (Chapter 3). However, it has not been possible to assess the potential importance of this

difference to the fine specificities because of the strict requirement of a trimethylammonium functionality in all haptens (Leon and Young, 1971). However, the hybridization experiments have established that this difference is not sufficient to require a light chain of a different subclass for each of these proteins.

Functional similarities among these antibodies, as determined by NMR binding studies, are in good agreement with the results of the hybridization experiments (as discussed above for M603 and W3207). Thus the observation that, for example, heterologous hybrids of T15 and M167 or of T15 and M511 exhibit little or no PC affinity is in agreement with their vastly different binding sites, notably the electrostatic character of their phosphate subsites. From this point of view, the hybrids of M167 and M511 would be expected to (in analogy to M603 and W3207) exhibit high PC and choline affinities but this has not yet been tested. However, the binding interactions between hapten and antibody of M603 and W3207 on the one hand and of T15 on the other are also quite similar. Therefore, it is not too surprising to find that one of the heterologous hybrids of T15 and W3207 also has appreciable PC affinity.

Based on the present results, the proposal that L1-H3 complementarity is the dominant factor in determining chain compatibility (Padlan et al., 1976; Vrana et al., 1977) appears to be an oversimplification since no correlation between sequence homology in these regions and the ability to form PC-binding hybrid immunoglobulins is observed.

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PART II

^{19}F NMR STUDIES OF TRIFLUOROACETONYLATED IMMUNOGLOBULINS AS A PROBE OF ANTIBODY CONFORMATION

CHAPTER 6

BACKGROUND

Immunoglobulin Effector Functions

Upon combination of antigen and antibody *in vivo*, a variety of physiologically significant reactions may be initiated. These "effector functions", whose natures are dependent on the immunoglobulin class and subclass, are triggered by the Fc portion of the antibody after combining with antigen in the Fab region. Effector functions range from activation of the complement cascade to the triggering of various cell types through cell surface immunoglobulin receptors.

Activation of the classical complement pathway may occur *in vivo* when either a single IgM (Borsos and Rapp, 1965a, 1965b; Ishizaka *et al.*, 1968) or a minimum of two adjacent IgG molecules (Borsos and Rapp, 1965a; Cohen, 1968; Humphrey and Dourmashkin, 1965) bind to the antigenic determinants on a cell surface. This allows the first complement component to bind directly to the immunoglobulin(s) and thereby initiate the triggering of the complement cascade. This mechanism eventually results in destruction of the antigenic cell by the direct lytic action of the terminal complement components although the biological consequences of the release of cleavage

products of the intermediate complement components may be of quantitatively greater importance in target cell destruction.

As a second example of antibody effector functions, reaginic antibody (IgE), when bound to blood basophils or tissue mast cells, can, upon cross-linking by appropriate antigen, cause the release of various vasoactive amines from these cells (Ishizaka, 1970; Ishizaka et al., 1969; Osler et al., 1968). These released amines act on specific target cells to give rise to the classical allergic and hypersensitivity reactions.

Interaction of antigen with the surface immunoglobulins of B lymphocytes (IgD and IgM) can trigger various cellular events including the differentiation of these cells into antibody-secreting plasma cells. Under different conditions, these B lymphocytes can be rendered tolerant to the same antigen (Vitetta and Uhr, 1975; Warner, 1974; Marchalonis, 1975).

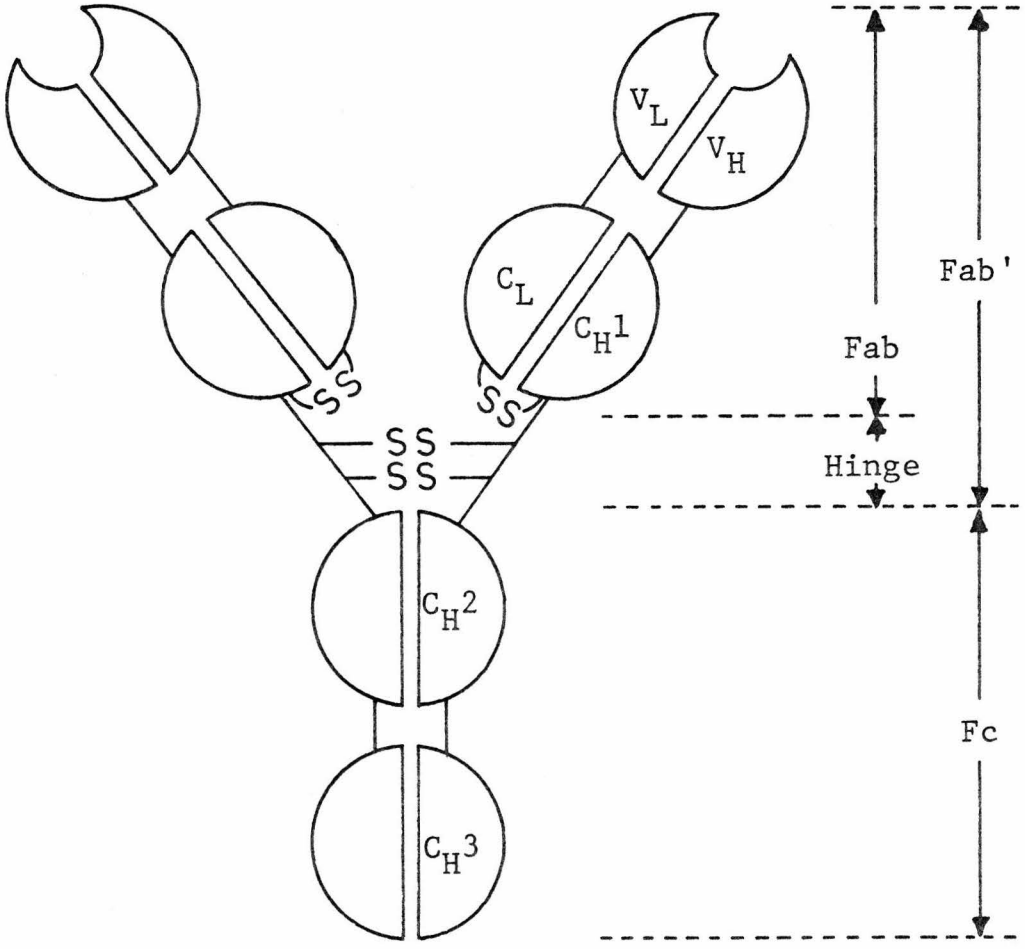
Antibody Domain Functions

Antibodies have been shown by a variety of physical techniques, including X-ray crystallography, to consist of a series of homologous, globular regions

or domains (Huber et al., 1976; Dorrington and Tanford, 1970; Nezlin et al., 1970). Each domain encompasses an approximately 110 amino acid stretch (homology unit), one each from the light and heavy chains (Fab domains) or two from the two identical heavy chains (Fc region) (Figure 22). The 3-dimensional structure of each homology unit, as well as some features of the amino acid sequence, are very similar and lead to the conclusion that these homology units have arisen by repetitive gene duplication (Hill et al., 1966; Singer and Doolittle, 1966). Because each domain in the antibody structure is relatively independent and because some antibody functions appear to be localized to a single domain, Edelman proposed the domain concept which states that each immunoglobulin domain has evolved to perform a unique and specific purpose (Edelman et al., 1969; Edelman, 1970). This has been well established in the case of the antigen binding function of the variable domain; the evidence for specific domain localization of various effector functions is briefly reviewed below.

Figure 22

Schematic illustration of a human IgG1 molecule showing the domain structure.


V_L
V_H
C_L
C_H1
S S
S S
S S
S S
C_H2
C_H3
Fab'
Fab
Hinge
Fc

It has been known for some time that C1q binds to both IgG and IgM and that the binding site is located in the Fc region (Hyslop et al., 1970; Augener et al., 1971; Mackenzie et al, 1971). The evidence is now quite good that in the case of IgG, C1q binds directly to the C_H2 region. For example, Fabc fragments of rabbit IgG (antibody missing the C_H3 domain) have essentially normal complement fixing ability (Connell and Porter, 1971; MacLennan et al., 1974; Ovary et al., 1976) and intact C_H2 fragments likewise possess activity equivalent to that of Fc fragments (Ellerson et al., 1972; Yasmeen et al., 1976). Kehoe et al. (1972) had earlier shown that C1 activation could take place using a 7000 dalton fragment from the C_H2 region although its molar activity was only 3% that of the Fc fragment (Kehoe et al., 1974). Fab or C_H3 fragments in all cases lack complement activating ability. Similar studies on enzymatic fragments of IgM have suggested that the C1 binding site is in the C_H4, or C-terminal, domain (Hurst et al., 1974, 1975).

The precise nature of the complement-binding site is still unknown, but it can be considered to

arise either from a specific amino acid sequence or a particular 3-dimensional conformation. The first possibility has been shown unlikely by studies which, in the case of IgG, show no apparent correlation between C_H2 sequence and the ability of certain subclasses to fix complement (Kehoe et al., 1974). On the other hand, the ability of reduced and alkylated β_2-microglobulin (which exists as a random coil) to fully fix C1 (Isenman et al., 1975a) argues against a specific conformational requirement. To explain these conflicting results, it has been suggested that the structural features for the C1 binding site consist of a sequence requirement which is modulated by changes in tertiary structure (Isenman et al., 1975b).

Most studies have concluded that it is the C_H3 domain of IgG that is bound by the Fc receptor found on the surface of various cell types (monocytes, macrophages, K cells, neutrophils) and is responsible for triggering their cytophilic activity (Okafor et al., 1974; MacLennan et al, 1974; Minta and Painter, 1972; Ramasamy et al., 1975) although others have implicated the C_H2 region in this process (Alexander et al., 1976; Wisløff et al., 1974). A decapeptide has been

isolated from the C_H3 region which completely inhibits binding of cytophilic antibodies to mononuclear cells (Ciccimarra et al., 1975). However, other recent studies suggest that an intact Fc region may be required for optimal binding to Fc receptor (Ovary et al., 1976; McNabb et al., 1976). Klein et al. (1977) showed that on a molar basis C_H3 is 10% as effective and C_H2 is 1% as effective as intact IgG in binding to Fc receptor on murine T cells. The C_H4 domain of IgM has been shown to contain both complement fixing ability and cytophilic activity for human lymphocytes (Conradie and Bubb, 1977). The domain hypothesis may therefore be an oversimplification encouraged by the fortuitous localization of some, but not all, antibody functions to a particular domain.

