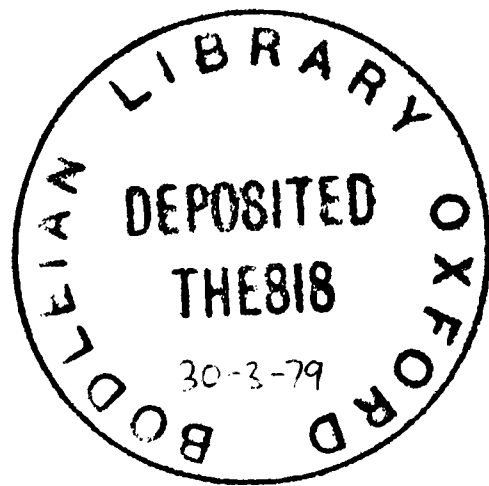




STRUCTURAL STUDIES ON ANTIBODIES

Steven K. Dower

Worcester College, Oxford.

This thesis was submitted in partial fulfilment of
the requirements for the degree of D.Phil.,

January 1979.

ABSTRACT

Structural Studies on Antibodies.

Steven K. Dower, Worcester College, Oxford.

A thesis submitted for the degree of D.Phil.

Michaelmas Term, 1978.

1. Data from ^1H nuclear magnetic resonance studies on the Fv fragment of protein 315, a Dnp-binding BALB/c mouse IgA(λ_2) myeloma protein, have been used to refine a predicted structure of the combining site of the protein. The Dnp-binding subsite in the modified structure is composed of the side chains of three aromatic amino acids Trp 93_L, Tyr 34_L and Phe 34_H. A fourth aromatic amino acid residue is close to the side chain-NH-CH₂-group, this is Tyr 33_H. The antibody-hapten binding is a simple encounter process, which causes no extensive conformation change in the Fv fragment.
2. A method for paramagnetic structural studies has been devised using Dnp derivatives with oligophosphate side chains, which create a specific manganese binding site on the Fv fragment-hapten complex. The distances from the bound metal ion to the imidazole side chains of two of the three histidine residues of protein 315 have been determined.
3. ^1H nuclear magnetic resonance has been used to study the histidine residues of the Fv fragment of protein 315. It has been shown that one of the three histidine residues (102_H) is close to the combining site, but that this residue does not participate directly in binding haptens. ^{31}P nuclear magnetic resonance studies have shown the presence of a positively charged amino acid side chain near the entrance of the combining site of the Fv fragment. This residue has been identified as Arg 95_L.
4. The mode of binding of trinitrophenyl derivatives to the Fv fragment has been studied by ^1H nuclear magnetic resonance. It is concluded that these haptens, when bound to the Fv fragment, make contacts with the same amino acid side chains as Dnp derivatives.

This thesis contains work previously reported in the following publications:-

1. Dower, S.K., Gettins, P., Jackson, W.R.C., and Dwek, R.A., (1978), Biochem. J., 169, 179-188.
2. Dower, S.K., and Dwek, R.A., (1978), In "Biomolecular Structure and Function", pp.295-328, Eds. Agris, P., Loepky, D., and Sykes, B.D., Academic Press, New York, San Francisco, London.
3. Dower, S.K., Wain-Hobson, S., Gettins, P., Givol, D., Jackson, W.R.C., Perkins, S.J., Sunderland, C.A., Sutton, B.J., Wright, C.E., and Dwek, R.A., (1977), Biochem. J., 165, 207-225.
4. Dwek, R.A., Wain-Hobson, S., Dower, S., Gettins, P., Sutton, B., Perkins, S.J., and Givol, D., (1977), Nature (London) 266, 31-37.
5. Wain-Hobson, S., Dower, S.K., Gettins, P., Givol, D., McLaughlin, A.C., Pecht, I., Sunderland, C.A., and Dwek, R.A., (1977), Biochem. J., 165, 227-235.

Acknowledgements

I would like to thank Dr. R.A. Dwek for his help, support and encouragement during the past three years.

The work reported in this thesis is part of a project on protein 315 in which several people have participated. I should like to thank the following in particular:-

Dr. S. Wain-Hobson, with whom I collaborated closely during the first two years of my work, and who taught me the use of the 270 MHz n.m.r. spectrometer.

Dr. C. Wright - who built the original model of protein 315 in Oxford.

Mr. P. Gettins - who carried out the model building study based on the n.m.r. data.

Mr. R. Jackson - who has made available to me his data on the light chain dimer of protein 315, prior to publication.

I would also like to thank the following, who, although not directly involved in the work reported here have provided me with help and discussion in this work:-

Dr. S. Easterbrook-Smith - for teaching me FORTRAN programming and helping to write and run the programs given in this thesis.

Drs. D. Gadian and D. Gorenstein for discussions and advice on the ^{31}P n.m.r. studies.

Drs. D.B. Dunn and R. Trim for advice on the synthesis and separation of the oligophosphate compounds, and for providing me with the facilities of the John Innes Institute, Norwich, for the trial separation procedures.

Dr. I.D. Campbell for many useful discussions on the design and interpretation of ^1H n.m.r. experiments.

Finally, I should like to thank the Medical Research Council and Worcester College, Oxford, for providing me with financial support for this work.

CONTENTS

Page

CHAPTER 1 : GENERAL INTRODUCTION	1
1. Introduction	1
2. The Immune Response	2
3. Monoclonal Proteins	4
4. Antibody Specialization in Relation to Effector Function: Classes and Subclasses	7
5. The Structure of Immunoglobulins	8
a) The four chain unit	8
b) The fundamental unit of immunoglobulin structure: the domain	8
c) Variable and constant regions	11
d) Patterns of structural variation in immunoglobulins: isotypes, allotypes and idiotypes: classes and subclasses	14
6. The Antibody Combining Site	
a) Introduction	19
b) Antibody-antigen binding studies	20
c) Covalent modification of antibody molecules	23
d) Physical studies of antibody-antigen interactions	25
e) X-ray crystallographic analyses of antibody variable regions	33
7. Protein 315: A Review	37
CHAPTER 2 : HIGH RESOLUTION ^1H N.M.R. STUDIES ON THE FV FRAGMENT OF PROTEIN 315. : THE BINDING OF 2,4 DINITRO- PHENYL DERIVATIVES	
1. Introduction	44
2. Materials and Methods	
a) Preparation of the Fv fragment of protein 315	47
b) Preparation of haptens	49
c) Preparation of n.m.r. samples	49
d) 270 MHz ^1H n.m.r. studies	50
e) Fluorescence measurements	50
f) Optical difference spectroscopy	51
g) Analysis of binding data	51
h) Lineshape analysis	52
i) Ring current calculations	52
3. Results	
a) The ^1H n.m.r. spectrum of the Fv fragment of protein 315	53
b) Spin-lattice relaxation time measurements of resonances in the Fv fragment spectrum	54

Page

c)	The effect of hapten binding on the ^1H n.m.r. spectrum of the Fv fragment	56
d)	The binding of N-Dnp-L-aspartate to the Fv fragment	
	i) Optical difference spectroscopy	58
	ii) ^1H n.m.r. studies	59
e)	Chemical exchange rates for the Fv fragment N-Dnp-glycine complex	63
4.	Discussion	
a)	Assignments for resonances in the Fv fragment spectrum	65
b)	The origin of secondary shifts in proteins	69
c)	The interpretation of the observed chemical shift changes - the Dnp subsite	75
d)	The mode of hapten binding	79

Appendices

I	Ring Current Calculations	83
II	Linewidth Calculations	85
III	Computer Programs	86

CHAPTER 3 : HIGH RESOLUTION ^1H N.M.R. STUDIES ON THE FV FRAGMENT OF PROTEIN 315. : THE USE OF A PARAMAGNETIC CENTRE TO OBTAIN DISTANCE INFORMATION

1.	Introduction	89
2.	Materials and Methods	
	a) ^1H n.m.r. studies	91
	b) Fluorescence measurements	91
	c) Preparation of samples	91
	d) Preparation of N-Dnp-2-aminoethyl phosphates	
	i) Preparation N-Dnp-2-aminoethyl phosphate	92
	ii) Preparation of N-Dnp-2-aminoethyldiphosphate and N-Dnp-2-aminoethyltriphosphate	93
	e) Manganese binding studies	95
	f) Analysis of manganese resonance broadening data	96
3.	Results	
	a) Binding studies	98
	b) High resolution ^1H n.m.r. studies	100
4.	Discussion	
	a) Reliability of the distance measurements	101
	b) Conclusions	103

CHAPTER 4 : CHARGED RESIDUES IN THE COMBINING SITE OF THE FV FRAGMENT OF PROTEIN 315

1.	Introduction	104
2.	Materials and Methods	
	a) Haptens	107
	b) Preparation of the Fv fragment of protein 315	108
	c) Protein and hapten solutions	108
	d) ^1H n.m.r. studies	108
	e) Fluorescence measurements	108
	f) ^{31}P n.m.r. studies	109
	g) Analysis of pH titration data	109
3.	Results	
	a) Binding studies	111
	b) The histidine residues of the Fv fragment of protein 315	112
	c) ^{31}P n.m.r. studies of hapten binding to the Fv fragment	113
4.	Discussion	
	a) The histidine residues of the Fv fragment	116
	b) ^{31}P n.m.r. studies	116
	c) Conclusions	121
	Appendices	
	I ^{31}P relaxation mechanisms	123
	II Changes in the pKa's of phosphate and phosphonate groups	125
	III Computer program	127

CHAPTER 5 : THE BINDING OF 2,4,6 TRINITROPHENYL DERIVATIVES TO THE FV FRAGMENT OF PROTEIN 315

1.	Introduction	128
2.	Materials and Methods	
	a) Preparation of solutions of Fv fragment from protein 315	129
	b) Synthesis of haptens	129
	c) Preparation of hapten and tryptophan solutions	130
	d) Fluorescence measurements	130
	e) Optical difference spectra	130
	f) ^1H n.m.r. studies	130
	g) Analysis of n.m.r. data	130

Page

3.	Results	
	a) Model compound studies	131
	b) Optical difference spectroscopy	132
	c) Binding studies	132
	d) High resolution ^1H n.m.r. studies	133
	e) Ring current calculations	135
4.	Discussion	135

CHAPTER 6 : GENERAL CONCLUSIONS

1.	N.m.r. and model building: an approach to antibody combining site structures: an assessment	138
2.	Summary of Structural data available for protein 315	140
3.	The mode of interaction of haptens with protein 315	141
4.	The relationship of the protein 315-hapten system to other Dnp-binding myeloma proteins and antibodies	144

BIBLIOGRAPHY	146
--------------	-----

Chapter 1

GENERAL INTRODUCTION

1. Introduction

Immunoglobulins, those proteins that mediate the humoral immune response, occur in serum as a complex mixture of different molecules. Despite this complexity, all immunoglobulins are variants on a common fundamental structure (Gally, 1973). The relationship between structural variation, and the diversity in antigen binding in this family of proteins is now clearly understood in terms of variations in the amino acid sequences of immunoglobulins (Capra and Kehoe, 1975; Potter, 1977). However, comparatively little three dimensional structural information is available. Clearly, a complete understanding of the nature of antibody diversity and specificity cannot be achieved on the basis of sequence comparisons alone. A knowledge of the three dimensional structures of a large number of antibody combining sites is also required. The best method for obtaining such data is X-ray crystallography (Blundell and Johnson, 1976). This method has been applied successfully to a few immunoglobulins (Davies, Padlan and Segal, 1975). It has contributed greatly to our understanding of the structural basis of the relationship between hypervariable regions and antibody specificity (Padlan, 1977), the significance of intradomain sequence homologies (Beale and Feinstein, 1976), the location of serological markers in the three dimensional structure, and the variations in the positioning of interchain disulphide bonds (Poljak, 1975).

Since the immunoglobulins occur as a large family of structurally related proteins, it is both reasonable and useful, to attempt to extrapolate from proteins for which a crystal structure is known, to the structures of proteins for which only the primary sequence is known. Such model building studies have been carried out, in an attempt to predict

the structure of antibody combining sites at high resolution (Padlan et al, 1976; Padlan and Vrana, unpublished data), and the structure of IgM at low resolution (Feinstein, 1974).

Clearly it would be best if such model building procedures could be made completely reliable. However, at this stage it is important to combine these with suitable experimentation, to assess the correctness of any structures produced. The best way to do this would be to independently determine the structure by X-ray crystallography and to compare this with the structure produced by model building. An alternative, compromise, approach is to employ spectroscopic methods in conjunction with a model building study, in the hope that the limited structural information provided by the former will be sufficient to dictate a unique structure, when applied as constraints on possible structures generated in the latter. The most useful spectroscopic methods for this purpose are the magnetic resonance methods, in particular ^1H nmr, since this provides information about hydrogen nuclei, which are ubiquitous in proteins.

The application of a combined model building and magnetic resonance approach to the elucidation of the structure of the combining site of the Dnp (dinitrophenyl)-binding mouse myeloma IgA (λ) protein 315, is described in this thesis.

2. The immune response

The adaptive immune response of vertebrates provides a defence against infection by a wide range of pathogens. The mechanism by which this is achieved can be separated into two stages. In the first of these, the foreign substance (or antigen) is detected by cells carrying receptors specific for that antigen (Raff et al, 1970; Klinman and Sigal, 1978). This event causes those cells to differentiate and proliferate into an expanded population of cells committed to the recognition of that antigen

(Cunningham, 1974). This stage also encompasses the acquisition of immunological memory (Burnet, 1959), which is manifested by an amplified response on subsequent contact with the same antigen. This is a statement of the clonal selection hypothesis (Burnet, 1959). The cells activated in this stage of the immune response are the small lymphocytes (Gowans, 1969). There are two types of small lymphocytes, classified according to the organ-dependence of their differentiatational pathway as B-cells (Bone-marrow or Bursa-dependent) or T-cells (Thymus-dependent). These two types of cells can be distinguished experimentally on the basis of cell surface structures, detected either as antigenic differences or by scanning electron microscopy (Chess and Schlossman, 1977). Small lymphocytes circulate in the blood/lymphatic system, and appear to be quite inert until antigenic experience (Gowans, 1969); they do not divide, synthesize DNA, phagocytose, or respond to chemotactic agents. The differentiatational response of the two cell types to antigen is, however, different. The cells produced by T-cell activation are cytotoxic blast cells that directly mediate cell killing, whereas the cells produced by B-cell activation are plasma cells that secrete antibodies (immunoglobulins that bind antigen) which can independently mediate cell killing.

Antibody mediated cell-killing, which requires B-cell independent effector systems, can be regarded as the second stage of the humoral immune response. This effector stage can take place in the absence of the plasma cells that originally produced the antibodies, whereas the T-cell dependent response requires the presence of the cytotoxic blast cells. Hence the former is referred to as the humoral immune response and the latter is referred to as the cellular immune response. It appears that these two types of immune response are not mutually exclusive or independent, since T-cell co-operation is required to produce activation of B-cells (Miller and Mitchell, 1969; Mitchison, 1971).

The normal immune response is highly heterogenous. It has been estimated that the total number of different antibodies produced in an immune response to a simple hapten (4-hydroxy-5-iodo-3-nitro-phenacetyl-R) in CBA/H mice is 3000-30000. In contrast to this diversity in antigen recognition, the second stage of the humoral immune response requires that antibodies bound to antigen be recognized by a few specialized effector systems. This suggests that, in order to fulfil this dual role, antibodies will show a pattern of structural variation in which a limited region of the protein is changed whilst much of the structure is conserved between different antibodies.

3. Monoclonal Proteins

The complexity of the mixtures of immunoglobulins obtained from pooled sera, whether non-immune or raised against an antigen, prevents isolation of pure proteins. There are, however, several means by which homogenous antibody-type proteins can be prepared.

The method of splenic focus transfer, devised by Klinman (Klinman, 1971, 1972; Pink and Askonas, 1974), can be used to prepare homogeneous antibody. This involves the transfer of small fragments of spleen from an animal in which an immune response has been raised, into in vitro culture. If splenic foci are isolated, clones of antibody synthesizing cells are obtained, and pure antibody can be obtained from these cells. The disadvantage of this procedure is that the antibody synthesizing clones have a limited life span (c 80 generations) (Kreth and Williamson, 1973), and thus the amounts of antibody obtained are small.

An alternative method relies on the occurrence of plasmacytomas. These are tumours of the small lymphocytes which produce cell lines similar to the plasma cells induced by the normal immune response. There are two important differences however; the plasmacytoma cells do

not have a limited life span, and since the neoplastic transformation is rare, only one type of protein is produced. Thus cell clones arise which persist and make pure antibody-type proteins. A lymphoproliferative component of this type has been found in several human diseases, such as multiple myelomatosis (Geschichter and Copeland, 1928), Waldenström's macroglobulinaemia (Waldenström, 1944), and hypergammaglobulinaemic purpura (Waldenström, 1943), all of which show symptoms of splenomegaly and elevated levels of serum globulin. The serum globulin produced by the plasmacytoma can be isolated during therapeutic plasmaphoresis, and provides large amounts of homogenous material suitable for structural analysis.

A more useful laboratory system was pioneered by Potter (1972) who showed that injection of a wide variety of adjuvant-like materials into the peritoneum of BALB/c mice produced plasmacytomas. These can be serially explanted into mice of the same inbred strain, or F_1 hybrids with other inbred strains, and thus can be maintained indefinitely. This system has been used to obtain a wide variety of myeloma proteins, and protein 315 was obtained by this method (Eisen, Simms and Potter, 1968). Myeloma proteins have contributed greatly to our understanding of antibody structure; all X-ray analyses have been performed on such proteins, as have many of the sequence analyses. There are, however, problems associated with the use of myeloma proteins as 'representative antibodies'. Since these proteins are produced by a neoplastic transformation, and not by antigenic stimulation, their antibody specificity (if any) is not known. This must be established by screening procedures. It has been established that antibodies raised against a particular antigen and myeloma proteins shown to bind that antigen by screening, share idiotypic (Rudikoff and Claflin, 1976; Granato, Braun and Vassali, 1974) (combining region) structures in common. This establishes that there are antibodies in normally raised antisera that are similar in combining region structure to myeloma proteins. It is not true, however, that the myeloma proteins are a random

sample of the total antibody population. Thus the majority of mouse proteins are of the IgA class. This is to be expected since most of the lymphocytes in the peritoneum are IgA-bearing (Potter, 1977). Further, the specificity distribution in both mouse and man is non random. Thus in man, many monoclonal proteins seem to have autoimmune specificities (Capra and Kehoe, 1975), e.g. cryoglobulins (anti-gammaglobulin). In mice, many of the myeloma proteins that have been produced, and that have been shown to bind antigens, are specific for determinants that would be common in the environment, such as phosphorylcholine (Potter, 1977), which would be a common component of bacterial glycolipids.

Monoclonal proteins of known specificity can be induced in the rabbit (Krause, 1970; Eichmann, Braun and Krause, 1971). Immunogens with simple repeated structures, such as bacterial cell wall polysaccharides, are capable of eliciting production of high levels of often only one antibody in some rabbits (Eichmann, Braun and Krause, 1971). The high response and restricted heterogeneity phenotypes are heritable characters.

An improved method for obtaining monoclonal proteins of defined antigenic specificity was demonstrated by Kohler and Milstein (1975, 1976). They adapted the cell fusion method of Harris (Harris and Watkins, 1965) to the production of hybrid cell lines from plasmacytoma and normal spleen cells activated by antigen. By selecting for cell lines producing spleen cell antibodies, it is possible to 'immortalise' particular antibody specificities and thus to obtain homogenous real antibodies of defined specificity. This method has been used with inbred mouse strains, where the hybrid cells can be propagated in vivo as described above.

TABLE 1 : Biological properties and activities of human immunoglobulins

Characteristics	IgG1	IgG2	IgG3	IgG4	IgA1	IgA2	IgM	IgD	IgE
Serum (mg./ml.)	5.12	2-6	0.5-1	0.2-1	0.5-2	0-0.2	0.5-1.5	0-0.4	0-0.002
Secretion	-	-	-	=	+	+	(+)	-	
Half-life in days	23	23	16	23	6	6	5	3	2
Synthesis (mg./kg./day)	25		3.4		24		7	0.4	0.02
Classic C	++	+	++	-	-	-	+	-	-
Alternate C	-	-	-	-	-	+	+	<u>±</u>	<u>±</u>
Macrophages	+	<u>±</u>	+	<u>±</u>	-	-	-	-	-
Neutrophiles	+	+	+	+	+	+	-	-	-
Lymphocytes	+	<u>±</u>	+	<u>±</u>	-	-	-	-	-
Staphylococcal A	+	+	-	+	-	-	-	-	-

Symbols used: -, negative; (+), occasional positive reaction;
±, weakly positive; ++, strongly positive; blank space, not tested.

Data from Spiegelberg, 1975.

4. Antibody specialization in relation to effector function;
classes and subclasses

Three types of immunoglobulin (IgG, IgA, IgM) can be distinguished in normal serum by electrophoresis (Heidelberger and Pederson, 1937; Tiselius and Kabat, 1939; Grabar and Williams, 1953). These can be demonstrated in all vertebrates from birds to man (Kubo, Zimmerman and Grey, 1973). In addition, two classes have been isolated, IgD (Rowe and Fahey, 1965), IgE (Ishizaka, Ishizaka and Hornbrook, 1966). The former of these has only been detected in man. On the basis of sequence analyses and by the use of serology, the IgG class is divisible into four subclasses; the IgA class into two subclasses; and the IgM class into two subclasses. (Section 5(d)).

Each class of immunoglobulin is presumed to be capable of expressing any antigenic specificity. But each shows a particular pattern of biological activity (Table 1). This is revealed in the different activities of different classes towards, e.g. the classical pathway of complement activation, IgM will activate this whereas IgA will not (Reid and Porter, 1975). IgA is found in high concentrations in secretions such as bile and colostrum, and has a particular role in what has been termed the secretory immune response (Tomasi and Grey, 1972). In secretions this immunoglobulin is in the form of a secretory dimer, in which two IgA monomers are complexed with one molecule of 'secretory piece' and one molecule of J chain (Halpern and Koshland, 1970). IgM normally occurs as a pentamer, associated with one molecule of J chain. (Section 5(d))

The existence of several functionally specialized classes of immunoglobulins provides a flexible immune defence system, in which the type of antibody elicited by a particular pathogen will be most suitable in responding to the mode of infection of that pathogen.

5. The structure of immunoglobulins

a) The four chain unit

All immunoglobulins, irrespective of class or species of origin, contain the same fundamental four chain unit. The variations on this structure which differentiate the various classes and subclasses of immunoglobulins are discussed in section 5(d). The studies which led to the elucidation of this structure were carried out on rabbit and human IgG (Porter 1959; Edelman, 1959; Fleischman, Pain and Porter, 1962; Fleischman, Porter and Press, 1963; Edelman and Poulik, 1961; Edelman and Gally, 1962; Edelman and Gally, 1964). These studies showed that the IgG molecule is composed of four chains, two heavy (H) and two light (L) chains, cross-linked by disulphide bonds. The two light chains are identical and each has a molecular weight of $c\ 2.5 \times 10^4$ and contain $c\ 220$ amino acids. The two heavy chains are identical (Nisonoff, Hopper and Spring, 1975) and each has a molecular weight of $c\ 5 \times 10^4$, and contain $c\ 450$ amino acids. It was also established that Bence-Jones proteins, which can be isolated from the urine of patients suffering from multiple myelomatosis, are free immunoglobulin light chains (Edelman and Gally, 1962).

All immunoglobulins also contain oligosaccharide groups, linked to the heavy chain of the four chain unit (Dawson and Clamp, 1968; Kornfeld and Kornfeld, 1976). The possible structural and functional significance of these components of immunoglobulin structure is discussed in section 5(d).

b) The fundamental unit of immunoglobulin structure: the domain

Porter (1959) showed that non-immune rabbit IgG could be proteolytically cleaved, with nearly total recovery of protein digested, as large fragments (Table 2). Extensive application of this method to immunoglobulins from many species and classes (Nisonoff, et al, 1960; Edelman et al, 1960; Givol, 1967; Turner and Bennich, 1968; Connell and Porter, 1971;

TABLE 2 : Proteolytic fragmentation of immunoglobulins

Fragment	Domains	Class	Species	Enzyme	References
Fab	$V_L C_L V_H C_H^1$	IgG	rabbit	papain	Porter 1959.
Fc	$(C_H^2, C_H^3)_2$	IgG	rabbit	papain	Porter, 1959.
Fd	V_H, C_H^1			papain	Björk and Tanford, 1971
Fab'	$V_L C_L V_H C_H^1$	IgG	rabbit	pepsin	Nisonoff <u>et al</u> , 1960.
pFc'	$(C_H^3)_2$	IgG	human	pepsin	Turner and Bennich, 1968.
Facb	$(V_L C_L V_H C_H^1 C_H^2)_2$	IgG	rabbit	plasmin	Connell and Porter, 1971.
Fv	$V_H V_L$	IgA	mouse	pepsin	Inbar, Hochman and Givol, 1972.
Fab'	$V_L C_L V_H C_H^1$	IgA	mouse	pepsin	Inbar, Rothman and Givol, 1971.
Fc	$(C_H^2 C_H^3)_2$	IgA	mouse	papain	Grey, Sher and Shalitin, 1970.
$(Fc\mu)_5$	$(C_H^2, C_H^3, C_H^4)_5 J$	IgM	human -	pepsin	Lin and Putnam, 1978
				papain	Onoue, Kishimoto and Takamura, 1968.
	C_H^2	IgG	human	trypsin	Eilerson <u>et al</u> , 1976.
Fce	$(CH_2 CH_3 CH_4)_2$	IgE	human	papain	Bennich and Johansson, 1971.

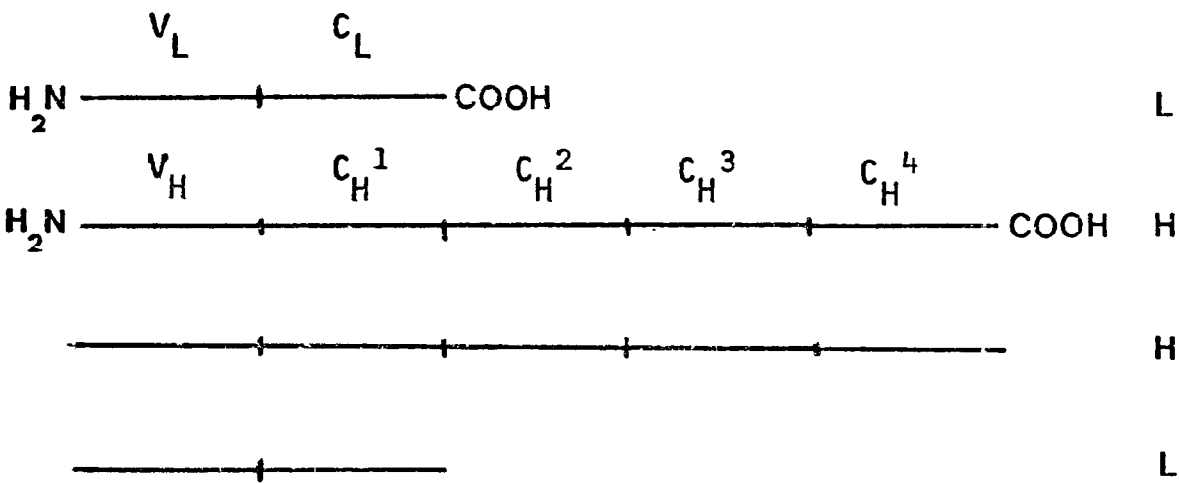
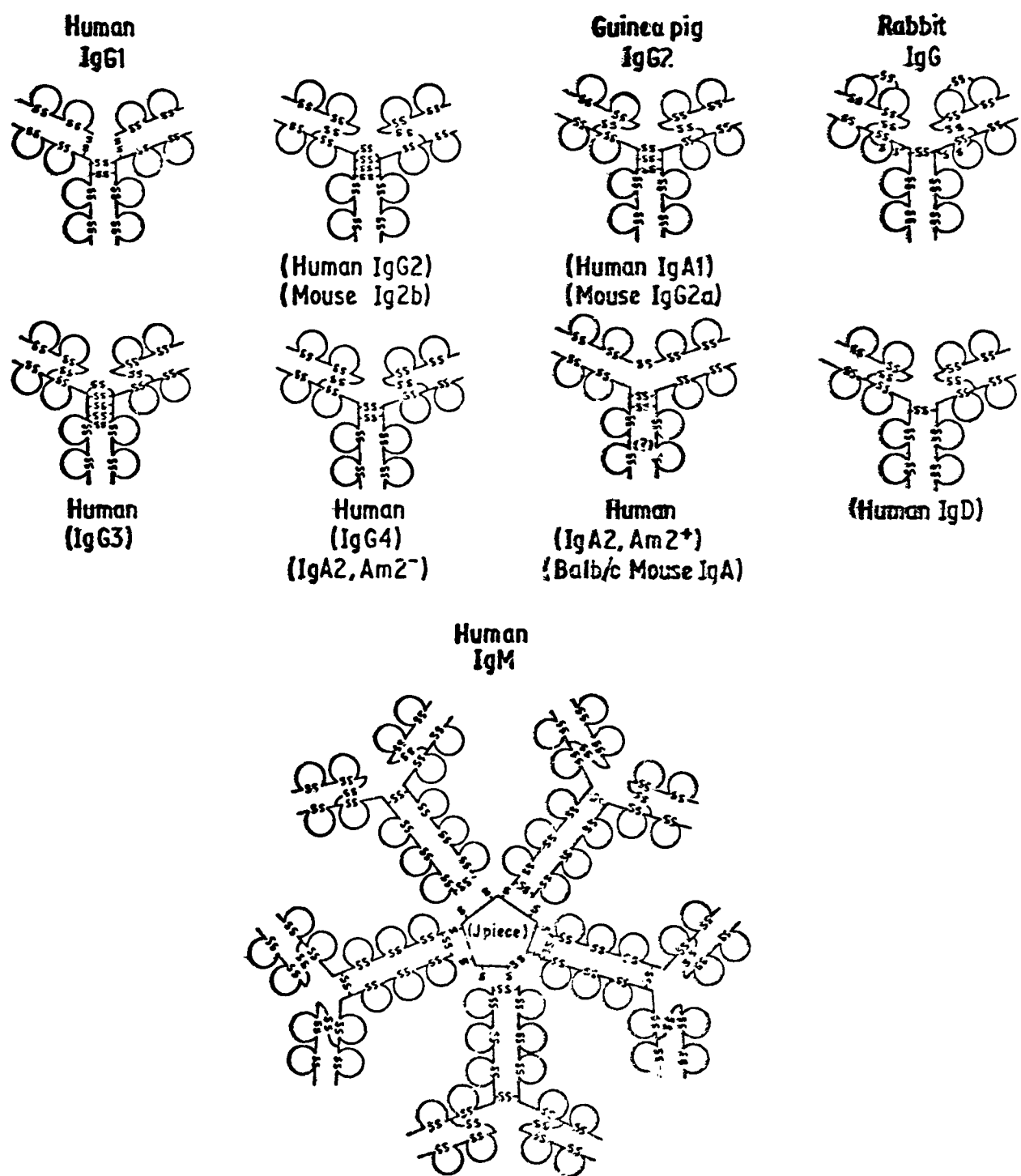


FIG. 1. Disulphide bonding patterns in immunoglobulins



The IgA is indicated as the 7s monomer, there are cysteine residues in the Fc region which form links to the J chain. Further in mouse IgA the number of disulphide bonds is not well established, the structure given is based on data of Grey, Abel and Zimmerman (1971).

Taken from Gally, 1973.

Ellerson et al, 1976; Kakimoto and Onouye, 1974; Sharon and Givol, 1976; Lin and Putnam, 1978; Laschinger and Connell, 1978; Inbar, Hochman and Givol, 1972) has demonstrated that these proteins consist of several proteolysis resistant regions, each containing two 11,000 to 12,000 molecular weight chain segments, which are non covalently associated. These chain fragments are linked in the complete molecule by regions of protease susceptible polypeptide chain. The patterns of fragmentation obtained with a variety of proteases and immunoglobulins are summarized in Table 2.

The total number and pattern of disulphide bonds in an immunoglobulin molecule is a characteristic of the particular class and species of origin of the protein (Milstein and Svasti, 1971; Gally, 1973; Nisonoff, Hopper and Spring, 1975). The disulphide bonds can occur between residues on the same chain (intra-chain), and between residues on different chains (inter-chain). It is the latter that show the marked variation between class and species (Figure 1).

The minimal pattern of intrachain disulphide bonds is highly conserved in all immunoglobulin molecules (Edelman et al, 1969; Gall and Edelman, 1970; O'Donnell, Frangione and Porter, 1970; Milstein and Svasti, 1971; Bourgois, Fourgereau, and Rocca-Sera, 1974; Beale and Feinstein, 1976), although in some cases additional bonds are present. (Strosberg et al, 1972; Strosberg et al, 1974; Kratzin et al, 1975.) The conserved disulphide-bonded cysteine residues occur between 50 to 70 residues apart in the primary sequence. There are four or five such pairs in heavy chains and two in light chains, and in all cases they are spaced at c110-120 residue intervals down the chain. This repeating pattern of disulphide bonded cysteine residues has been found to be a feature of the sequence as a whole (Beale and Feinstein, 1976; Natvig and Kunkel, 1973; Hill et al, 1966; Edelman, 1970). The length of the repeated chain segment established on the basis of sequence

FIG. 2 : The immunoglobulin domain structure

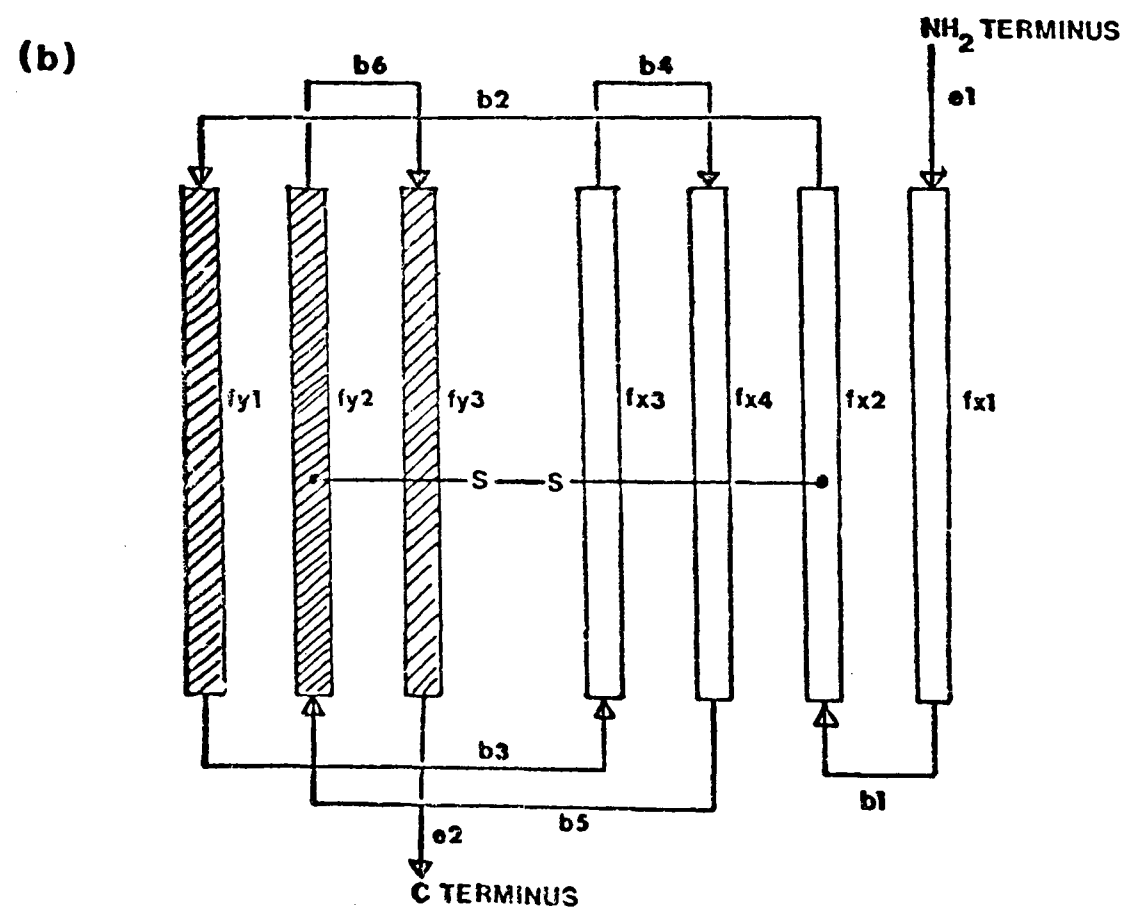
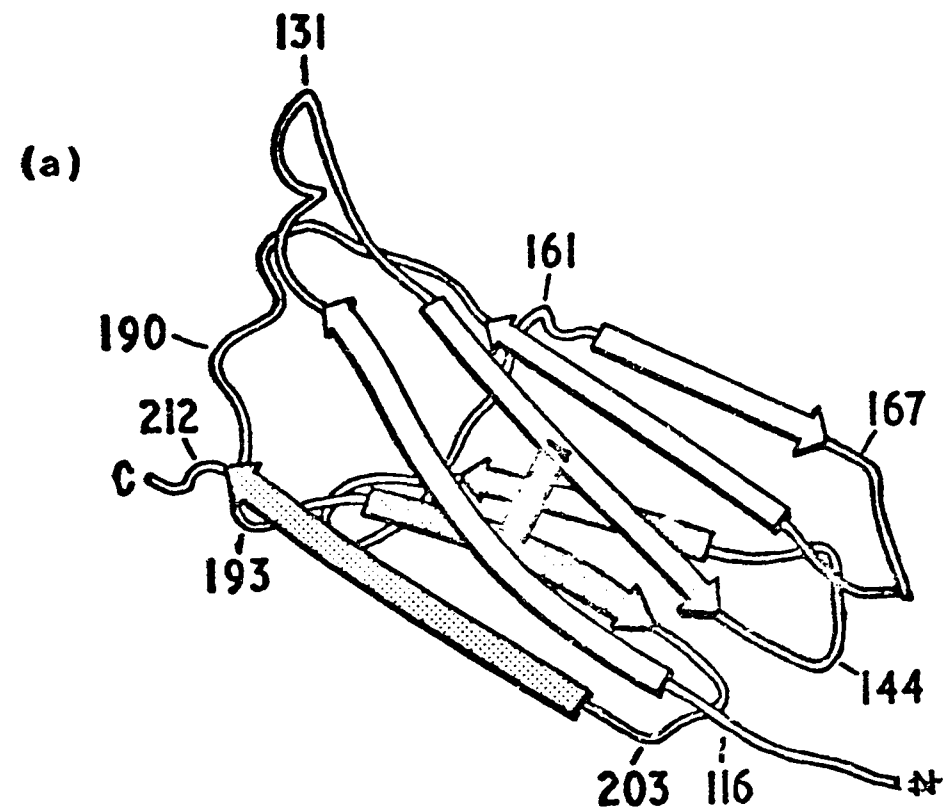


Fig. 2 :

- (a) The structure of the mcg light chain constant domain, taken from Edmundson et al (1975). The two layers of β sheet are indicated by bars, the strands of chain connecting them by the narrower lines. The three strands of face γ are shaded. The intradomain disulphide bridge is shown as a black bar.
- (b) Plan of the immunoglobulin domain showing the pattern of connectivity. The shaded strands of antiparallel β structure correspond to those in (a). Nomenclature is after Beale and Feinstein f = face, b = bend, e = end. The chain segments correspond approximately to sequence positions as follows:

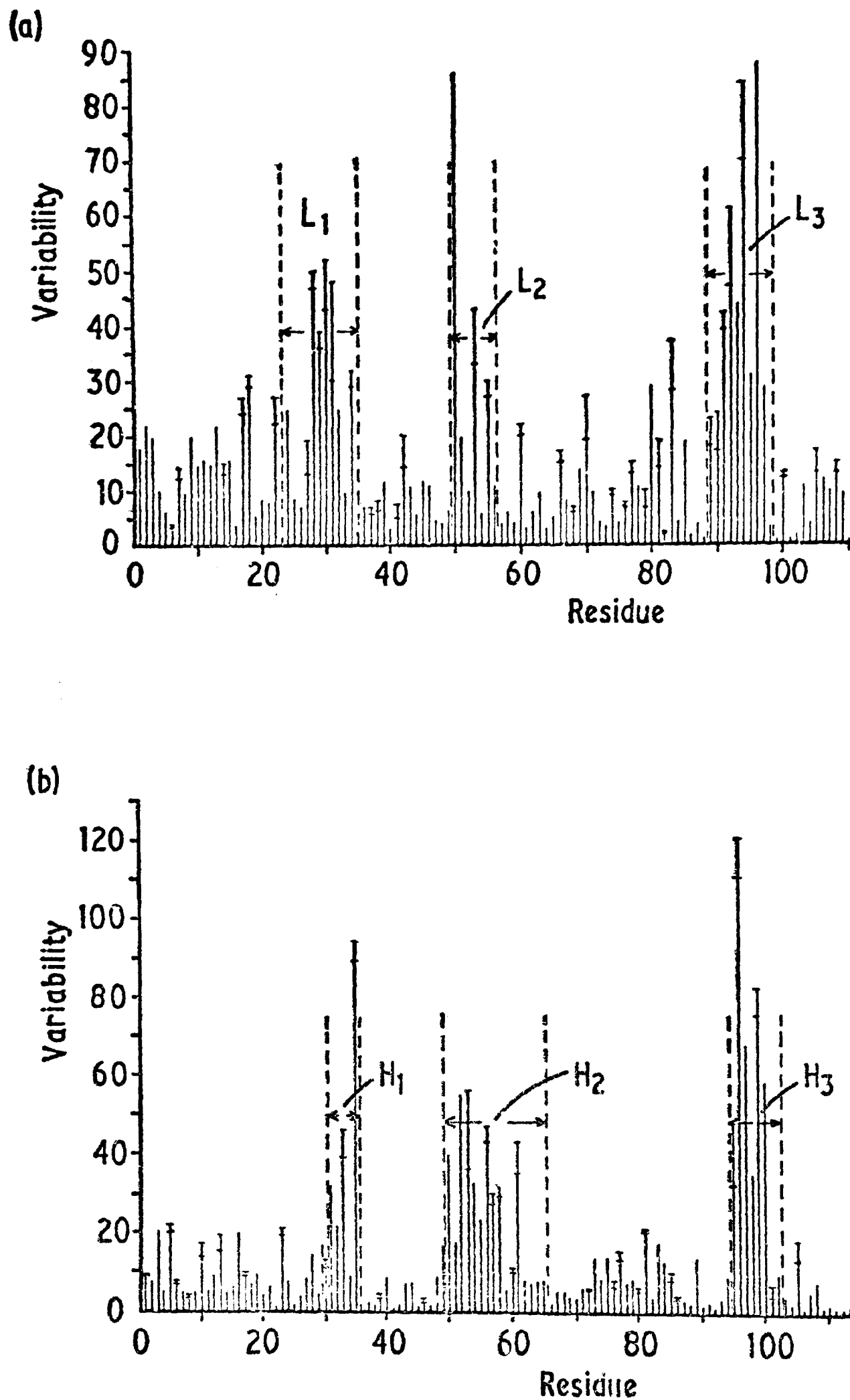
$e_1(1-7)$, $fx_1(8-15)$, $b_1(16-24)$, $fx_2(25-34)$, $b_2(35-42)$, $fg_1(43-49)$,
 $b_3(50-60)$, $fx_3(61-71)$, $b_4(72-83)$, $fx_4(84-91)$, $b_5(92-100)$, $fy_2(101-109)$
 $b_6(110-116)$, $fy_3(117-124)$, $e_2(125-129)$.

In the variable domain, segments at positions b_2 , b_4 and b_6 form the hypervariable regions.

comparisons, is similar to the length of the polypeptide chain segments isolated from the smallest well-defined proteolytic fragments, such as pFc' (Turner and Bennich, 1968) and F_v (Inbar, Hochman, and Givol, 1972). These data all support Edelman's domain hypothesis (Edelman and Gall, 1969; Edelman, 1970) which postulates that the homology regions all fold independently into similar three dimensional structures. This would give four or five of these in heavy chains (V_H, C_H1, C_H2, C_H3, C_H4) depending on the class, and two in light chains (V_L, C_L). Thus both types of chain, in all immunoglobulin molecules, have the same overall structure, which is a linear array of independently folded domains, with limited interdomain longitudinal contact, linked by less structured regions of polypeptide chain. These domains are associated in pairs in the whole molecule, as shown in Table 2; V_H with V_L, C_H1 with C_L, C_H2 with C_H2, C_H3 with C_H3, and C_H4 with C_H4. This view of immunoglobulin structure has been confirmed by X-ray studies (see Section 6(e) and Edmundson et al, 1975). The 'immunoglobulin fold' structure of an individual light chain constant domain is shown in Fig. 2, and described in the legend.

Beale and Feinstein (1976) have used the common three-dimensional domain structure to provide a rationale for interpreting the pattern of inter-domain sequence homologies. The segments of chain that participate directly in the β -structure (Fig. 2), have more highly conserved sequences than those of the loops of chain connecting these segments, either with each other, or with the next domain. Thus comparison of the sequences of a human IgG (NEW), mouse IgA (Proteins 104E and 315), and pig constant light domains (λ) (Nivotny and Franek, 1975), shows that the sequence homology in the β region of the domain is 67%, whereas in the non- β -sheet loops, the homology can be as low as 6%. This pattern of marked variation in the non- β -loop sequences, without disturbing the basic immunoglobulin fold, a general feature of immunoglobulin structure, is most striking in the N terminal domains of heavy and light chains, which are thus called the variable regions (V regions).

FIG. 3 : Hypervariability in immunoglobulin variable regions



Taken from Kabat, Wu and Bilofsky, (1976). The pattern of sequence variability in (a) Light chain variable domains and (b) Heavy chain variable domains is illustrated. The sequences compared are from all species for which data were available in 1976. The ordinate variable is calculated from:

$$\text{variability}(x) = \frac{\text{no. of different amino acids in position } x}{\text{no. of times the most frequent amino acid occurs at } x.}$$

c) Variable and constant regions

Peptide mapping studies of Bence-Jones proteins (Hilschmann and Craig, 1965; Dreyer and Bennett, 1965) provided the first evidence that most of the sequence variability of immunoglobulin chains is confined to the N terminal domain. Two Bence-Jones proteins, Roy and Cum, showed no similarity in the peptides from this region. Comparison of the sequences in this region show that no two light chains have the same sequence here (Kabat, Bilofsky and Wu, 1976). The sequences of the C terminal domains of light chains, on the other hand, fall into two well defined groups that correspond to the two types of serologically defined light chains K and λ (Korngold and Lipari, 1956). A similar pattern has been found for heavy chains, again variability is confined to the N-terminal domain (Edelman, 1970, Beale and Feinstein, 1976; Kabat, Bilofsky and Wu, 1976). Hence the Fc region sequences of proteins of the same class or subclass show a high degree of homology.

If light chain variable region sequences are compared for K or λ chains, then the pattern of distribution of V regions sequences allows the proteins to be grouped into distinct subsets on the basis of characteristic substitutions or deletions (Capra and Kehoe, 1975; Potter, 1977; McKean, Bell, and Potter, 1978; Barstad et al, 1978). This pattern differs between λ and K chains and from species to species. This shows that λ and K constant regions are associated with different sets of variable regions. If, however, the N terminal sequences of heavy chains are compared, no distinction can be drawn between these sequences in proteins from different classes. The sequence data suggest that all heavy chains share the same set of variable regions. Evidence in support of this view was obtained by the isolation from a patient suffering from multiple myelomatosis, of two proteins T11. One of these was an IgM and the other an IgG, but both had identical light chains and identical heavy chain variable regions (Wang et al, 1969; Wang et al, 1970). It has been demonstrated that during B-cell differentiation in chickens, a sequential

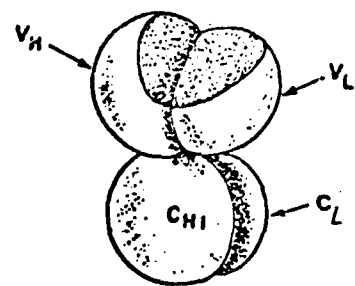
expression of IgM and IgG occurs. (Cooper, Lawton and Kincade, 1972)

On the basis of the early studies on Bence-Jones proteins, Dreyer and Bennet (1965) argued that the variable and constant regions of immunoglobulin chains were coded for by separate structural genes. This view was supported by the subsequent characterization of the heavy chain system, and has been confirmed by analyses of the structure of mouse light chain (Tonegawa et al, 1977 ; Tonegawa et al, 1978; Rabbitts, 1978; Seidman et al, 1978) genes.

The total number of V region genes present in the B-cell population of an adult animal is very large (Kreth and Williamson, 1973; Pink and Askonas, 1974). The origin of this diversity is not clearly established. Hypotheses range from those that postulate encoding of all the information as heritable genes (Germ Line), to those that postulate the existence of only a few such genes, the diversity being created by mutation during lymphocyte differentiation (Somatic Mutation). (Williamson, 1976; Capra and Kehoe, 1975; Cunningham, 1974; Milstein, 1967) The pattern of sequence variation found in proteins belonging to a particular V region set (V_λ , V_K , or V_H) is consistent with either viewpoint. The truth probably lies 'inelegantly' (Gally, 1973) somewhere in between the two extremes.

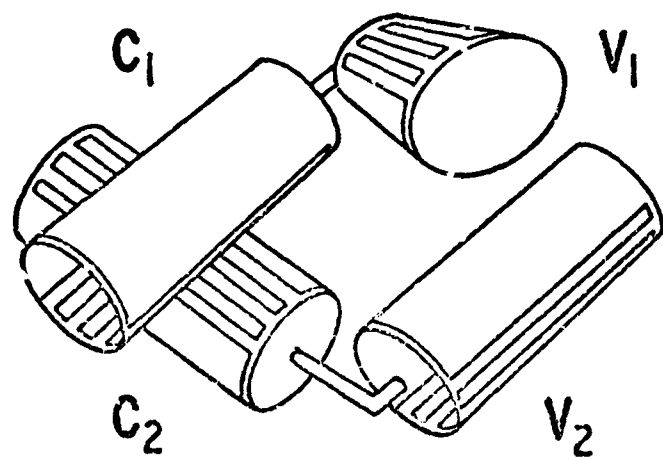
Within the V region sequence, the variability can be seen to be non randomly distributed with respect to sequence position (Fig. 3). This is to be expected if the domain structure is to be preserved. (Beale and Feinstein, 1976) However, the hypervariability is confined to only some of the non β -sheet regions of the sequence (Kabat and Wu, 1970; Wu and Kabat, 1971), specifically at positions 24-34, 50-56 and 89-97 in V_L domains; and 31-35, 50-56, (81-85) and 95-102 in V_H domains. X-ray analyses show that the hypervariable segments from both V_H and V_L domains are apposed in the three dimensional structure, on the surface of the V region facing away from the constant region (Fig. 4) (Edmundson et al, 1975; Segal et al, 1974; Poljak et al, 1972). That this region forms

FIG. 4 : Schematic Fab region structure showing location of the antibody combining site



The shaded area indicates the approximate location of the antibody combining site.

FIG. 5 : Rotational allomerism in the Fab region structure



The hatched areas correspond to face x in Fig. 2. The structure is based on that of the mcg light chain dimer (Edmundson et al, 1975)

the combining site of these proteins is confirmed by affinity labelling studies (Ray and Cebra, 1972; Koo and Cebra, 1974; Strausbach et al, 1971; Givol, 1974), and X-ray analyses (Edmundson et al, 1974; Epp et al, unpublished data; Amzel et al, 1974; Segal et al, 1974). The affinity labelling studies show that the residues labelled by these reagents are located in hypervariable segments. The X-ray studies show that the bound haptens are located in contact with amino acid residues that are part of the hypervariable segments.

A further difference between the V and C regions is seen in the way in which the domains in different chains form pairs. (Edmundson et al, 1975) The V domains associate with the three strand β -sheet faces apposed, whereas the C domains associate with the four strand β -sheet faces apposed (Fig. 5). Edmundson termed this phenomenon 'rotational allomerism', and has suggested that the reason for this may be to allow the formation of a binding cavity for antigen, between the two apposed V domains. This argument is based on the structure of Mcg, which is a Bence-Jones (Light chain) dimer, and does not apply so strongly to the Fab fragment structures, where the binding cavities are smaller (Amzel et al, 1974; Segal et al, 1974).

Thus in summary the pattern of V region variability can be seen to be one of multiple substitutions and deletions in some of the non β -sheet loops, against a background of conserved domain structure. This conserved structure, termed the 'immunoglobulin fold' by Poljak (1975), provides a framework to which a wide range of hypervariable structures can be attached. This provides a structural basis for the dual role of antibodies, in which it is necessary to generate a wide range of antigen binding sites on a background of common structure.

d) Patterns of structural variation in immunoglobulins: isotypes, allotypes, and idiotypes: classes and subclasses.

The pattern of structural variation in immunoglobulins can be described either chemically or serologically. The latter classification is defined in terms of three types of antigenic determinant on the immunoglobulin molecule. These are allotypes, idiotypes and isotypes.

The allotypic determinants on the immunoglobulin molecule are found to fall into sets which behave as mendelian alleles. (Kindt, 1975.) A simple example of this phenomenon is the human K Inv system, which involves point substitutions at positions 153 and 191 (Ropartz, Lenoir and Rivat, 1961).

Idiotypes are determinants on individual immunoglobulin molecules, for example, myeloma proteins, that are unique to the protein (Oudin, 1974). Thus an antiserum raised by interspecies immunisation with a myeloma protein, and then absorbed with closely related proteins (of the same allotype and isotype) will be reactive to idiotypic determinants on that immunoglobulin (Capra and Kehoe, 1975). Such antiidiotype antisera can also be raised by intraspecies immunisation (Lynch et al, 1972). These determinants are associated with the hypervariable regions (see Section 5(c)). Evidence in support of this view is provided by the observation that antiidiotype antisera and antigens bind competitively to antibodies (Brient and Nisonoff, 1970).

Isotypic determinants are those present in all individuals of a species. These are the determinants that differ between immunoglobulin of different classes and subclasses (Nisonoff, Hopper and Spring, 1975).

The structural variations that characterize the class and subclass of an immunoglobulin molecule can be described in terms of sequence variations in the C_H regions. These variations in sequence can be subdivided into three categories.

- i) Variation in the number of C_H domains. In IgM (μ) and (ϵ) immunoglobulins there is an extra (C_H4) domain (Bennich and Johansson, 1971; Bennich, Milstein and Secher, 1973; Watanabe et al, 1973; Putnam et al, 1973), compared with IgA, IgG and IgD.
- ii) Variation in the interdomain chain segment between C_H1 and C_H2 . This region is termed the hinge region, since it is here that the segmental flexibility of immunoglobulin molecules largely reside (Feinstein and Rowe, 1965; Lafferty and Oertlis, 1963; Valentine and Green, 1967; Pilz, Kratky and Karush, 1974; Pilz, Schwarz and Palm, 1977). This region is usually longer than other interdomain sequences, and varies widely in structure between immunoglobulins of different classes and subclasses. Since the hinge region contains the major site(s) of proteolytic cleavage in the immunoglobulin molecule, variations in hinge region structure cause variations in the susceptibility of immunoglobulins to proteases (Gergly, Medgyesi and Stanworth, 1967; Jefferis et al, 1968; Gergly, Fudenberg and Van Loghem, 1970). The majority of interchain disulphide bonds are concentrated in the hinge region (Frangione, Milstein and Franklin, 1969; Milstein and Svasti, 1971) and vary markedly both in number and position between immunoglobulins of different classes and subclasses (see Fig. 1).
- iii) Intradomain structure in the C_H region. Beale and Feinstein (1976) have compared the sequences of C_H regions on the basis of the domain concept (section 5(b)). They suggest that the pattern of homologies between domains indicates that individual domains are functionally specialized, pointing out that the sequences of the same domains in immunoglobulins of the same subclass in different species show a higher degree of homology than the sequences of different domains in the same molecule.

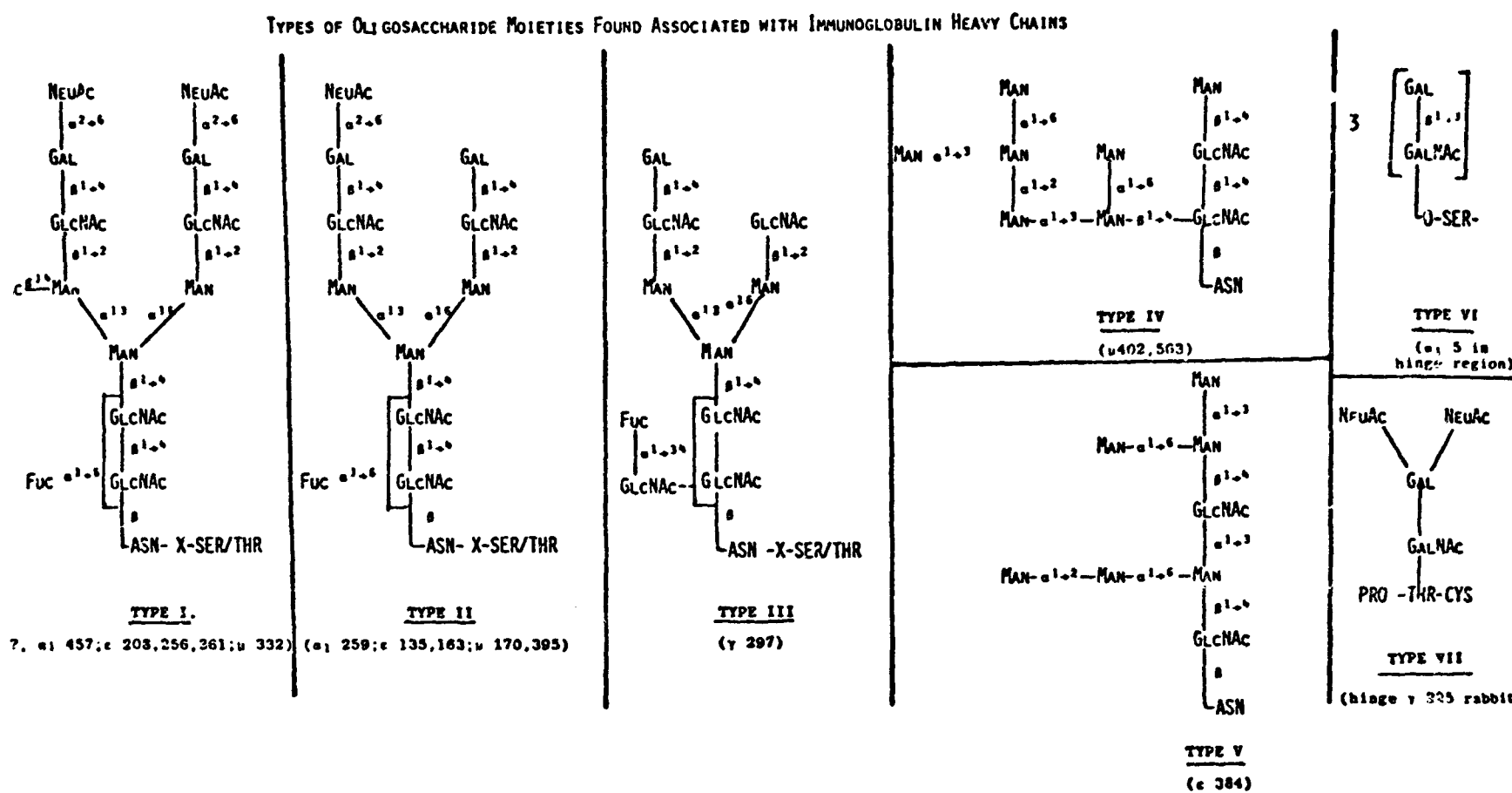
There are three further structural differences between immunoglobulins of different classes and subclasses:

i) Polymerization of the four chain unit. Immunoglobulins of the IgA and IgM classes occur as oligomers. IgM invariably occurs as pentamers in which the IgM four chain subunits are cross linked via disulphide bonds between the C μ 4 domains (Fig. 1). IgA as isolated from serum is present as a range of oligomer sizes (Tomasi and Grey, 1972). In secretory fluids, however (bile, colostrum, semen, etc.), this occurs in the form mainly of a secretory dimer (Heremans, 1974).

ii) Association with other polypeptides. The IgM pentamer and the IgA secretory dimer contain a polypeptide, J (joining) chain (Koshland, 1975). This is synthesized in the plasma cells that produce the immunoglobulin chains, it occurs as one mole per mole of oligomer, and is considered to control the oligomerization process (Halpern and Koshland, 1971; Mestecky et al, 1977; Wilde and Koshland, 1978). A glycoprotein component, the secretory component is associated with the IgA secretory dimer (Halpern and Koshland, 1971). This c 70,000 molecular weight component contains c 9% carbohydrate (Tomasi & Calvanico, 1968) and is tightly bound to the IgA component of the complex (Brandtzaeg, 1977). The secretory component may be associated with the IgA secretory process, that is with the passage of the IgA across the mucosal layer. Evidence in support of this hypothesis has been obtained from histochemical studies, in which immunofluorescence located the secretory component in the mucosal cells but not in IgA secreting plasma cells (Tourville et al, 1969).

iii) Oligosaccharide components of immunoglobulins. All immunoglobulins are glycoproteins. The oligosaccharide is attached to the polypeptide by an N-acetyl glucosamine-asparagine transglycosylase, which is specific for the sequence Asn-X-Ser/Thr. (Nisonoff, Hopper, and Spring, 1975; Kornfeld and Kornfeld, 1976; Shimizu, Putnam and Paul, 1971). The structures of several immunoglobulin oligosaccharides are illustrated in

FIG. 6 : Types of oligosaccharide moieties found associated with immunoglobulin heavy chains.



Class A structures include types I-III and are complex, multi-branched oligosaccharides. Class B structures include types IV-VII and are simple, core-type (IV and V) or mucin-type (VI and VII) oligosaccharides.

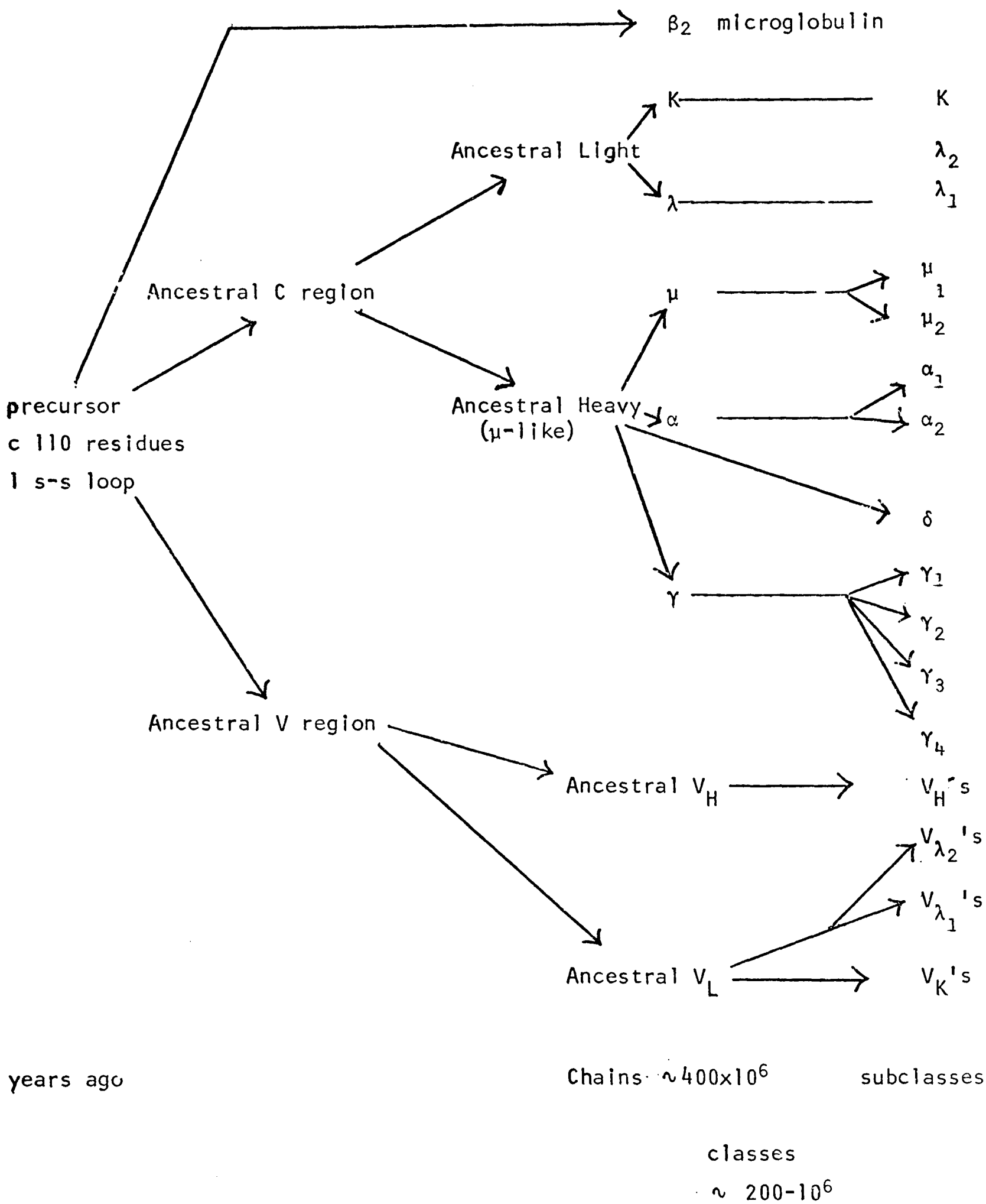
Taken from Winkelhalke, J. (1978).

Fig. 6. If the correct triplet of amino acids occurs in the hinge region of the immunoglobulin then this will be the major site of attachment of oligosaccharide, although there are examples of attachment of oligosaccharide to the Fab regions of myeloma proteins if this triplet occurs in the variable region (Press and Hogg, 1970; Sox and Hood, 1970). A structural role for the oligosaccharide has been proposed on the basis of the one Fc fragment high resolution crystal structure so far determined, in which it is observed to lie between the two C_H2 domains closely apposed to the protein surface (Human IgG1 Kol, Diesenhofer et al, 1976).

Some correlation between the structural differences outlined above and the functional differences outlined in section 4 can be made. The extra domain in the IgM has been suggested by Feinstein (1974) to be necessary for the formation of the stable pentamer. The oligomeric structure of immunoglobulins of the IgM and IgA classes may act to increase the affinity of these antibodies for antigens (Karush, 1962; Hornich and Karush, 1972). This would be consistent with appearances of IgM antibodies early in the immune response (Benedict, Lawson and Nik-Khan, 1963; Nossal et al, 1964). Clearly the differential interaction of the different immunoglobulin classes with effector systems must be a function of differences in the tertiary structures of the C_H regions. At present, however, there is insufficient three dimensional structure data to pinpoint the important differences. Attempts to correlate sequence differences with functional differences must be treated with caution. For example, Low, Liu and Putnam (1976) could detect no obvious correlation between sequences of the C_H2 domain, and the capacity to bind Clq (the first component of complement) (Reid and Porter, 1975), in human immunoglobulins of the IgM, IgG, IgA and IgE classes.

Finally, the pattern of structural homology found in these molecules suggests that this family of proteins has arisen by a process of divergent evolution (Fig. 7.) (Milstein and Pink, 1970). The most probable scheme

FIG. 7 : A scheme for the evolution of immunoglobulins



Is one in which the present proteins arose by a process of gene duplication and fusion from a primitive c 100-120 amino acid precursor protein. This view is supported by the existence of membrane protein, β_2 microglobulin (Berggard and Bearn, 1968) which is of approximately the same size as an immunoglobulin domain (100 amino acid residues) and shows a significant degree of homology with immunoglobulins (Peterson et al, 1972). This pattern of evolution is supported by studies on the phylogeny of immunoglobulins (Kubo, Zimmerman and Grey, 1973; Marchalonis, 1977).

6. The Antibody Combining Site

a) Introduction

In the previous section the location of the antibody combining site in the immunoglobulin molecule was discussed in terms of the sequence and three dimensional structure of the protein. The antibody combining site is formed by the apposition of amino acid residues in the six hypervariable segments of the variable region, three in the V_H and three in the V_L domain. In this section the interaction of antigens with the antibody combining site is discussed, in order to examine the functional aspects of antibody specificity. Most of the data in this section have been obtained from studies on antibody-hapten interactions; haptens are small molecules linked by chemical reaction to immunogenic macromolecules. Pure anti-hapten sera can be obtained either by absorbing the total antiserum with the unmodified carrier protein or by affinity chromatography (Farah, Kern and Eisen, 1960). Antibody-hapten interactions are simpler to study than, for example, the interaction of antibody with a protein antigen. Typical examples of such haptens are the 2,4-dinitrophenyl (Dnp) and 2,4,6-trinitrophenyl (Tnp) groups, which can be attached to amino groups on proteins, using 2,4-dinitro-fluorobenzene and 2,4,6-trinitrobenzenesulphonic acid.