Possibility of Conformational Changes in Antibodies

Two fundamentally different models have been proposed to explain how antigen binding can trigger effector functions in a spatially distinct region of the antibody molecule (Metzger, 1974). The first model holds that antibody aggregation is a sufficient

signal so that previously univalent antibody becomes multivalent to a third component (e.g. Clq). The increased binding affinity of the third component, due simply to multipoint attachment, is responsible for effector function initiation. An alternative model proposes that antigen binding triggers a conformational change in the antibody molecule which is transmitted from the binding site to the Fc region. A third, but related, model suggests that the spatial arrangement of antigenic determinants on a multivalent antigen forces the antibody "arms" open, thereby exposing a previously hidden effector site. The conformational change model is, from a molecular viewpoint, the more interesting as it may allow allosteric antibody behaviour. The experimental evidence for such a model has been indirect and controversial but will be reviewed below in order to summarize existing knowledge pertaining to this problem.

Early attempts to monitor antibody conformational changes upon antigen (or hapten) binding suggested that a decrease in antibody size may be a common feature. Binding of lactose to specific rabbit antibody (IgG)

resulted in a small decrease in the sedimentation coefficient of the antibody (Warner et al., 1970). Similarly a 10% volume decrease was observed upon binding of tetra-D-alanine to rabbit anti-poly(D-alanyl) antibodies using the technique of small-angle X-ray scattering (Pilz et al., 1973). This size decrease was specific for the Fc region as no such change was observed with Fab' or $(Fab')_2$ fragments from the same antibody system (Pilz et al., 1975). A 2-3% volume decrease in rabbit IgG resulted upon occupation of 50% of the binding sites by a specific p-azophenyl-β-lactoside hapten. In this latter, small-angle X-ray scattering study, a change of the overall T-shape of the antibody molecule was ruled out, suggesting that the volume decrease was due to a generalized contraction (Pilz et al., 1974).

Apparent changes in the hinge region have also been observed. The most direct evidence stems from the electron microscope study of Feinstein and Rowe (1965) which shows an increase in the angle between the two Fab arms of the antibody after combining with antigen. Nevertheless, artifacts due to sample

preparation are difficult to rule out. As a result of the fluorescence studies of Tumerman et al (1972) it has been proposed that combining with antigen results in a more rigid interlinking of Fab and Fc subunits in the intact antibody.

Other studies have yielded multiple evidence for antibody conformational changes although the nature and location of these changes is often poorly defined. A decreased susceptibility to chymotrypsin digestion has been shown for rabbit anti-benzenearsonate and trimethylanilinium antibodies in the presence of hapten (Grossberg et al., 1965). However, it is difficult from these studies to rule out a protective effect limited to the Fab region (Metzger, 1970). Similarly, the changes in the circular dichroic spectra of several homogeneous rabbit antibodies seen on combination with carbohydrate haptens have not been shown to arise from regions other than the Fab fragments (Holowka et al., 1972). A more intriguing study has been presented by Liberti et al. (1972a, 1972b). These authors studied hydrogen exchange rates of sheep antibodies and $(Fab')_2$ fragments to poly($Glu^{60}Ala^{30}Tyr^{10}$). Besides the expected blocking of 23-24 hydrogens per

molecule of Fab due to the presence of antigen, preferential exchange of certain protons was observed as the antibody to antigen ratio was increased. This change was identical in intact antibody and $(Fab')_2$ and has been ascribed by these authors as arising from significant conformational changes near the hinge region due to the stresses imposed by large complex formation. Similar results including changes in circular dichroic spectra were observed in the case of sheep antibodies in another study (Callahan et al., 1973). An increase in binding sites for Staphylococcus aureus Protein A (known to be located in the Fc) was observed upon antigen binding to rabbit IgG (Grov, 1973). In another study, solvent perturbation spectra revealed an increase in exposure of aromatic chromophores to solvent in the rabbit antibody - human blood group glycoprotein M system (Gizler and Morawiecki, 1973). Other indirect evidence for antigen-induced conformational changes has been obtained by Raman spectroscopy (Painter and Koenig, 1975) and light scattering techniques (Marrack and Richards, 1971). An interesting observation is that of Carrico et al. (1974) who raised antisera to a "pepsin site", an

antigenic site produced on $(Fab')_2$ formation that is not present on intact antibody. A similar antigenic site was serologically detected when antibody combined with antigen. Such studies would tend to support a mechanism whereby a normally hidden effector function determinant is made accessible after antigen binding.

Kinetic studies of antibody-hapten reactions usually yield close to diffusion-controlled association rate constants (Froese and Sehon, 1965; Froese, 1968) and no difference in rate constant is observed between intact antibodies and Fab fragments (Levison et al., 1971; Pecht et al., 1972; Skubitz et al., 1977). Determination of the association rate constants of several trifluoromethyl aromatic haptens to MOPC 315 by the novel technique of perturbation mixing resulted in similar rate constants of 1-3 x 10^8 M^{-1} sec^{-1} for each of the intact antibody, Fab' and Fv fragments (Kooistra, 1977). These results render a protein conformation change unlikely since the latter generally occur with rate constants of 10^2-10^4 M^{-1} sec^{-1} (Hammes, 1968).

The association rate constants for a variety of

antibody-antigen systems have also been determined. Typical results yield values for k_{on} of 2×10^5 M^{-1} sec^{-1} for the system fluorescein-labelled ovalbumin and rabbit anti-ovalbumin measured by fluorescence depolarization (Dandliker and Levison, 1968) and 4.5×10^5 and 5.8×10^6 M^{-1} sec^{-1} for the association of rabbit Fab with cytochrome c and hemoglobin S respectively (Noble et al., 1972). However, the meaning of these latter numbers is difficult to evaluate in view of the lack of theoretical values for diffusion-controlled rate constants in protein-protein systems (Metzger, 1974).

If ligand binding to antibody is followed by a conformational rearrangement on the part of the antibody one might, under favorable conditions, expect to see two relaxation times when fast kinetic experiments are carried out (Pecht and Lancet, 1976). These relaxation times would correspond to the fast associative step and the slower conformational change. Such behaviour has been observed for two anti-DNP antibody systems (Froese, 1968; Barisas et al., 1977). Similarly, T-jump relaxation experiments of 19S IgM antibodies have revealed, apart from the fast step,

a slow relaxation time attributable to hapten binding (Haustein et al., 1977). However, all these experiments suffer somewhat from the fact that heterogeneous antibodies were employed; such systems might easily display kinetic heterogeneity. It is therefore of great interest that temperature jump experiments have yielded kinetic evidence for a hapten-induced conformational transition in reduced and alkylated MOPC 460, a homogeneous mouse myeloma IgA with DNP specificity (Lancet and Pecht, 1976). A detailed analysis of the results supports a mechanism in which the antibody molecule exists in one of two interconvertible states with differing hapten affinities. Upon hapten binding the equilibrium is shifted towards the better binding state. However, in preliminary experiments (Lancet and Pecht, 1976) similar relaxation behaviour was observed with Fab fragments suggesting that this interconversion may not extend to the Fc region. Nevertheless, such results are in accord with a model in which effector functions are initiated by allosteric antibody behaviour.

Thompson and Hoffmann (1971, 1974a, 1974b) and Kuo et al. (1976) have studied the binding of C1 by

rabbit anti-erythrocyte antibodies (IgG) and observed this binding to exhibit homotropic cooperativity. From a detailed analysis of the binding behaviour they concluded that antibodies are allosteric proteins (Hoffmann, 1976) in the formal sense of the term (Monod et al., 1965). In their formulation, IgG antibodies form clusters on the erythrocyte surface and can, as a whole, exist in one of two interconvertible states, with differing affinities for C1. Binding of C1 to such a cluster shifts the equilibrium of all antibodies in that cluster to the higher affinity state. In such a scheme, antigens act both as allosteric activators of C1 binding and as the means by which allosteric IgG clusters are formed. Cooperative hapten binding has been observed in several antibody systems (Zimmering et al., 1967; Matsukura et al., 1971; Matsuyama et al., 1971; Carayon and Carella, 1974) as well as in the L_2 dimer of MOPC 315 (Lancet et al., 1977) although the physiological significance of this latter observation is not apparent.

Perhaps the most convincing evidence for antigen-induced conformational changes in the Fc region of antibodies has come from a study of the circular polarization of luminescence (CPL) (Jaton et al.,

1975; Schlessinger et al., 1975; Pecht et al., 1977), a technique which monitors protein conformation in the vicinity of tryptophan residues. Such studies have shown that binding of RNase to anti-RNase and poly-(DL-alanyl)-poly(L-lysine) to anti-poly(D-alanine) causes CPL spectral changes in both Fab fragments and intact antibody. However, the changes observed with intact antibody are different from, and more pronounced than, those seen with Fab fragments and therefore are likely to arise from changes in the Fc region. Moreover, binding of the "loop" of lysozyme, a monovalent antigen, to specific antibody resulted in conformational changes attributed to the Fc region but was not accompanied by complement activation. Binding of the dimeric antigen bis-"loop", on the other hand, resulted in distinctly different CPL spectra as well as the ability to fix complement. Similar results were observed with different-sized oligosaccharides binding to homogeneous anti-type III pneumococcal antibody (Jaton et al., 1975, 1976). The small hapten phosphorylcholine did not cause CPL changes upon binding to McPC 603 or McPC 603 Fab'. These results indicate that binding of large but

monovalent antigens can cause conformational changes within the Fc region but that these alone are not sufficient to generate the effector functions, which are dependent on the binding of at least a divalent antigen. In a study of 19S IgM with specificity for the phenyl-β-lactoside hapten, however, binding of monovalent antigen did result in complement fixation (Brown and Koshland, 1975). These authors, showed that monovalent antigen bound with lesser affinity than hapten, a difference attributed to activation of the Fc complement site by binding energy in the case of the monovalent antigen.

Importance of Disulfide Bonds

In the case of the CPL experiments, reduction of the inter-heavy chain disulfide bonds completely abolished both the CPL changes attributed to the Fc region and the complement fixing ability. Changes in the CPL spectrum due to the Fab fragments were unaffected (Schlessinger et al., 1975; Jaton et al., 1975). It is well established that Fc-dependent effector functions are, in the intact antibody, dependent on the integrity of the hinge region inter-heavy chain disulfide bonds, in the cases of IgG (Schur

and Christian, 1964; Isenman et al., 1975b; Michaelson et al., 1975), IgM (Frank and Humphrey, 1969; Bubb and Conradie, 1975) and IgE (Takatsu et al., 1975). However, Fc fragments lacking intact inter-heavy chain disulfide bonds exhibit complement fixing ability similar to the intact antibody complexed with antigen (Plaut et al., 1972; Yasmeen et al., 1976). This is true even for IgG subclasses in which the intact antibody complexed with antigen does not fix complement (Isenman et al., 1975b). The hinge region disulfide bonds therefore are not directly involved in C1 binding but their integrity appears to be required for interactions between Fab and Fc. One postulate, based upon the observation that intact hinge region disulfide bonds infer a certain conformational rigidity to the antibody molecule (Lapanje and Dorrington, 1973; Romans et al., 1977), is that the intact disulfide bonds prevent the Fab region from sterically blocking access to the C1 binding site (Sledge and Bing, 1973). Differences in complement fixing ability between different immunoglobulin classes and IgG subclasses may reflect the many markedly different hinge region sequences (Michaelson

and Natvig, 1974) which may allow this hypothetical Fab-Fc interaction to occur to varying degrees (Isenman et al., 1975b).