In this section the observed pattern of specificity will be described. This will then be related to the structural features of the antibody combining site described in section 5(c). An important insight into the nature of antibody specificity was described by Talmage (1959), who pointed out that if antibodies are produced by a clonal selection mechanism, then a highly specific antiserum can be composed of antibodies that are much less specific. This arises because the antibodies are induced if they bind the immunogen. If each antibody also binds a range of related ligands, this pattern differing between different antibodies, then specificities for such related ligands will be diluted out in the total antiserum.

It is not a corollary of this hypothesis that the antibody molecules carry several spatially distinct sites for structurally completely unrelated ligands. This latter argument has been advanced by Richards and co-workers (see later), who have used data from studies on myeloma proteins to support the view that antibodies are 'multispecific'.

b) Antibody-Antigen binding studies

Two fundamental features of the antibody-antigen interaction have been investigated using data obtained from binding studies. These are the nature of the specificity of antibodies for a single chemical group (a hapten), and the size of the site for antigens composed of several such groups.

Landsteiner and co-workers (Landsteiner, 1944) studied the interaction of haptens with antibody preparations, using series of haptens of closely related structure to inhibit the precipitin reaction between antisera and hapten-carrier conjugates. By this method the relative affinities of antisera for many haptens can be established. This work was continued by Pauling, Pressman, Nisonoff and others (Nisonoff, Hopper and Spring, 1975), and it was established that whole antisera were highly specific for haptens. For example, the affinities of rabbit anti-p-phenylazobenzoate antiserum for molecules as similar as m-nitrobenzoic acid and p-nitrobenzoic acid differ by an order of magnitude (Nisonoff and Pressman, 1957). These comparative data are a reliable index of affinity of such complex mixtures of molecules for haptens. It is, however, difficult to attribute any exact meaning to the absolute values of the average affinity (K_0) of antisera for antigens, since the value obtained for K_0 depends on the form of the population distribution assumed for the affinity constants of the component antibodies. These can range from 10^3 - 10^{11} M⁻¹ l. (Karush, 1962; Werblin and Siskind, 1972; Erwin and Aladjem, 1976). Further, the average affinity and the distribution of affinities change

during the course of an immune response (Eisen and Siskind, 1964; Steiner and Eisen, 1967; Werblin and Siskind, 1972).

The size of the antibody combining site has been investigated by the use of homologous series of oligomeric haptens (Nisonoff, Hopper and Spring, 1975). These include glucose oligomers, synthetic polypeptides (e.g. poly-alanyl derivatives), and hapten carrier conjugates of the form Dnp(L-lysine)_n . The conclusions from these studies can be summarized in two points. Firstly, that the antibody combining site can accommodate more than one monomeric unit, for example, in the case of glucans up to 5-6. Secondly, that one of these monomeric units is bound more strongly than the others. This is found to be the terminal group of the antigen. This group is termed the immunodominant group. It would be the interaction of this group with the antibody that the early workers studied.

On the basis of the studies discussed above, the combining site of antibodies which bind antigens composed of several structurally distinct groups, can be regarded as containing several distinct subsites. In the case of protein antigens, where the antibody may recognize part of the antigen composed of several different amino acid side chains (Arnon, 1973), the subsites may be structurally diverse. A well characterized example of this kind is the interaction of antibodies with the 'loop' of lysozyme (Arnon and Sela, 1969). It could be argued that such antibodies are multispecific. However, many enzymes which are considered to be monofunctional show a similar pattern of functionally specialized subsites, for example, lysozyme (Chipman and Sharon, 1969), and the serine proteases (Blow, 1971). It is thus difficult to see how the idea of multispecificity can be directly translated into a useful structural concept, which can clarify the nature of antibody-antigen interactions (Richards et al, 1975).

Since it is possible to dissect the antibody-antigen interaction into interactions at subsites, it follows that some or all of these subsites may be localized on either the V_H domain or the V_L domain. A direct

experimental approach to this problem can be made because H and L chains can be separated and recombined with substantial recovery of antigen binding activity (Roholt, Radzinski and Pressman, 1965). Thus a variety of recombinant antibody combining sites can be generated, including; light chain dimer, heavy chain dimer, and recombinants between heavy and light chains of different antibody preparations (heterologous). If heterogenous antisera are dissociated and then allowed to reassociate, approximately half of the original activity can be recovered (Porter and Weir, 1966; Roholt, Radzinski, and Pressman, 1965). In a number of studies where H and L chains of heterogenous preparations were separated and the activity of the isolated chains was investigated, varied results were obtained (Porter and Weir, 1966). In some cases the L chain retained activity, in others the H chain, whilst often the activity was completely lost. The myeloma proteins of the BALB/c mouse provide a better system for studying this problem, since a number of structurally related homogenous myeloma proteins have been obtained (Potter, 1977). Thus, for example, Barstad et al (1978) have studied recombinants between phosphorylcholine myeloma proteins of the TEPC 15 group. This group of myeloma proteins show closely related V_H structures but less closely related V_L structures, all the recombinants show significant retention of phosphorylcholine binding activity. It is concluded that the hapten binding is located on the heavy chain. This is supported by the X-ray structure of one of these proteins, McPc603 (section 6(e)). In contrast a group of glucan binding myeloma proteins, which include J 558 and MOPC 104E show very closely related light chains (Cesari and Weigert, 1973); the role of the heavy chain in this system is poorly defined at present, however, due to lack of sequence data. Protein 315 (section 7) provides the best comparative data between a light chain dimer and an intact combining site. This protein binds Dnp haptens, and the dimer of the light chain retains significant affinity for Dnp haptens.

Two points can be established from these types of studies. Firstly, that the specificity for antigens is not exclusively associated with either chain, rather the relative contributions vary from system to system. Secondly, that the specificity of a particular V_H or V_L domain is to some extent independent of the other part of the site. This view is supported by the evidence discussed above for the phosphorylcholine binding proteins, where changing the structure of the light chain does not abolish hapten binding, or even markedly affect the specificity. The detailed structural basis for antigen binding has been established for two myeloma proteins, for which high resolution X-ray structures are available (section 6(e)).

c) Covalent modification of antibody molecules

Two experimental approaches to antibody specificity fall under this heading, affinity labelling and chemical modification. These methods have been used to identify the types of amino acid residues present in antibody combining sites, and, in conjunction with sequence analyses, to determine the sequence positions of amino acid residues that participate in the combining site. Both methods use reagents which selectively modify amino acid residues in antibodies. The set of modifiable residues is further restricted in the case of affinity labels by the site-directed nature of the reagent, which is a hapten or hapten derivative (Givol, 1974).

The elucidation of antibody specificity by chemical modification relies on the capacity of amino acid residue specific reagents to diminish or abolish hapten or antigen binding. Since, in general, many residues on the protein are modified, the site specific subset is located by demonstrating hapten or antigen protection against inactivating modification. An example of this kind of investigation is that of Pressman and coworkers (Freedman, Grossberg and Pressman, 1968) on rabbit anti-p-azobenzenearsonate antibodies. It was shown that the antibody population could be subdivided into proteins having a hapten-protectable, activity-linked arginine residue (40-50%),

and those having an equivalent lysine residue (30-50%). It was also concluded that all of the antibodies in the population had two identical combining sites by these criteria.

Affinity labelling studies, in conjunction with sequence analyses, have been used to determine which sections of the variable region sequence participate in the antibody combining site (Givol, 1974). For example, Koo and Cebra (1974) showed that a lysine residue in the second hyper-variable region of the H chain of guinea pig anti-p-azobenzeneearsonate IgG₂ antibodies, was in or near the combining site, since this residue was labelled by N(1-C¹⁴)-bromoacetylmono(p-azobenzeneearsonate)-L-tyrosine. This reagent is an example of a label in which the reactive group, in this case bromoacetyl, is part of the hapten side chain. This type of reagent offers the opportunity of using a homologous series of derivatives with side chains of different lengths to search for nucleophilic amino acid residues in the antibody combining site. The most extensive application of this approach is to the mouse myeloma protein 315 and is discussed in section 7.

An example of a reagent which carries the reactive group in the immunodominant determinant, is m-nitrobenzenediazonium fluoroborate (MNBDF). This has been used as a hapten analogue in anti-Dnp antibodies and Dnp-binding myeloma proteins (Wofsy, Metzger and Singer, 1962; Singer and Doolittle, 1966; Goetzl and Metzger, 1970), where it has been found to label primarily tyrosine residues in both the heavy and light chains. In the case of protein 315, some controversy exists as to whether the tyrosine residue labelled, 34_L, is a hapten contact residue or merely close to the combining site (Richards et al, 1974; Hadier and Metzger, 1971). This illustrates a problem in the interpretation of affinity labelling data, namely that extent of modification of a particular amino acid residue, is not necessarily representative of the ground state of the antibody reagent complex. Since the structure of this complex may not present suitable

side chains for reaction. This problem can be avoided by the use of photoactivated affinity labels, such as the 4-azido-2-nitrophenyl group (NAP) (Fleet, Knowles and Porter, 1972). This type of reagent has two advantages. Firstly the reagent can be used to raise specific antibodies, since it is stable in the dark. Secondly, the nitrene produced by photolysis is extremely reactive, and will label the closest amino acid residue, irrespective of type. This reagent has been used to label rabbit antibodies raised against it. However, the contact residues in the antibodies of this population seemed to be highly heterogenous, as the recovery of labelled peptide was low (13% of total label incorporated in phe-cys-ala-Arg, in positions 91-94 of the heavy chain).

Affinity labelling studies with bivalent reagents (Hadler and Metzger, 1971; Givol et al, 1971), reacted with the mouse myeloma protein 3i5, have shown that both heavy and light chains participate in the antibody combining site. Since the chains can be cross linked by these reagents. This is a useful independent confirmation of the results of X-ray studies (See section 6(e)).

d) Physical Studies of antibody - antigen interactions

Three aspects of the antigen (or hapten)-antibody interaction have been studied by physicochemical methods; the kinetics and thermodynamics of the binding process, the environment of bound antigen or hapten, and the effect of antigen binding on the antibody molecule. This last aspect has been reviewed in detail by Metzger (1974, 1978) and will therefore, be summarized only briefly below. The discussion of the first two aspects will concentrate on antibody hapten interactions and assess the significance of the data in relation to the structural features of the site. The major contribution to our understanding of the structural basis of hapten binding by antibodies has been made by X-ray crystallographic studies. Since it is necessary to examine these results in detail, the data are

discussed in section 6(e).

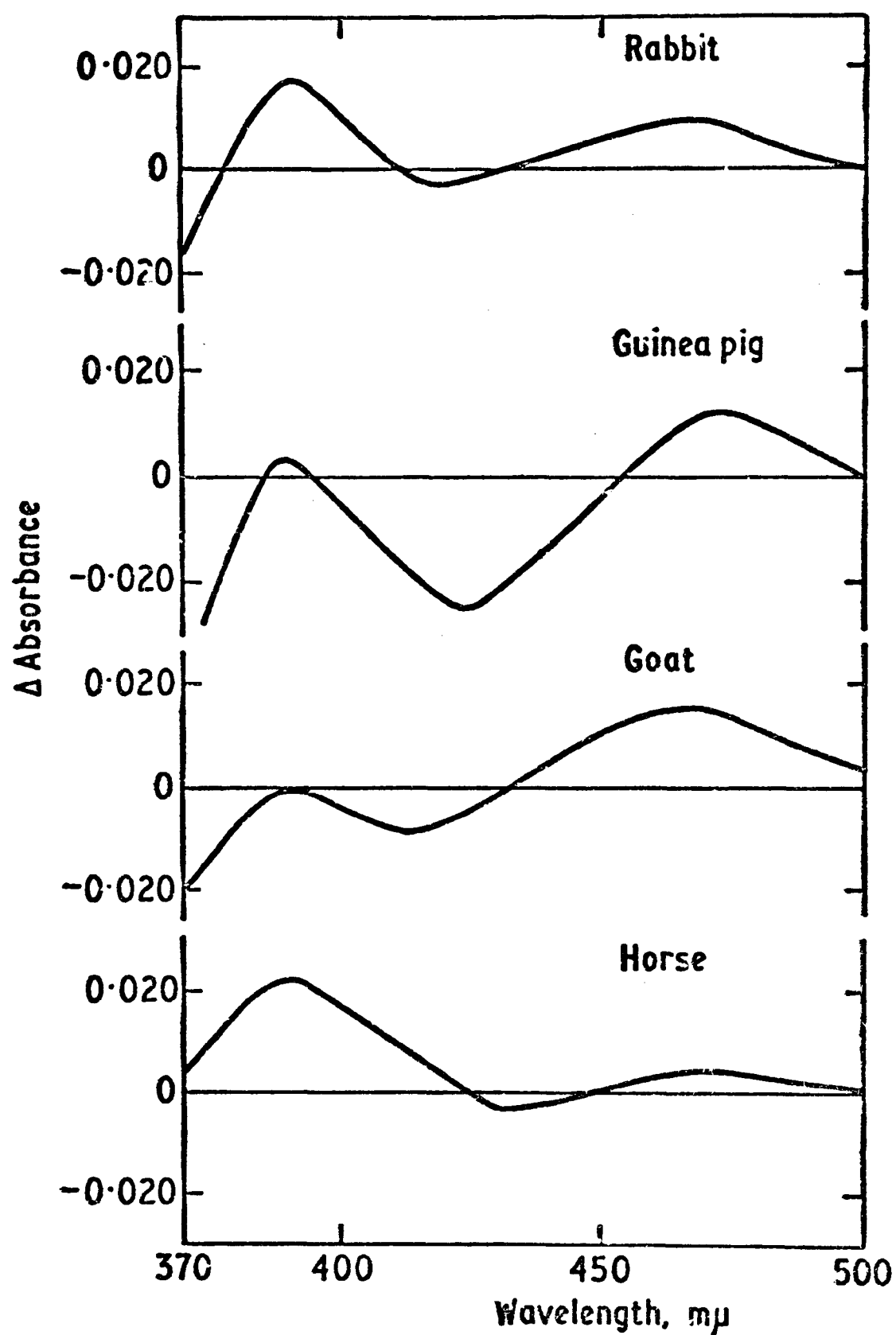
The kinetics of antibody-hapten interactions have been studied using the stopped-flow and temperature jump techniques (Sturtevant et al, 1961; Day et al, 1963; Froese, Sehon and Eigen, 1962; Froese, 1968; Kelly, Froese, Sehon, 1971; Haselkorn et al, 1974; Hammes, 1974; Hammes and Wu, 1974; Froese and Sehon, 1975). The major conclusions from these studies can be summarised in three points:

1) The association rate for haptens to antibodies (10^7 - 10^8 $\text{m}^{-1}\text{sec}^{-1}$) are close to the diffusion controlled limit for a 7s immunoglobulin ($D=3.8 \times 10^{-7} \text{cm}^2 \text{sec}^{-1}$) and a small molecule ($D \approx 10^{-5} \text{cm}^2 \text{sec}^{-1}$) of $c \approx 10^9$ $\text{m}^{-1}\text{sec}^{-1}$. Whether the difference of 10 - 10^2 between theory and experiment is significant is not clear, since the theoretical calculation makes no allowance for steric factors (Sehon, 1963).

2) For a particular antibody preparation the differences in affinity for different haptens arise from differences in the rates of dissociation (or lifetime) of the protein-ligand complex.

3) The association-rates for Fab fragments and haptens were found to be of the order of 2-fold faster than the rate of association of the hapten with the whole 7s antibody (Kelly, Sehon, Froese, 1971). This, however, was not confirmed by Barisas, Singer and Sturtevant (1975). These data are consistent with a diffusion controlled simple encounter mechanism for antibody-hapten binding. The very slow dissociation rates found with some antibody preparations, which are reflected in the high affinity of these proteins for antigens, correlates with the biological role of these molecules which is "irreversible" antigen binding under conditions where the antigen concentrations can vary widely. The free energy of the antigen-antibody reaction can be dissected into its components of enthalpy and entropy by use of VanT'Hoff plots or by direct calorimetric methods (Barisas, Sturtevant and Singer, 1971; Barisas, Singer and Sturtevant, 1972). The two methods, applied to the binding of ϵ -N-Dnp L-lysine to rabbit anti-Dnp antibodies.

FIG. 8 : Optical difference spectra obtained on binding Dnp to induced anti-Dnp antibodies from several species



The difference spectra are obtained by subtracting the spectrum of Dnp from that of the same concentration of hapten in the presence of equimolar antibody. All the spectra show a characteristic red shift.

Data of Little and Eisen, 1967.

give values for ΔH of $-17.5 \text{ Kcal m}^{-1}$ (VanT'Hoff) and $-22.8 \text{ Kcal m}^{-1}$ (microcalorimetric titrations). That the difference in these values was due to heterogeneity in the antibody preparation, is suggested by the finding that the two values are within experimental error for the two myeloma proteins 315 and 460 (Johnson, Barisas and Sturtevant, 1974). No attempt has been made to correlate the thermodynamics of hapten binding with structural features of the site. This is reasonable since the values of entropy changes, in particular, are dependent on several factors, one of the most important of which is the interaction of the components of the equilibrium with the solvent water.

Spectroscopic methods have been used to monitor the environment of the antibody-bound haptens, the conformation of antibody molecules (Dorrington and Tanford, 1970; Cathou and Dorrington, 1975), and conformation changes (Metzger, 1974, 1978). The most useful structural approach for defining the properties of the antibody combining site, has been the use of haptens as spectroscopic "probes" of their bound environment. The methods that have been used include, absorption spectroscopy, fluorescence, circular dichroism (CD), optical rotatory dispersion (ORD),

electron spin resonance, and nuclear magnetic resonance.

Characteristic effects (a red shift and hypochromism) on the absorption spectrum of Dnp and Tnp haptens (Little and Eisen, 1967), folic acid (Rubenstein and Little, 1970) and fluorescein (Voss and Watt, 1977), when bound to specifically raised rabbit antibodies can be reproduced with tryptophan but not with tyrosine or phenylalanine. This indicates that tryptophan is probably a contact residue in the combining sites of all these antibodies. In the case of anti Dnp-antibodies Little and Eisen (1967) have shown that essentially similar optical difference spectra are produced with anti-Dnp antibodies from several species (Fig. 8). This suggests that interaction with a tryptophan residue is a widely distributed feature of anti-Dnp antibody combining sites. Since, however, the

magnitude of the effect could vary between different components of the immune serum, the data do not show that all anti-Dnp antibodies have this structural feature.

Fluorescent haptens have been used to investigate the hapten environment in the antibody combining site. This method is capable of giving a limited amount of information about the polarity of the site and the rate of motion of the bound hapten. Fluorescence energy transfer experiments can also be used to determine distances between chromophores. For example, Bunting and Cathou (1973) have determined that the distance from a bound hapten, dimethyl aminonaphthalenesulphonyl-L-lysine to a fluorescein label located at the reduced interheavy chain disulphide bond in rabbit IgG. It was concluded that the distance was approximately 80Å. Comparison of this distance with dimensions of the Fab fragment determined by X-ray crystallography ($< 92\text{\AA}$), led to the suggestion that the chromophore was $< 12\text{\AA}$ inside the protein surface. Since this is a small difference between two large numbers the significance of this distance is questionable. It has been suggested on the basis of fluorescence enhancement of anti-4 anilinonaphthalene-1-sulphonate and related haptens when these bind to specific rabbit antibodies that the bound environment is more hydrophobic than bulk water (Yoo et al, 1970; Nakamura et al, 1970). Fluorescence depolarization studies have shown that the rotational correlation time for ξ -N-dansyl-L-lysine bound to λ specific rabbit antibodies is consistent with the value determined from the Stokes-Einstein equation. The value for whole antibody is much shorter than the theoretical value, however, suggesting internal flexibility of the molecule. The hapten appears from these studies to be rigidly bound (Yguerabide, Epstein and Stryer, 1970).

Circular dichroism (CD) can be induced in the spectrum of Dnp, Tnp and fluorescein when these haptens bind to specific antibodies (Athey and Cathou, 1977; Cathou and Dorrington, 1975). With one exception

(see section 7) no attempt has been made to obtain any structural information from the induced CD of bound haptens. Thus the technique mainly provides a useful method for determining qualitatively whether two sites are the same or different. Resonance raman is a potentially more informative technique, since it can be used to observe particular vibrational transitions in a selected chromophore (Carey, 1978). Carey and coworkers have examined the binding of Dnp ligands to rabbit anti Dnp antibodies (Carey, Froese and Schneider, 1973) and to mouse myeloma proteins (Kumar et al, 1978). The interesting feature of the conclusions from the latter study is that the different Dnp derivatives of the general structure DnpNHR showed variations in the effects of binding on the 2 and 4 nitro group of the haptens. This indicates that in these myeloma proteins the side chain may alter the interaction between the immunodominant group and the protein. The problem is that no theoretical treatment exists to relate structural changes to these changes in the resonance raman spectrum.

Electron spin resonance (ESR) studies have utilized Dnp haptens which have nitroxide-containing groups attached to the hapten by spacer groups (2,4 DnpNH-R-1 oxyl 2,2,6,6,tetramethyl-4-piperidiny1). By preparing a set of homologous haptens in which the length of the spacer is varied. A plot of correlation time (estimated from the ESR spectrum) against spacer length for bound spin-label hapten can be constructed (Hsia and Piette, 1969; Piette et al, 1971; Wong et al, 1974). This changes from that typical of immobilized spin label for the shortest derivative (N-(1 oxyl 2,2,6,6, tetramethyl-4-piperidiny1)-2,4 dinitrobenzene) to that for free spin label (Stryer and Griffith, 1965 Hsai and Piette, 1969) for the longest (N-2,4 Dinitrophenyl-(1 oxyl 2,2,6,6, tetramethyl-4-piperidiny1)-caproate. The region of transition is assumed to be the protein/solvent interface. Thus in the case of anti Dnp antibodies (Hsai and Piette, 1969) or Dnp-binding myeloma proteins (Hsai and Little, 1973; Wong et al, 1974; Sutton et al, 1977) the depth estimated for the binding

site is $\sim 12\text{\AA}$.

Nuclear magnetic resonance (n.m.r.) methods are potentially the most informative spectroscopic techniques, since the signals in the n.m.r. spectrum arise from individual nuclei in the sample (Dwek, 1973). The applications of n.m.r. to the characterization of hapten-antibody interactions have been limited to a few studies. Those involving interactions of protein 315 with haptens will be described in section 7. The earliest report is that of Burgen et al (1967) who studied the effect on the ^1H resonance of the trimethyl ammonium hapten of binding to rabbit p-amino phenoxycholine antibodies. They demonstrated a linear relationship of the barrier to rotation of the methyl protons of the bound hapten, and the free energy of hapten-antibody binding. This study provides information of similar quality to that obtained with the optical methods described above. This illustrates that the power of n.m.r. methods, in that there is a detailed correlation between the signals in the n.m.r. spectrum and the environments of individual nuclei in the sample, cannot be fully exploited in heterogenous systems of this type. Recent studies have therefore concentrated on myeloma proteins. Goetze and Richards have used ^31P and ^{13}C n.m.r. to monitor the interaction of phosphorylcholine and related haptens with five proteins of the TEPC 15 group of BALB/c myeloma proteins (Goetze and Richards, 1977 a,b; Goetze and Richards, 1978) (Proteins TEPC 15, W3207, McPC 603, MOPC 167 and MOPC 511). Data have been obtained on the exchange rates for the choline trimethylammonium and phosphate group, and the environment of the bound hapten has been explored by obtaining ^{13}C and ^{31}P data and by determining the effect on the pK_a 's of the phosphate group of binding to the proteins. Since the crystal structure of one of these proteins, McPC 603 (Section 6(e)) has been determined and most of the hapten interactions have been shown to be made with the heavy chain, for which sequence data are available, it was possible to correlate to some extent the variations in hapten environments with

the variation in H chain structure.

Similar studies have been carried out by Gettins et al (1977) using ^{31}P and ^1H n.m.r. to study the phosphoryl choline/McPC 603 interaction. Richards and coworkers have also studied the interaction of ^{19}F labelled Dnp and Tnp analogues with the three Dnp-binding BALB/c mouse myeloma proteins MOPC 315, XRPC 25 and MOPC 460 (Kooistra and Richards, 1978; Hardy and Richards, 1978; Kooistra, Hardy and Richards, unpublished data). In contrast to the results of Carey and coworkers (Kumar et al, 1978) the results of these studies indicate that the mode of binding of the tri-fluoromethyl nitro ring is unaffected by substitution of different side chains. ^1H n.m.r. data has also been obtained for the human Bence Jones protein REI in the laboratory of Dr. K. Wüthrich, but no published reports are as yet available.

Data have been obtained on the dimensions of the antibody combining site by using the probe-spacer method described above for spin-labelled haptens. Two types of approach have been utilized to monitor the binding of antibodies to bivalent Dnp haptens in which the distance between the Dnp groups is varied. In one report Wilder, Green and Schumaker (1975) detected the formation of cross linked oligomers by ultracentrifugation and concluded that with bivalent haptens of the form $\text{DnpNH}(\text{CH}_2)_n\text{NHDnp}$ only if the extended length exceeded $20\text{\AA}$ did significant dimerization of antibody (rabbit anti-Dnp) occur. Thus the combining site was estimated to be c 10 ($20 \div 2$) $\text{\AA}$ deep. Rosenstein and Richards (1976) have carried out studies directed towards the same end by measuring the retention of proteins MOPC 315 and MOPC 460 on affinity columns composed of hapten-spacer-sepharose. These gave values for the depth of the Dnp binding site of c $11\text{\AA}$ in protein 315 and c $9\text{\AA}$ in protein 460. These are consistent with values obtained by other methods (see section 7).

The information obtained from these studies gives some insight into the nature of antigen-antibody binding. It is fast, close to the diffusion

controlled limit, and appears to be described by a simple encounter type of mechanism. The combining site of the antibody molecule is a pocket or groove which is sufficiently rigid so that the rate of motion of bound hapten groups is not significantly faster than the overall rate of motion of the Fab region of the immunoglobulin molecule. The overall depth of the site is sufficiently small for it to be contained within the hyper-variable region of the structure (see section 6(e)).

There is no indication from any of the data discussed above that there is an antigen induced conformation change in the antibody molecule. However, monitoring the environment of the bound hapten using the hapten spectroscopic properties would not reveal this, and there is one report on the kinetics of hapten binding which shows a conformational equilibrium which is perturbed on hapten binding, in an Fab' fragment (Lancet and Pecht, 1976). Considerably more data pertinent to this question have been collected by using physicochemical techniques to monitor the effects on the antibody molecule of antigen binding (Metzger, 1978). The biological significance of this effect is related to the dual role of the antibody molecule.

It is necessary for the biological role of antibodies, that the effector systems of the immune response be activated by antigen-complexed antibody, but not by free antibody. The effector systems interact with the Fc region of the antibody molecule (an exception appears to be the alternate complement pathway, Götze and Müller-Eberhardt, (1976)), which is distant from the antigen binding site. How, then, does the binding of antigen affect the effector interactions of the antibody molecule?

Three hypotheses have been proposed to account for the link between the antigen binding and effector functions of the antibody molecule. These involve allosteric, distortive and aggregational mechanisms respectively. The first two mechanisms involve an antigen-induced conformational change in the antibody molecule, the last does not. Thus considerable effort has

been invested in attempting to demonstrate, by a variety of physico-chemical methods, that there is an antigen induced conformational change in antibody molecules (Metzger, 1978). It would appear that for some but not all antibody-antigen systems that there is a change in the state of the antibody molecule when antigens bind. This, in itself, does not discriminate between the three mechanisms, since all three could be simultaneously operative. Nor does this prove that conformational changes are functionally important in effector system activation by immune complexes. In order for this to be achieved it is necessary to demonstrate that the observed conformational changes can be directly correlated with the altered activity of antibody towards effector systems. There are considerable technical problems in obtaining reliable evidence on this last point, since even the simplest immune effector system, the complement system is a multiprotein proteolytic cascade system with two alternative modes of activation by immune complexes (Reid and Porter, 1975; Götze and Muller-Eberhardt, 1976; Fothergill and Anderson, 1978). The present view, as outlined by Metzger, is that aggregation of antibody is probably a prerequisite for the activation of complement, but that contributions from mechanistic elements of the other two types cannot be ruled out.

e) X-ray crystallographic analyses of antibody variable regions

The method of X-ray crystallography is the single most powerful technique available for elucidating the structure of proteins. At present, X-ray diffraction data are available for eight myeloma proteins; these studies are summarized in Table 3. These data have revealed the detailed structural basis for hapten binding to two myeloma protein Fab' fragments (from McPC 603 and NEW) and one V_L dimer (REI). More limited data are available on one light chain dimer (Mcg).

The Fab' fragment from the human myeloma protein NEW, binds menadione ($K_A \approx 10^3 \text{m}^{-1}$ $\downarrow$) and its derivative, vitamin $K_1\text{OH}$ ($K_A = 1.7 \times 10^5 \text{m}^{-1}$ $\downarrow$). In

TABLE 3 : X-ray crystallographic analyses of immunoglobulin structures

Protein	Type	Resolution	Hapten Complex	Resolution
NEW	human Fab' (γ_1, λ)	2.0 Å	yes	3.5 Å
McPC 603	mouse Fab' (α, K)	3.1 Å	yes	4.5 Å
REI	human $(V_L)_2 (K)$	2.0 Å	yes	not known
AU	human $(V_L)_2 (K)$	2.5 Å	no	--
Mcg	human $(L)_2 (\lambda)$	3.5 Å	yes	6.0 Å
Mcg	H_2L_2	preliminary	no	--
KOL (Fc)		3.5 Å	--	--
KOL	human $H_2L_2 (\gamma_2, K)$	4.0 Å	no	--
Dob	human $H_2L_2 (\gamma_1, K)$	6.0 Å	no	--
Zie	human $H_2L_2 (\gamma_2, K)$	preliminary	no	--

References:

1) NEW: Rossi and Nisonoff, 1968; Rossi et al, 1969; Poljak et al, 1972a,b; Amzel et al, 1974; Poljak et al, 1974.

2) McPC 603: Rudikoff et al, 1972; Padlan et al, 1973; Segal et al, 1974.

3) REI: Epp et al, 1974; Epp et al, 1975; Huber R., private communication.

4) Au: Fehlammer et al, 1975.

5) Mcg: Edmundson et al, 1970; Ely et al, 1973; Schiffer et al, 1973; Edmundson et al, 1974; Edmundson et al, 1975; Ely et al, 1978; Firca et al, 1978.

6) KOL: Colman et al, 1976; Diesenhofer et al, 1976a,b; Huber et al, 1976; Palm and Colman, 1974; Palm, 1974.

7) Dob: Sarma et al, 1971.

8) Zie: Ely et al, 1978.

the structure of the complex with this latter hapten the bound hapten is located in a shallow groove 15Å long 6Å wide and 6Å deep. The bound hapten forms contacts with residues on both the heavy and the light chains. The most important contact residues are gly 29_L, Asn 30_L, Tyr 93_L, Arg 95_L, residues 102-104 of the heavy chain for the naphthoquinone ring and Trp 47_H, Tyr 50_H, Asp 58_H for the phytyl side chain. A seven residue deletion on the C terminal side of the L₂ hypervariable segment (Table 4) removes this segment from the site. It can be seen for this antibody that the hapten aromatic ring makes contacts mainly with the light chain, whilst the side chain of the hapten interacts mainly with the heavy chain. Comparison of the complexed and uncomplexed structures reveals no conformation changes when hapten binds.