X-Ray Structures

Ultimately, an answer to the antibody conformational problem must come from X-ray studies. The 3-dimensional structures of several Fab' and Fc fragments (Poljak et al., 1974; Segal et al., 1974; Deisenhofer et al., 1976a, 1976b) and Bence-Jones proteins (Schiffer et al., 1973; Epp et al., 1974; Fehlhammer et al., 1975) as well as two intact immunoglobulins (Huber et al., 1976; Silverton et al., 1977) have been obtained to varying resolution. It is noteworthy that in the cases of the two Fab' fragments with known ligand specificity (protein New binding a γ-hydroxy derivative of vitamin K_1 and McPC 603 binding phosphorylcholine) no changes in the crystal structures wer observed on hapten binding (Amzel et al., 1974; Padlan et al, 1973). Nevertheless, the possibility of such a change, perhaps induced only by the binding of larger antigens, cannot be ruled out. Poljak et al. (1974) point out a possible mechanism whereby conformational changes may occur within

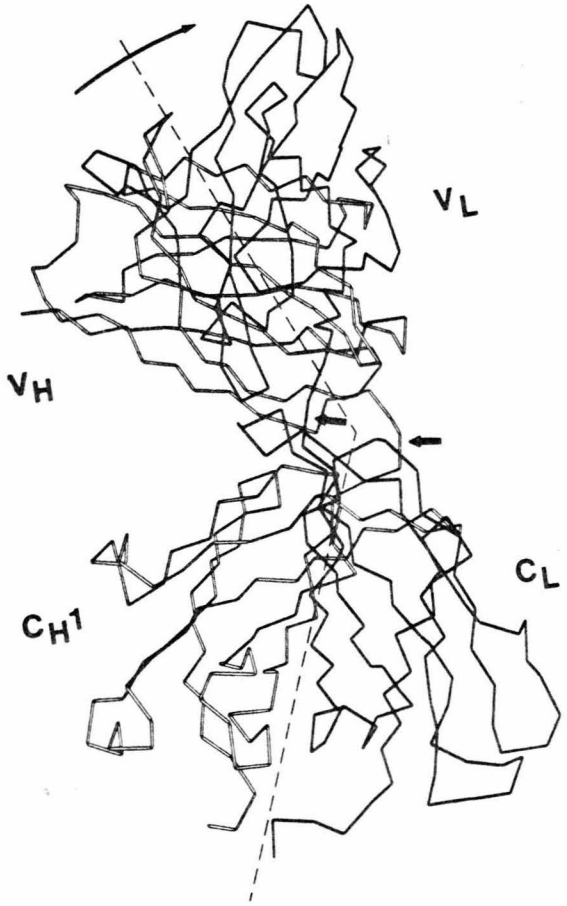
the McPC 603 Fab' fragment. In this protein, as well as in Fab' New, the angle between the major axes of the C_L and V_L subunits is greater than 90^o whereas the corresponding angle between the V_H and C_H1 subunits is less than 90^o. In Bence-Jones proteins, the two light chains adopt different conformations such that one of them mimics the heavy chain of an Fab' fragment (Schiffer et al., 1973). A conformational change could occur by a hinge-like movement in the switch region (the V-C contact region) and, because of the inequality of the above angles, would lead to relative movement of the subunits and exposure of some new side chain residues (see Figure 23). Very similar types of changes are observed by crystallographic studies upon oxygenation of hemoglobin (Perutz et al., 1968).

A more comprehensive conformational change model has come from X-ray studies of the human IgG molecule Kol (Huber et al., 1976). This structure shows little or no longitudinal contacts (contacts between adjacent homology units on the same polypeptide chain) and should therefore exhibit considerable flexibility. Furthermore, the structure has

Figure 23

A view of the α-carbon backbone of Fab' New. This figure and the following description is taken from Poljak et al. (1974).

"The V and C_1 domains, the L chain (open line), the Fd' chain (solid line) and the local, approximate 2-fold axes (broken lines) relating the V_L to the V_H subunit and the C_L to the C_H1 subunit are shown. The two short arrows indicate the switch region of both chains. The longer arrow indicates a possible relative motion of the V and C_1 domains (see text)."


V_L
V_H
C_{H1}
C_L

an overall "Y" shape and allows no Fab-Fc contact except through the extended hinge region sequence. It is suggested that upon ligation the antibody molecule "stiffens up" through the formation of longitudinal inter-domain contacts. This change would also involve folding of the hinge region peptide so as to allow C_H1-C_H2 contact thereby providing a direct mechanism for transfer of a signal arising in the Fab region to the Fc region. This model has the benefit of being in agreement with many previously-discussed solution studies in which an overall decrease in volume and/or flexibility of the antibody molecule upon ligation seemed to be indicated. The quaternary structure of the Fab' region in the isolated fragment differs from that in the intact immunoglobulin and therefore it was suggested that the isolated Fab' is already in the liganded conformation (Huber et al., 1976). It is of interest that myeloma protein Dol, a human IgG with a 15 residue deletion in the hinge region has an overall "T"-shaped structure and shows significant contact between Fab and Fc region, largely through the carbohydrate groups (Silverton et al., 1977). Thus, different antibodies may crystallize in differing conformations, all of which are attainable in solution.

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CHAPTER 7

^{19}F NMR STUDY OF POSSIBLE CONFORMATIONAL CHANGES IN MOUSE IMMUNOGLOBULINS

INTRODUCTION

NMR is an extremely versatile and sensitive technique for studying the structure and conformation of macromolecules. The method of covalently attaching to macromolecules small reporter groups which then serve as NMR probes is well established (Huestis and Raftery, 1971, 1972a; Paselk and Levy, 1974). The reporter group contains an NMR-sensitive nucleus such as ^{19}F, ^{31}P, ^{13}C etc. and by monitoring its signal the problem of analyzing the complicated proton NMR spectra of macromolecules can be circumvented. Fluorine, because of its small size and high sensitivity, is an ideal candidate for incorporation into such reporter groups and indeed NMR studies of proteins containing covalently bound trifluoroacetyl groups have been successfully employed in the detection of ligand-induced environmental changes in the vicinity of the fluorine probe and in the elucidation of the molecular basis of such changes (Huestis and Raftery, 1971, 1972b).

In this study it was attempted to search for possible antigen-induced conformational changes in two mouse IgA2 immunoglobulins by such an NMR method.

Mouse IgA2 molecules contain six labile disulfide bonds (Tomasi and Grey, 1972) which were reduced and trifluoroacetonylated. It is known that reduction and alkylation of these disulfide bonds does not affect the gross antibody structure or affect its antigen binding properties (Dorrington and Smith, 1972) and perturbation of the native protein structure is therefore considered to be minimal. Furthermore, the disulfide bonds are strategically located so as to allow probing of the environments by NMR labels located in the Fab and Fc regions as well as the important hinge region. Hapten, multivalent protein antigens and cellular antigens were used in this study since it is doubtful whether hapten-antibody combination can result in expression of effector function (and thereby, presumably, Fc conformational changes) (Schlessinger et al., 1975). Two immunoglobulins, TEPC 15 with specificity for phosphorylcholine (PC)[1] and MOPC 315 with specificity for 2,4-dinitrophenyl (DNP) groups were used.

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[1]Abbreviations used are: PC, phosphorylcholine; DNP, dinitrophenyl; SRBC, sheep red blood cells; BSA, bovine serum albumin.

MATERIALS AND METHODS

Materials

Unreduced MOPC 315 protein purified by ion-exchange chromatography was a gift from D. Kooistra. ^{14}C-Iodoacetamide (56 mCi/mmole) was obtained from New England Nuclear and diluted with non-radioactive iodoacetamide (Sigma) before use. 3-Bromo-1,1,1-trifluoropropanone was purchased from PCR Specialty Chemicals, Gainesville, Florida. Sheep red blood cells in Alsevier's solution were obtained from Davis Labs, Davis, California; 2,4-dinitrofluoro-benzene was purchased from Pierce Chemical Co., and dithiothreitol was obtained from Sigma.

Optimization of Reaction of Mouse IgA with CF_3COCH_2Br

Unreduced MOPC 315 protein at a concentration of 1.5 mg/ml in 0.2 M Tris, pH 8.6 buffer was reduced by treatment with 0.01 M dithiothreitol for one hour at room temperature. The pH was then adjusted to the desired value by the addition of 0.02 M Tris, pH 7.3 buffer. Neat 3-bromo-1,1,1-trifluoropropanone was then added slowly and with stirring to the protein solution and the pH kept constant by the dropwise

addition of 1 N NaOH. The amount, number of aliquots of reagent and the length of incubation were varied. To terminated the reaction, the pH was adjusted to 8.0 and ^{14}C-iodoacetamide (5×10^6 cpm) was added to a final concentration of 0.035 M. After 45 minutes at 0^oC the reaction mixtures were quantitatively transferred into dialysis bags and dialized three times against a large excess of 0.02 M borate, 0.15 M NaCl, 1 mM EDTA, pH 8.0 buffer to remove the excess radioactive reagent.

After completion of dialysis, the content were quantitatively transferred into volumetric flasks and made up to volume with buffer. One ml samples were then counted on a liquid scintillation counter so that the counting error was always less than 5%.

Preparation of Trifluoroacetonylated TEPC 15

Serum or ascites fluid from mice carrying plasmacytoma TEPC 15 was brought to 45% saturation by the addition of saturated ammonium sulfate. The resultant precipitate was removed by centrifugation and dialyzed exhaustively against 0.2 M Tris, pH 8.6. The protein solution was then reacted with 3-bromo-1,1,1-trifluoropropanone under the optimal conditions

as determined in the previous experiment. After dialysis, trifluoroacetonylated TEPC 15 was specifically isolated by passage of the mixture through a phosphorylcholine-Sepharose affinity column.

Preparation of Fab' and Fab Fragments

Fab' fragments of trifluoroacetonylated TEPC 15 were prepared in a similar fashion to that described previously for iodoacetamide-alkylated immunoglobulins (Inbar et al., 1971).

Fab fragments were produced by a method based on the procedures of Grey et al. (1970) and Nisonoff et al. (1960). To a solution of trifluoroacetonylated TEPC 15 (6 mg/ml) in 0.09 M phosphate, 0.05 M NaCl, 2 mM EDTA, pH 7.5 buffer, papain (17 units/mg, 2X crystallized from Sigma) was added to give an enzyme: antibody ratio of 1:50 (w/w). Dithiothreitol was immediately added to give a final concentration of 0.01 M and digestion was allowed to proceed for one hour at 37°C. Reaction was stopped by the addition of a 10% excess of solid iodoacetamide and stirring of the mixture for one hour at 4°C. The solution was then dialyzed against borate-buffered saline,

pH 8.0 and protein with binding activity isolated by passage of the mixture through the PC affinity column. SDS gel electrophoresis of this protein (Fairbanks et al., 1971) showed considerable amounts (∿ 40%) of undigested immunoglobulin. The Fab fragments were therefore separated from undigested material by gel filtration on Sephadex G-100.

Preparation of Phosphorylcholine-Conjugated Bovine Albumin (PC-BSA)

Bovine albumin fraction V (0.25 g, 3.6 μmoles) (Nutritional Biochemical Corporation, Cleveland, Ohio) was dissolved in 50 ml 0.02 M borate, 0.16 M NaCl, 1 mM EDTA, pH 9.2 buffer and to this was added 70 μmoles of a solution of p-diazoniumphenylphosphorylcholine prepared as described previously (Chesebro and Metzger, 1972). The solution was stirred at room temperature for one hour and then at 0°C for 2 hours. After exhaustive dialysis against the above borate buffer, the yellow protein solution was concentrated by ultrafiltration to a concentration of 40 mg/ml and stored frozen.

Preparation of Dinitrophenyl-Sensitized Sheep Red Blood Cells (DNP-SRBC)

The procedure was based on that of Thompson and Hoffmann (1971, 1974). Sheep blood in Alsevier's solution (10 ml) was washed 4 times with cold 0.01 M borate, 0.14 M NaCl, pH 9.5 buffer. After each washing the cells were packed by centrifugation for 10 minutes at 500 rpm. After the last washing, the packed cells were diluted with the same buffer to make a 5% (v/v) suspension. To this was added 1% of the volume of 2,4-dinitrofluorobenzene:acetone (1:165, v/v). The suspension was allowed to stand at room temperature for 75 minutes with occasional stirring. The dinitrophenyl-sensitized sheep red blood cells (DNP-SRBC) were then washed 5 times with an excess of veronal-buffered saline (Rapp and Borsos 1970) containing 1 mM Mg^{2+} and 0.15 mM Ca^{2+}. Except for the 75 minutes of reaction time, all steps were carried out in the cold ($4^{o}C$) and cold buffers were used throughout. A control sample was prepared in an identical fashion except that the 2,4-dinitro-fluorobenzene was replaced with water.

Preparation of Trifluoroacetonylated MOPC 315

This was carried out in a manner identical to that used for the TEPC 15 protein. Immunospecific purification was carried out by means of a dinitrophenyl-lysine-Sepharose affinity column (Goetzl and Metzger, 1970).

NMR Samples

Protein samples were concentrated to 2-3 ml by ultrafiltration on a PM-10 membrane and clarified by centrifugation.

For NMR samples involving sheep red blood cells, 1.5 ml packed SRBC in veronal-buffered saline were added to 1 ml of a concentrated solution of trifluoroacetonylated MOPC 315 in the same buffer. After thorough mixing, the tube was sealed and spectra run within 18 hours. Sample tubes were either left non-spinning during data accumulation or were spun slowly enough (0-4 rps) so that no packing of the cells resulted. ^{19}F NMR spectra were obtained on a Varian XL-100-15 spectrometer at 94 MHz and an ambient probe temperature of 30 ± 1°C.

RESULTS AND DISCUSSION

Quantitation of the number of trifluoroacetonylated residues per mole protein was facilitated by the use of radioactive iodoacetamide which, like 3-bromo-1,1,1-trifluoropropanone, reacts almost exclusively with free sulfhydryl groups at near neutral pH (Means and Feeney, 1971). The reduced antibody was first allowed to react with the trifluoroacetonylating reagent and was then exposed to the more reactive iodoacetamide. The number of iodoacetamide-labelled groups could be determined directly by radioactive counting. The difference between the maximal number of iodoacetamide-reactive groups observed (in the absence of CF_3COCH_2Br) and the number of iodoacetamide-reactive groups observed in the individual experiments was taken to be equal to the number of trifluoroacetonylated groups.

The results of these experiments are give in Table VII. A maximum number of 13.3 sulfhydryl groups could be labelled with ^{14}C-iodoacetamide after reduction of the labile disulfide bonds of MOPC 315. This is in reasonable agreement with the known secondary structure of mouse IgA2 which is known to contain

Table VII

Quantitation of Antibody Sulfhydryl Groups

Labelled by CF_3COCH_2Br Under Various Conditions

Sample	pH	Excess CF_3COCH_2Br[a]	No. of aliquots	Time[b]	Groups Labelled[c]
1	8.0	—	—	—	0
2	7.2	5x	1	1 hr.	10.9
3.	7.2	5x	3	1 hr.	11.9
4	8.0	5x	1	5 hrs.	9.8
5	8.0	50x	1	5 hrs.	10.5
6	8.0	5x	3	1 hr.	13.1
7	8.6	5x	1	5 hrs.	10.1
8	8.6	25x	1	5 hrs.	9.9

[a] Excess of CF_3COCH_2Br over free SH groups in solution.

[b] Reaction time per aliquot.

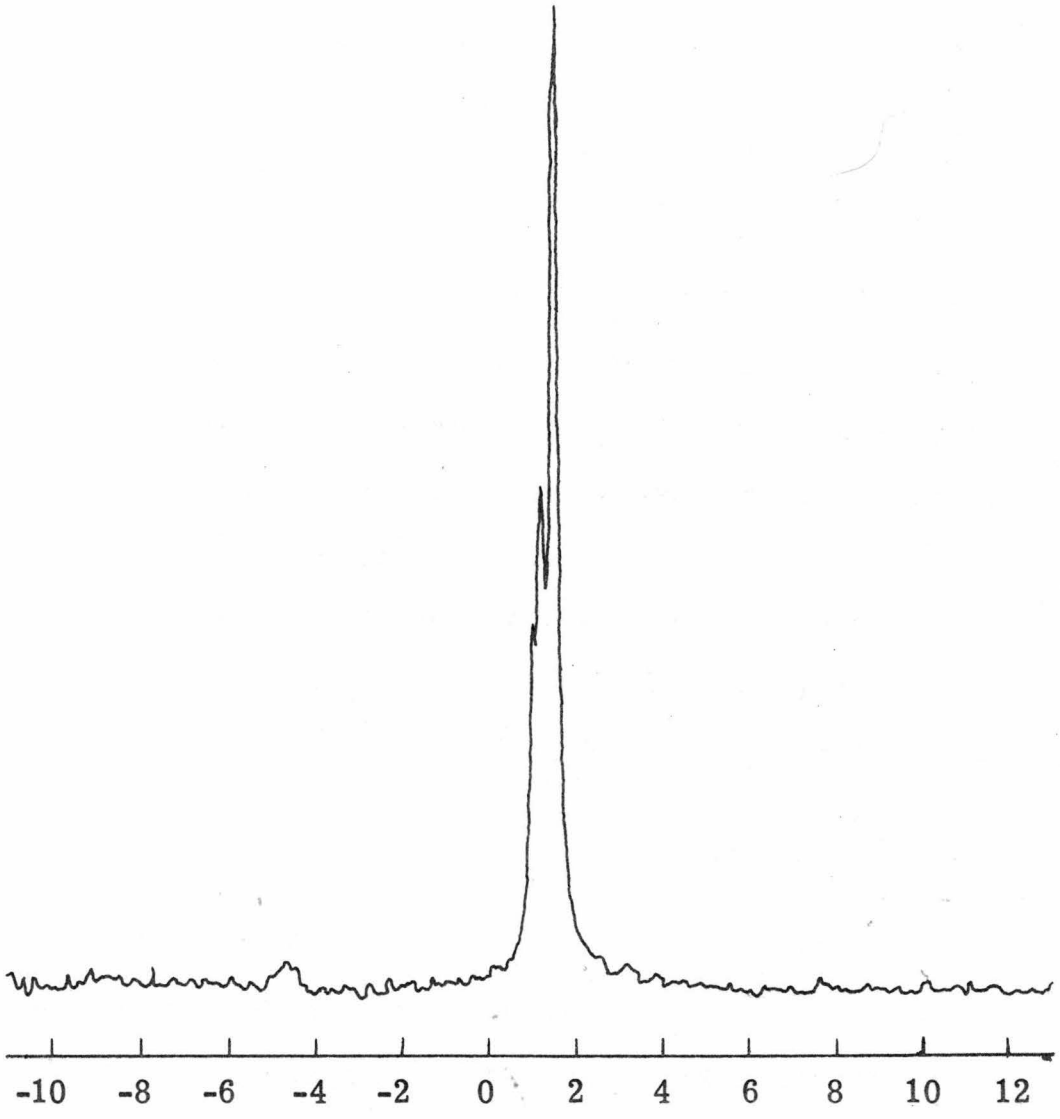
[c] Number of trifluoroacetonylated groups per antibody molecule.

six labile disulfide bonds per molecule (Tomasi and Grey, 1972). Reaction with other amino acid residues (histidine and/or those containing amino groups) is likely to account for the observed labelling of 13.3 rather than the expected 12 groups/antibody molecule. Under optimal conditions (sample 6 of Table VII), 13.1 groups per antibody molecule could be trifluoroacetonylated. Thus, reaction of immunoglobulin with 3-bromo-1,1,1-trifluoropropanone or iodoacetamide results in a quantitatively similar degree of antibody modification. These optimal conditions were subsequently used for the routine trifluoroacetonylation of TEPC 15.

The ^{19}F-NMR spectrum of trifluoroacetonylated TEPC 15 is shown in Figure 24. The observed spectrum can be readily divided into two groups of resonances with a chemical shift difference between them of 6 ppm. The low-field resonance (5 ppm downfield from CF_3COOH) always accounted for less than 10% of the total integrated area of the fluorine signal (although the precise percentage was somewhat variable between samples prepared at different times) and was present in the spectra of intact antibody, Fab' and Fab fragments. For these reasons, the low field

Figure 24

^{19}F NMR spectrum of trifluoroacetonylated TEPC 15. The sample contained 40 mg/ml protein in 0.02 M borate, 0.16 M NaCl, 1 mM EDTA, pH 8 buffer. An acquisition time of 0.3 sec was used and 13,000 transients were collected.



δ, ppm from CF_3COOH

resonance was tentatively assigned to the partially fluorinated amino termini of the light and heavy chains. The variability in intensity of this peak might arise from small variations in reaction pH of differently prepared samples and the pH-sensitivity of the amino group alkylation reaction (Means and Feeney, 1971).

The high field multiplet (1.5 ppm upfield from CF_3COOH) consits of at least five partially overlapping signals spread over a range of 50 Hz (Figure 25a). The intensity ratios of these peaks were remarkably constant among protein samples prepared at different times. Since the mouse IgA2 molecule is known to contain six labile disulfide bonds (Tomasi and Grey, 1972), quantitative modification should result in six resonances in the ^{19}F NMR spectrum. The observed spectrum can be reconciled with this structure if one allows for the overlap of two peaks so that one of the resonances remains unobserved.