The Fab' fragment of the mouse myeloma protein McPC 603 binds phosphorylcholine with an affinity of $1.7 \times 10^5 \text{ m}^{-1}$. The bound hapten is located in a deep pocket (12Å deep x 15x20Å) at the front of the variable region, and most of the hapten interactions are with the heavy chain. The phosphate group interacts with the positively charged side chains of Arg 52_H and lys 54_H and hydrogen bonds to the -OH of Tyr 33_H. The trimethyl ammonium group is located close to (~5Å) the carboxyl of glu 36_H. The methylene groups of the phosphorylcholine will be in contact with the aromatic ring of Tyrosine 33_H. Hence in this protein the binding is mainly to the heavy chain. Again, no conformation changes were detected on hapten binding in this system. However, since the phosphate group binding site is occupied by a sulphate in the apoprotein, this cannot be taken as unequivocal proof that there are no such changes on hapten binding. The Mcg light chain dimer structure appears to form rather atypical complexes with a number of aromatic haptens. The binding cavity formed by the two V_L domains is 25Å deep and crystallographic data show that haptens can bind at any one of three quite different locations, one of which lies inside the framework region of the protein. It has also been shown that

TABLE 4 : Alignment of Variable Region Sequences

Light Chains		10	
K	REI	Asp-Ile-Glu-Met-Thr-Gln-Ser-Pro-Ser-Ser-Leu-Ser-Ala-Ser-Val	
K	M603	Asp-Ile-Val-Met-Thr-Gln-Ser-Pro-Ser-Ser-Leu-Ser-Val-Ser-Ala-	
	REI	Leu-Gln-Ala-Gly-Val-Pro-Ser-Arg-Phe-Ser-Gly-Ser-Gly-Ser-Gly-	
	M603	Arg-Glu-Ser-Gly-Val-Pro-Asp-Arg-Phe-Thr-Gly-Ser-Gly-Ser-Gly-	
λ	NEW	PCA-Ser-Val-Leu-Thr-Gln-Pro-Pro- 0 -Ser-Val-Ser-Gly-Ala-Pro-	
λ	Mcg	PCA-Ser-Ala-Leu-Thr-Gln-Pro-Pro- 0 -Ser-Ala-Ser-Gly-Ser-Leu-	
λ	315	PCA-Ala-Vla-Vla-Thr-Glu-Glu- 0 -Ser-Ala-Leu-Thr-Thr-Ser-Pro-	
	NEW	Arg- 0 - 0 - 0 - 0 - 0 - 0 - 0 -Phe Ser-Val-Ser-Lys-Ser-Gly-	
	Mcg	Arg-Pro-Ser-Gly-Val-Pro-Asp-Arg-Phe-Ser-Gly-Ser-Lys-Ser-Gly-	
	M315	Arg-Ala-Pro-Gly-Val-Pro-Val-Arg-Phe-Ser-Gly-Ser-Leu-Ile-Gly-	
		60	70
Heavy Chains		70	
α	M603	Glu-Val-Lys-Leu-Val-Gly-Ser-Gly-Gly-Gly-Leu-Val-Gln-Pro-Gly-	
γ	NEW	PCA-Val-Gln-Leu-Pro-Glu-Ser-Gly-Pro-Glu-Leu-Val-Ser-Pro-Gly-	
α	M315	Asp-Val-Gln-Leu-Gln-Glu-Ser-Gly-Pro-Gly-Leu-Val-Lys-Pro-Ser-	
	M603	Thr-Thr-Glu-Tyr-Ser-Ala-Ser-Val-Lys-Gly-Arg-Phe-Ile-Val-Ser-	
	NEW	Thr-Ser-Asp-Thr-Asp-Thr-Pro-Leu-Arg-Ser-Arg-Val-Thr-Met-Leu	
	M315	Ser-Asn-Tyr-Gly-Asn-Pro-Ser-Leu-Lys-Asn-Arg-Val-Ser-Ile-Thr-	
		60	70

References for sequence determinations:

1.	REI:	Epp <u>et al</u> , 1974.
2.	M603 light chain:	Padlan <u>et al</u> , 1976.
	heavy chain:	
3.	NEW light chain:	
	heavy chain:	

Gly-Asp-Arg-Val-Thr-Ile-Thr-Cys-Gln-Ala-Ser-Gln- 0 - 0 - 0 - 0 - 0 - 0 -Asp-Ile
 Gly-Glu-Arg-Val-Thr-Met-Ser-Cys-Lys-Ser-Ser-Gln-Ser-Leu-Leu-Asn-Ser-Gly-Asn-Gln-
 Thr-Asp-Tyr-Thr-Phe-Thr-Ile-Ser-Ser-Glu-Gln-Pro-Asp-Glu-Ile-Ala-Thr-Tyr-Tyr-Cys-
 Thr-Asp-Phe-Thr-Leu-Thr-Ile-Ser-Ser-Val-Gln-Ala-Glu-Asp-Leu-Ala-Val-Tyr-Tyr-Cys-
 Gly-Gln-Arg-Val-Thr-Ile-Ser-Cys-Thr-Gly-Ser-Ser-Ser-Asn-Ile- 0 - 0 - 0 -Gly-Ala-
 Gly-Gln-Ser-Val-Thr-Ile-Ser-Cys-Thr-Gly-Thr-Ser-Ser-Asp-Val- 0 - 0 - 0 -Gly-Gly-
 Gly-Gly-Thr-Val-Ile-Leu-Thr-Cys-Arg-Ser-Ser-Thr-Gly-Ala-Val- 0 - 0 - 0 -Thr-Thr-
 Ser-Ser-Ala-Thr-Leu-Ala-Ile-Thr-Gly-Leu-Gln-Ala-Glu-Asp-glu-Ala-Asp-Tyr-Tyr-Cys-
 Asn-Thr-Ala-Ser-Leu-Thr-Val-Ser-Gly-Leu-Gln-Ala-Glu-Asp-Glu-Ala-Asp-Tyr-Tyr-Cys-
 Asp-Lys-Ala-Ala-Leu-Thr-Ile-Thr-Gly-Ala-Gln-Thr-Glu-Asp-Asp-Ala-Met-Tyr-Phe-Cys-

Gly-Ser-Leu-Arg-Leu-Ser-Cys-Ala-Thr-Ser-Gly-Phe-Thr-Phe-Ser-Asp- 0 -Phe-Tyr-Met-
 Glx-Thr-Leu-Ser-Leu-Thr-Cys-Thr-Gly-Ser-Thr-Val-Ser-Thr-Phe-Ala- 0 -Val-Tyr-Ile-
 Gln-Ser-Leu-Ser-Leu-Thr-Cys-Ser-Val-Thr-Gly-Ser-Tyr-Ile-Thr-Ser-Gly-Tyr-Phe-Trp-
 Arg-Asp-Thr-Ser-Gln-Ser-Ile-Leu-Tyr-Leu-Gln-Met-Asn-Ala-Leu-Arg-Ala-Gly-Asp-Thr-
 Val-Asn-Thr-Ser-Lys-Asn-Gln-Phe-Ser-Leu-Arg-Leu-Ser-Ser-Val-Thr-Ala-Ala-Asp-Thr-
 Arg-Asp-Thr-Ser-Glu-Asn-Gln-Phe-Phe-Leu-Lys-Leu-Asp-Ser-Val-Thr-Thr-Gln-Asn-Thr-

4. McG: Fett and Deutsch, 1974.
5. 315 light chain: Dugan et al, 1973.
 heavy chain: Francis et al, 1974.

Notes:

The sequences are numbered as for protein 315.

Ile-Lys-Tyr-Leu-Asn-Trp-Tyr-Gln-Gln-Thr-Pro-Gly-Lys-Ala-Pro-Lys-Leu-Leu-Ile-Tyr
 Lys-Asn-Phe-Leu-Ala-Trp-Tyr-Gln-Gln-Lys-Pro-Gly-Gln-Pro-Pro-Lys-Leu-Leu-Ile-Tyr-
 Gln-Gln-Tyr-Gln-Ser-Leu-Pro-Tyr-Thr-Phe-Gly-Gly-Gly-Thr-Lys-Leu-Gln-Ile-Thr-Gly-
 Gln-Asn-Asp-His-Ser-Tyr-Pro-Leu-Thr-Phe-Gly-Ala-Gly-Thr-Lys-Leu-Glu-Ile-Lys-Arg-
 Gly-Asn-His-Val-Lys-Trp-Tyr-Gln-Gln-Leu-Pro-Gly-Thr-Ala-Pro-Lys-Leu-Leu-Ile-Phe-
 Tyr-Asn-Tyr-Val-Ser-Trp-Tyr-Gln-Gln-His-Ala-Gly-Lys-Ala-Pro-Lys-Val-Ile-Ile-Tyr-
 Ser-Asn-Tyr-Ala-Asn-Trp-Ile-Gln-Gln-Lys-Pro-Asp-His-Leu-Phe-Thr-Gly-Leu-Ile-Gly-
 Gln-Ser-Tyr-Asp-Arg-Ser- 0 -Leu-Arg-Val-Phe-Gly-Gly-Gly-Thr-Lys-Leu-Thr-Val-Leu-
 Ser-Ser-Tyr-Glu-Gly-Ser-Asp-Asn-Phe-Val-Phe-Gly-Thr-Gly-Thr-Lys-Val-Thr-Val-Leu-
 Ala-Leu-Trp-Phe-Arg-Asn- 0 -His-Phe-Val-Phe-Gly-Gly-Gly-Thr-Lys-Val-Thr-Val-Leu-

100

Glu-Trp-Val-Arg-Gln-Pro-Pro-Gly-Lys-Arg-Leu-Glu-Trp-Ile-Ala-Ala-Ser-Arg-Asn-Lys-
 Val-Trp-Val-Arg-Gln-Pro-Pro-Gly-Arg-Gly-Leu-Glu-Trp-Ile-Ala-Tyr-Val-Phe-Tyr-His-
 Asn-Trp-Ile-Arg-Gln-Phe-Pro-Gly-Asn-Lys-Leu-Glu-Trp-Leu-Gly-Phe-Ile-Lys-Tyr-Asp-
 Ala-Ile-Tyr-Tyr-Cys-Ala-Arg-Asn-Tyr-Tyr-Gly-Ser-Thr-Trp-Tyr-Phe-Asp-Val-Trp-Gly-
 Ala-Val-Tyr-Tyr-Cys-Ala-Arg-Asx-Leu-Ile-Ala- 0 - 0 -Gly-Cys-Ile-Asx-Val-Trp-Gly-
 Ala-Thr-Tyr-Tyr-Cys-Ala-Gly-Asp-Asn-Asp-His- 0 - 0 -Leu-Tyr-Phe-Asp-Tyr-Trp-gly-

100

Glu-Ala-Ser-Asn

Gly-Ala-Ser-Thr

His-Asn-Asn-Ala

Glu-Val-Asn-Lys

Gly-Thr-Ser-Asp

Arg

Gly

Gly

110

Gly-Asn-Lys-Tyr

Gly- 0 - 0 - 0

Gly- 0 - 0 - 0

Ala

Gln

Gln

110

ligands induce a conformation change in the protein. Since this light chain dimer does appear to be exceptional, it is difficult to know how much weight to place on data obtained from binding studies with it, although in its overall conformation it resembles other immunoglobulin-type molecules closely.

In the case of REI it has been shown that the bound hapten molecule (N-Tnp-2-aminoethylphosphate) forms a stacking complex with Tyr 93, a nitrogroup is close to Asn 36 and Tyr 36, Gln 91 and Tyr 97 are also close to the site. It is surprising that the affinity of the hapten for this protein is so low (10^1 - 10^2 M⁻¹) when the structural complementarity between the protein and the hapten is apparently very good. A possible explanation which could account for this, is that there is no tryptophan residue in this combining site. It has been shown (see section (d)) that many (if not all) anti polynitrophenyl antibodies have a tryptophan as part of the combining site, thus the absence of such a residue could account for the low affinity.

If the structures of the two Fab fragment combining sites are compared it can be seen that the relative contributions of the heavy and light chains are different in the two cases. In the case of NEW the interactions with the light chain dominate, in McPC 603 the interactions with the heavy chain dominate. This difference arises from the changes in the lengths of the hypervariable loops. The depth of the site is also changed by such insertions and deletions, thus the L1 loop in McPC 603 is 6 residues longer than that in NEW largely accounting for the deeper site in the former. Whilst the overall shape of the combining surface can be altered by changing the lengths of the hypervariable segments, it appears that the conformations of the polypeptide backbone of segments of the same length are similar (Padlan, 1977). Thus the extensive structural homology seen for framework residues (section 5(b)), is continued to a lesser extent into the hypervariable segments that form the antibody combining site.

TABLE 5: Alignment of hypervariable sequences

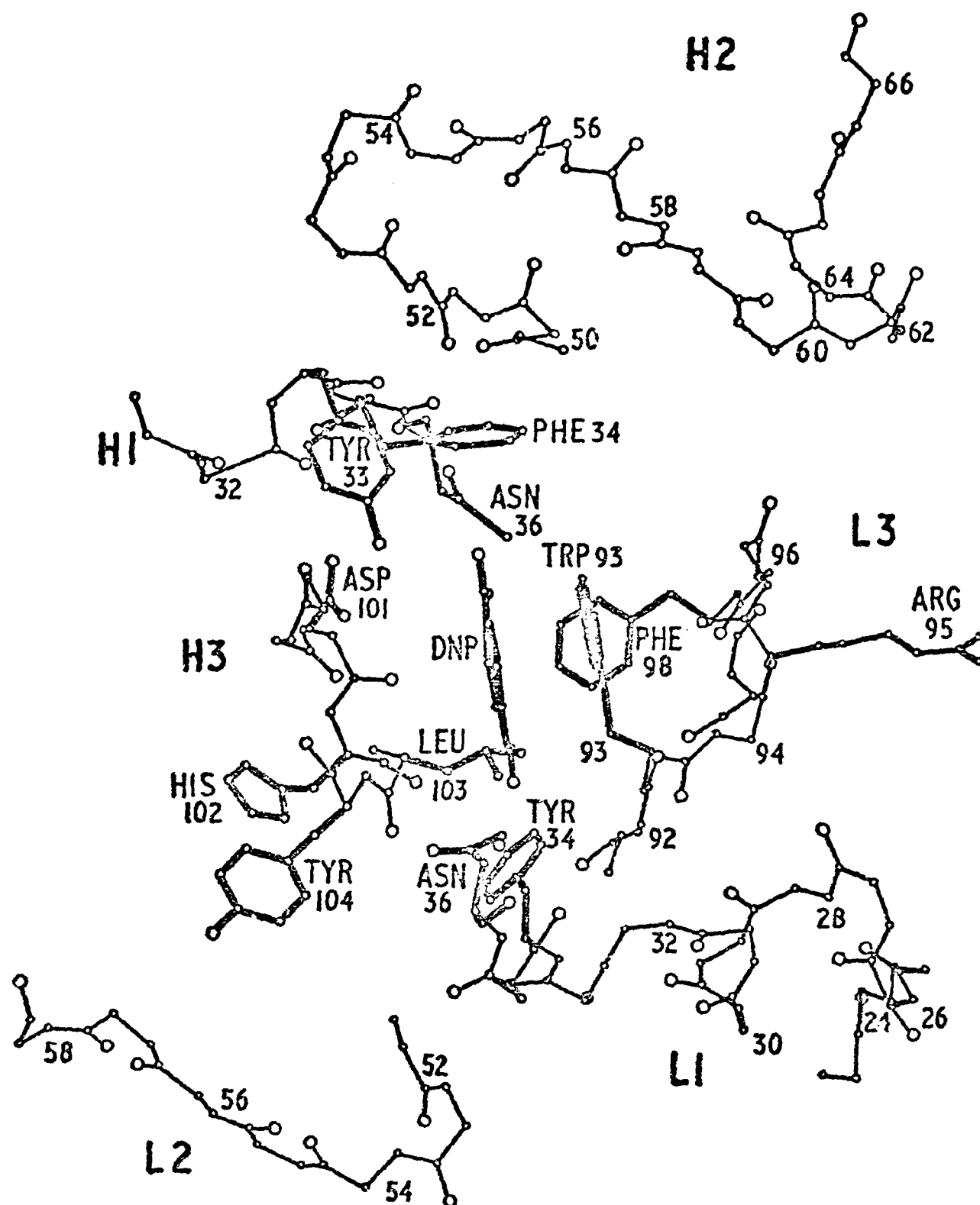
Light Chains	24	26	30	32	34	36
L1	K REI	Ala Ser gln- 0 - 0 - 0 - 0 - 0 - 0	-Asp-Ile-Ile-Lys-Tyr-Leu-Asn [*]			
	K M603	Ser-Ser-gln-Ser-Leu-Leu-Asn-Ser-Gly-Asn-Gln-Lys-Asn-Phe-Leu-Ala				
	λ New	gly-Ser-Ser-Ser-Asn-Lle- 0 - 0 - 0	-gly-Ala-gly-Asn-His-Val-Lys			
	λ Mcg	gly Thr Ser Ser Asp Val- 0 - 0 - 0	-gly-gly-Tyr-Asn-Tyr-Val-Ser [*]			
	λ M315	Ser Ser Thr gly Ala Val- 0 - 0 - 0	-Thr-ThrSer-Asn-Tyr-Ala-Asn			
L2		50	52	54	56	
	K REI	<u>Ile-Tyr-glu</u> -Ala-Ser-Asn-leu-glu-				
	K M603	Ile-Tyr-gly-Ala-Ser-Thr-Arg-glu-				
	λ NEW	Ile-Phe-His-Asn-Asn-Ala-Arg- 0 -				
	λ Mcg	Ile-Tyr-glu [*] -Val-Asn-lys-Arg-Pro-				
-3	λ M315	Ile-gly-gly-Thr-Ser-Asp-Arg-Ala				
		91	93	95	97	99
	K REI	<u>gln-gln-Tyr-gln-Ser-Leu-Pro-Tyr-Tyr-Phe</u>				
	K M603	gln-Asn-Asp-His-Ser-Tyr-Pro-leu-Thr-Phe				
	λ NEW	gln-Ser-Tyr-Asp-Arg-Ser- 0 -Leu-Arg-Val-				
	λ Mcg	<u>Ser-Ser-Tyr-glu-gly-Ser-Asp-Asn-Phe-Val-</u>				
	λ M315	Ala-leu-Trp-Phe-Arg-Asn- 0 -His-Phe-Val-				

TABLE 6 : Model building procedures for protein 315 combining site

Hypervariable segment	Based on backbone conformation of	Other comments
L 1	Mcg	λ -type and same length
L 2	McPC 603	—
L 3	McPC 603	Modified to allow for more H-bonding in protein 315
H1	McPC 603	—
H2	McPC 603/NEW	Sequence in 3 residues longer in 603 than 315 which is the same length as H2 of NEW.
H3	McPC 603	Could have used the structure of NEW for the segment 103-105.

The framework structure (fx and fy segments, see Fig. 3) was built from the coordinates of McPC 603 Fab'. This gives the starting and finishing coordinates for the hypervariable segments.

FIG. 9 : Predicted combining site of protein MOPC 315



This is obtained from the co-ordinates of Padlan et al (1976), which have been very kindly made available by Dr. E. Padlan. The hypervariable loops of the heavy (H) and light (L) chains, and the hapten position relative to the two chains, are indicated.

The hypervariable segments of chain identified on the basis of sequence variations in immunoglobulins are indicated in Table 4 and shown in Table 5. Recently this terminology has been replaced by "complementarity determining residues" (see, for example, Potter, 1977). As shown in Table 5, those sequences that line the binding site are not identical with the hypervariable sequences. Further, when the contacts of the bound hapten with the protein are examined, it is clear that some contact amino acid residues are not located in the hypervariable sequences (for example Trp 47_H in the NEW Fab' framework. Thus the relationship between sequence variability in antibody molecules, the structure of the site and the mode of ligand binding is not as simple as direct comparisons of only two structures might suggest. The dependence of the tertiary structure on many weak non covalent interactions of amino acid side chains distant from one another in the primary sequence indicates that several compensating changes in sequence may have to occur to produce a change at one point in the molecule. Further, these considerations show that, the assignment amino acid residues to the role of "complementarity-determining" can only come from a three dimensional structure.

The very similar conformation of the polypeptide chain backbones of the hypervariable loops has led to the view that it should be possible to extrapolate from the X-ray structures (Table 3) that are known, using primary sequence data alone. The proteins for which this has been done are given in Potter (1977). The structure of the combining site of the polynitrophenyl binding mouse myeloma protein 315 (sequences in Table 4) has been predicted by a model building study (Padlan et al, 1976) and is shown in Figure 9. Table 6 shows which hypervariable segment conformations were used to predict the conformations of the six segments in protein 315. The framework regions of the protein 315 variable domains were constructed from the coordinates of M603. The coordinates for the structure in Figure 9 are given in the back of this thesis. These coordinates have been

checked using a diamond model building program by Padlan et al (1976). An attempt to further refine this structure by energy minimization calculations has been made.

7. Protein 315: a review

Protein 315 is a Dnp-binding IgA secreted by the plasmacytoma cellline MOPC 315 (Eisen, Simms and Potter, 1968). The plasmacytoma was induced in a seventh generation C57BL to BALB/c backcross mouse that had been selected for the C57BL C_H allogroup, and which was thus heterozygous at the C_H loci. Protein 315 was shown to be of the A12 allotype, which is a BALB/c C_H gene product. The protein as isolated from serum consists of 7s IgA monomer and a variety of oligomers containing J chain (Underdown, Simms and Eisen, 1971) which are synthesized in the plasmacytoma cells (Parkhouse, 1972; Della Corte and Parkhouse, 1973). The protein can be prepared as 7s monomer containing no J chain by reduction and alkylation.

The protein was shown to contain typical IgA subunits each containing two heavy and two light chains. The overall conformation of the 7s monomer and the oligomeric forms of protein 315 was examined by electron microscopy, and shown to contain typical Y shaped antibody subunits (Green, Dourmashkin and Parkhouse, 1971; Dourmashkin, Virella and Parkhouse, 1971). The 7s subunit could be fragmented with trypsin (Underdown, Simms and Eisen, 1971) and pepsin (Inbar, Rotman and Givol, 1971) to give Fab' fragments. The 7s monomer contained two combining sites and the Fab' fragment contained one. A low molecular weight component was present in the peptic digest, and Inbar, Hochman and Givol (1972) showed that by lowering the pH to 3.5 (see Chapter 2) peptic digestion yielded an Fv fragment that consisted of the variable domains of the heavy and light chains (Hochman, Inbar and Givol, 1973), carried

all the idiotypic determinants of the 7s monomer (Wells, Fudenberg and Givol, 1973), and retained full antigen binding activity (Inbar, Hochman and Givol, 1972). It is not known why the V/C switch region of this protein is pepsin susceptible. Interest centred on this protein since it showed affinities for Dnp ligands (e.g. 10^7M^{-1} for $\epsilon\text{-N-Dnp-L-lysine}$) that approached those of induced anti Dnp antisera, and provided a useful laboratory system since the plasmacytoma can be propagated in vivo (see section 3). The sequences of both the V_L (Schulenberg et al, 1971) and the V_H domains (Francis et al, 1974) were determined, and it became clear that the protein 315 light chain was a new type (λ_2).

The availability of a high affinity Dnp-binding homogeneous immunoglobulin offers the possibility of gaining insight into the structural basis for the specificity of the extensively studied anti-Dnp immune response. If the study of protein 315 is to be established as relevant to this problem, it is important to consider what relationship exists between the structure of the V region of protein 315 and those of induced mouse anti Dnp antibodies. Anti-idiotypic antibodies to protein 315 were prepared syngeneically (Sirisinha and Eisen, 1971) and xenogeneically (Brient, Haimovitch and Nisonoff, 1971), and shown to bind competitively with $\epsilon\text{-N-Dnp-L-lysine}$ to protein 315. Granato, Braun and Vassali (1974) used the syngeneic antiserum to search for cross reactivity between protein 315 and BALB/c mouse anti-Dnp antibodies. They found that 45% of the anti-315 idiotype antiserum bound to the anti Dnp antibodies and that the anti Dnp response was partially suppressed in protein 315 immunized mice, which had anti 315 idiotype antibodies in the humoral circulation. Some doubt was cast on the first of these findings when it was shown that only 60% of the syngeneic antiserum could compete with $\epsilon\text{-N-Dnp-L-lysine}$ for protein 315. No cross reaction between protein 315 and a BALB/c anti Dnp preparation of the unstated origin was found with this purified "anti-site"

preparation (Hellman, Shrier and Givol, 1976). The failure to detect the cross reaction may possibly be understood in the light of recent results (Reth, Hämmerling and Rajewsky, 1978; Cotner and Eisen, 1978). It has been shown that the primary immune response to NP in C57BL mice is restricted to a set of heteroclitic λ containing antibodies, hyperimmunization produces a more diverse set of predominantly K containing antibodies. It has similarly been shown in BALB/c mice that 90% of the anti Dnp antibodies formed in primary response, contain λ_2 chains that partially cross react with a rabbit anti V_L315 serum. This presumably contains mostly anti-framework antibodies. It is amongst such early anti Dnp antibodies that the search for idiotypic cross reactions should be undertaken. The expectation that some such evidence of structural similarity might be found is strengthened by the results of Tonegawa et al (1978) which show that the protein 315 V_L sequence differs at only four positions from the sequence of the protein encoded by a (probably 'the') BALB/c mouse V λ_2 germ-line gene. If this is considered in conjunction with the arguments of Seidman et al (1978), that the mutation distance between the germ-line V genes and those expressed in the adult B cell population is small, then it would seem that the V_L domain of protein 315 is similar in structure, to that of other λ_2 BALB/c mouse anti-Dnp antibodies.

The binding of a wide variety of Dnp derivatives, Tnp derivatives and other aromatic haptens to protein 315 has been studied in the laboratory H.N. Eisen (Eisen, Simms and Potter, 1968; Eisen et al, 1970; Underdown, Simms and Eisen, 1971; Michaelides and Eisen, 1974). In general it was found that binding of all these haptens produced quenching of the protein fluorescence, and a red shift in the hapten absorption maximum. The capacity of protein 315 to discriminate between different aromatic ligands seemed low compared with that of, for example, induced anti Dnp antibodies. In particular it was noted that menadione and Dnp derivatives cross-reacted with protein 315 (Michaelides and Eisen, 1974). This was taken

as evidence in support of the concept of multispecificity. However, it was subsequently shown that this was also a property of whole antisera to Dnp (Johnston and Eisen, 1976). The thermodynamics of binding of Dnp and Tnp haptens to protein 315 has been studied by Sturtevant and coworkers (Johnston, Barisas and Sturtevant, 1974) by methods described earlier (6(d)), who concluded that the thermodynamics of hapten binding could be reproduced by L-tryptophan alone. This and the other data discussed above suggest that at least one tryptophan residue in protein 315 is present in combining site.

The kinetics of binding of Dnp derivatives to protein 315 have been studied by the temperature jump method (Pecht, Givol and Sela, 1972; Pecht, Haselkorn and Friedman, 1972; Haselkorn et al, 1974), who found that nitro groups in the 6, 2 and 4 positions are needed for high affinity, that a positively charged residue is located near the entrance to the site, and that the rate of association of haptens with the protein is too slow to be accounted for as a simple diffusion controlled reaction.

The overall dimensions of the combining site of protein 315 have been studied by several methods; ultracentrifugation (Wilder, Green and Schumaker, 1975), electron spin resonance (Piette et al, 1971; Hsia, Wong and Kalow, 1973; Hsia et al, 1973; Hsia and Little, 1973; Wong et al, 1974; Dwek et al, 1975a,b; Sutton et al, 1977), fluorescence polarization spectroscopy for a series Dnp-spacer-acridine (Pecht, 1974) and affinity chromatography (Rosenstein and Richards, 1976). The results of these studies indicate that the Dnp binding site of protein 315 is a pocket 9-12Å in depth and c 16x9Å at the mouth. In all these studies it is assumed that the Dnp group acts as an "anchor", i.e. the mode of binding of this portion of the hapten is not affected by the structure of the side chain, as the length of the spacer group is varied.

The binding haptens to the Fv, and fragments Fab and the 7s IgA of protein 315 has been studied by circular dichroism (Rockey et al, 1971;

Glaser and Singer, 1971; Inbar, Givol and Hochman, 1973; Freed, Rockey and Davis, 1976; Orin et al, 1976). The results of these studies have been interpreted as showing that polynitrophenyl derivatives form a stacked complex with at least one tryptophan residue in the combining site of protein 315. Further it was shown that both fragments and the intact IgA induce the same circular dichroism in the hapten spectrum, indicating that the combining site is unaltered by the presence of the constant regions. This latter conclusion is consistent with the small differences in affinity for Dnp haptens between the three species (Givol, 1974). It is also supported by the results of ^{19}F and ^1H nmr studies (Kooistra and Richards, 1978; Morris, Givol and Dwek, 1978).

Both affinity labelling (Goetzl and Metzger, 1970a,b; Hadler and Metzger, 1971; Weinstein, Wilchek and Givol, 1969; Haimovich, Givol and Eisen, 1970; Haimovich, Eisen and Givol, 1971, Givol et al, 1971; Haimovich et al 1972) and chemical modification (Klostergaard et al, 1977a,b; Klostergaard et al, 1978) studies have been applied to the investigation of the types of aminoacids and the regions of the sequence that are involved in the combining site of protein 315. The use of MNBDF (section 6(c)) has shown that a tyrosine at position 34_L (Table 4) is close to the bound hapten. This residue is also labelled by the bromoacetyl derivatives used by Givol and coworkers. Some of these affinity labels also react with a lysine residue at position 52_H, this labelling being most marked with α -N-Bromoacetyl- ϵ -N-Dnp-L-lysine (BADL) in which the bromoacetyl group is c 16Å from the Dnp group. Since the combining site is c 12Å deep this suggests that this residue is peripheral to the site. This is confirmed by the chemical modification studies by Klostergaard et al in which maleylation of this residue does not significantly reduce the affinity of the protein for ϵ -N-Dnp-L-lysine. A more direct role is suggested for an arginyl residue by a chemical modification data from these workers,

glyoxalation of one arginine residue abolishes the affinity for Dnp-aminocaproate which protect this residue (see Chapter 4). Independent evidence for the participation of a tyrosine residue was found from iodination studies by Klostergaard et al (1978). They showed that one tyrosine could be protected by ϵ -N-Dnp-L-lysine from iodination and that this is the same tyrosine which is labelled by N-bromoacetyl N'-Dnp ethylenediamine (BADE) (Givol et al, 1971), 34_L on the light chain. Both iodination and BADE labelling of this residue markedly reduce the affinity of protein 315 for haptens, whereas neither the amination by MNBDF (Goetzl and Metzger, 1970) nor nitration of this residue (Gavish, Jackson, Givol, Zakut and Dwek, unpublished data) affect the affinity of the protein significantly.

It has been suggested (Padlan et al, 1976) that a component of the total affinity of protein 315 for Dnp haptens is the formation of hydrogen bonds between 2 and 4 nitro groups of the hapten and amino acid side chains on the protein. From figure 9 it can be seen that residues Asn 36_L and Asn 36_H are proposed as filling this role. Three attempts have been made to search for the interactions of the nitro groups with the protein using resonance raman spectroscopy (Kumar et al, 1978), F^{19} n.m.r. on Dnp analogues with the trifluoromethyl group replacing the nitro groups (Kocistra and Richards, 1978) and a similar set of derivatives in which a chlorine atom was substituted for the nitro group (Gettins, Givol and Dwek, 1978). Whilst the interpretation of the data is not unequivocal, the results of these studies show that replacement of either nitro group with a group (CF_3 or Cl) not capable of forming a hydrogen bond leads to loss of c 1.5 Kcal/M of binding energy. (p.80)

Finally, both the light chain dimer (Schechter, Ziv and Licht, 1976; Licht et al, 1977) and the V_L dimer of protein 315 (Gavish, Givol and Dwek, 1978) have been prepared. It has been shown that both proteins retain significant affinity for Dnp ligands ($K 10^2-10^4 m^{-1}$ $\downarrow$), and that similar

spectroscopic changes (red shift and protein fluorescence quenching) are observed when Dnp ligands bind. It was inferred that most of the important contacts between the Dnp ring and the protein lay on the light chain. It is, however, difficult to work with the isolated heavy chain or the V_H fragment of protein 315, since these species aggregate (Givol, private communication). Thus it is not possible to show that the heavy chain of protein 315 does not bind Dnp derivatives.

The data presented in Chapters 2-5 of this thesis represent an attempt to continue the structural characterization of the combining site of protein 315. The model of Padlan et al (1976) is used as a basis for ^1H and ^{31}P n.m.r. studies which provide data that are applied to the determination of the mode of hapten binding; and to characterizing the involvement of particular amino acid residues in the hapten binding site.

Chapter 2

HIGH RESOLUTION ^1H N.M.R. STUDIES ON THE FV FRAGMENT OF PROTEIN 315 : THE BINDING OF 2,4 DINITROPHENYL DERIVATIVES

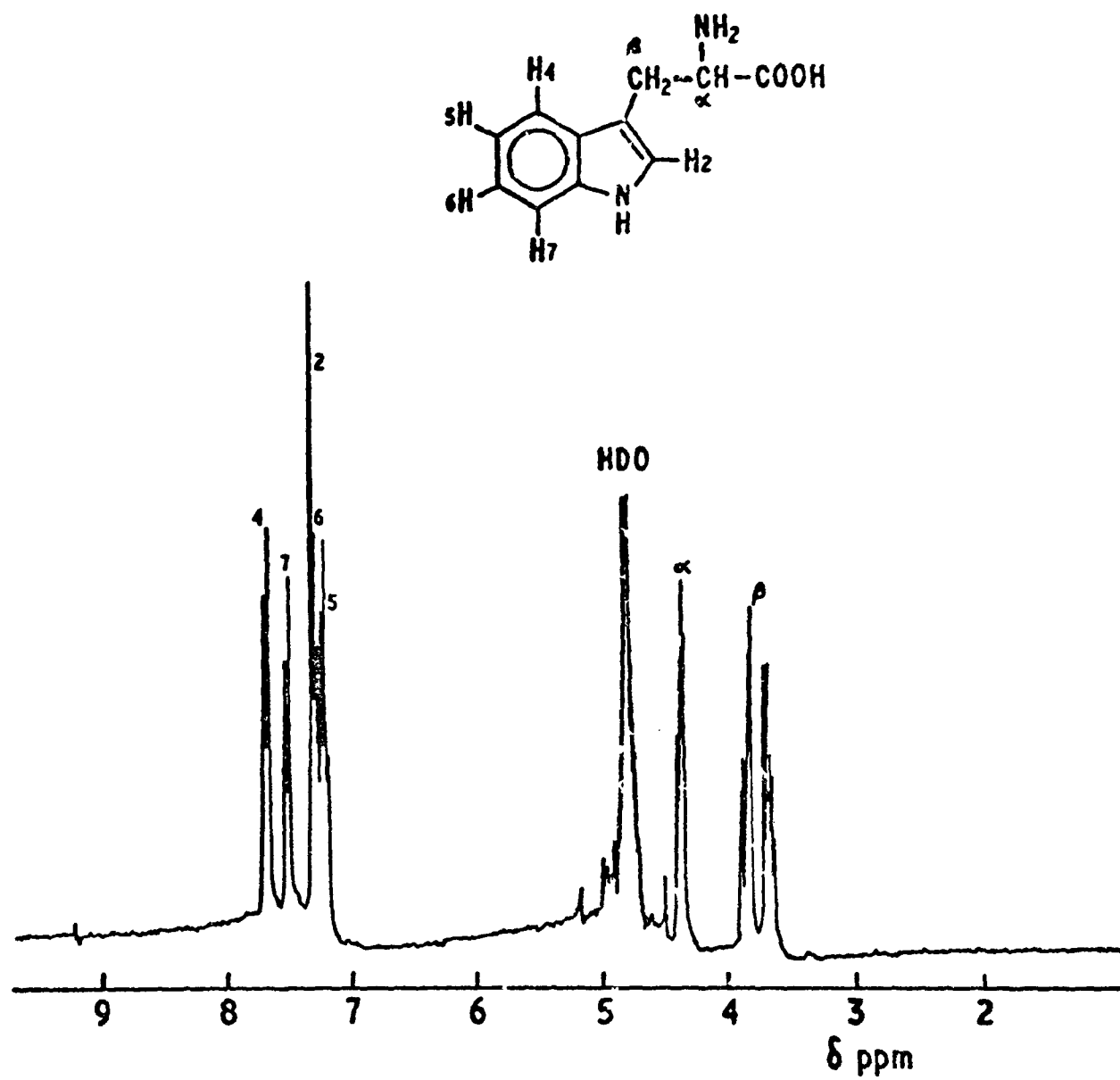
1. Introduction

This chapter describes the application of high resolution ^1H n.m.r. to the study of the interactions of Dnp derivatives with protein 315. This type of approach is an extension to the spectroscopic studies reviewed in sections 6(d) and 7. High resolution ^1H n.m.r. offers advantages over spectroscopic methods such as circular dichroism or fluorescence, in that the information content of the spectra is higher. Use of ^1H n.m.r. also permits the reciprocal effects of ligand on protein and protein on ligand to be monitored. High resolution ^1H n.m.r. is not a useful method for ab initio structure determination. It is best used in conjunction with a crystallographically determined structure. The predicted structure (Padlan et al, 1976) of the combining site protein 315 (see Chapter 1, section 6(e) and figure 9) is thus used as a close approximation to the true structure of this region of the protein in designing and interpreting the ^1H n.m.r. experiments. The data obtained from these experiments can then be used to provide criteria for testing how accurate this prediction is.

The information content of protein and small molecule ^1H n.m.r. spectra is essentially the same. The far greater complexity of the former presents the investigator with far greater difficulties in obtaining information from the spectra. The strategy for interpreting these spectra will, therefore, be briefly reviewed.

The ^1H n.m.r. spectrum is composed of resonances arising from individual ^1H nuclei in the sample. This is illustrated in Fig. 1 which shows the resolved and assigned spectrum of L-tryptophan. The resonance arising from a particular ^1H nucleus is characterized by five parameters. These

FIG. 1 : The 270 MHz ^1H n.m.r. spectrum of L- tryptophan



Spectrum recorded for 200 scans on 10 mM L-tryptophan, $\text{pH}^* 7.0$ in D_2O , with intersequence time of 20 seconds.

are:

- 1) frequency, described as the chemical shift in parts per million (ppm) of the spectrometer operating frequency;
- 2) intensity;
- 3) coupling constant(s), described in Hz, which cause the resonance to assume a multiplet appearance;
- 4) spin-spin relaxation time T_2 , which is determined directly from the resonance singlet linewidth, and,
- 5) spin-lattice relaxation time T_1 , which is determined from the rate at which a nucleus loses magnetization subsequent to radiofrequency excitation.

L-Tryptophan is one of the twenty one amino acids that commonly make up proteins. Thus, to a first approximation, the ^1H n.m.r. spectrum of a protein consists of a superposition of many spectra of the type shown in figure 1. However, changes in the chemical shifts, the resonance linewidths (T_2 's) and, to a less marked extent, the coupling constants, change the appearance of the protein n.m.r. spectrum so that it ceases to resemble this simple superposition (Dwek, 1973; Campbell, 1977). The changes in chemical shift arise from the creation of particular magnetic environments within the highly structured protein molecule. Secondary shifts caused by the protein tertiary structure can be determined by comparing the chemical shifts of the resolved and assigned resonances in the protein ^1H n.m.r. spectrum, with those for the same resonances when the protein is in a random coil conformation. Standard compilations are available for these latter chemical shift values (Sternlicht and Wilson, 1967; McDonald and Phillips, 1969; Roberts and Jardetsky, 1970; Bundi and Wüthrich, 1978).

Coupling constants between vicinal ^1H nuclei in aliphatic systems change as the average conformation about the C-C bond linking them is changed (Fischman et al, 1977). These thus may also change between the

random coil and globular conformations. The effects on the linewidths of resonances arise from two mechanisms in proteins containing no paramagnetic centre. The first of these is dipolar H^1-H^1 coupling, which, since it is correlation time dependent, is in turn dependent on the molecular weight of the protein. The greater the molecular weight of the protein the longer is the correlation time for ^1H nuclei in it, thus the T_2 's for these ^1H nuclei are shorter, and the linewidths are greater. The second mechanism is chemical exchange; if an ^1H nucleus explores different chemical environments at a rate comparable to the changes in chemical shift it experiences in passing between them, then the linewidth of the resonance from this ^1H nucleus will be increased. Thus the motions of amino acid residues within the anisotropic magnetic environments in the protein molecule can lead to chemical exchange resonance broadening of resonances in the protein spectrum (Wagner, De Marco and Wüthrich, 1976; Campbell et al, 1976). Since the non-exchangeable side chain ^1H resonances of proteins with no paramagnetic centre occur within a range of 10 ppm, the increase in linewidths produced by these mechanisms causes extensive overlap of resonances in the spectrum leading to problems of resolution. Further, since most of the ^1H - ^1H coupling constants in amino acid side chains are less than 10 Hz, as the linewidths approach this value problems in determining coupling constants will lead to problems of assignment. The same considerations with regard to chemical shifts, linewidths, and coupling constants, apply to the changes in the ^1H n.m.r. spectrum of a ligand when it binds to a protein.

Since it is these effects which result from the particular three dimensional structure of the protein, and the motions within that structure, it is from the secondary shifts and linewidth changes that information about features of the protein structure may be obtained. A strategy for studying proteins using ^1H n.m.r. can thus be outlined.

1) The ^1H n.m.r. spectrum of the protein is broken down into the subspectra arising from particular types of amino acid side chain. This is termed first stage assignment and is performed using the coupling patterns of amino acid side chains.

2) The several subspectra assigned to a particular type of amino acid residue are each assorted to each particular residue in the sequence, e.g. each of three phenylalanines in horse cytochrome c (Cookson et al, 1978). This is termed second stage assignment.

If a particular resonance in the ^1H n.m.r. spectrum of a protein can be assigned to a particular ^1H nucleus in the molecule, then the secondary shift and linewidth, and the responses of these to perturbations, such as ligand binding, in the protein environment give information about the protein structure around that nucleus and its responses to perturbations. Similarly the changes in chemical shift, relaxation behaviour and coupling constants that a ligand experiences when it binds to a protein can give information about the binding process and the bound environment (Dwek, 1973).

2. Materials and Methods

a) Preparation of the Fv fragment of protein 315

The Fv fragment of protein 315 was prepared from the 7s IgA monomer extracted from the ascites fluid of tumor bearing F_1 BALB/c/DBA mice, by a modification of the method of Inbar et al (Inbar, Hochman and Givol, 1972). The ascites fluid was fractionated, reduced and alkylated, as described by Inbar et al (1972). The reduced and alkylated 7s IgA was purified by absorption to an ϵ -N-Dnp-L-lysine-sepharose column. Two alternative methods were used to elute the 7s IgA:

i) The IgA was eluted with 0.1M ammonia. As fractions were collected the pH was rapidly adjusted to 8.2 using solid ammonium bicarbonate, fractions with an optical density at 280 n.m. > 0.05 were pooled and dialyzed over night against 10 litres of 0.05 M sodium acetate pH 4.7, 0.15 M sodium chloride.

ii) The IgA was eluted from the ϵ -N-Dnp-L-lysine-sepharose column using a solution of 0.05 M Dnp-glycine pH 7.0. Fractions with an $\epsilon_{360} > 0.1$ were pooled and passed down a Dowex (1-X8; 200-400 mesh; Sigma Chemical Company, Poole, Dorset) column to remove the Dnp-glycine. Fractions with an optical density at 280 n.m. > 0.05 were pooled and dialyzed against acetate buffer.

In both cases the protein was subsequently concentrated to 10mg/ml, and digested with pepsin at 37°C, at an enzyme:substrate ratio of 1:50, for three hours. The pH was adjusted to 3.6 with 1M acetic acid, and digestion was continued at 37° for three hours. The pH of the solution was adjusted to 7.2 with 2M Tris and the solution was dialysed against 10 litres of 0.05M Tris buffer pH 7.4, 0.15M sodium chloride overnight. After dialysis the solution was centrifuged at 5000 rpm for 30 min in an MSE 18 centrifuge. The volume was reduced to 10ml by ultrafiltration. The sample was applied to a 3x80cm G-75 column and eluted with pH 7.2 Tris buffer at a flow rate of 50ml/hr, 10ml fractions were collected. The Fv fragment eluted at 200mls. The G-75 column revealed the presence of soluble aggregates of IgA present in the ammonia eluted protein preparation, there was little of this material in the Dnp-glycine eluted preparation. The fractions containing the Fv fragment were pooled, concentrated to 10-20mg/ml. dialysed against 10 $^1\text{H}_2\text{O}$ overnight and against 4x100ml of $^2\text{H}_2\text{O}$ for 2 hours each. The $^2\text{H}_2\text{O}$ solution of Fv fragment was lyophilized for 24 hrs and stored at -20°C. The purity of the material was checked by SDS gel electrophoresis (1% sodium dodecylsulphate, 10%

acrylamide) using reduced and alkylated protein. The activity was checked by protein fluorescence quenching titration with ϵ -N-Dnp-L-lysine.

The yields obtained with the Dnp-glycine method were 2-3mg of Fv per ml of ascites fluid (8-10 mg of 7s IgA). The yields obtained with the ammonia elution method, which has the advantage of being rapid, were lower by (10-40%) and more variable, due to loss of IgA as aggregated material. It is curious that the Fv fragment prepared by the ammonia method crystallizes while the Fv fragment prepared by the Dnp glycine method does not (A. Aschaffenberg, private communication). The activity and polyacrylamide gel mobility of these two preparations are the same.

b) Preparation of haptens

ϵ -N-Dnp-L-lysine and N Dnp-L-Aspartate were purchased from the Sigma Chemical Company. N-Dnp-glycine and 2,4 dinitrobenzene were purchased from B.D.H. Chemicals, Poole, Dorset. 2,4 dinitrophenol was purchased from Hopkins and Williams, Romford, Essex. L-Tryptophan and L-tryptophan methyl ester were purchased from Fisons, Loughborough, Leics. N-Dnp-2-aminoethylphosphate was prepared as described in Chapter 3. N-Dnp-amino methylphosphonate was prepared as described in Chapter 4.

c) Preparation of n.m.r. samples

Samples for ^1H n.m.r. spectroscopy were prepared by dissolving the lyophilised protein in $^2\text{H}_2\text{O}$ containing 0.15 M sodium chloride. The concentration of protein was determined by measuring the optical density at 280 nm (1mg/ml solution = 1.5 at 280 nm). Solutions of Dnp-L-aspartate, Dnp-glycine and L-tryptophan were prepared by dissolving the solids in $^2\text{H}_2\text{O}$, the concentrations were checked by optical density: solid sodium chloride was added to a concentration of 0.15 M. The pH* of the protein solutions was either determined as the uncorrected meter reading using a radiometer glass electrode, or by measuring the chemical shifts (relative

to DSS, see below) of the histidine C2-resonances (see Chapter 4). The pH* of other solutions was measured with the glass electrode..

d) 270 MHz ^1H n.m.r. spectroscopy

270 MHz ^1H n.m.r. spectra were recorded using a Bruker spectrometer modified in this laboratory, and an Oxford Instruments superconducting magnet. Data were collected in the fourier transform mode, using a Bruker BNC-12 computer. The solvent ^2H resonance was used to obtain an internal field frequency lock. Free induction decays were accumulated over 2x2K data points, using quadrature detection, giving an acquisition time of 0.513 secs, for a 4000 Hz sweep width (interpoint time 125 μ sec). Spectra were obtained from a free induction decay accumulated for 2000 scans, with a repetition rate of 0.55 secs and a 70° pulse angle. All spectra were deconvoluted, to enhance signal to noise using an exponential line-broadening function. This adds 2.5 Hz to the linewidth (timeconstant = -4 sec, acquisition time 0.513 sec) of resonances.

Chemical shifts are reported as parts per million (ppm) from the methyl resonance of DSS (2,2 dimethylsilapentane sulphonate (sodium salt)), used as an external standard as a saturated solution in $^2\text{H}_2\text{O}$.

All measurements were performed at a probe temperature of 303 °K, maintained by an air flow temperature controller. An air flow was also used to spin the sample.

e) Fluorescence measurements

The binding of haptens to the Fv fragment of protein 315 was measured by the method of quenching of the protein fluorescence (Eisen et al, 1970). Data were collected on a Perkin-Elmer/Hitachi MPF/2A spectrofluorimeter, with a 298°K thermostatted cell compartment. Protein fluorescence was excited at 280 nm and observed at 340 nm. The correction for trivial attenuation of incident and emitted light was made using a blank containing

L-tryptophan methyl ester of a concentration which gave a similar fluorescence intensity to that of the protein solution. All protein fluorescence titrations were performed in triplicate. Reported values of hapten dissociation constants (K_D) were obtained from titrations in which the protein concentration was adjusted to approximately K_D . All solutions were contained 0.05 M pipes (piperazine- NN' -bis-(2-ethanesulphonic acid)) and 0.15 M sodium chloride, pH was adjusted to 5.0-7.0 using 5 M NaOH and determined using a glass electrode.

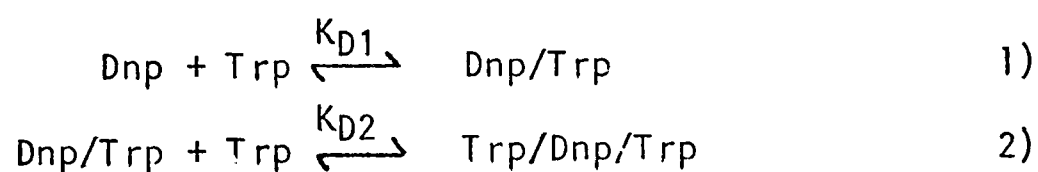
Data were fitted to theoretical curves using a simple FORTRAN program BIND written in this laboratory.

f) Optical difference spectroscopy

Absorption difference spectra were recorded on a Carey 17 recording spectrometer, with Hellma QS tandem cells.

g) Analysis of binding data

i) Model compound studies: The binding of N-Dnp-L-aspartate (Dnp) to L-tryptophan (Trp) in H_2O cannot be analyzed as formation of a simple binary complex, but is consistent with the following model:-



Self association of N-Dnp-L-aspartate is sufficiently weak ($K_D < 1 \text{ M}$) for these equilibria to be neglected, under the conditions of the experiments (see section 3 (c)).

ii) The binding of hapten to tryptophan results in changes of chemical shift, $\Delta\delta_{\text{obs.}}$, of the hapten and tryptophan resonances. Under conditions of fast exchange:

$$\Delta\delta_{\text{obs.}} = \bar{X}_{\text{bin.}} \Delta\delta_{\text{bin.}} + \bar{X}_{\text{ter.}} \Delta\delta_{\text{ter.}} \quad 3)$$

where $\bar{X}_{\text{bin.}}$ and $\bar{X}_{\text{ter.}}$ are the fractions of hapten or tryptophan bound in the binary and ternary complexes respectively, and $\Delta\delta_{\text{bin.}}$ and $\Delta\delta_{\text{ter.}}$ are the corresponding fully bound shifts. A simple iterative procedure was used to calculate theoretical curves for a range of values of K_D and fully bound shifts. These curves were then compared with the experimental data.

Antibody-binding studies. Hapten binding to antibody is sufficiently tight to satisfy the relationship $[\text{hapten}], [\text{protein}] \gg K_D$. Hence $[\text{hapten}]_{\text{bound}} = [\text{protein}]_{\text{total}}$ when $[\text{hapten}]_{\text{total}} > [\text{protein}]_{\text{total}}$. Thus:

$$\frac{\Delta\delta_{\text{obs.}}}{\Delta\delta} = \frac{[\text{protein}]_{\text{total}}}{[\text{hapten}]_{\text{total}}}$$

and a plot of $\Delta\delta_{\text{obs.}}$ against $1/[\text{hapten}]_{\text{total}}$ will yield the fully bound hapten chemical shift.

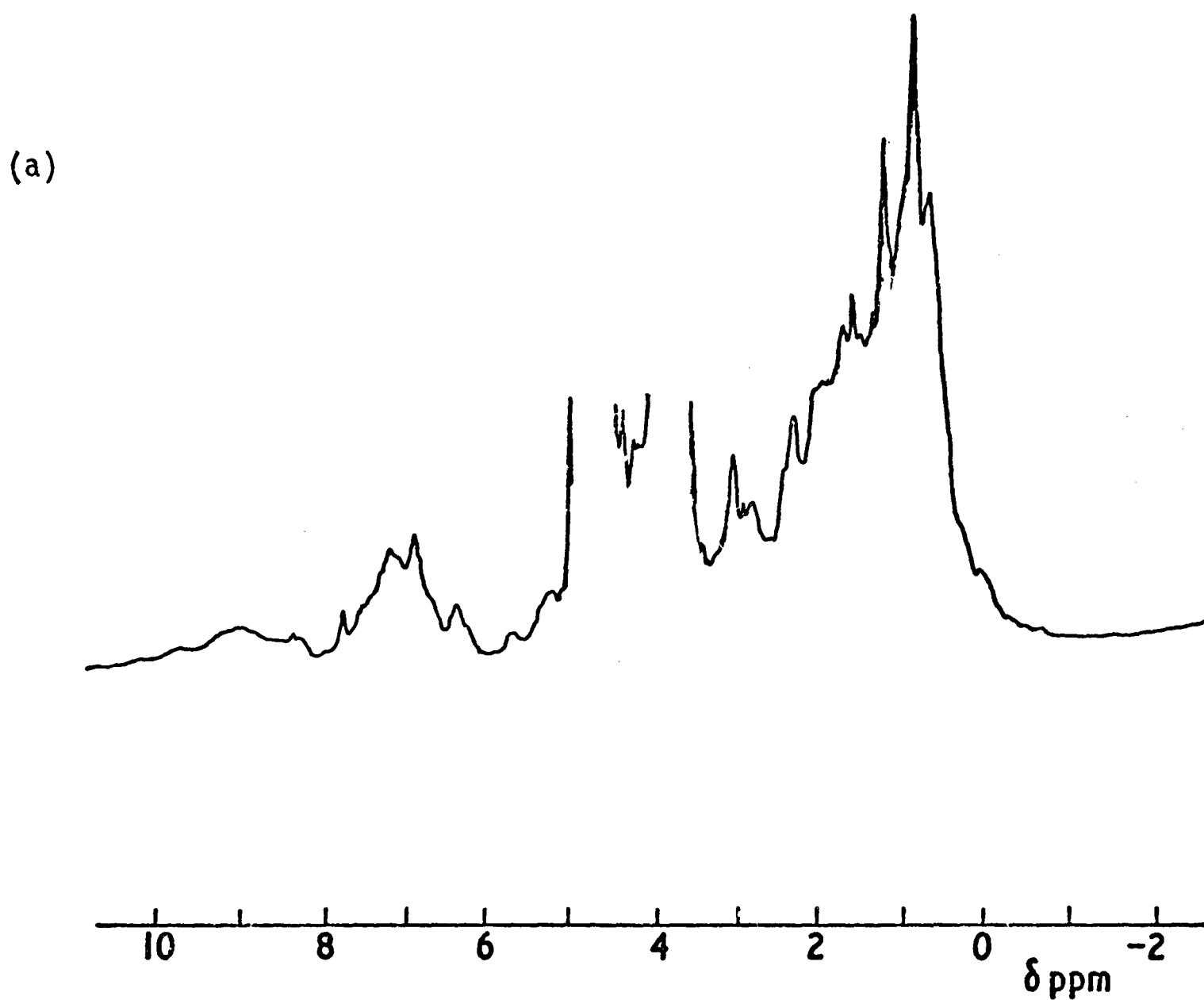
h) Lineshape analysis

This was carried out as described in Appendix II.

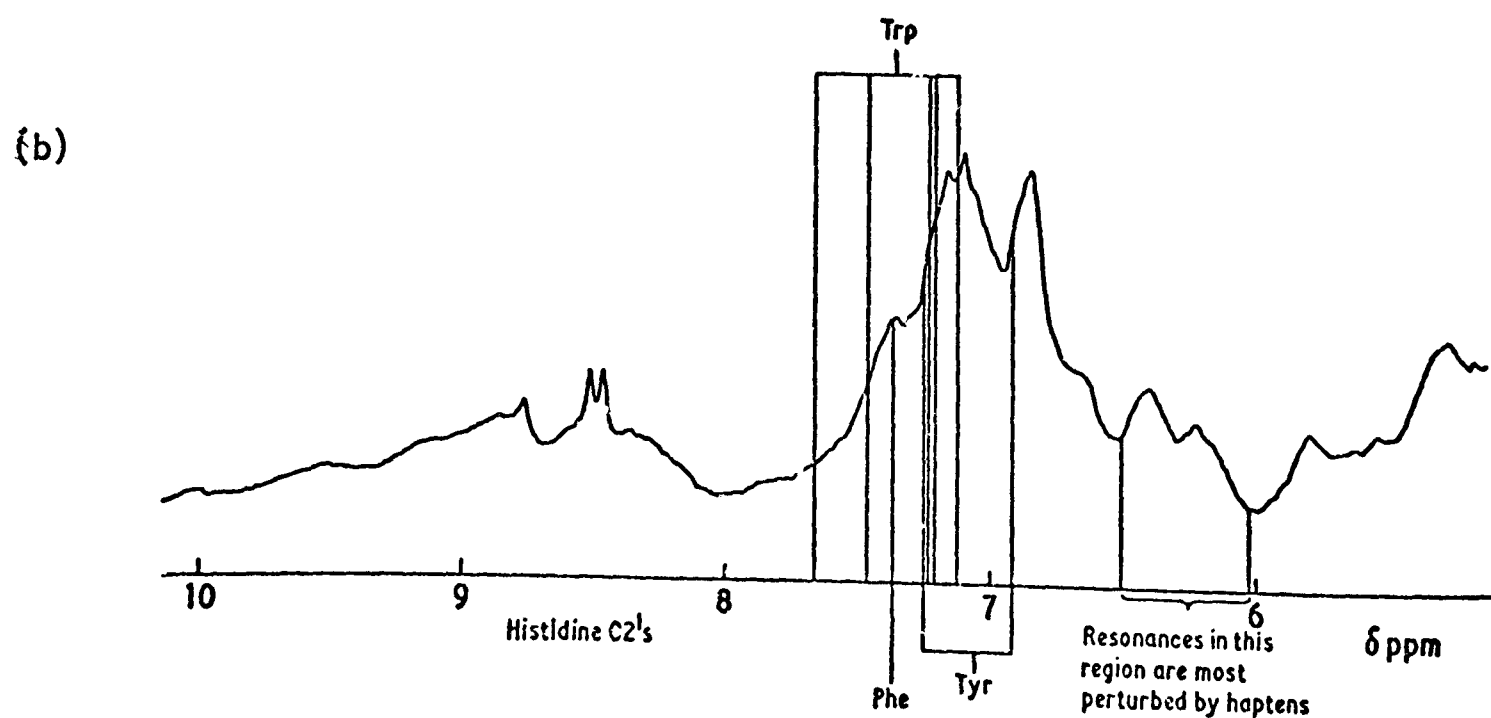
i) Ring current calculations

These were carried out using the Johnson-Bovey (1968) equation as discussed in Appendix I.

FIG. 2 : The 270 MHz ^1H n.m.r. spectrum of the Fv fragment of protein 315.



a) 2mM Fv fragment pH* 8.07, 0.15 M NaCl:other conditions as in internals and methods section.



Aromatic amino acid region of 270 MHz n.m.r. spectrum of the Fv fragment, pH 5.0 other conditions as in a).

3) Results

a) The ^1H n.m.r. spectrum of the Fv fragment of protein 315

Figure 2(a) shows the 270 MHz ^1H n.m.r. spectrum of the Fv fragment of protein 315. Little detail can be seen in this spectrum, since it is poorly resolved. The Fv fragment has a molecular weight of 25,250 (Inbar, Hochman and Givol, 1972). Since the protein can be expected to be expected to be approximately globular ($40 \times 40 \times 50 \text{\AA}$) on the basis of structural homology between antibody molecules (Chapter 1, sections 5 and 6(e)), the value for the rotational correlation time ($7 \times 10^{-9} \text{sec}$) calculated from the Stokes-Einstein equation:

$$\tau_R = \frac{M\bar{V}}{RT} \eta \quad 5)$$

(M - molecular mass, $\bar{V}$ - partial specific volume, η - bulk viscosity) which treats the molecule as a rigid sphere of uniform density, should be a good estimate for the correlation time of the protein. This agrees approximately with the value calculated from Gd(III) proton relaxation enhancement data of $4.2 \times 10^{-9} \text{sec}$ (Burton et al, 1977). The expected linewidth for a resonance arising from an ^1H nucleus which is $2.5 \text{\AA}$ from two nearest neighbour ^1H nuclei in a system with a correlation time of this order, should be 6-7 Hz (Dwek, 1973). One of the three histidine H2 resonances, that assigned to His 97_L, has a linewidth which is considerably larger than this (c 30 Hz) at pH*'s far from the pK of the histidine residue, indicative of some anisotropic motion of this residue relative to the protein environment.

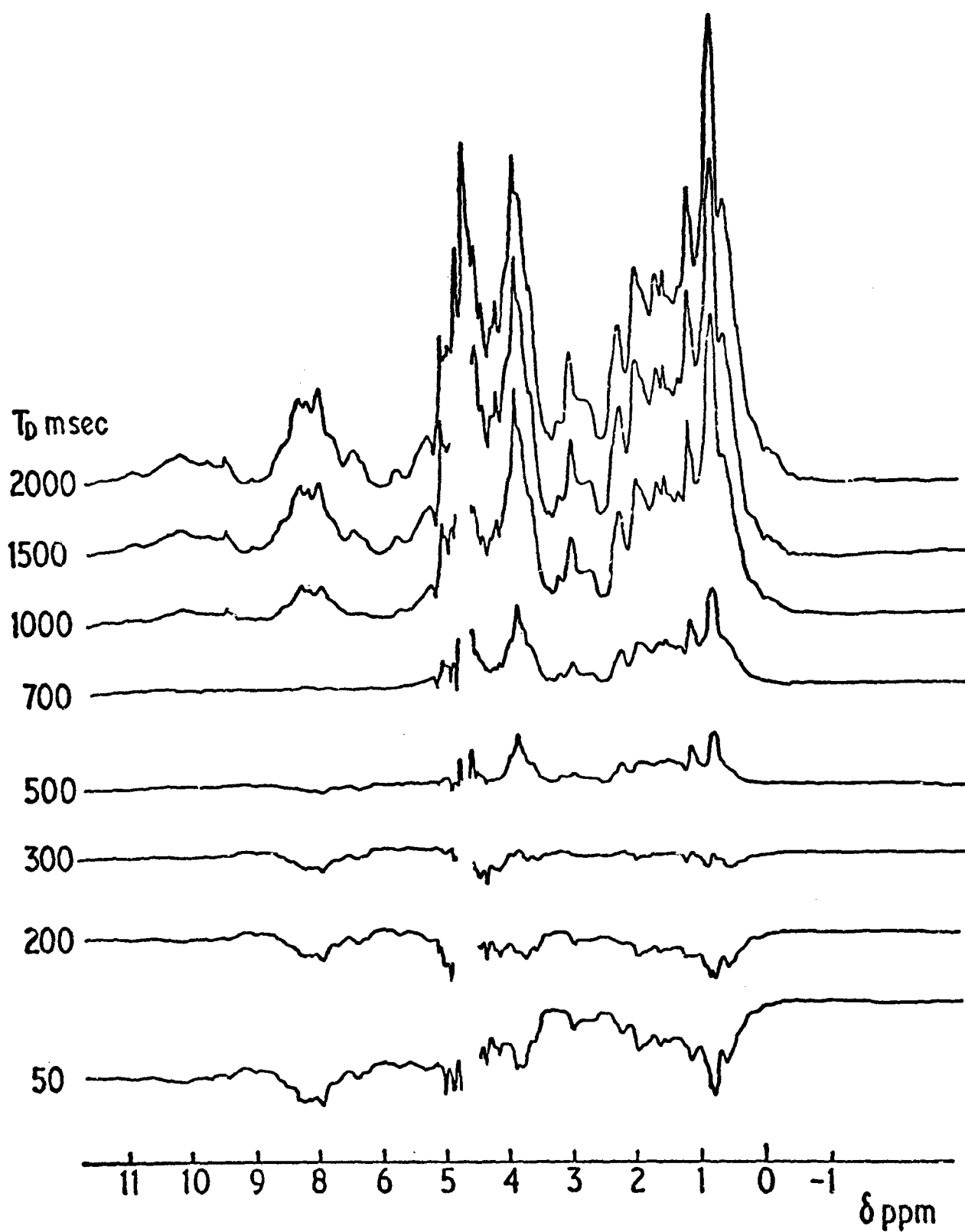
The protein spectrum contains several resonances in the region 6-6.5 ppm (Fig. 2(b)). These are unusually high field chemical shifts for aromatic amino acid resonances side chain (normal range 6.5-8 ppm), and far too low field chemical shifts for aliphatic amino acid side chain resonances (normal range 4.5-1 ppm). This suggests that the resonances in this

TABLE 1 : Amino Acid Composition of the Fv Fragment of Protein 315

<u>Aromatic amino acids</u>	<u>number¹</u>	<u>number of ¹H nuclei²</u>
Tyr	10	40
Trp	6	30
Phe	12	60
His	3	6
		136
<u>Aliphatic amino acids</u>		
Ala	12	36
Arg	7	42
Asn	10	20
Asp	12	24
Cys	4	8
Gln	7	28
Glu	10	40
Gly	26	--
Ile	9	81
Leu	19	171
Lys	8	64
Met	1	7
Pro	9	54
Ser	20	40
Thr	26	104
Val	15	105
β CH ₂ groups of aromatic residues		824
-	31	62
<u>α CH groups</u>		
-	--	226

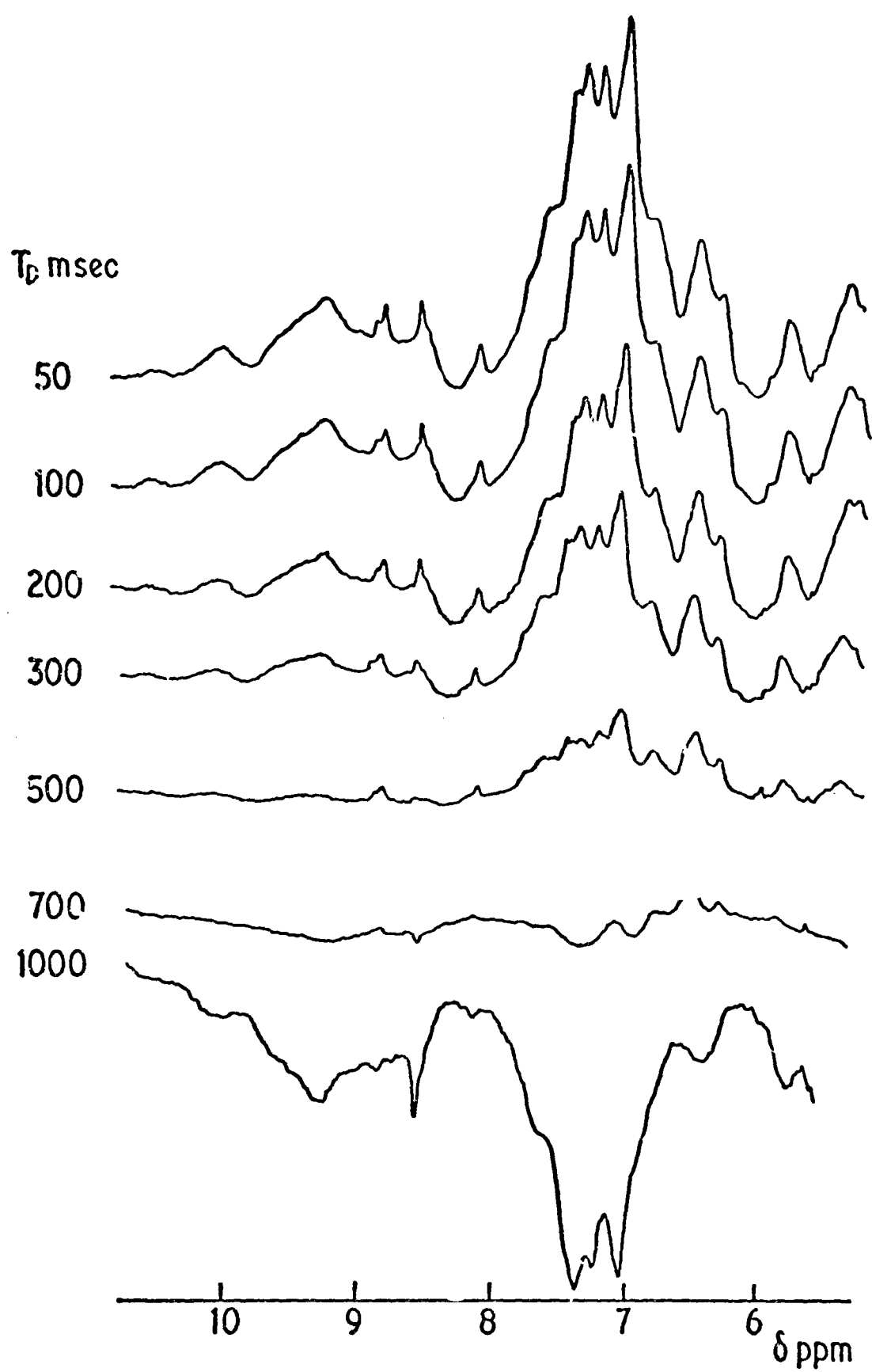
- 1) These numbers are based on the sequence data given in Ch. 1, Table 4.
2) Only non-exchangeable ¹H are given.

FIG. 3(a) : T_1 sequence on the Fv fragment of protein 315



1.39 mM, Fv pH* 6.9, 0.15M NaCl $T = 303^\circ \text{K}$ spectrum recorded with a $\Pi - \tau - \Pi/2$ pulse sequence with intersequence time of 4 seconds. Delay times are shown in Fig.