Three of the six labile disulfides are inter-heavy chain bonds in the hinge region of the molecule, one is formed between the C-terminal residues of the light chains, and two are localized in the Fc region (Tomasi and Grey, 1972). Partial assignment of the

Figure 25

High field region of ^{19}F NMR spectra of trifluoroacetonylated TEPC 15 and proteolytic fragments.

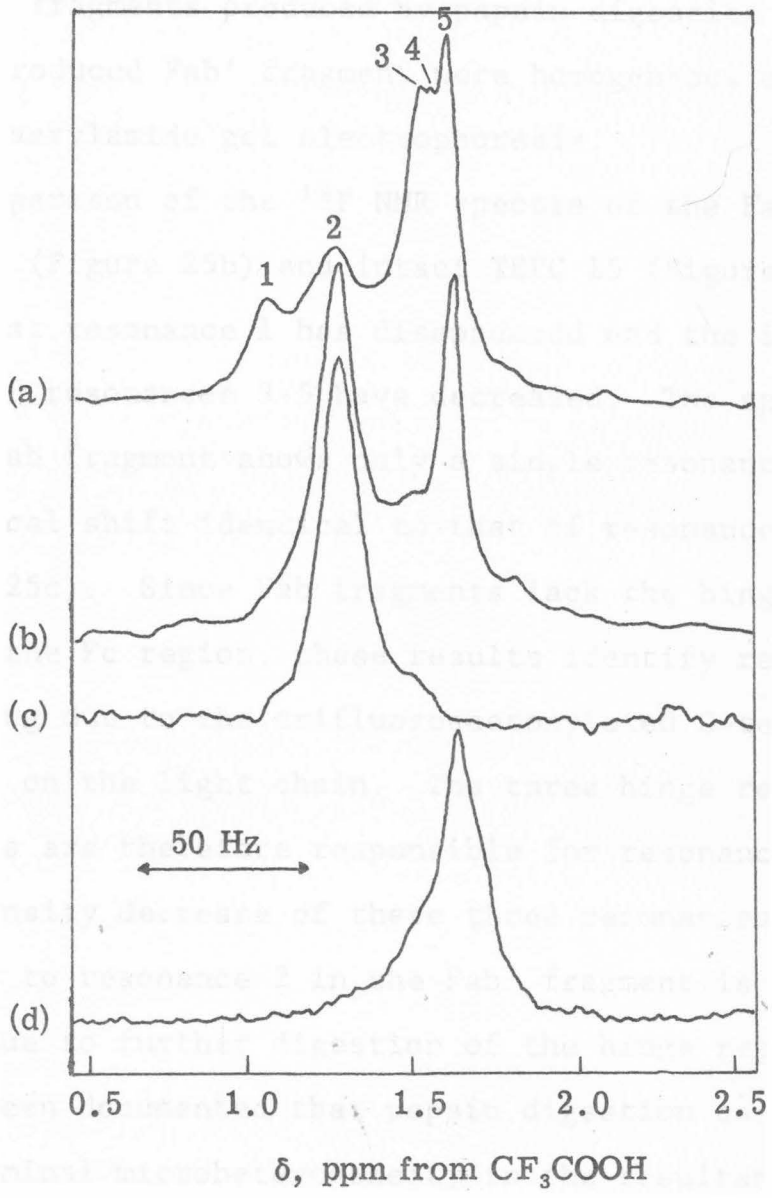
(a) Intact TEPC 15

(b) Fab' fragment

(c) Fab fragment

(c) TEPC 15 after treatment with 1% SDS

All spectra were taken at protein concentrations of 25-50 mg/ml in borate-buffered saline, pH 8.


1
2
3
4
5
(a)
(b)
(c)
(d)
50 Hz
0.5
1.0
1.5
2.0
2.5
δ, ppm from CF3COOH

resonances to specific sulfhydryl groups was made possible by enzymatic digestion of the immunoglobulin. Both the fragments produced by papain digestion and the pepsin-produced Fab' fragment were homogeneous on SDS-polyacrylamide gel electrophoresis.

Comparison of the ^{19}F NMR spectra of the Fab' fragment (Figure 25b) and intact TEPC 15 (Figure 25a) shows that resonance 1 has disappeared and the intensities of resonances 3-5 have decreased. The spectrum of the Fab fragment shows only a single resonance of chemical shift identical to that of resonance 2 (Figure 25c). Since Fab fragments lack the hinge as well as the Fc region, these results identify resonance 2 as being due to the trifluoroacetonylated C-terminal cysteine on the light chain. The three hinge region cysteines are therefore responsible for resonances 3-5. The intensity decrease of these three resonances relative to resonance 2 in the Fab' fragment is most likely due to further digestion of the hinge region. It has been documented that pepsin digestion can result in C-terminal microheterogeneity in the resultant Fab' fragment (Utsumi and Karush, 1965). The greater intensity of resonance 5 relative to resonances 3 and 4 would identify this signal as arising from the most

N-terminal of the three hinge region cysteines. Resonance 1 must therefore belong to one of the alkylated cysteine residues of the Fc region. The expected signal due to the second cysteine in the Fc region may overlap with resonance 1. Assignment of resonances 1-5 is schematically illustrated in Figure 26.

The ^{19}F NMR spectrum of SDS denatured immunoglobulin (Figure 25d) shows a single peak. This is consistent with complete denaturation of the molecule and attainment of an identical chemical environment for all the trifluoroacetonyl groups.

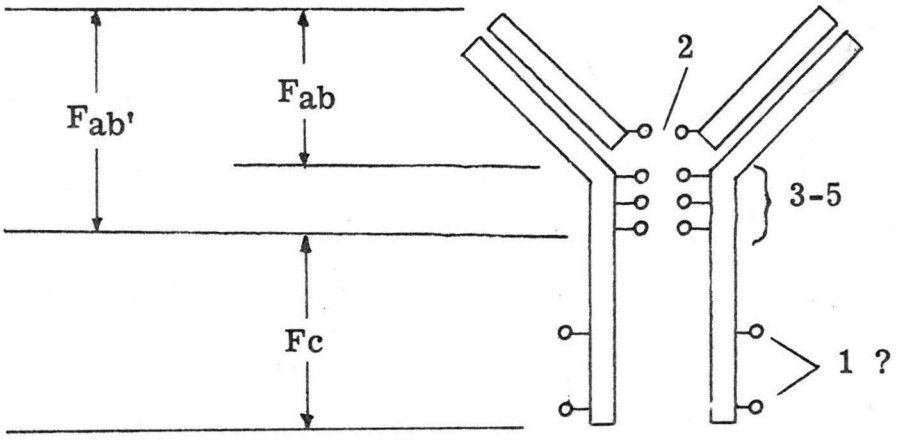
Several interesting features of Figure 25 may be noted. All the resonances of the intact protein or enzymatic fragments occur at identical or lower field strength than the single resonance of the denatured protein and there is a good correlation between linewidth and distance downfield from this signal. The narrow linewidths($\sim$ 7 Hz) of resonances 3-5 and the similarities in chemical shift of these resonances to that of the denatured protein suggest a relatively hydrophilic environment and little or no motional restriction of these trifluoroacetonyl groups. This is in agreement with all known evidence regarding the

Figure 26

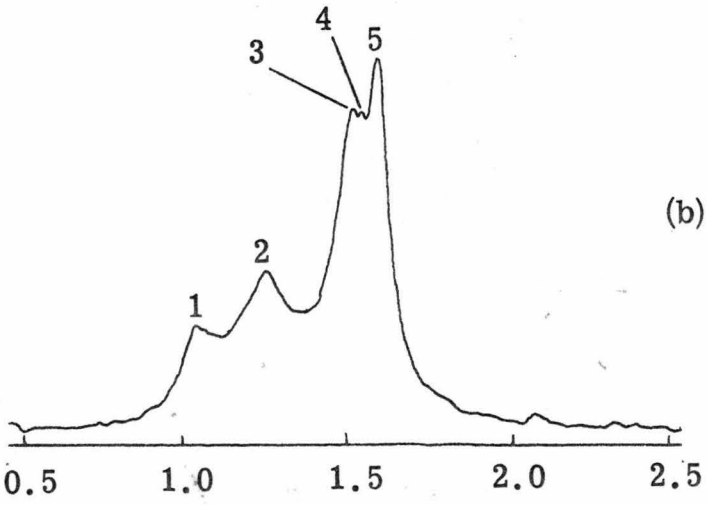
Proposed relationship between ^{19}F NMR spectrum and structure of trifluoroacetonylated TEPC 15.

(a) Structure of mouse IgA2 (Tomasi and Grey, 1972).

(b) ^{19}F NMR spectrum.



(a)



(b)

hinge regions of immunoglobulins which are known to be unusually susceptible to proteolytic attack and may serve as flexible hinges around which the Fab "arms" rotate (Tumerman et al., 1971). Furthermore, X-ray diffraction studies of IgG have shown this region to exhibit little or no tertiary structure (Huber et al., 1976) and it is almost certain that in solution amino acid residues comprising this region experience a solution-like environment.

Resonances 1 and 2 are shifted downfield from the others and exhibit noticeably greater linewidths (15-20 Hz). A plausible explanation is that these resonances arise from trifluoroacetonyl labels that experience a more hydrophobic environment. This is in agreement with our observation that the ^{19}F NMR signal of CF_3COCH_2Br dissolved in a series of organic solvents of decreasing dielectric constants shifts progressively downfield. The larger linewidths of resonances 1 and 2 suggest greater motional restriction for these than for the hinge region labels, implying that the former are likely located in a more interior region of the molecule. Of interest in this regard is resonance 2 whose chemical shift and linewidth are unchanged on forming Fab fragment. This indicates