3(b)



Aromatic amino acid region of 3(a) shown expanded x4 vertically and x2 horizontally.

region of the protein arise from aromatic amino acid side chain protons which are in unusual environments in the protein. From the amino acid composition of the protein and the spin-lattice relaxation times for resonances in this region (see below) there are 15-20 ^1H resonances in this region of the spectrum (Table I).

b) Spin-lattice relaxation time measurements of resonances in the Fv fragment spectrum

The spin lattice relaxation times were measured by the inversion-recovery pulse sequence $\Pi - T_D - \Pi/2$. The behaviour of nuclear magnetization is described by the Bloch equations:-

$$\frac{dM_z}{dt} = \frac{dI}{dt} = \frac{I_\infty - I}{T_1} \quad 6)$$

when I is intensity. Integration of this between $t=0$ $I = -I_\infty$ and $t = T_D$ $I = I_D$ gives:-

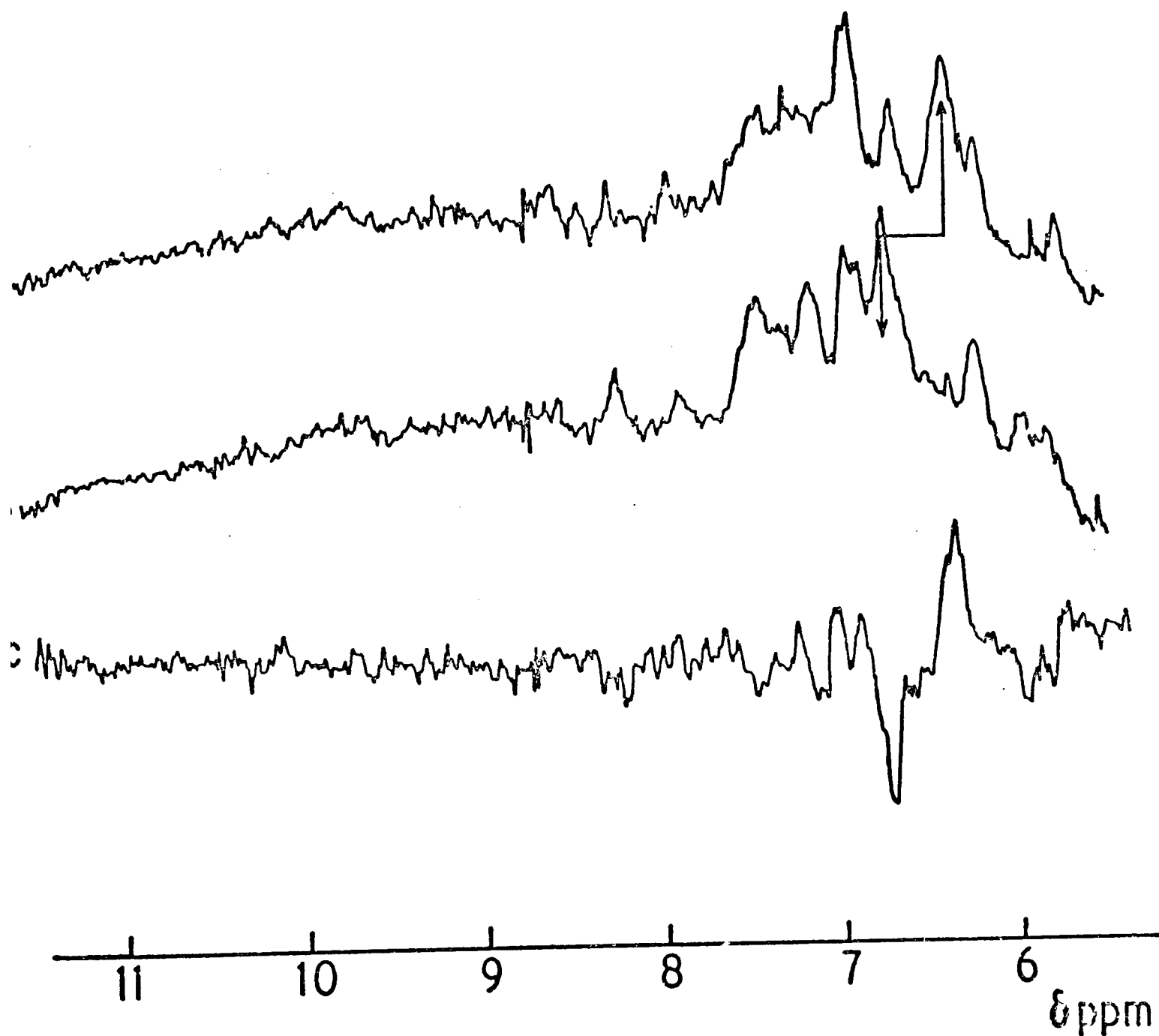
$$I_D = I_0 (1 - 2 \exp(-T_D/T_1)) \quad 7)$$

This describes the behaviour of the resonance intensity as a function of the delay time T_D . The application of this to the Fv fragment of protein 315 is shown in Fig. 3. It can be seen that across the entire range from 4.5-0 ppm the intensity reaches 0 at $T_D = 300$ msec. Since from 7):-

$$T_1 = T_D^0 / \log_e 2 \quad 8)$$

when T_D^0 is the delay time at which a resonance reaches a null. It follows that the T_1 values for aliphatic acid ^1H nuclei are distributed about a mean of 450 msec. It is not possible to explore the distribution more precisely as the poor resolution, and thus extensive overlap in the spectrum, prevents observation of single resonances. Similarly an approximate mean value for the T_1 's of the aromatic amino acid ^1H nuclei is

IG. 4 : T_1 difference spectrum of the Fv-fragment with N-Dnp-L-aspartate



A - 2 mM Fv fragment pH* 6.8, 0.15 M NaCl T = 303° K, delay time = 500 msec, intersequence time 4 seconds.

B - 2 mM Fv fragment pH* 6.8 with 2 mM N-Dnp-L-aspartate, recorded under the same conditions as A.

C - is spectrum A - spectrum B

800-900 msec. Those resonances in the range 6.5-6 ppm have an average T_1 of the order of 1300 msec. All these values are shorter than the value for T_1 calculated from the equation:-

$$\frac{1}{T_1} = \frac{3}{10} \frac{\gamma^4 \hbar^2}{6r^6} [J(\omega) + 4J(2\omega)] \quad 9)$$

(Dwek, 1973) using two protons at a distance of 2.5Å and a correlation time of 7×10^{-9} sec, which is 6.7 sec. This shows that relaxation mechanisms other than isotropic dipolar coupling are operating in this system (Coates et al, 1973; Kalk and Berendsen, 1976).

The estimated T_1 values can be used to determine to what extent resonances in the F_v fragment spectrum are saturated under conditions of normal data collection (section 2(d)). Using the treatment of Freeman, Hill and Kaptein (1972) it can be shown that with a 70° pulse angle and a pulse repetition rate of 0.55 sec the relative intensities of resonances with T_1 values of 450, 800 and 1300 msec will be, 100%, 70% and 50%. Thus caution must be exercised in interpreting intensities in the normal ^1H n.m.r. spectra of the F_v fragment.

Finally, the use of partially relaxed spectra offers the possibility of spectral simplification. This is shown in Fig. 4. This shows the effect on the aromatic region of a $T_D = 500$ msec partially relaxed F_v fragment spectrum of adding equimolar N-Dnp-L-aspartate. It can be seen that the only significant change in the spectrum is that a resonance at 6.32 ppm shifts downfield by 0.37 ppm. This could be due to a marked effect on the T_1 's of two different resonances, but (Table 3) this corresponds to the unperturbed chemical shift and change in chemical shift of a resonance that is affected by hapten binding in the normal ^1H n.m.r. spectrum of the F_v fragment. The aliphatic (-1 - 4.5 ppm) region of the difference spectrum is not shown in Fig. 4, since no effects are seen here. The partially relaxed spectra also shows that no marked effects on the T_1 's of any ^1H nuclei in the protein are caused by the bound ligand.

FIG. 5 : Difference spectra of the Fv fragment with several haptens

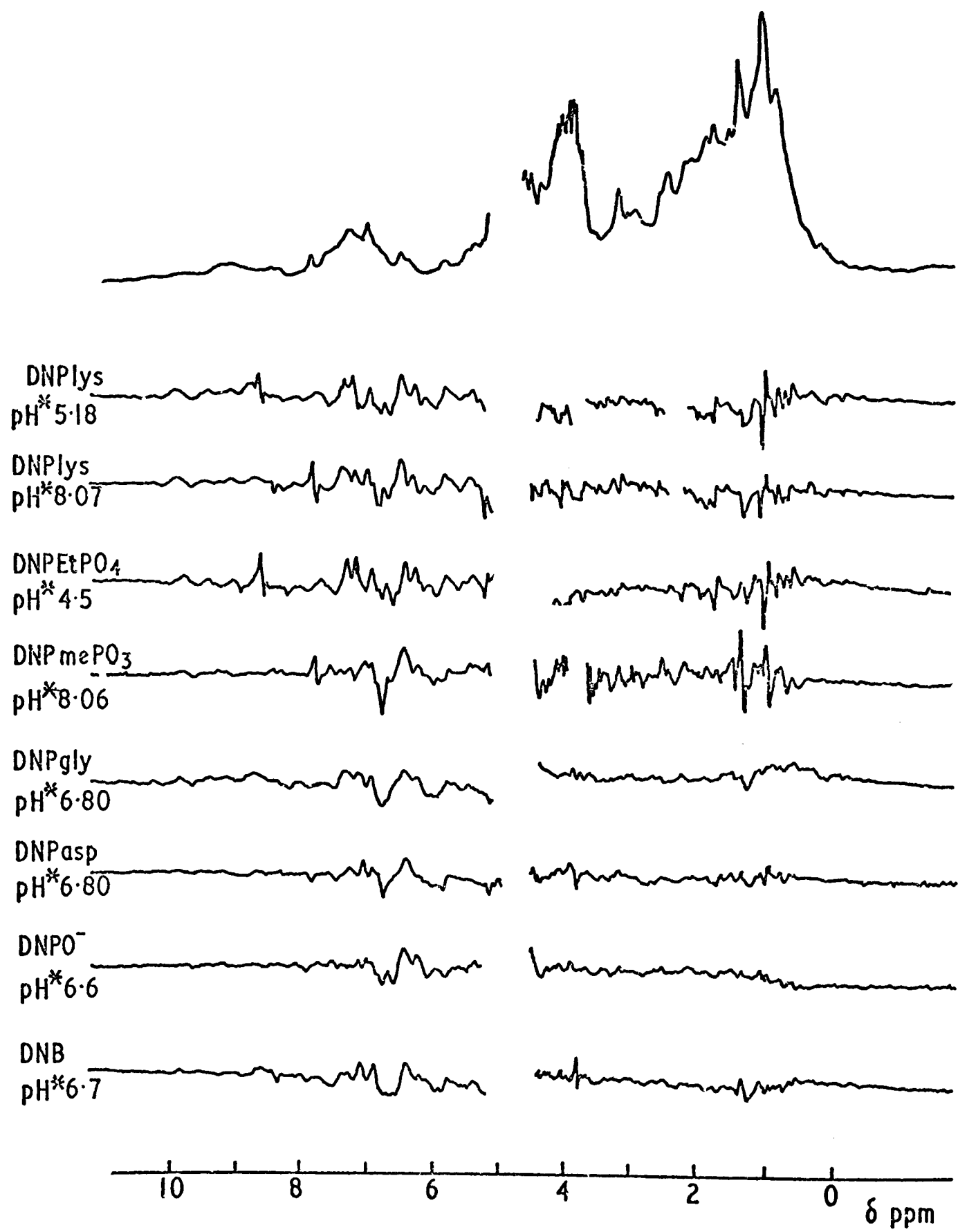


Fig. 5.

All difference spectra 2x the Fv fragment spectrum in vertical scale, and were generated by subtracting the spectrum of the protein-hapten complex from that of the protein alone. In all cases the Fv fragment concentration was 1-2 mM and the samples with hapten contained equimolar hapten. Other conditions as given in the methods section. The spectra were scaled with respect to one another by subtracting the Fv alone spectrum of each pair. The data for 2,4 dinitrophenol and 2,4 dinitrobenzene were collected by S. Wain-Hobson.

c) The effect of hapten binding on the ^1H n.m.r. spectrum of the Fv fragment: Difference spectroscopy

In order to be able to determine the chemical shift changes that occur when a ligand binds to a protein, it is necessary for the rate of ligand dissociation from the protein to be sufficiently large to produce conditions of fast exchange for chemical shifts (see below). If this is not the case then it is only possible to observe the initial and final chemical shift positions of perturbed resonances. Further one cannot determine which of the resonances in the protein hapten complex arise from the ^1H nuclei of the bound ligand. Such difference spectra give information of a qualitative nature similar to that given by, for example, circular dichroism (Chapter 1 section 6(d)), in that the modes of interaction of different ligands can be compared. The major advantage of the ^1H n.m.r. method, however, is that the extent of the spectral perturbation can be quantified in terms of the number of ^1H nuclei perturbed. Shown in Figure 5 are the 270 MHz ^1H n.m.r. difference spectra generated by subtracting the spectrum of an Fv fragment-hapten complex from that of the Fv fragment. All seven haptens are Dnp derivatives of the form Dnp-NHR. The spectra are arranged in order of binding affinity from $\epsilon\text{-N-Dnp-L-lysine}$ (highest) to 2,4 dinitrobenzene (lowest). This correlates approximately with the size of the substituent on the DnpNH group, in that as this increases so does the binding affinity (see Chapter 4).

The pattern of chemical shift changes produced in the aromatic (6-8 ppm) region of the protein spectrum appears to be similar in all seven cases, apparently consisting of a small diminution in the intensity of the spectrum in the range 6.8-7.5 ppm and changes in chemical shifts of resonances in the region 6-6.6 ppm. The diminution in intensity is due to a decrease in the T_2 's of some ^1H nuclei in the protein when hapten binds. The chemical shift changes are due to a perturbation of the environment of some protein ^1H nuclei when the hapten binds. There are also differences in the aliphatic (0-4.5 ppm) region of the protein spectrum, these vary more widely between

FIG. 6 : A comparison of the effect of ligands on the 270 MHz ^1H n.m.r. spectra of the Fv fragment and of Lysozyme

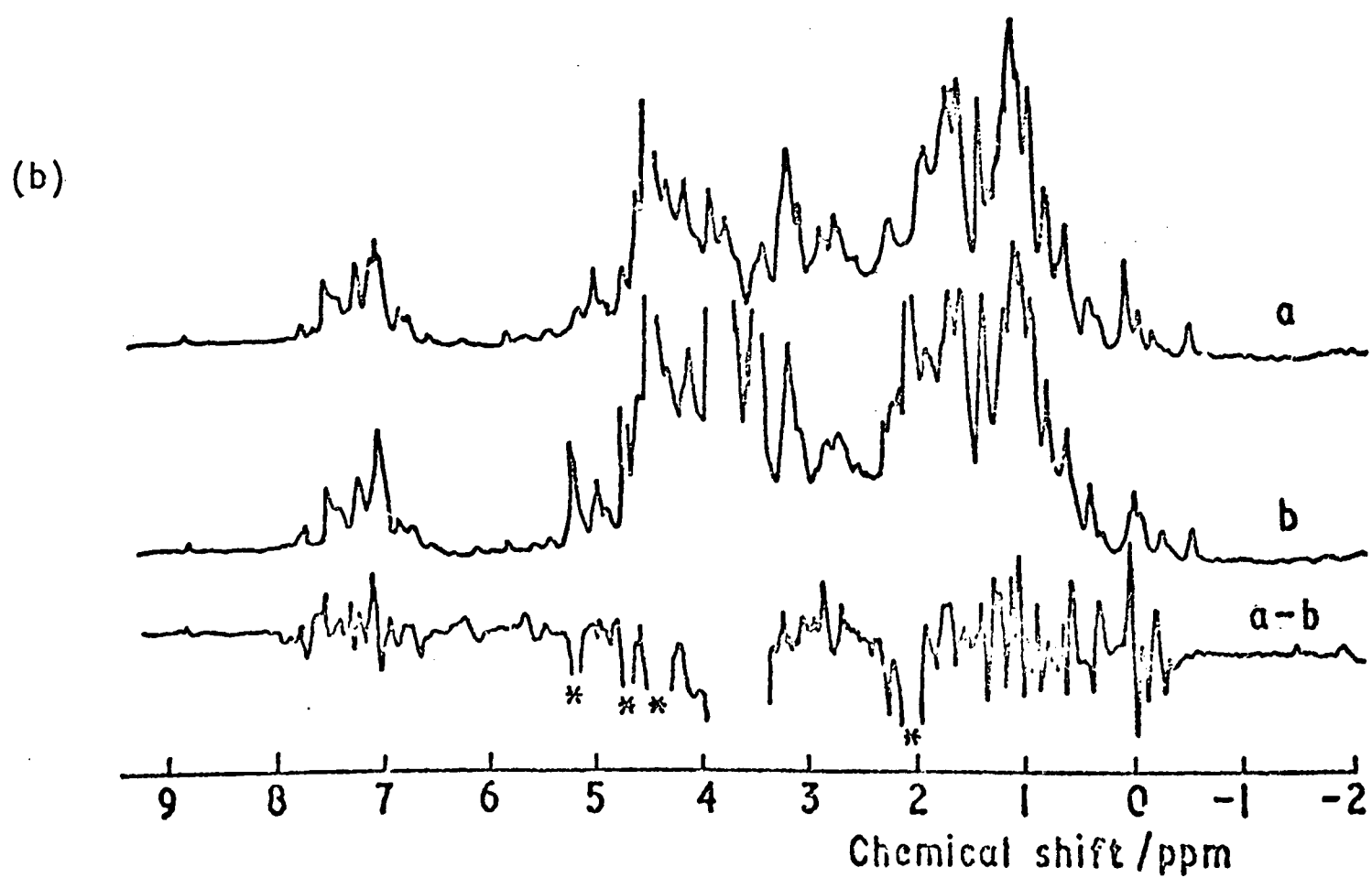
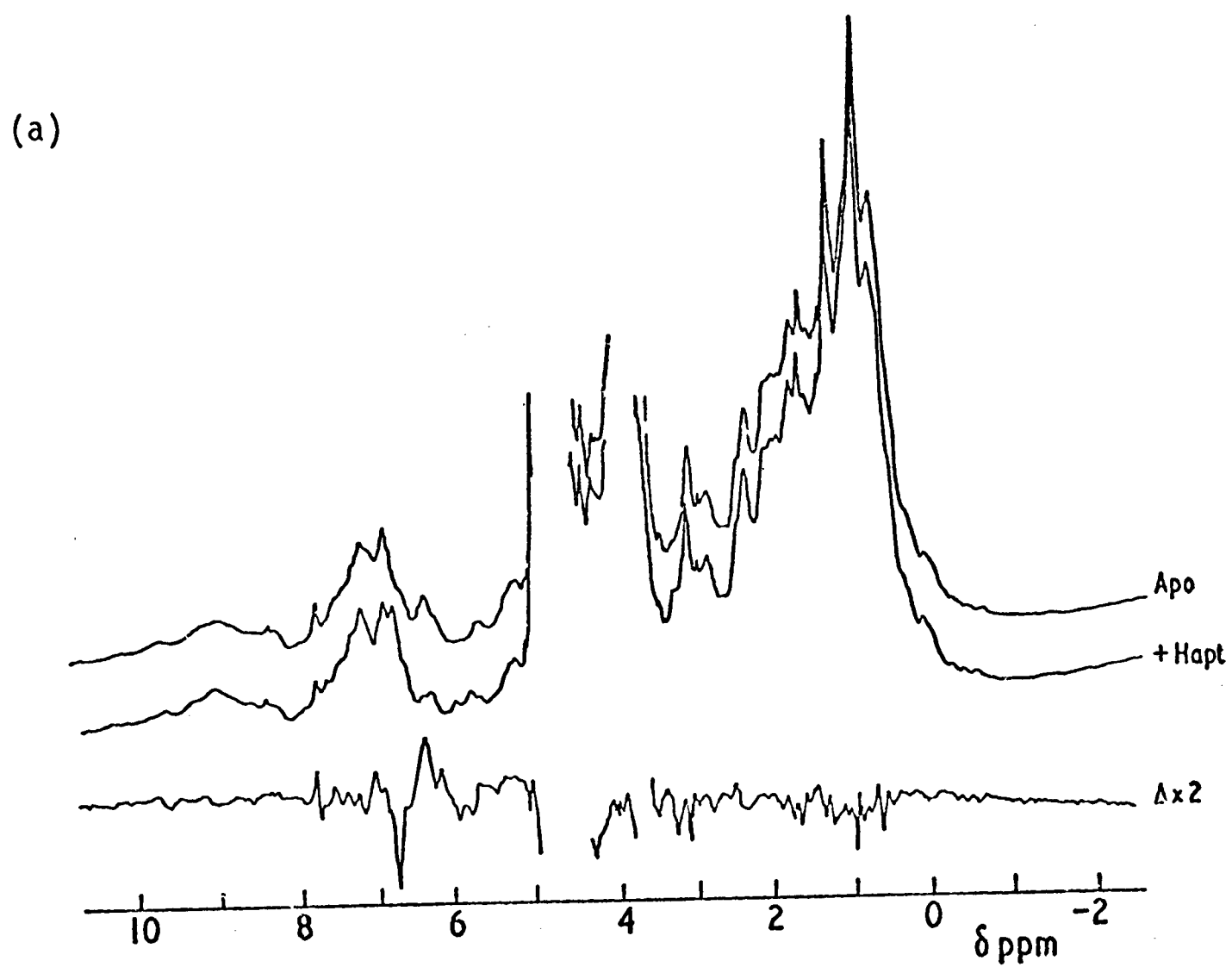


Fig. 6.

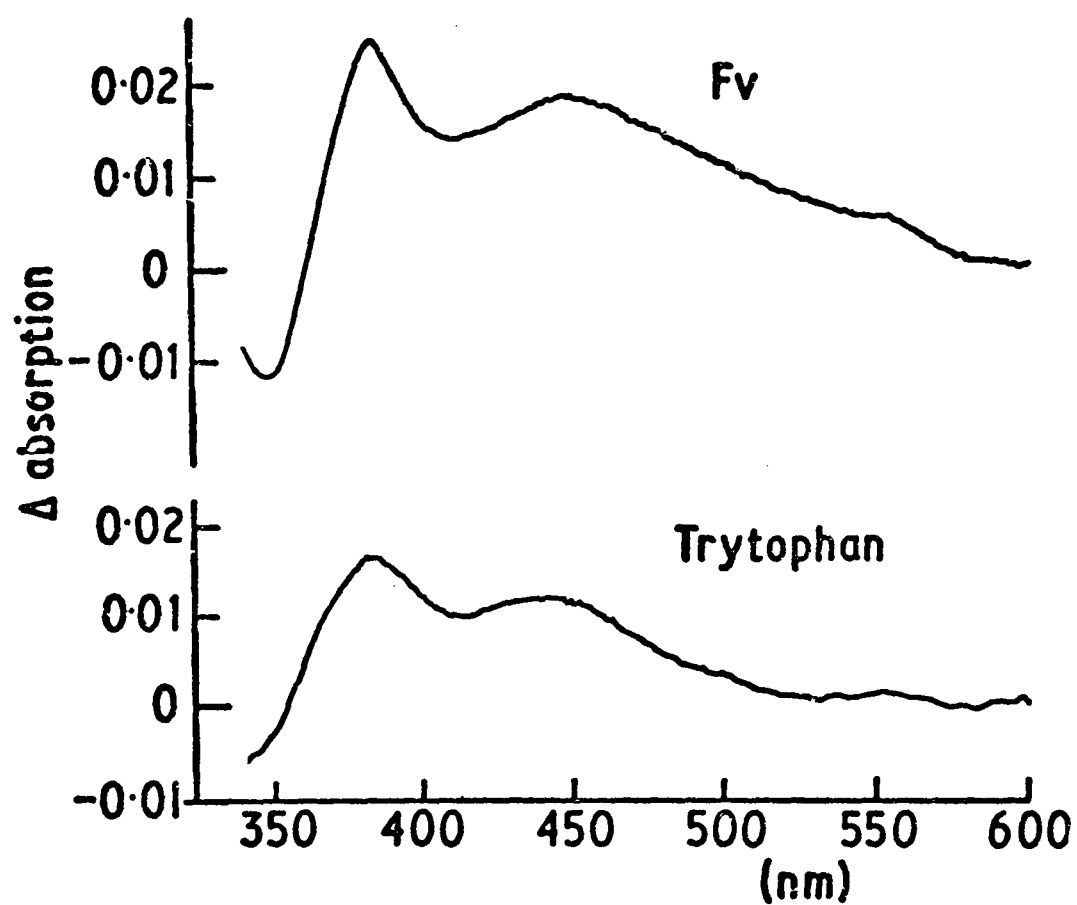
- a) The effect on the ^1H n.m.r. spectrum of 2 mM Fv fragment at pH* 8.07 of the addition of equimolar N-Dnp-methyl phosphonic acid - the difference spectrum is x2 vertical scale of the 270 MHz n.m.r. spectrum.

- b) The effect on the ^1H n.m.r. spectrum of 5 mM lysozyme, pH* 4.5 of the addition of 20 mM β -methyl-N-acetylglucosamine. The resonances indicated by asterisks arise from the ligand. The difference spectrum is on the same scale as spectra a and b.

haptens. The intensity in this region of difference spectra is, however, in all eight cases, less than 1% of the total intensity in this region of the ^1H n.m.r. spectrum of the Fv fragment. Thus it is difficult to be sure that such a small difference is real. The differences in the aromatic amino acid spectrum are however of the order of 10-15% of the total intensity in this region in all eight cases; these are thus a more reliable representation of the hapten induced changes of the ^1H n.m.r. spectrum of the protein. The similarity in these difference spectra shows that all seven haptens perturb the environments of the same protein ^1H nuclei on binding. The similarity in the difference spectra produced by ϵ -N-Dnp-L-lysine binding at two widely different pH^* 's show that the mode of hapten binding is unaffected by the ionization state of residues on the protein. This is consistent with the pH^* dependence of the Fv fragment ^1H n.m.r. spectrum (chapter 4) in which only the resonances of the three histidine residues are significantly affected in the pH^* range 5-8.5. This shows that there are no pH^* dependent conformational changes in the Fv fragment.

Fig. 6 compares the changes in the ^1H n.m.r. spectra of lysozyme in which ligand binding causes a conformational change, and the Fv fragment, when ligands bind to these proteins. The changes in the lysozyme spectrum induced by the binding of β -methyl NAG are extensive, as indicated by the difference spectrum, which is on the same scale as the two spectra being subtracted. The difference spectrum produced by the binding of N-Dnp-methylphosphonate to the Fv fragment represents only 1.4% of the total intensity in the spectrum. This corresponds to 13 perturbed ^1H nuclei. This estimate can be corrected using the T_1 data discussed in the previous section, since most of the intensity in the difference spectrum arises from aromatic amino acid ^1H resonances and most of the intensity in the Fv fragment spectrum arises from aliphatic amino acid ^1H resonances. Further, the partially relaxed difference spectrum (Fig. 4) shows that most of the ^1H nuclei that contribute to the difference spectrum have T_1 values close

FIG. 7 : Comparison of the difference absorption spectra of Dnp-aspartate on interaction with either the Fv fragment or tryptophan.



Conditions were: Dnp-aspartate, 70 μ M; Fv fragment, 40 μ M; Trp, 20 mM; pH 6.8; Pipes buffer, 40 mM; NaCl, 0.15M. $T = 298$ K

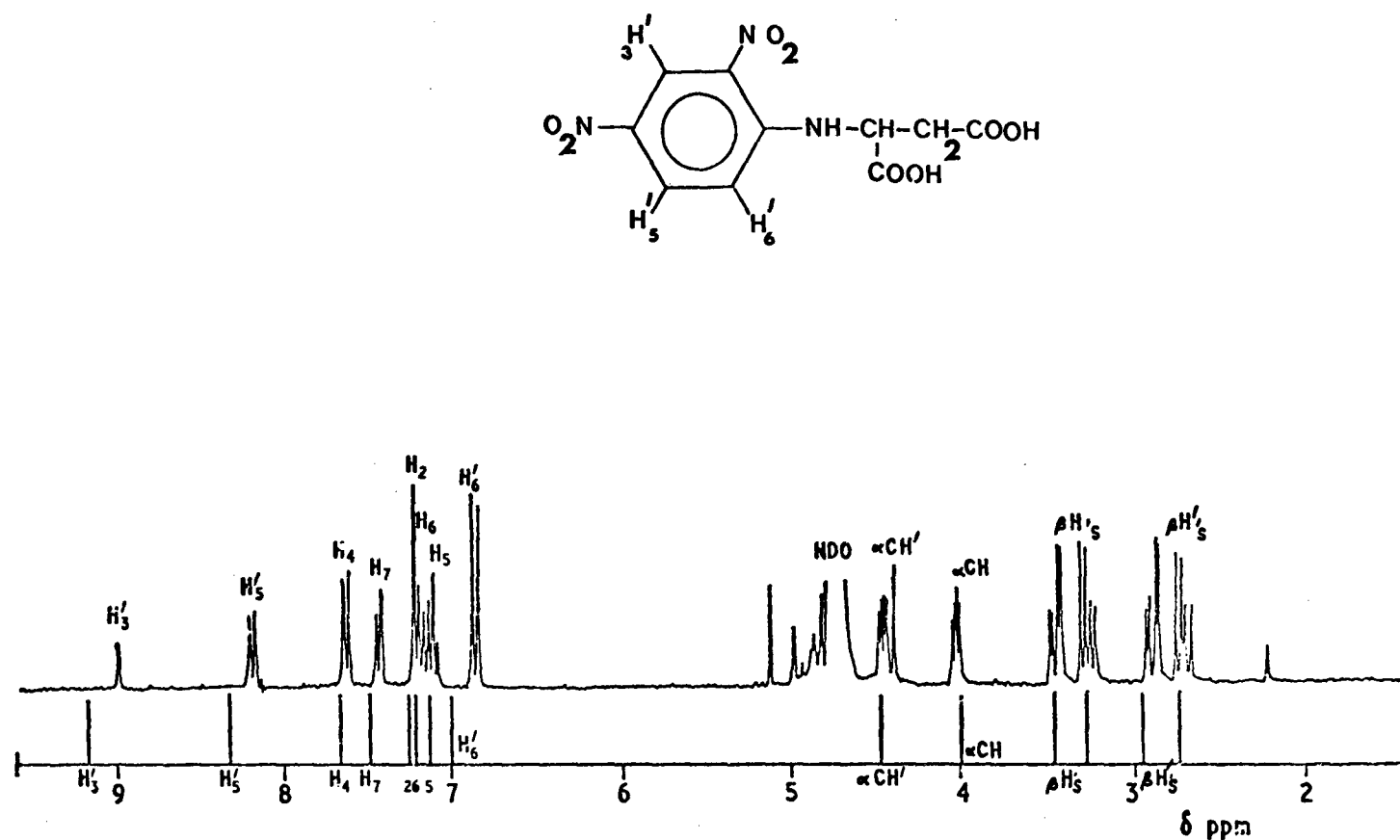
to the mean value of 800 msec for the aromatic amino acid ^1H nuclei in the Fv fragment. Thus a corrected intensity of 20 perturbed ^1H nuclei is appropriate. This small number, which is representative of all the haptens studied (Fig. 5), indicates that if the binding of hapten to the Fv fragment does induce a change in protein conformation then that change is confined to a few amino acid side chains. These are most probably in close proximity to the bound hapten. Thus the small perturbation, suggesting a local effect on the protein, confined to the region of the hapten binding site, taken in conjunction with the similar difference spectra produced by all seven haptens, supports the view that all the haptens interact with the same amino acid residues on the protein.

d) The binding of N-Dnp-L-aspartate to the Fv fragment

i) Optical difference spectroscopy

As discussed in Chapter 1 sections 6(d) and 7, it has been shown that the binding of polynitrophenyl haptens to anti-Dnp antibodies produces characteristic changes in the absorption spectrum of these haptens (a red shift and hypochromism) (Little and Eisen, 1967) and that this effect can be reproduced by tryptophan but not by other aromatic amino acids. This method was used to demonstrate that protein 315 bound these haptens (Eisen, Simms and Potter, 1968). Further, Freed, Rockey and Davis (1976) concluded that the circular dichroism induced on the binding of N-Dnp and N-Tnpaminocaproate to protein 315 could only be accounted for by short range interactions between the chromophoric group of the hapten and tryptophan(s) in the combining site of the protein. The optical difference spectra produced on interaction of N-Dnp-L-aspartate with L-tryptophan and with the Fv fragment of protein 315 are shown in Fig. 7. The similarity of these two difference spectra suggests that the N-Dnp-L-aspartate interacts with a tryptophan residue in the combining site of the Fv fragment

FIG. 8 : ^1H n.m.r. spectrum at 270 MHz of a mixture of Dnp-aspartate and tryptophan



The shifts on complex-formation are all upfield for the aromatic protons. The shifts of the ^1H resonances in the free compounds are indicated. Conditions were 10mM-Trp, 10mM-Dnp-aspartate, 150mM-NaCl, pH 6.0, and $T = 303$ K.

to produce a structure for this pair of aromatic rings, similar to that in aqueous solution.

ii) ^1H n.m.r. studies

The Interaction of N-Dnp-L-aspartate with L-tryptophan was monitored by observing the ^1H n.m.r. spectra of mixtures of the two compounds. This is illustrated in Fig. 8, which shows the spectrum of a sample containing 10 mM N-Dnp-L-aspartate and 10 mM L-tryptophan. It can be seen that the resonances assigned to the aromatic groups of both molecules all experience small upfield shifts. These effects can be quantified by performing suitable titrations and examples of the changes in the chemical shifts of these resonances are shown in Fig. 9(a). These data cannot be fitted by binary complex (Dnp-Trp) formation alone. The simplest model consistent with the data is given in section 2(g) and involves formation of a ternary complex Trp-Dnp-Trp. Using this to generate theoretical curves, the data are fitted by $K_{D1} = 43 \pm 8 \text{ mM}$, $K_{D2} = 170 \pm 30 \text{ mM}$ ($K_{D1} \approx 0.25 K_{D2}$) and the values for the fully bound chemical shift changes given in Table 2. These values for K_D give a ΔG° for binary complex formation of $-2.79 \text{ Kcal M}^{-1}$, this value is higher than the values determined by Johnston, Barisas and Sturtevant (1974) and Little and Eisen (1967) of approximately -0.7 Kcal/mole . These authors, however, assumed that only binary complex formation occurred for the system L-tryptophan-Dnpaminocaproate. It is difficult to estimate the fully bound chemical changes for the Dnp accurately because of the formation of the ternary complex. However, the chemical shift ratios do not alter as a function of Dnp saturation with tryptophan, hence these ratios can be used to determine the structure of the binary complex. The shift changes could arise from three sources; changes in the solvent water structure around the two compounds when the complex is formed; changes in the intramolecular electron distribution either as a result of the presence of the other ring

system or as a result of the possible changed solvent structure, and through space ring current (Appendix I) or chemical shift anisotropy effects from the two nitro groups of the N-Dnp-L-aspartate. It has been concluded, however, from a number of studies on the interaction of polynitrophenyl derivatives with electron donating compounds, that the major source of the observed shift changes is the ring current effect (Foster and Fyfe, 1969).