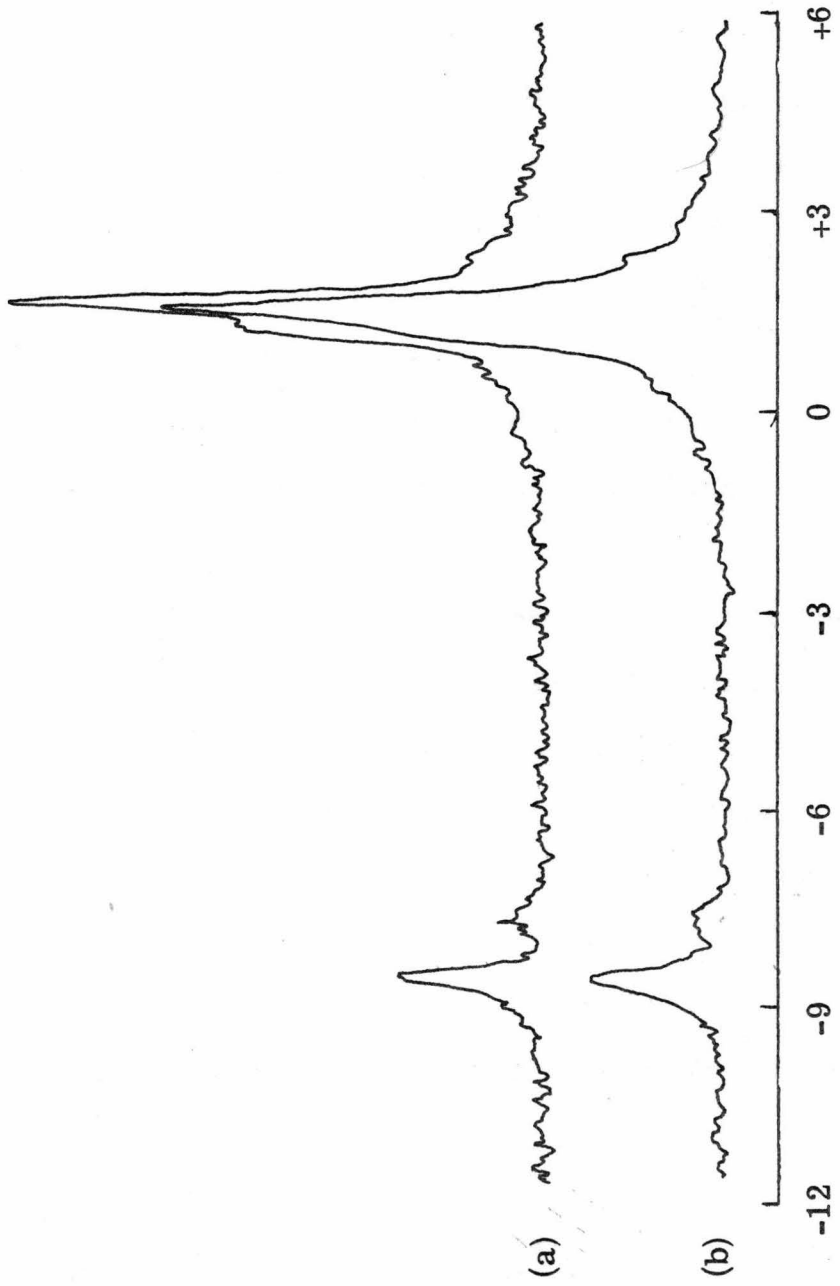
that the location of the inter-light chain disulfide bond is intrinsically hydrophobic and that this property is independent of the presence of the Fc fragment and hinge region. X-ray studies of a human immunoglobulin indicate that access of solvent to this region may indeed by restricted (Silverton et al., 1977). The ^{19}F NMR signals of the trifluoroacetonyl labels therefore are sensitive to differences in chemical environment.

The ^{19}F NMR spectra of the antibody and its enzymatic fragments were identical in the presence and absence of saturating quantities of the hapten PC. Similarly, the multivalent antigen PC-BSA also did not cause any spectral changes when soluble complexes of it and TEPC 15 were examined. No pH-dependent spectral changes were observed in the range pH 5.6-8.0.

The ^{19}F NMR spectra of trifluoracetonylated MOPC 315 both bound to DNP-SRBC and in the presence of control SRBC are shown in Figure 27. The resolution in these spectra is considerably lower than in those not involving cellular samples, due to the high viscosities of these samples and the fact that the sample tubes were spun very slowly. The spectra are generally

Figure 27

^{19}F NMR spectra of trifluoroacetonylated MOPC 315 in the presence of (a) DNP-SRBC, (b) control SRBC. Protein concentration was 26 mg/ml and cell concentration approximately 50% (v/v) in veronal-buffered saline, pH 7.2.


(a)
(b)
-12
-9
-6
-3
0
+3
+6
δ, ppm from CF_3COOH

similar to those of the TEPC 15 protein (Figure 24) with the exception that there are two low field resonances at ∿ -8 ppm instead of the single low field resonance at -5 ppm seen with TEPC 15. This feature was found to be a consistent difference between the spectra of these two immunoglobulins. However, even in this case where cellular antigens were used, no significant spectral changes could be observed between free and cell-bound antibody.

In view of the demonstrated sensitivity of trifluoroacetyl labels to nearby conformational (Huestis and Raftery, 1971, 1972a) and ionic (Huestis and Raftery, 1972b) changes, it seems likely that no major conformational changes occur in the vicinity of these labels on antigen binding. It therefore seems probable that, in this antibody system, no major conformational change is transmitted to the Fc region upon binding of antigen. Since multivalent antigens were also employed, the documented inability of antibodies to exhibit effector functions on binding of small, univalent haptens (Schlessinger et al., 1975; Jaton et al., 1976) was not a factor in the outcome of these experiments. A real possibility is that IgA, unlike other immunoglobulin classes, transmits no

conformational changes to the Fc region as it is generally accepted that IgA exhibits no Fc-dependent effector functions. (Some recent results indicate that IgA may, after all, activate complement through the classical pathway via its Fc region (Iida et al., 1976; Burritt et al., 1977).) This explanation would entail major structural differences between immunoglobulins of different classes, perhaps in the important hinge region. Although it is known that immunoglobulin sequence homology is weakest in the hinge region (Low et al., 1976), this possibility is difficult to evaluate. At the time this study was carried out, no monoclonal antibodies of other classes with known antigen specificity were available to directly test this possibility.

A more likely explanation for the lack of observation of any conformational change in these experiments is the fact that the inter-heavy chain disulfide bonds of the native molecule had been reduced and alkylated. Thus, in analogy to what is observed with IgG and IgM (Shur et al., 1964; Michaelson et al., 1975; Isenman et al., 1975; Frank and Humphrey, 1969), reduction of these bonds in IgA may prevent the transmission of an antigen-induced signal in the Fab

region to the Fc region or, less likely, destroy the structural integrity of the complement binding site. The effect of hinge-region disulfide bond reduction and alkylation, though of drastic biological consequences, must be of a subtle nature as it has no detectable effect on antigen binding properties or on the general conformation of the immunoglobulin (Dorrington and Smith, 1972).

It may be noted that changes in the circular dichroic spectrum (Morris et al., 1974) and various optical parameters (Pollet et al., 1974; Riesen et al., 1976) have been observed upon hapten binding to several mouse myeloma proteins and a human IgM Waldenström with specificity for PC. These changes were observed also in the reduced and alkylated proteins as well as in Fab' fragments indicating small structural changes that in this case are restricted to the vicinity of the binding pocket. The inability to detect such changes by ^{19}F NMR suggests that they do not extend to the C_L-C_H1 domain, which contains the trifluoroacetonyl label closest to the combining site.

Recently, kinetic evidence for a hapten-induced change in the DNP-binding myeloma protein MOPC 460

(reduced and alkylated) has been obtained by T-jump experiments. In agreement with the above conclusion, these changes appear also to be localized to the Fab region (Lancet and Pecht, 1976).

The lack of any pH-dependent changes in the ^{19}F NMR spectrum in the region pH 5.6-8.0 is in agreement with the results of Pollet and Edelhoch (1973) who, on the basis of fluorescence experiments, concluded that no major structural changes occur in the TEPC 15 Fab' fragment in the range pH 3-11.

Conclusions

Reduction and trifluoroacetonylation of mouse immunoglobulin inter-chain disulfide bonds results in the covalent incorporation of six environmentally-sensitive ^{19}F reporter groups. The chemical shifts and linewidths of the ^{19}F NMR signals reflect their varying degrees of exposure to solvent and indicate that the C-termini of the light chains, and the labelled cysteines of the Fc region, are in a less hydrophilic environment than the exposed hinge region. From the lack of observable ^{19}F NMR spectral changes upon binding of trifluoroacetonylated antibodies to haptens and multivalent antigens, it is concluded

that reduced and alkylated mouse IgA2, upon binding of antigen, does not exhibit conformational changes extending to the C-terminus of the Fab or to the Fc region.

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Proposition I

The term complement refers to a group of 11 serum proteins which occur naturally in inactive form. After stimulation by antibody-antigen complexes these proteins may undergo a complex sequence of reactions which terminates in the destruction of the antigenic cells through membrane lysis.

The current mode of complement-mediated cell lysis involves three functional units, each utilizing different complement components (Müller-Eberhard, 1972). The first unit has affinity for the immune complex via specific sites on the antibody. After binding to antibody, this recognition unit acts enzymatically on an activation unit allowing it to bind to the cell membrane. This second unit serves to enzymatically activate the third or attacking unit which, after binding to the cell surface, causes membrane damage. Selective destruction of the target cell is assured by the extreme lability of many of these proteins unless they are bound to a cell surface. The membrane attack complex is the only known mechanism of blood plasma which is capable of impairing biological membranes (Kolb and Müller-Eberhard, 1975a) and for this reason the

nature of its action has attracted great interest. One of the oldest concepts of complement hemolysis is the hypothesis that the terminal complement components contain phospholipase A activity, acting through enzymatic digestion of membrane lipids (Fischer, 1964; Smith and Becker, 1968) but this concept has lacked recent experimental confirmation (Humphrey, 1972; Inoue and Kinsky, 1970; Lachmann et al., 1973). It is now believed that complement exerts its lytic effect through a detergent action (Müller-Eberhard, 1970).

The attack complex (MW $\sim$ 1 x 10^6) can be formed either in solution or on a membrane surface and is known to contain C5b, C6, C7, C8 and C9 in a 1:1:1:1:3 ratio (Kolb and Müller-Eberhard, 1975a). The solution complex also contains an unidentified protein, termed S-protein, which is believed to be the serum equivalent of a hydrophobic membrane acceptor site (Podack et al., 1976, 1977). The use of specific antisera has allowed Kolb et al. (1972) to deduce the approximate positions of the individual components with respect to each other and to the cell surface. C5b, C6 and C7 are bound directly to the

cell surface and form an "adaptor" for C8 which binds to this trimolecular complex. C9 is believed to bind largely to C8. A search for unique structural features among members of the C5b,6,7,8,9 complex has served to focus attention on C8. This molecule consists of 3 polypeptide chains (α,β,γ) of roughly similar size. Radioiodination in solution results in localization of 90% of the radioactivity to the β chain (Kolb et al., 1976) suggesting that it encapsulates the other chains. This has led Müller-Eberhard (1975) to postulate that the attack complex functions by insertion of the hydrophobic α and/or γ chain of C8 into the membrane bilayer as a result of a conformational change in C8 induced by binding to the C5b,6,7 complex. The role of C9, which is known to increase the degree of C8-induced hemolysis (Hadding and Müller-Eberhard, 1969; Kitamura and Inai, 1974), would be to enhance the transition occurring in C8. Since neoantigens of the soluble complex were also detected on the target cell-bound complex, it was suggested that the membrane attack complex is primarily located on the cell surface as opposed to the interior of the membrane (Kolb and Müller-Eberhard, 1975b).