A further problem is introduced by the certainty that the two rings can move rapidly relative to one another on the n.m.r. time scale. Thus the chemical shifts in the binary complex are an average for an unknown population distribution of structures. Given these considerations, the structure shown in Fig. 10 can be taken to represent only the approximate average structure of the binary complex. This structure was calculated from the shift ratios for the H'_3 , H'_5 and H'_6 of the Dnp ring using RSEARCH, a FORTRAN program (Perkins, 1977), which is based on the Johnson-Bovey equation (1958) (Appendix I). Since the shift ratios cannot be used to determine the interplanar distance in the complex, values for this were taken from the crystallographic structures for the 2,4,6 trinitrobenzene-skatole complex (Hanson, 1964) (3.3 Å) and the tryptophan- 2,4,6 trinitrophenol complex (Gartland, Freeman and Bugg, 1974) (3.4 and 3.5 Å). The search gave two sets of solutions, one of which is shown in Fig. 10, the other has the Dnp ring centre located over the five membered ring of the tryptophan, since the latter gives very small (< 0.05 ppm) shifts for all the tryptophan protons but H_2 , the former was selected as being more representative of the time averaged structure. The closest interplanar distance (3.3 Å) was chosen as this gave the lowest value for the root mean square error in the RSEARCH program. The structure can only be taken as indicating that the Dnp and indole rings have considerable overlap with the Dnp ring located approximately over the centre of the indole ring. More importantly the experimentally determined chemical shift changes will be similar to the contribution that each ring will make to the chemical shift change of the

FIG. 9 : ^1H chemical shift changes in a mixture of N-Dnp-L-aspartate and L-tryptophan

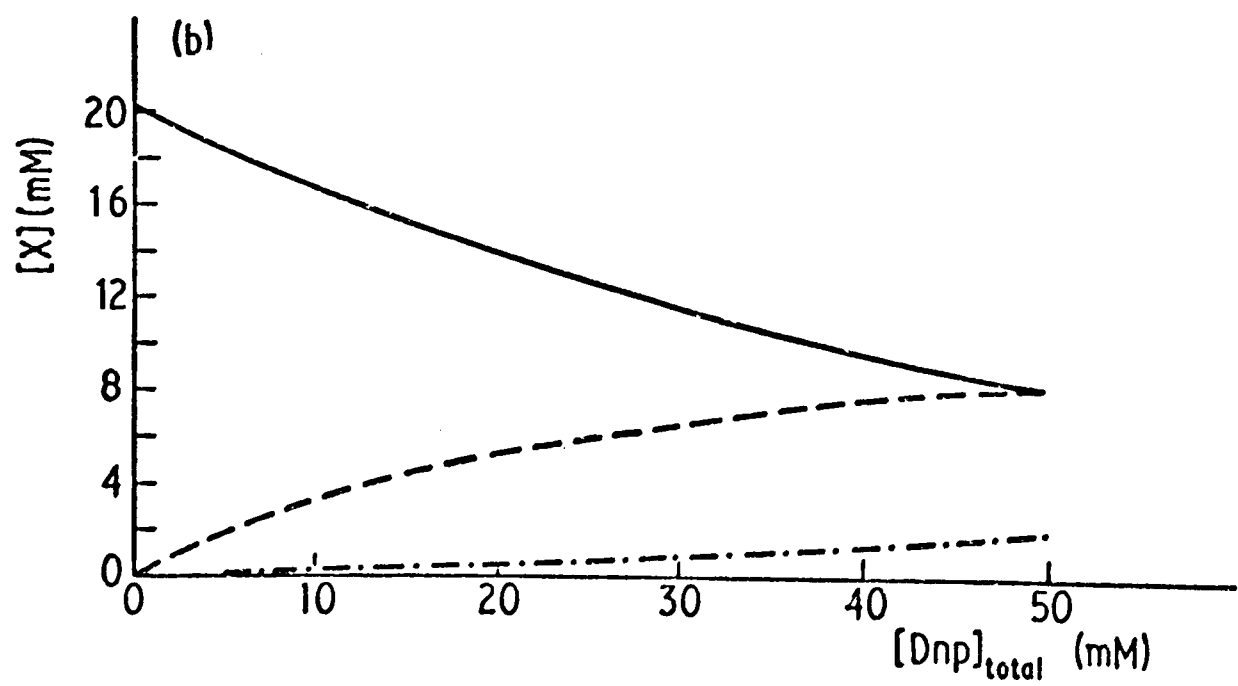
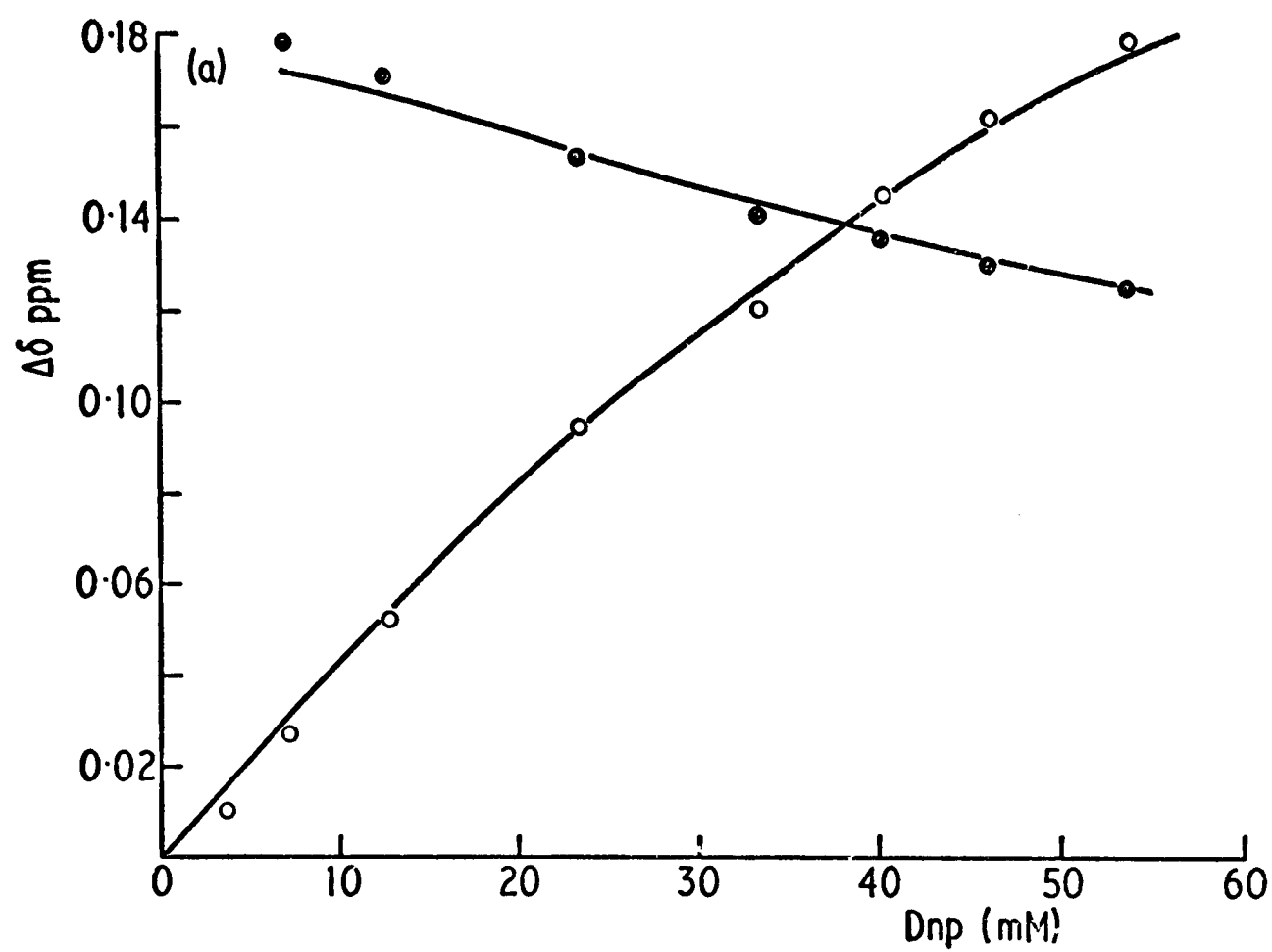


Fig. 9.

- a) Variation in the chemical shift changes of the H'_3 resonance of N-Dnp-L-aspartate ($- \bullet -$) and the H_7 ($- \circ -$) resonance of L-Tryptophan with increasing N-Dnp-L-aspartate concentration. The tryptophan concentration was 20 mM, $T = 303^\circ \text{ K}$, $[\text{NaCl}] = 0.15 \text{ M}$ and $\text{pH}^* = 6.0$. The H'_3 shift is corrected to infinite dilution of N-Dnp-L-aspartate. The continuous curves were calculated by a simple FORTRAN program TWOSITE using the model described in section 2(g), with the parameters $\Delta\delta(H_7) = 0.28 \text{ ppm}$, $\Delta\delta(H'_3) = 0.53 \text{ ppm}$, $K_{D1} = 40 \text{ mM}$, $K_{D2} = 160 \text{ mM}$.

The data are an average of three experiments.

- b) Variation in the concentrations of the components $\text{Trp}(-)$, $\text{Trp} - \text{Dnp}(- - -)$ and $(\text{Trp})_2(\text{Dnp}) (- \bullet -)$ with total Dnp concentration. The curves were calculated using the parameters from (a).

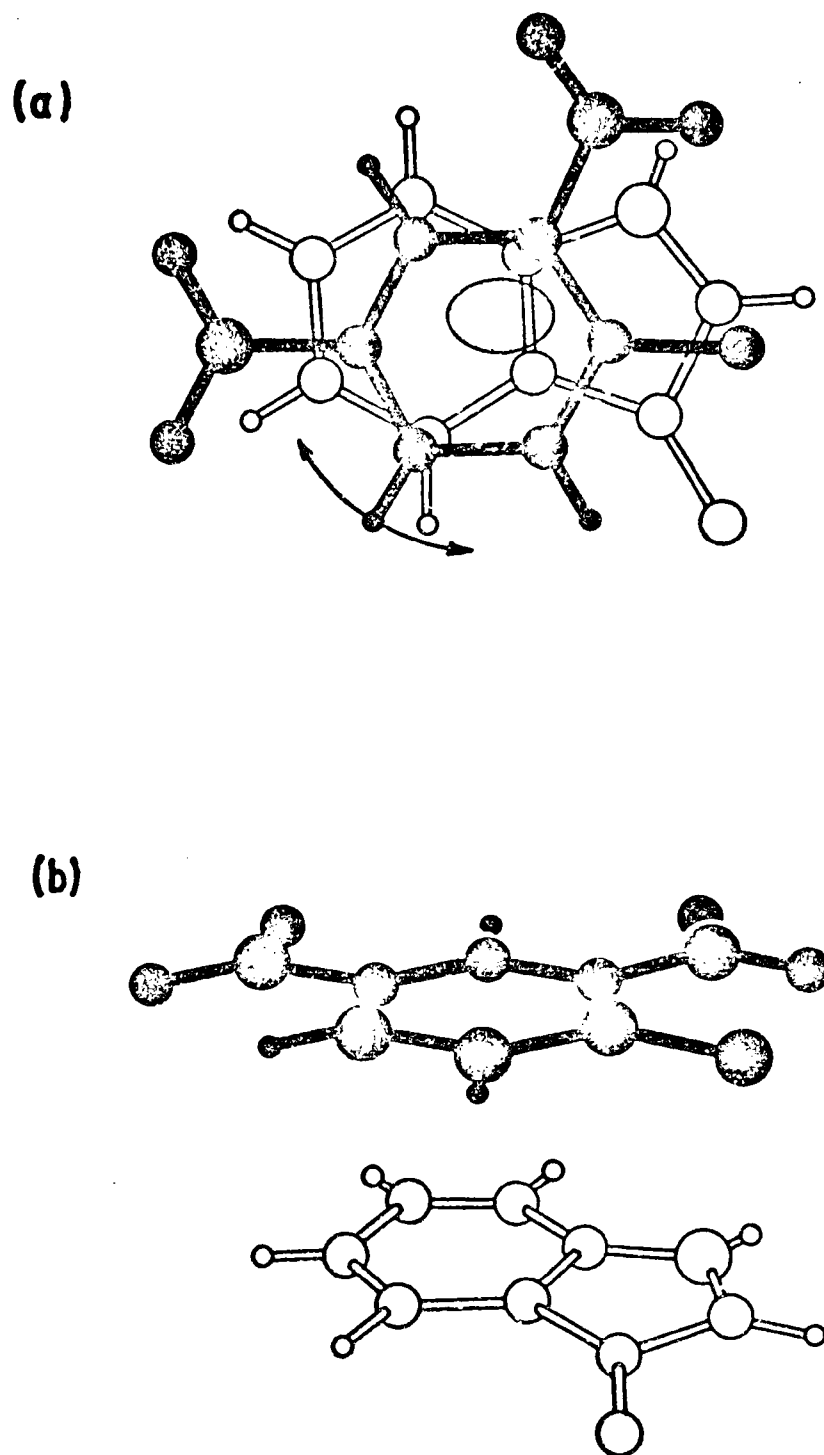
TABLE 2 : Experimental and calculated bound shifts (ppm) and shift ratios of the aromatic protons of the Dnp-aspartate-L-tryptophan complex

Proton	Shift		Shift Ratios	
	Experimental	Calculated	Experimental	Calculated
Dnp-Asp H ₍₃₎	0.35-0.55	0.71	1.98	1.86
Dnp-Asp H ₍₅₎	0.22-0.35	0.43	1.26	1.13
Dnp-Asp H ₍₆₎	0.17-0.27	0.38	1.00	1.00
Trp H ₍₂₎	0.19	0.10	0.96	0.37
Trp H ₍₄₎	0.23	0.29	1.21	1.07
Trp H ₍₅₎	0.18	0.23	0.92	0.85
Trp H ₍₆₎	0.19	0.27	1.00	1.00
Trp H ₍₇₎	0.28	0.53	1.34	1.96

The experimental shift(s) are those determined for the fully bound complex at pH6.0 and *T* = 303 K. The calculated shifts were derived from the Johnson-Bovey (1958) equation (see the Appendix) for the structure obtained from the computer program RSEARCH, and shown in Fig. 10. The shifts are the changes in position of the protons from those in the free compound, and the shift ratios for both compounds are quoted with respect to their H₍₆₎ protons.

These values are based on data collected by Dower, S.K., Jackson, R., and Gettins, P.

FIG. 10 : Structure of the Dnp-aspartate and tryptophan complex



The two views are obtained from a computer search of the possible structures, with a separation between the two aromatic rings of $3.3 \text{ \AA}$ which give calculated ring-current shifts in agreement with those obtained experimentally. The shifts are calculated by the Johnson-Bovey (1958) equation, modified to take into account the aromatic nature of the indole ring. The two views represent (a) a vertical view down the z-axis and (b) that vertical view rotated 30° about the z-axis and 60° about the x-axis. The ellipse and the arrows indicate the extent of the family of solutions, of which the structure shown is a member.

FIG. 11 : Difference spectra from the 270 MHz ^1H n.m.r. spectrum of the Fv fragment resulting from addition of N-Dnp-L-aspartate

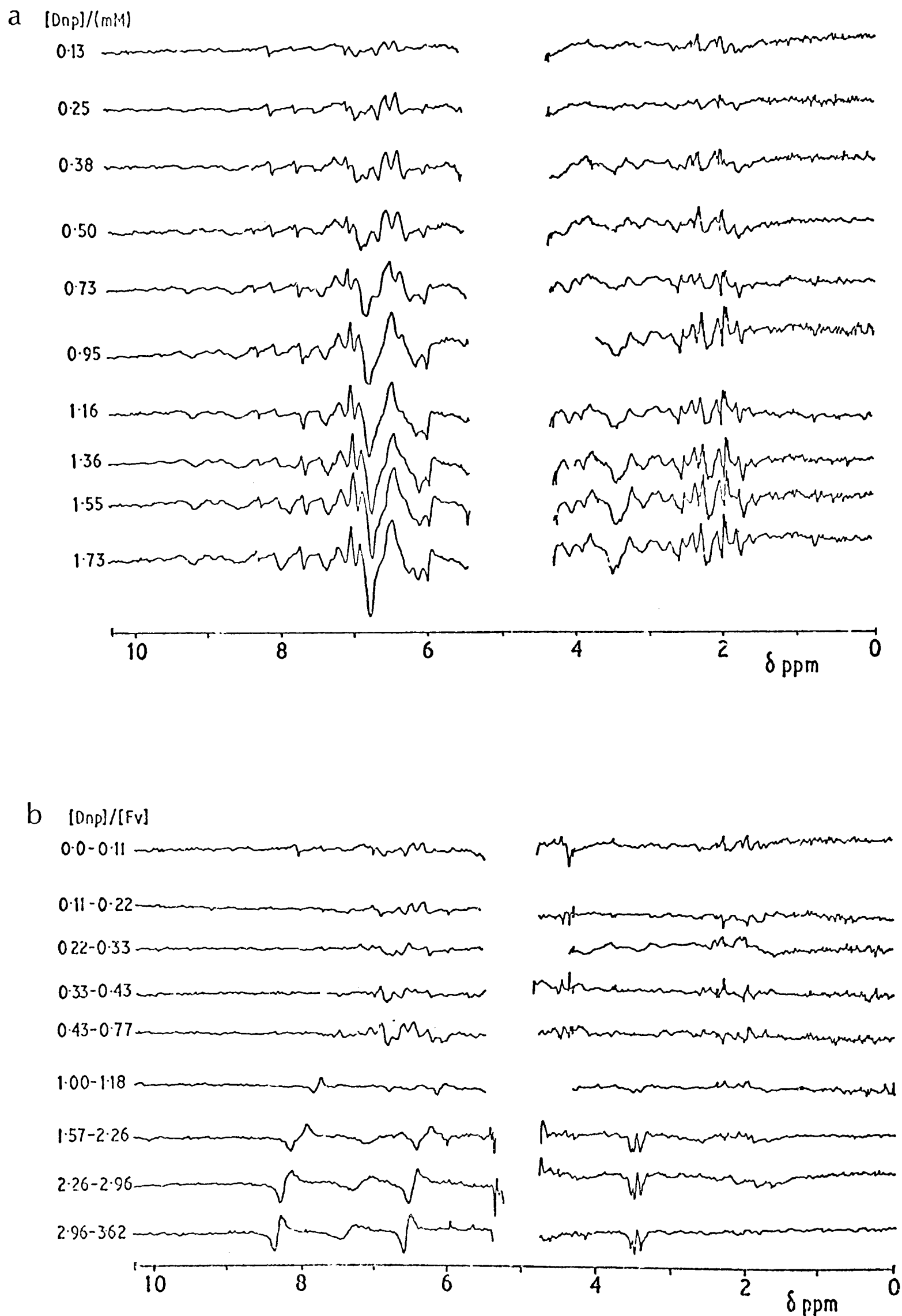


FIG. 11.

The sample was 1.06 mM Fv fragment pH* 6.9.

- a) Spectra were obtained by subtraction of the spectrum of a sample containing the concentration of N-Dnp-L-aspartate indicated from that of the Fv fragment alone.
- b) Spectra were obtained by subtracting the spectra of samples as indicated, note that when the ratio of [Dnp]/[Fv] exceeds 1:1 only the hapten resonances continue to change chemical shift, as the protein is saturated.

other, when N-Dnp-L-aspartate binds to the Fv fragment, if the same type of structure is formed by the Dnp and a tryptophan indole side chain in the protein.

Since it is possible that Little and Eisen (1967) had failed to detect complex formation between Dnp and tyrosine or phenylalanine by optical difference spectroscopy because spectral properties of Dnp are unaltered by binding to these amino acids, ^1H n.m.r. spectroscopy was also used to search for complex formation between these compounds. The spectra of solutions containing 10 mM N-Dnp-L-aspartate and either 10 mM phenylalanine or 10 mM tyrosine, recorded at 90 MHz, pH* 7 and 303° K showed no shift changes in the resonances of either component. It was therefore concluded that N-Dnp-L-aspartate did not form a stacked complex with these amino acids.

The binding constant of N-Dnp-L-aspartate to the Fv fragment was measured by protein fluorescence quenching at pH* 7.0 and 303° K in the presence of 0.15 M pipes buffer and 0.15 M NaCl, the binding constants was 24.5 μM . This hapten binds weakly to the Fv fragment (Chapter 4, Table 1) and was thus used to obtain quantitative ^1H chemical shift change data, since it is in fast exchange for chemical shift (see section (e)).

The effect on the ^1H n.m.r. spectra of both the Fv fragment and N-Dnp-L-aspartate can be determined by varying the hapten concentration present in a solution containing the protein. Because the ^1H n.m.r. spectrum of the protein is too poorly resolved to permit the observation of single resonances, it is necessary to use difference spectra to follow the changes in the protein spectrum. It has been shown that few protein resonances are affected by the binding of haptens (section (c)), so that use of this method affords great simplification of the spectra. Figs. 11(a) and (b) show the changes in the hapten and Fv fragment spectra caused by the binding of the hapten to the protein. The two sets of spectra, generated as described in the legend to Fig. 11, permit the total effect on the ^1H n.m.r. spectrum and the progressive changes in it to be monitored

simultaneously. In the serially subtracted spectra (Fig. 11(b)), it can be seen that when the hapten concentration exceeds that of the protein, only the chemical shifts of hapten resonances continue to change. Since the protein concentration is 1.06 mM, and the dissociation constant of N-Dnp-L-aspartate under the conditions of the n.m.r. experiment is 24.5 μ M (see below), when the hapten concentration reaches 1.06 mM the protein will be saturated. Thus after equivalence the concentration of bound hapten does not change. This allows the chemical shift change for the hapten resonances between water and the protein combining site to be determined as described in 2(g).

The chemical changes for hapten and protein obtained from the fast-exchange experiment are presented in Table 3. Using the observed fully bound shift change for the H₃ resonance of the hapten, a binding constant of 24.5 μ M was determined by curve fitting using the data of Fig. 11 which is plotted in Fig. 12. No attempt was made to curve fit the protein shift change data since the changes in chemical shift (Table 3) for the protein resonances are small and close to the linewidths for the protein resonances (estimated 15-25 Hz = 0.06-0.1 ppm). The difficulty that this leads to in interpreting the intensities of resonances contributing to the difference spectra is discussed in Appendix II. The protein chemical shift changes are thus accurate to only 0.06 ppm. The difference spectra given in Fig. 11 show that most of the protein resonances perturbed by the binding of N-Dnp-L-aspartate arise from aromatic amino acid residues. Some resonances in the aliphatic amino acid region of the spectrum are perturbed, but all the chemical shift changes are too small to be quantified. As shown in Table 3, some of the aromatic amino acid resonances show chemical shift changes greater than 0.3 ppm. The chemical shift changes for the hapten are, in contrast, large. The use of these two sets of data to obtain information about the bound environment is discussed in Section 4.

FIG. 12 : The changes in chemical shift of ligand and protein resonances when N-Dnp-L-aspartate binds to the Fv fragment.

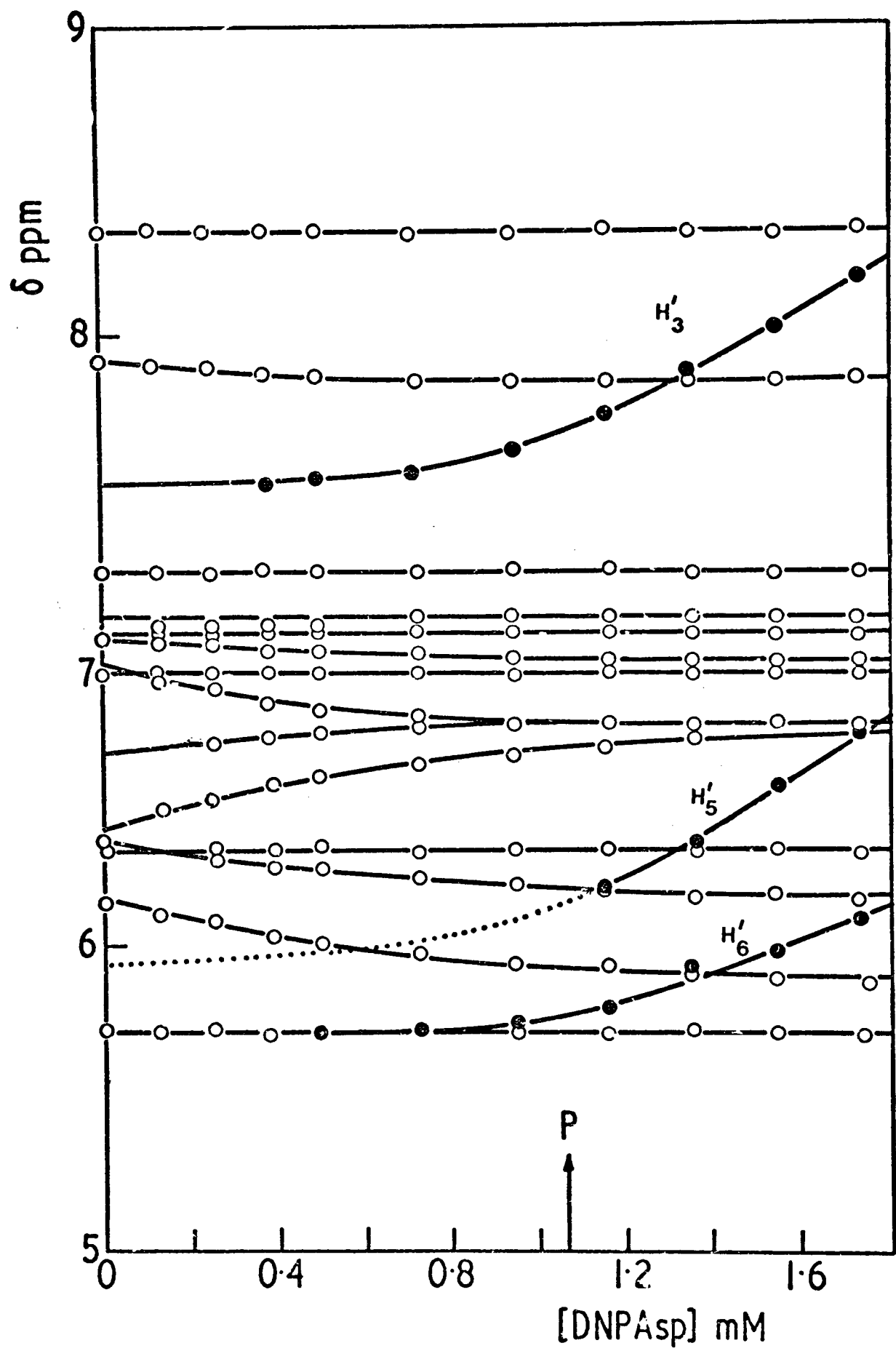


Fig. 12.

The data are those obtained from the spectra given in Fig. 11, the aliphatic region is not shown as no variation in chemical shift could be detected in this region of the protein spectrum, as discussed in the text. The hapten curves (—●—) are calculated using the data given in Table 3 and a binding constant of $24.5 \mu\text{M}$. The protein curves (—○—) are drawn by hand, as discussed in the text. The curves for H_5 and H_6 are extrapolated from the binding constant and chemical shift changes.

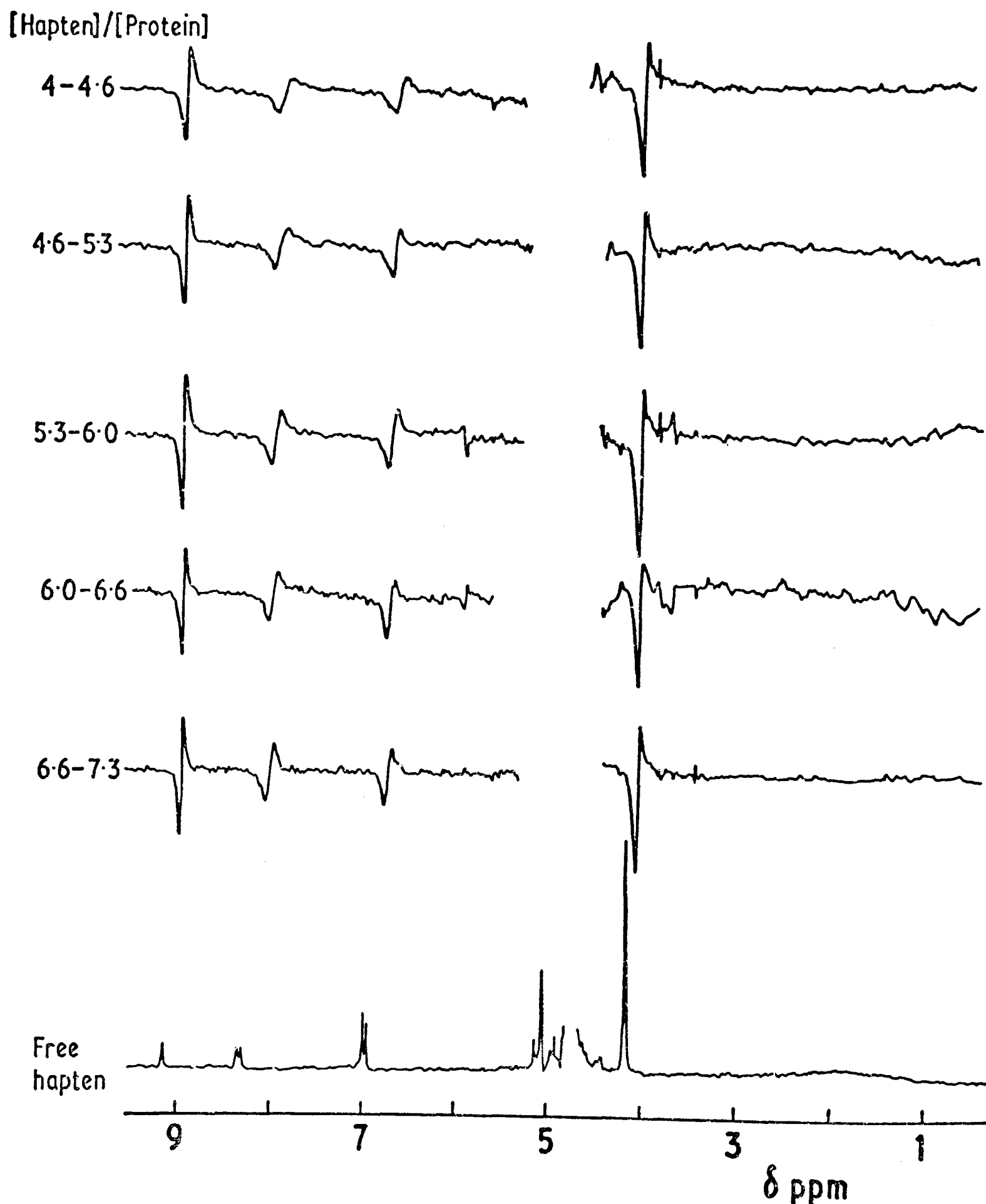
TABLE 3 : Chemical shifts($\Delta\delta$) of resonances perturbed on binding of Dnp-aspartate to fragment Fv from protein 315 at pH6.9

Hapten resonances	δ_0 (p.p.m.)	$\Delta\delta$ (p.p.m.)	No. of protons
H ₍₃₎	9.18	+1.68	1
H ₍₅₎	8.36	+2.3	1
H ₍₆₎	7.04	+1.31	1
-CH ₂ -	2.76	+0.1	
(Complex pattern with three main peaks)	2.91	+0.2	2
	2.99	+0.2	
-CH-	Not observable		
Protein resonances			
5.9 His C ₍₂₎	7.82	+0.06	1
—	7.00	Broadens out	~2
5.9 His C ₍₄₎	6.95	+0.07	1
—	6.90	+0.19	1
—	6.62	-0.08	1
—	6.37	-0.29	2
	6.33	+0.17	<2
	6.16	+0.25	2
Peak decreases as hapten is added	7.20		~2
Peak increases as hapten is added	7.42	Poss:0.22	2

Measurements are made at 270 MHz and $T = 303$ K and in the presence of 0.15 M-NaCl. Shifts were measured from the sodium salt of DSS as an external standard.
 — denotes a downfield shift change; + denotes an upfield shift change.

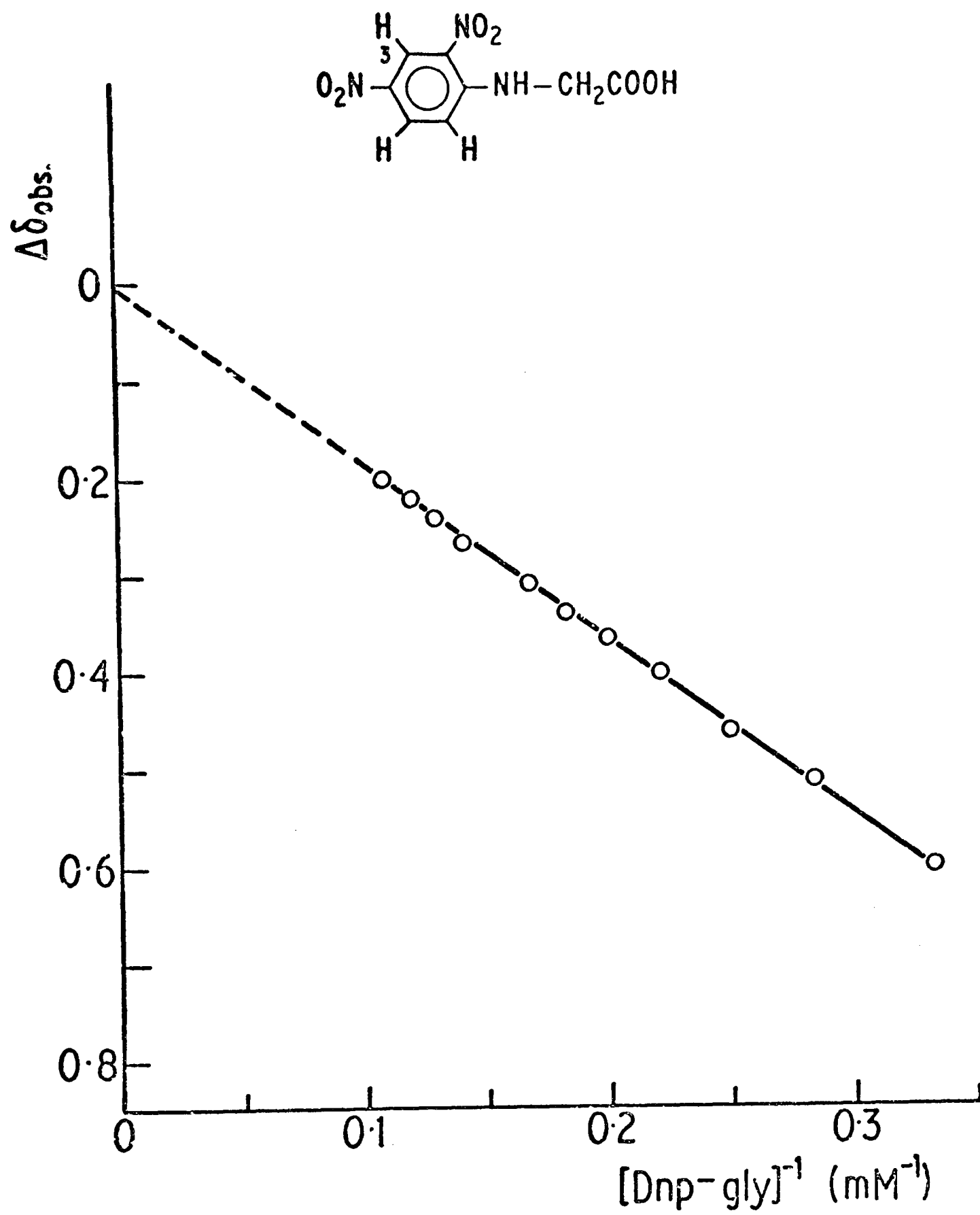
Conditions as for Fig. 11.