A somewhat different mode of complement action, termed the "doughnut model", has been proposed by Mayer (1972). In this model, the polypeptide chains of several of the terminal complement components insert into the membrane forming a rigid, hollow structure with a hydrophilic interior and hydrophobic exterior which allows small molecules to directly exchange with the cell interior through the central channel. This model is in accord with electron microscope studies of complement lesions which show a dark central portion (diameter 100 Å) surrounded by a light ring (Humphrey and Dourmashkin, 1969) which can be interpreted as arising from a hydrophobic ring with a hydrophilic interior (Mayer, 1972). Further evidence for such an insertion hypothesis comes from solvent elution and enzymatic stripping experiments which show that insertion starts after formation of the C5b,6,7 complex and that polypeptide chains from C5b, C7, C8 and C9 become inserted in the bilayer (Hammer et al., 1975, 1977, Bhakdi et al., 1975). Extensive insertion of hydrophobic polypeptides is also suggested by the observed release of intact membrane phosphilipids (Kinoshita et al., 1977).

Although the models proposed by Müller-Eberhard and Mayer both feature insertion of hydrophobic polypeptides into the bilayer, they differ fundamentally in the 3-dimensional arrangement of the components of the lytic complex and its degree of embeddedness in the membrane. It is proposed that chemical cross-linking experiments followed by SDS gel electrophoretic analysis be employed to deduce the protein-protein contacts in the complex (Davies and Stark, 1970) and thereby differentiate between these two, or alternate, structures. Complement-mediated lysis of liposomes containing the Forssman antigen is readily achieved (Haxby et al., 1968; Kinsky et al., 1969; Inoue et al, 1971) which means that systems completely free of protein except for antibody and complement can be used and the employment of cross-linking reagents specific for protein functional groups will ensure that only proteins of interest will react. The cross-linked proteins can be identified by prior radioiodination of the purified components (Podack et al., 1976) or through the use of cleavable, heterobifunctional cross-linking reagents such as 3-[(p-azidophenyl)dithio]propioni-

midate (Das et al., 1977). In this latter experiment a given complement protein is first allowed to react with the imidate end of the reagent, allowed to complex with the other proteins, and cross-linking achieved by photoactivation of the azidophenyl group. The cross-linked proteins can be isolated (gel filtration or preparative electrophoresis), cleaved and identified by standard techniques. Cross-linking of the membrane-bound complex can also be carried out using water-soluble reagents such as gluteraldehyde, bisdiazobenzidine or diimidates of varying length or water-insoluble reagents such as hexamethylenediisocyanate and 1,5-difluoro-2,4-dinitrobenzene (Means and Feeney, 1971; Hucho et al., 1975) and thereby potentially differentiate between protein subunits in contact with each other in the aqueous phase as opposed to the interior of the lipid bilayer. Furthermore, cross-linking experiments of the soluble complex (containing S-protein) may indirectly indicate which polypeptides interact strongly with the membrane lipids in the cell-bound complex since these are expected to be in closest contact with S-protein in the soluble complex (Podack et al., 1976, 1977).

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Proposition II

The noncytotoxic degranulation of basophils and tissue mast cells, resulting in the release of biologically active amines, may be initiated by several biologically relevant substances including IgE-antigen complexes (Osler et al., 1968; Ishizaka et al., 1969) and the anaphylatoxic complement components C3a and C5a (Cochrane and Müller-Eberhard, 1968). The first step in the IgE-mediated reaction involves binding of IgE to a cellular receptor specific for the Fc region (Ishizaka et al., 1970a,b). Subsequent events in the release mechanism are triggered by the binding of specific antigen or anti-IgE to the cell-bound immunoglobulin.

On the basis of a large body of evidence it has been possible to dissect this release mechanism into several distinct steps. One of the earliest events following (and perhaps synonymous with) antigen binding to receptor-bound IgE is the Ca^{2+}-independent activation of a serine esterase with chymotrypsin-like specificity (Humphrey et al., 1963; Perera and Mongar, 1963; Becker and Austen, 1966). In the absence of Ca^{2+}, this activated state decays and leaves the cell unre-

sponsive to further stimulation (Lichtenstein, 1971). In the presence of Ca^{2+}, histamine is released via an energy-requiring mechanism (Ranadive and Cochrane, 1971).

Studies of the mast cell - IgE interaction have been greatly facilitated by the availability of several transplantable murine mast cell tumors. These include a rat basophilic leukemia cell line and a mouse mastocytoma line both of which have been adapted to cell culture (Kulczycki et al., 1974; Mendoza and Metzger, 1976). By treatment of these cells with Nonidet P-40 it has been possible to quantitatively solubilize IgE binding activity (Conrad et al., 1976) and large amounts of purified receptor are now obtainable by affinity chromatography of detergent-solubilized membrane preparations over IgE-Sepharose (Rossi et al., 1977). This had led to preliminary physical characterization of the IgE receptor (Conrad et al., 1976; Kulczycki et al., 1976; Rossi et al., 1977; Newman et al., 1977) and allows experiments designed to prove other possible functions of the receptor molecule to be undertaken.

Since it is likely that the IgE receptor, or components closely associated with the receptor in the cell membrane, exhibit serine protease activity, it is proposed that such activity be quantitated using potent

serine protease substrates such as N-carbobenzoyl-L-lysine p-nitrophenyl ester and N-benzoyl-L-phenylalanyl-L-valyl-L-arginine p-nitroanalide, the latter being considerably more sensitive than previously used substrates (Bing, 1969). The use of synthetic substrates should allow precise measurements of the kinetics and stoichiometry of the activation process. Such studies are perhaps most readily carried out using previously described cell-free particles (Carson et al., 1975) or liposomes resulting from cell sonication and can be carried out in the presence or absence of IgE and anti-IgE. The purified receptor will be assayed similarly. If proteolytic activity is limited to membrane fragments, suggesting that the enzyme is a molecule distinct from the receptor, attempts to copurity it with the receptor can be made by first cross-linking the receptor with any closely associated membrane proteins in situ, prior to detergent solubilization. SDS gel electrophoretic analysis of the enzymatically active protein, isolated before and after activation, can be carried out to determine if autocatalysis of the enzyme occurs during the activation process.

Such studies should help to clarify not only the molecular basis of mast cell triggering but, since all cell systems involved in noncytotoxic mediator secretion have certain key features in common, should also shed light on the more general phenomenon of how cells are triggered via their membrane receptors.

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Proposition III

C-reactive protein (CRP) is a trace constituent of normal serum whose concentration is increased as much as 1000-fold during the acute phase of inflammatory reactions (Anderson and McCarty, 1950; Hedlund, 1961). Although measurement of CRP levels has long been used as an index of acute inflammation, its biological purpose is still unknown. CRP has the unique ability to precipitate pneumococcal C-polysaccharide, lecithin and sphingomyelin in a Ca^{2+}-dependent reaction (Tillett and Francis, 1930; Abernathy and Avery, 1941; Kaplan and Volanakis, 1974). In addition, it exhibits many properties normally associated with antibodies such as agglutination (Gal and Miltenyi, 1955), opsonization (Kindmark, 1971) and the ability to activate the classical complement pathway (Kaplan and Volanakis, 1974; Osmand et al., 1975). Other properties attributed to CRP include the abilities to stimulate leukocyte migration (Wood, 1951), facilitate phagocytosis by reacting with bacterial surfaces (Hokama et al., 1962; Kindmark, 1971, 1972), induce lymphocyte-blast transformation (Hornung and Fritchi, 1971) and enhance lymphocyte cytotoxicity (Hornung, 1972).

CRP has been isolated from serum of man, monkey, rabbit and dog (Riley and Coleman, 1970) but only the proteins from man and rabbit have been extensively characterized (Gotschlich and Edelman, 1965; Bach et al., 1977). Rabbit CRP is composed of five identical subunits of MW 22,900 associated through non-covalent forces although the intact protein may be somewhat heterogeneous with respect to number of subunits (Bach et al., 1977). The human protein is highly similar. Inhibition studies have shown that CRP binds specifically the phosphorylcholine determinant of the substances it precipitates (Volkanis and Kaplan, 1971; Heidelberger et al., 1972). Equilibrium dialysis has shown one binding site per subunit and a phosphorylcholine association constant of 1.0-1.3 x 10^6 M^{-1} at 5°C for both proteins (Bach et al., 1977; Osmand, 1972). The partial amino acid sequences of both proteins are highly similar and, provocatively, show a distant but significant homology with the C_H3 domain of human IgG and the human HLA H chain (Osmand et al., 1977). This has led to the postulate that these three groups of proteins share a distant, common evolutionary origin (Osmand et al., 1977).

CRP can be isolated from acute phase sera in high yield (> 0.6 mg/ml, Osmand et al., 1975) and is rapidly and easily purified to homogeneity by affinity chromatography and gel filtration (Kaplan and Volanakis, 1974; Osmand et al., 1975). This now allows detailed physical studies requiring large amounts of pure protein to be carried out. It is proposed that the binding site interactions between phosphorylcholine and CRP be investigated using ^{13}C and ^{31}P NMR in analogy to similar studies involving phosphorylcholine-binding mouse myeloma immunoglobulins (Goetze and Richards, 1977, 1978). Such studies would be of interest for the following reasons: (i) The binding site structures of phosphorylcholine-specific antibodies from different mammalian species may be generally similar as evidenced both by the strict conservation of functionally important residues (Riesen et al., 1976) and various binding properties. These antibodies are likely therefore to represent a generalized "best fit" of the immunoglobulin structure to the phosphorylcholine moiety which has been preserved in the germ-line of mammals because of its optimum specificity (Potter et al., 1976). The high phosphorylcholine affinity of CRP (similar to that of antiphosphorylcholine antibodies) likely represents an example of convergent evolution as CRP has a structure unlike that of immunoglobulins

(Gotschlich and Edelman, 1965). This therefore affords an opportunity to study how high phosphorylcholine affinity has independently evolved in two structurally different protein systems. It is interesting that the fine specificity of CRP for phosphorylcholine analogs is similar to that of some mouse antibodies (Kaplan and Volanakis, 1974). (ii) The binding site of CRP differs importantly from that of mammalian antibodies in that Ca^{2+} must be bound prior to combining with phosphorylcholine. In addition, CRP, unlike mouse antibodies, is able to bind some phosphate esters not containing a quaternary nitrogen (Gotschlich and Edelman, 1967). Thus, in spite of certain similarities, there are also important differences which should make a comparison of binding site architectures of great interest. (iii) A detailed knowledge of the CRP binding site may yield information concerning the biological function of this protein.

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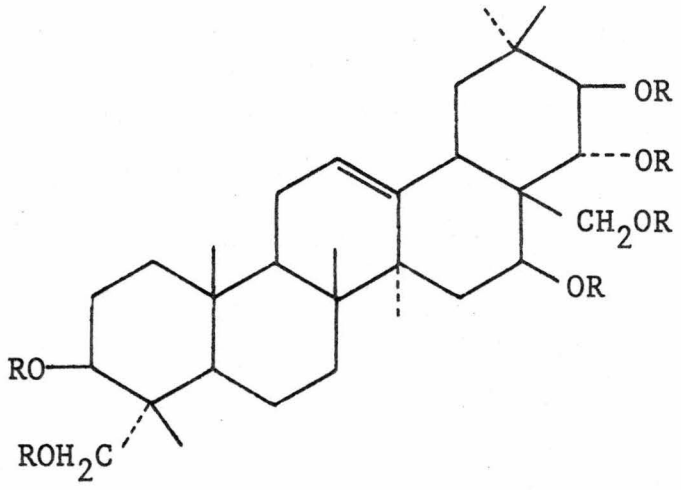
Proposition IV

The current view of the human taste sense is that it consists of four different modalities (salt, sour, sweet and bitter) each with its particular type of receptor (Pfaffmann et al., 1971). It is thought that the reversible binding of stimuli to cell surface receptors results in a local reorientation of the taste cell membrane leading to nerve impulses (Beidler, 1962, 1967).

Sweet taste can be elicited by a variety of molecules including various carbohydrates, D-amino acids, halogenated carbon compounds, inorganic salts and miscellaneous organic compounds (Schallenberger and Acree, 1971) as well as certain proteins (Morris and Cagan, 1972; van der Wel, 1972). In spite of this, the evidence is strong that there is only one type of sweet receptor in mammals (Schoonhoven, 1973).

The leaves of the Indian plant Gymnema sylvestre have long been known to specifically suppress the sensitivity to sweet substances. Chewing of the leaves, or application of acqueous extracts to the tongue, temporarily reduces the sensitivity to sweet substances while leaving other taste sensations unaffected.

Kurihara (1969, 1971) has isolated the active substance (gymnemic acid A_1) which was characterized as



The six R groups represent formic, acetic, n-butyric, isovaleric, tiglic and glucuronic acids in unknown positions. Gymnemic acids A_2 and A_3 represent degradation products of A_1 formed by liberation of esterified acids and exhibit progressively less activity. These compounds can be separately isolated. Gymnemic acid (GA) was found to suppress the sweet taste of all classes of sweet-tasting substances tested (except chloroform). It has been suggested that the inability to abolish

the sweet taste of $CHCl_3$ is due to the high vapor pressure and lipid-dissolving qualities of this compound which may be related to a different mechanism of sweet induction (Kurihara et al., 1968).

An understanding of the mode of action of gymnemic acid would be helpful towards elucidating the mechanism of the stimulation of taste receptors. There is however no general agreement on a possible mechanism. Because of its specificity in abolishing only the sweet response, GA almost certainly does not act as an anesthetic in suppressing the nerve function of taste cells. Warren and Pfaffmann (1959) showed that the degree of sweet sensitivity suppression of GA is equivalent for equi-sweet concentrations of sucrose and saccharin. This, as well as the glycoside structure of GA, suggests that GA competes for binding to the sweet receptor site on the taste membrane. This does not explain however why GA itself is tasteless. In addition, this hypothesis leaves unexplained the findings of Warren et al. (1969) who reasoned that if binding is competitive, an adaptation to high concentrations of sweets before GA application should inhibit the suppressing effect of GA. This was not observed, and the authors suggested that either GA binds non-competitively to another site which influences

the sweet receptor site or GA may interfere with intermediate cell reactions leading to nerve stimuli. Further complicating the issue are observations that GA does not inhibit the sweet response in all mammals (Hellekant, 1976) and that application of GA to the olfactory area of tortoises abolished their neural responses to olfactory stimuli (Tucker, 1969). It is clear that an unequivocal answer to the question of whether or not GA acts by competitively binding to the sweet receptor is a necessary first step in our understanding of the mode of action of this molecule.

Putative sweet receptors have been isolated from tongue epithelium of several mammals including cattle (Dastoli and Price, 1966; Price, 1971) and rat (Hiji and Sato, 1971). The bovine protein has been purified to near homogeneity and has a molecular weight of 150,000 and an isolectric point of 9.1 (Dastoli et al., 1968). The dissociation constants of the interaction of this protein with several sugars and amino acids were determined (a range of 6-660 mM) and found to parallel the taste thresholds of these compounds. It is proposed that the interaction of GA with this protein be studied as a means of determining if GA acts by blocking the

sweet receptor *in vivo*.

The major difficulty foreseen in this study is the possible low affinity (if any) of GA for the receptor proteins. This would make it difficult to quantitate the binding by conventional means such as equilibrium dialysis. A suitable alternative however is gel filtration which, based on the relative elution volumes of macromolecule and ligand, can be used to quantitate binding (Ackers and Thompson, 1965). In this case it would perhaps be most convenient to determine the binding affinity of GA indirectly by its ability to inhibit the binding of a radiolabelled sugar such as sucrose or fructose to the protein (Hiji and Sato, 1971). Alternatively, the receptor protein can be covalently attached to a solid support such as polyacrylamide (Schnapp and Shalitin, 1976) and the ability of GA to inhibit labelled sugar binding to the resin used to calculate a binding affinity. It will also be possible to determine whether the lower sweet suppressing activities of gymnemic acids A_2 and A_3 are due to lower binding affinities or due to other, more subtle effects.

This study will directly answer the question of whether or not GA functions by competing with sweet-tasting substances for the sweet receptor and thereby allow rational experiments, designed to further examine its mode of action, to be carried out.

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PROPOSITION V

Chemical carcinogens either directly, or enzymatically, yield reactive electrophiles which interact covalently with unknown critical macromolecules in the initiation of cancer. Many of the critical steps in oncogenesis including the intracellular course of the carcinogenic electrophile, the identity of the macromolecular target, and the functional consequences of the interaction remain poorly understood. Nevertheless, it is clear that despite their reactivity, sufficient amounts of the activated carcinogens survive the presence of high concentrations of intracellular nucleophiles to allow them to reach their targets (Miller and Miller, 1974).

During malignant transformation induced by aminoazo dyes or 2-acetylaminofluorene in rat liver or by polycyclic aromatic hydrocarbons in mouse skin and cell culture (Sorof et al., 1969, 1970; Tasseron et al., 1970; Kuroki and Heidelberger, 1972), carcinogen derivatives interact covalently with a small number of basic proteins in the cell cytosol. In particular, a single liver protein serves as the principal protein target of azocarcinogens during

the induction of hepatocarcinogenesis in the rat (Sorof et al., 1963; Sorof and Young, 1973). This interaction is highly specific with respect to protein, carcinogen and organ (Mainigi and Sorof, 1977). The target protein has been partially characterized (Sorof et al., 1973; Sani et al., 1974) but its identity and function remain unknown. The carcinogen-protein complex (h_2-5S azoprotein) is present mainly in the liver cytosol and in smaller amounts in nuclei (Bakay and Sorof, 1969; Bakay et al., 1969). No h_2-5S azoprotein or target protein is detected in the cytosol of differentiated liver tumors induced by aminoazo carcinogens but normal levels are present in hepatomas induced by other carcinogens (Mott et al., 1973).

The h_2-5S azoprotein has been isolated 88-91% pure in 50 mg quantities and characterized as a dimer of identical, noncovalently bonded subunits of MW 44,000 each (Sorof et al., 1974). Each subunit contains an average of one bound dye molecule. When assayed after denaturation and proteolysis, the azodye is apparently covalently attached to the degraded protein (Sorof and Young, 1967; Sorof et al., 1974).

Since laboratory manipulations of the complex might cause covalent bonding, the mode of binding *in vivo* is not certain. However, it has been speculated that the native complex may contain activated azocarcinogen (Ketterer *et al.*, 1975). This is an important issue since covalent *in vivo* binding would suggest that h_2-5S target protein modification may result in cancer whereas noncovalent binding might suggest that the target protein serves to protect and/or transport the azocarcinogen inside the cell. This latter possibility is lent some support by the discovery that ligandin is a specific *in vivo* target of some carcinogens (Litwack and Morey, 1970; Singer and Litwack, 1971). The role of ligandin in cells is still unknown but there are suggestions that it may serve as an intracellular transporting agent of hydrophobic molecules including steroids and carcinogens (Litwack *et al.*, 1971).

A recent, provocative study (Mainigi and Sorof, 1977) using the hepatocarcinogen 3'-methyl-4-dimethylaminoazobenzene has suggested that the azocarcinogen and protein moieties of the native azoprotein are noncovalently linked and have different biological activities. This was deduced from

the differences of the in vivo half-life of the bound azo dye in the complex (2.5 ± 0.25 days) and the protein moiety of the complex (8.7 ± 1.6 days). In addition, binding of azocarcinogen extends the half-life of the protein (presumably due to assuming a more compact conformation) since the target protein has a half-life of only 3.3 ± 0.2 days. Because after protein denaturation the azo dye appeared to be covalently bound to the protein, these authors suggested that the native complex contains azo dye in an activated state capable of yielding a reactive electrophile. They further postulated that the conformationally-altered protein of the azoprotein complex might determine the specificity of the reaction of the hydrophobically-bound azocarcinogen with critical nuclear macromolecules (Mainigi and Sorof, 1977). It is proposed that some predicted features of this model be directly tested in vitro.

One of the possible functions of the target protein is to protect the activated azocarcinogen from intracellular nucleophiles and other metabolic deactivation mechanisms. The intracellular reactions of azocarcinogens are understood in great

detail (Arcos and Argus, 1974) and it is known that reductive cleavage of the azo bond is the most important detoxifying mechanism for such compounds (Mueller and Miller, 1949). The ability of the azoprotein to protect the bound carcinogen from cleavage by azo reductase will be determined in vitro. Azo reductase activity can be assayed using liver slices or isolated microsomal fractions of tissue homogenates (Miller and Miller, 1953). The enzyme system requires riboflavin adenine dinucleotide and NADPH as cofactors as well as oxidizable substrate (Mueller and Miller, 1949, 1950). The azocarcinogen is conveniently quantitated spectrophotometrically after extraction with 20% trichloroacetic acid in 1:1 acetone-ethanol (Mueller and Miller, 1948). If reductive cleavage of the azo bond of the azoprotein is observed in this system, it will rule out both a covalent azocarcinogen-target protein bond (and therefore rule out modification of target protein as the initiating event in oncogenesis) and a carcinogen-shuttling role for the h_2-5S protein as proposed by Mainigi and Sorof (1977). On the other hand, if the azocarcinogen remains intact, the

hypothesis of Mainigi and Sorof will be strengthened. Either result should be of importance towards the eventual elucidation of a molecular mechanism of chemical oncogenesis.

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