FIG. 13(a) The effect of the Fv fragment on the 270 MHz ^1H n.m.r. spectrum of N-Dnpglycine



The samples contained 1.5 mM Fv fragment, pH* was 6.8, concentrations of N-Dnp-glycine as indicated. The spectra were generated as indicated in the figure

(b)



The chemical shift change of the H_3 resonance of N-Dnpglycine in the presence of 1.5 mM Fv fragment. The data from figure 13(a) are represented as linear plot as described in section 2(g).

TABLE 4 : Change in chemical shift of hapten resonances perturbed on binding of haptens to the Fv fragment from protein M0PC 3,5

Hapten	Proton	Shift change (p.p.m.)	pH*
N-Dnp-L-aspartate	H ₍₃₎	1.68	6.9
	H ₍₅₎	2.30	
	H ₍₆₎	1.30	
	B-CH ₂ -	0.16	
N-Dnp-glycine	H ₍₃₎	1.21	6.8
	H ₍₅₎	2.20	
	H ₍₆₎	1.77	
	α-CH ₂ -	1.00	

Measurements were made at 270 MHz, T=303°K, in the presence of 0.15 M-NaCl.

Finally the difference spectra in Fig. 11(a) indicate that some resonances in the region 7-7.3 ppm do not change chemical shift when the haptens bind, but rather appear to broaden. This has been noted as a general feature of hapten difference spectra, and would appear to indicate the alteration of some anisotropic motion in the protein in or near the hapten binding site, when hapten binds (See Section 4).

e) Chemical exchange rates for the Fv fragment N-Dnp-glycine complex

The serially subtracted ^1H n.m.r. difference spectra obtained when excess concentrations of N-Dnp-glycine were added to the Fv fragment of protein 315 are shown in Fig. 13(a). Both the chemical shift changes and the line widths for the hapten ^1H resonances were measured as the ratio of hapten/Fv fragment was varied. A graphical method was used to determine the total hapten shift changes as illustrated in Fig. 13(b) which shows the data for the H₃ resonance (Table 4). The observed linewidths are given in Table 5, which also shows the calculated exchange contribution to the linewidths.

The calculated linewidth contributions were determined by use of a fast exchange binary complex treatment (Campbell and Dobson 1978). The equilibrium of hapten (H) with the Fv fragment is written as:



and the fast exchange condition is:-

$$\tau_m^{-1} \gg 2\pi \Delta\omega_0 \quad (11)$$

when $\Delta\omega_0$ is the chemical shift change in Hz, and τ_m is the chemical exchange time in seconds. This equation can be written:

$$k-1 \gg 2\pi \Delta\omega_0 \quad (12)$$

and under these conditions the observed linewidth for a resonance arising from the hapten is given by:-

TABLE 5 : Linewidths of hapten resonances in the Fv fragment
N-Dnpglycine system

[N-Dnpglycine] (mM)	$\bar{X}_B$	H ₃		H ₅		H ₆		α -CH ₂	
		$\Delta\nu$	$\Delta\nu_m$	$\Delta\nu$	$\Delta\nu_m$	$\Delta\nu$	$\Delta\nu_m$	$\Delta\nu$	$\Delta\nu_m$
4	0.38	57	30	--	98	--	63.5	43	20.3
5.5	0.27	18	29	51	97	49	63	33	20
6.0	0.25	25	28.2	41	95	41	61	29	19.5
7.0	0.21	21	26.8	46	88	25	57	21	18.2
8	0.19	14	25.2	45	84	41	54	19	17.4
9	0.17	15	23.6	37	78	35	51	17	16.2

The experiment was performed at pH* 6.8 with 1.5 mM Fv fragment. $\Delta\nu$ is the observed linewidth in the Hz corrected for the exponential linebroadening. The values of $\Delta\nu_m$ are the expected exchange contribution to the linewidths in Hz calculated as described in the text. Both values are the linewidth at half height as defined by $\Delta\nu = (\pi T_2)^{-1}$

$$\frac{1}{T_{2\text{obs}}} = \frac{\bar{X}_F}{T_{2F}} + \frac{\bar{X}_B}{T_{2B}} + 4\pi^2 (1 - \bar{X}_B)^2 \bar{X}_B \frac{\Delta\omega^2}{k-1}$$

When B and F represent bound and free environments. The last term in this expression is the exchange contribution to the linewidth. From the data of Basilkorn et al (1974) and Johnston, Barisas and Sturtevant (1974), the appropriate value for $k-1$ under the conditions of the n.m.r. experiment is calculated as $6.6 \times 10^3 \text{ sec}^{-1}$. Using this value for $k-1$ and the values for $\Delta\delta_0$ given in Table 4, the values for $\Delta\nu_m$ given in Table 5 were calculated. In the cases of H_5 and H_6 the measured linewidths are expected to be an underestimate of the true linewidth since these resonances show an ortho coupling constant of 8.8 Hz. In the cases of H_3 and the α -carbon linked methylene protons the contribution from coupling is small (≈ 1 Hz) and is thus a negligible contribution to the overall measured linewidths. Under the conditions of the n.m.r. experiment, the exchange contribution should dominate the T_2 of the averaged hapten resonances unless the bound linewidth is very large. Since the apparent linewidths are less than the predicted exchange contribution no estimate of this latter value can be made. The discrepancies between the measured and calculated linewidths reflect the difficulties in determining the baseline positions and thus the linewidth for the broad resonances, thus only qualitative conclusions can be drawn. It can be seen that for those resonances with the smallest chemical shift differences (H_3 and $\alpha \text{ CH}_2$) the agreement between the calculated and experimental linewidths are within a factor of two (r.m.s. differences $H_3 : 0.34$ and $\alpha \text{ CH}_2 : 0.2$) and it is therefore concluded that these data are consistent with the binary complex formation by a simple encounter.

4) Discussion

a) Assignments for resonances in the Fv fragment spectrum

Since it is not possible to resolve spin-spin coupling for resonances in the Fv fragment spectrum, it is not possible to perform first stage assignments in this protein for any resonances except those assigned to histidine residues (See Chapter 4). Therefore, any assignments made must be based on the method of selective perturbations of the system. Such perturbations include binding of metal ions or ligands; changes in pH* and temperature; and changes in covalent structure (Dwek, 1973). Selected amino acid side chains in the protein can be covalently modified by chemical reagents as described in section 6(c) of Chapter 1. This method has the advantage that the modification of particular amino acid residues can be independently confirmed by sequence analysis. A particularly useful method for obtaining assignments in the ^1H n.m.r. spectrum of a protein is selective $^2\text{H}/^1\text{H}$ substitution (Roberts et al, 1977). This has the advantage over other chemical modifications that the protein structure surrounding the modified residue(s) is likely to be unperturbed. A further approach to the assignment problem in the Fv fragment is the separation of the V_L and V_H domains which compose this molecule. This isolated V_L domain exists as a dimer in solution (section 7, Chapter 1). The isolated V_H domain (sec. 7, Chapter 1), aggregates and is insufficiently soluble for ^1H n.m.r. studies. The V_L dimer can, however, be studied by ^1H n.m.r.

A comparison of the selective perturbation of the ^1H n.m.r. spectra of the Fv fragment and the V_L dimer of protein 315, caused by the binding of haptens to these proteins is shown in Table 6. It can be seen that resonances at 6.16, c.6.3, 6.37, 6.90, c 7.00, c 7.2 and c 7.33 appear to be perturbed similarly in both the Fv fragment and (V_L) ^1H n.m.r. spectra when haptens bind. Further, similar large shift changes are produced in the hapten spectrum on binding to both species. This suggests, firstly that those

TABLE 6 : A comparison of the effects of hapten binding on the ¹H
n.m.r. spectra of the F_v fragment and the V_L dimer of
protein 315

F _v + Dnp-Aspartate		V _L dimer + Dnp-Aspartate		V _L dimer + Dnp glycine	
δ ₀	Δδ	δ ₀	Δδ	Δδ ₀	Δδ
6.16	+0.25	6.15	+0.23	6.14	+0.27
6.33	+0.17	6.23	+0.30	6.21	+0.36
6.37	-0.29	6.36	-0.08	6.38	-0.11
6.62	-0.08	6.58	+0.16	6.62	+0.55
6.90	+0.19	6.73	+0.18	6.72	broadened
6.95	+0.07	6.90	< +0.15 or broadens	6.87	broadened
7.00	broadens	7.06	< +0.15 or broadens	7.04	-0.17
7.2					
7.2	decreases	7.22	-0.10	7.22	-0.16
7.42	increases	7.45	-0.15	7.45	-0.20
				7.63	+0.13

Notes

- 1) Conditions for the F_v fragment data are as given in Fig. 11
- 2) The data on the V_L dimer were collected by R. Jackson

resonances that are present in the ^1H n.m.r. difference spectra obtained on hapten binding to both proteins probably arise from the same ^1H nuclei which can thus be assigned to light chain amino acids. There do not appear to be any significant perturbations in the Fv fragment ^1H n.m.r. spectrum which are not also present in the difference spectra obtained with the V_L dimer when N-Dnp-L-aspartate binds. This observation suggests that few (if any) heavy chain aromatic amino acid resonances are perturbed when haptens bind. It is also striking that the unperturbed chemical shifts of the resonances that are affected by haptens should be so similar in the two species. This suggests that the environments of the light chain amino acid side chains in the vicinity of the bound hapten polynitrophenyl group, are not significantly perturbed by the substitution of the V_H domain by a V_L domain. The hypervariable segments of the light chain of protein 315 contain one Tyr (34_L) one Trp (93_L) and two phenylalanines (94_L and 98_L). It is therefore these residues that would be expected to contribute to the difference spectrum caused by hapten binding to both the Fv fragment and the V_L dimer, as the conformational changes consequent on this event are small. It must be pointed out that this relies on the assumption, consistent with the predicted structure of Padlan et al (1976), that no residues from constant segments of the V_L domain are in contact with the bound hapten. However, in Fab NEW, for which a crystal structure is available, a Trp (47) in the V_H domain has been shown to be in contact with the ligand side chain (Poljak, 1975) (see Chapter 1, section 6(e)).

The method of selected ^1H substitution of ^2H protein which affords extensive spectral simplification in bacterial proteins (Roberts et al, 1977) cannot be applied to protein 315, since this is a mammalian protein. It has, however, proved possible to obtain a 60% reduction in the intensity of tryptophan resonances in the Fv fragment spectrum by using a deuterated diet for the mice in which the tumour is grown (Gettins, 1979). Comparison of the effects on the ^1H n.m.r. spectra of this protein caused by

N-Dnp-L-aspartate with those caused by addition of the same hapten to the Fv fragment spectrum indicate few, if any differences (Gettins, 1979). In particular, none of the resonances in the region 7.0-6.5 ppm, which are affected by hapten binding, are diminished in intensity in the modified protein. A possible exception is the resonance(s) at 6.90 ppm which may arise from one or two ^1H nuclei, and which experience a shift of 0.19 ± 0.05 ppm on hapten binding (Table 3). The spectroscopic comparisons of the interaction of the Fv fragment and L-tryptophan with N-Dnp-L-aspartate discussed in section 3(d), indicate that the indole side chain of a tryptophan and the Dnp group of the hapten, should form a 'stacked' type of interaction in the Fv fragment combining site. If this is the case then there is an apparent discrepancy between these results and those of the ^2H substitution, since all the ^1H resonances of the tryptophan indole group should experience upfield shifts of 0.2-0.3 ppm, which would be observable in the ^1H n.m.r. difference spectra (Appendix II).

Three possible explanations could account for the failure to detect this perturbation. Firstly, it could be that the structure of the Dnp/indole pair on the combining site is different from the average solution structure. Although this is extremely likely, both the experimental chemical shift changes, and those calculated using the Johnson-Bovey equations, suggest that any relative structure which led to ring current effects on the shift of only one or two of the tryptophan ^1H resonances would give chemical shift changes of > 0.5 ppm on those affected. A second possibility is that the binding of the N-Dnp-L-aspartate to the Fv fragment causes some perturbations of the environment of the tryptophan ^1H nuclei other than that caused by through space ring current effects. Thirdly, it is possible that anisotropic motion of residues close to the tryptophan indole cause exchange broadening of the ^1H resonances arising from it, such that these are too broad to be observed. Preparation of $[^2\text{H}]\text{Trp V}_L$ dimer from $[^2\text{H}]\text{Trp}$ protein 315 may provide additional information

to resolve these possibilities, since the V_L domain of protein 315 contains only two tryptophan residues rather than the six in the Fv fragment.

Whatever the cause may be, the conclusion is that the perturbation of a set of tryptophan indole resonances, which is expected to be caused by the binding of N-Dnp-L-aspartate, is not observed.

Nitration of tyrosine residues can be used to obtain assignments in protein ^1H n.m.r. spectra (Snyder et al, 1975). This method has been applied to the Fv fragment and the V_L dimer of protein 315 (Jackson, 1979). Only one of the two tyrosines in the V_L domain is nitrated by treatment of the V_L dimer with tetranitromethane, this is Tyr 34_L. It has been suggested as the basis of affinity labelling studies (sections 6(c) and 7 of Chapter 1), that this residue is present in the combining site of protein 315. The effects on the ^1H n.m.r. spectrum of the Fv fragment and the nitrotyrosine 34_L Fv fragment, of the binding of N-Dnp-L-aspartate (Jackson, 1979) have been compared. No significant differences in the difference spectra for the two proteins can be detected. It is, therefore, not possible, at present, to conclude from these data where the Tyr 34_L resonances occur in the spectrum of the Fv fragment.

If the Fv fragment of protein 315 is reacted with tetranitromethane Tyr 33_H is the residue most rapidly labelled (Wain-Hobson, 1977). The nitrated protein spectra obtained in the initial n.m.r. studies were, however, too poorly resolved to be able to conclude if any features of either the ^1H n.m.r. spectra, or difference spectra obtained with the binding of N-Dnp-L-aspartate and N-Dnpglycine were changed in the nitro Tyr 33_H-Fv fragment as compared with the unmodified protein.

Further chemical modifications that have been carried out (Klostergaard et al, 1977a, b, 1978) could be used in conjunction with ^1H n.m.r. spectroscopy to attempt to obtain assignments. The difficulties in obtaining

assignments with the tyrosine nitrations, however, indicate that it will be difficult to interpret the results with these three modifications; glyoxalation of arginines, maleylation of lysines and iodination of tyrosines since these are less specific than the nitration and in the case of glyoxalation and iodination, the activity of the protein is abolished.

At present there is one tentative assignment in the ^1H n.m.r. spectrum of the Fv fragment of protein 315. This is a resonance at 6.90 ± 0.05 ppm, from one or two ^1H nuclei of an indole side chain of a tryptophan which is present on the light of protein 315. The severe problems encountered in interpreting the ^1H n.m.r. spectrum of this protein indicate that it would be profitable to turn to other nuclei, such as ^{19}F and ^{13}C to obtain further structural information. The success of the ^2H substitution suggests that it may be possible to use ^{19}F labelling and ^{13}C isotopic enrichment to prepare protein samples with simple spectra. Whilst interpretation of the chemical shift changes for these nuclei are more complicated than for protons, such studies offer the possibility of determining directly which of the three types of aromatic amino acid residues are in contact with the bound hapten.

b) The origin of secondary shifts in proteins

In the light of the discussion in section a) it is clear that in studying the formation of the complex between dinitrophenyl derivatives and the Fv fragment of protein 315, only the hapten resonances have assignments to particular ^1H nuclei. Given the limitations that this places on the use of the n.m.r. data to obtain structural information, it follows that the application of n.m.r. to elucidating the structural features of the Fv fragment combining must rest on the assumption that the

structure predicted by Padlan et al (1976), is a close approximation to the true structure. This is discussed further in section (d).

When an organic molecule is transferred from water to a hydrophobic site inside a protein, a number of potential sources of chemical shift change exist. Initially these may be divided into through space effects and effects on the internal electron distribution of the molecule. The through space effects acting directly at the ^1H nucleus will be those that give rise to changes in the magnetic field strength. These changes will arise mainly from the magnetic anisotropies of groups containing double bonds, of which the strongest are ring current effects (Jackman and Sternhell 1969, Emsley, Feeney and Sutcliffe, 1966; and see Appendix I). In proteins, therefore, the groups which give rise to magnetic anisotropies are carbonyl groups, peptide groups, carboxyl groups, Arg guanido groups and the aromatic ring systems of Tyr, Trp, Phe and His. Effects on the shielding of proton resonances from the ligand can also arise from changes in intramolecular electron distribution, which arise from local electric field effects. Two origins for this can be expected. The first is the direct effect of electric charges on the polarization of bonds in the ligand. The second is a change in the reaction field (Buckingham, 1960) of the ligand on binding to the protein. The former will arise from the presence of negatively charged carboxyl groups or positively charged amino or guanido groups close to the bound ligand. The latter arises from a dipole induced polarization in the solvent surrounding a solute which has a dipole moment. This would be expected to be present in water or $^2\text{H}_2\text{O}$ and largely absent in the protein which cannot be polarized by reorientation of the "solvent" as the protein is structured. Estimates of these effects, for highly polarized solutes e.g. methyl cyanide (Lumbroso, Wu, Dally, 1963) and for substituted benzenes (Buckingham, 1960) suggest that the effects are in the range 0.1-0.3 ppm. Data of Wain-Hobson (1977), suggest that for N-Dnp-L-aspartate the chemical shift of the H_3 and H_5 protons relative to

DSS as an internal standard are independent of the solvent to within ± 0.1 ppm, whereas the chemical shift of the H_6 proton experiences a downfield shift of up to 0.3 ppm as the solvent polarity decreases, for the set D₂O (pH* 3.5), Acetic acid, methanol, acetone. Only in the case of aromatic solvents such as benzene are large upfield shifts seen, in particular for electron deficient aromatic derivatives such as nitrobenzenes. Schaefer and Schneider (1960) have shown that solvation of 2,4 dinitrobenzene in benzene gave rise to upfield shifts of :-



This was interpreted as arising from the benzene diamagnetic anisotropy.

The theory relating the electric field gradient to the chemical shifts produced on a proton is described by Schweizer et al (1964). This contains two terms one of which described the effect on the S orbital of the hydrogen atom, and is dependent on $(E)^2$ when E is the electric field, the second of which is proportional to E_z the field gradient along the C-H bond. Thus:-

$$\Delta\sigma = a E_z + bE^2 \quad (14)$$

$\Delta\sigma$ is shielding in ppm

$$\Delta\delta = \frac{aq\cos\theta}{r^2} - \frac{bq^2}{r^4} \quad (15)$$

when q is -1 for one electron unit negative charge and θ is the angle that the radius joining the charge to the ^1H nucleus makes with the C-H bond. Roughly a positive charge at the carbon atom will shift the resonance of a hydrogen bonded to it downfield by 10 ppm. Thus negative charges located $90^\circ < \theta < 180^\circ$ will produce downfield shifts for the resonance. Application of this treatment leads to values in agreement with

TABLE 7 : Effects of pH* on the chemical shifts of resonances in the 270 MHz ^1H n.m.r. spectrum N-Dnp-L-aspartate

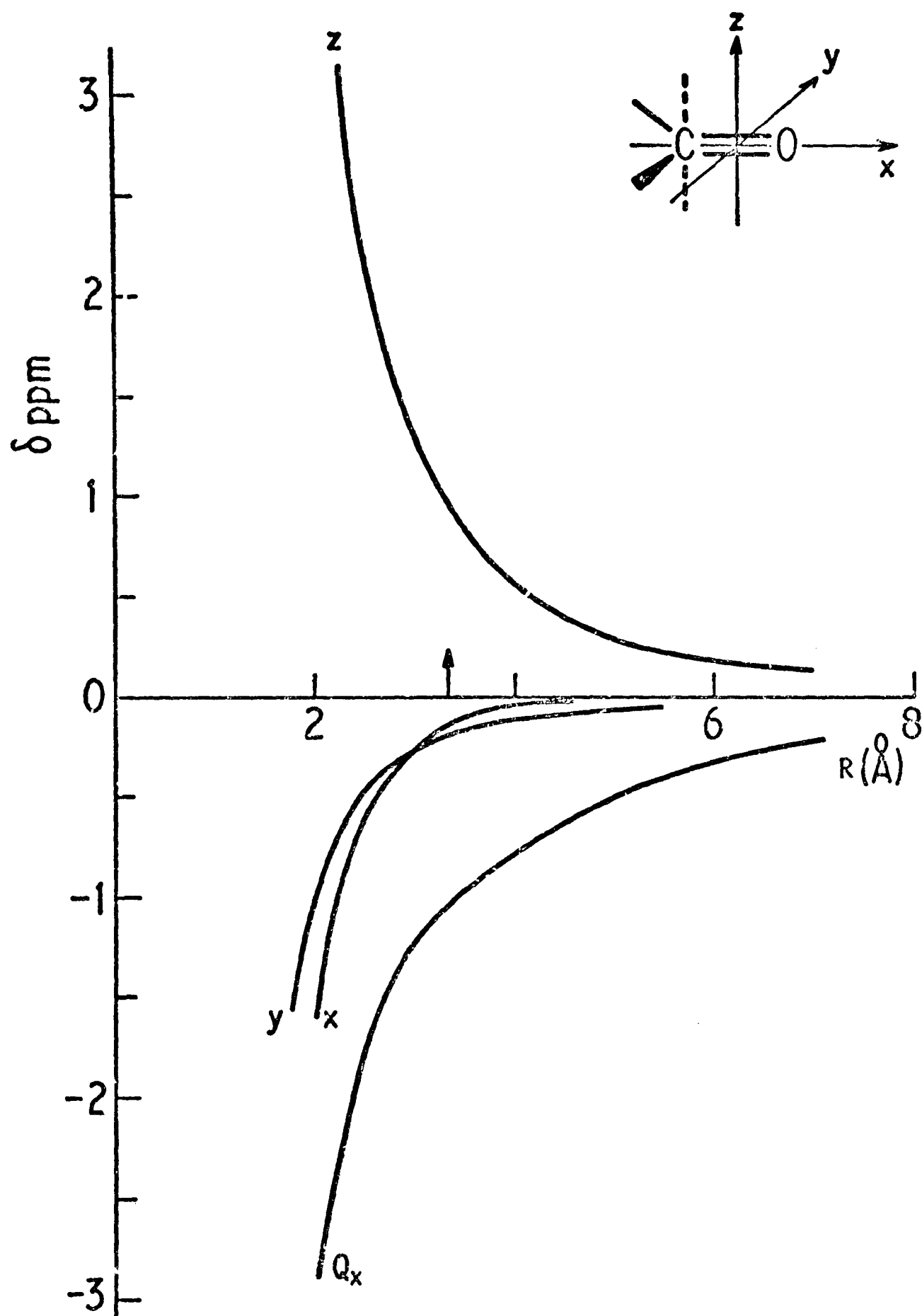
	H_3	H_5	H_6	αCH	βCH_2
pH* 2.1	9.18	8.38	7.15	4.80	3.16
pH* 12.5	9.17	8.35	7.02	4.53	2.85

Sample was 10 mM N-Dnp-L-aspartate in $^2\text{H}_2\text{O}$, at $T = 310^\circ\text{K}$

molecular orbital calculations for electron density distribution in heterocyclic ring systems (Veillard and Pullman, 1961).

In order to estimate the magnitude of charge effects, on the hapten resonances, the effect of the ionization of the carboxyl groups of N-Dnp-L-aspartate on the chemical shifts of the aromatic resonances in the spectrum were determined; the data are shown in Table 7 above. In this compound the average distances between the α carboxyl group and the three protons from the aromatic ring: H_3 , H_5 and H_6 , are 7.0, 6.2 and 4.2 Å respectively. No significant changes in the chemical shifts of the aromatic proton resonances are seen as the carboxyl groups of the compound ionize. It must be pointed out, however, that the effect of this ionization is composed of at least three contributions; a change in the through space chemical shift anisotropy, a change in the charge effect and a change in the bond electron density. The last of these should be insignificant in this system as it falls off rapidly as the number of bonds between the carboxyl group and the ^1H nucleus considered. The relative contributions of the other two mechanisms cannot be experimentally distinguished in this system. The comparison of a large number of model compounds of defined stereochemistry should enable this to be achieved, as the geometric dependence of these effects is different. The calculated geometric dependence

FIG. 14 : The long range shielding from carboxyl and carbonyl groups



The lines x y and z show the effect of the diamagnetic susceptibility along the x y and z axes, using the values for χ_i given by Popie (1962). The line Q_x represent the effect of a single negative charge lying on the x axis calculated using

$$\Delta\delta = -12.5/R^2$$

when $\Delta\delta$ is in parts per million.

of the long range shielding effects due to a negative charge and to a carbonyl group are represented in Fig. 14. The theoretical curves calculated from the first term of equation 15 and the values for the diamagnetic anisotropy of a carbonyl group as defined by Pople (1962), suggest that a carbonyl group could give rise to shifts of approximately $-0.2 - 1$ ppm; whereas a carboxyl group could give rise to upfield or downfield shifts of ± 1 ppm, if the ^1H nucleus were in Van der Waals contact with the carboxyl group, depending on the orientation of the C-H bond with respect to the electric and magnetic fields surrounding the group. It is surprising in the light of these considerations that calculations for the secondary shifts of amino acid residues in proteins, which attempt to predict the values on the basis of ring current effects alone, produce reasonable correlation between predicted and calculated values. This probably reflects the relative infrequency of buried carboxyl groups in proteins, and the fact that most Van der Waals contacts are between amino acid side chains. (Sternlicht and Wilson, 1967; Cowburn et al, 1970; Campbell, Dobson and Williams, 1975; Perkins and Dwek, 1979; Perkins and Wüthrich, 1979.) Sternlicht and Wilson (1967) specifically consider electric field effects in relation to αCH in α helices and give the relation:

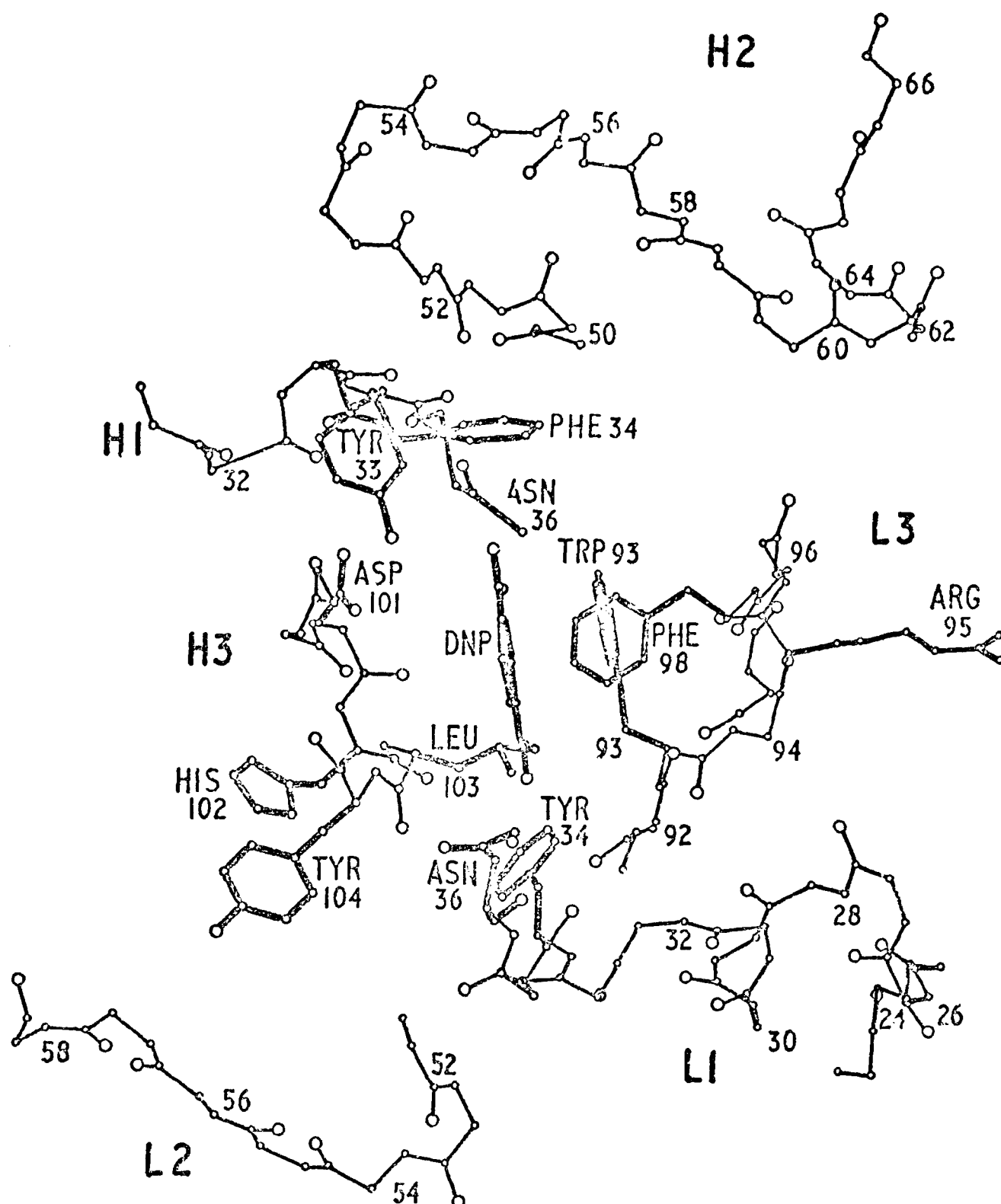
$$\sigma_E = -2.5 \times 10^{-12} E_z \quad 16$$

when E_z is in cgs. They conclude that this effect could be significant for these ^1H nuclei in lysozyme. However, for the methyl groups of aliphatic amino acid side chains they suggest that the major source of secondary shifts is probably the diamagnetic anisotropy of aromatic amino acid side chains. Perkins and Dwek have examined the correlation between observed secondary shifts in lysozyme, and those calculated from four different sets of native lysozyme coordinates using three different ring current equations (Pople, 1956; Johnson and Bovey, 1958; and Haigh and

Mallion, 1971). These calculations reveal that, particularly for some single proton resonances of aliphatic amino acid side chains, the secondary shifts calculated, vary markedly depending on the coordinate set used (e.g. γ CH of ile 98). This again stresses the problems in evaluating secondary shifts in proteins, since the shifts produced by diamagnetic anisotropies are localized and thus the dependence of shift on structure may be below the level of resolution that x-ray diffraction methods can provide. This leads to a further problem, that the time averaged solution structure may differ from that provided by crystallography. Thus McGammon, Gelin and Karplus (1977) have simulated the motion of Bovine pancreatic trypsin inhibitor, using a simple theoretical model and found an average root mean square displacement between the α -carbon coordinates of the time averaged dynamic structure and the crystal structure of c 1Å. This could have significant effects on the predicted n.m.r. spectrum. Further, if the state of the protein in solution is represented by a set of closely related conformations, then the non-linear nature of the magnetic field gradients could lead to a situation in which an ^1H nucleus experiences a large secondary shift in a state with a low population, thus resulting in an underestimate of the distance between it and the source of the shift, if this is not taken into account.

Thus it may be concluded that the interpretation of secondary shift in proteins is highly complex, even if this involves correlation of the secondary shifts of resolved and assigned resonances with those calculated from a known crystal structure. The complications arise from, multiple potential sources for observed secondary shifts, insufficient resolution in crystal structures, possible differences between the crystal structure and the average solution conformation, and effects of chemical exchange between different protein conformations.

FIG. 15 : Predicted combining site of protein MOPC 315



This is obtained from the co-ordinates of Padlan *et al* (1976), which have been very kindly made available by Dr. E. Padlan. The hypervariable loops of the heavy (H) and light (L) chains, and the hapten position relative to the two chains, are indicated.

c) The interpretation of the observed chemical shift changes -
the Dnp subsite

The shift changes for the hapten aromatic ring ^1H resonances observed when di and trinitrophenyl haptens bind to the Fv fragment of protein 315, are invariably large and upfield (Tables 3 and 4; Chapter 5; Wain-Hobson, 1977). The predicted combining site structure of Padlan et al (1976) was investigated to see if this could account for these effects, using a wire model built by C. Wright (1977) from coordinates supplied by E. Padlan (a microfiche copy of which is appended to this thesis). The structure is shown in Fig. 15. The structure prediction of Padlan et al used the evidence from crystal structures available (Chapter 1, Section 6(e)) and the backbone conformations of hypervariable segments of approximately the same length as those from protein 315, as summarized in Table 6 in Chapter 1. The model was initially constructed using these homologies alone and subsequently modified to include the experimental data available on protein 315. The data used were:-

- 1) the affinity labelling studies of Givol and coworkers, which had shown that the distance between the phenolic OH of tyrosine 34_L and the amino group of lysine 52_H was $\sim 15\text{\AA}$, since these residues could be cross linked by α -N-bromoacetyl- γ -N-Dnp-L-diaminobutyric N' bromoacetylhydrazide, in which the distance between the reactive groups was $15\text{\AA}$.
- 2) The close proximity of Tyr 34_L to the site was demonstrated by meta nitrobenzene fluoroborate and N-bromoacetyl-N'-Dnp-ethylene diamine labelling.
- 3) The spectroscopic evidence for the interaction with a Trp residue. This residue was identified as 93_L.
- 4) The overall dimension and polarity of the site as estimated from physical studies.

These data are all discussed in Chapter 1 section 7 and summarized in Chapter 6, Table 1.

The structure of the Dnp binding subsite predicted by Padlan et al, was a generally hydrophobic pocket in which the Dnp ring formed a parallel stacked complex with Trp 93_L, with an interplanar distance of 3.5-4.0Å. This distance seems too large by ~ 0.5Å if the evidence from crystal structures of model complexes is examined (section 3(c)). The other hydrophobic constant residues are leu 103_H and Phe 34_H. The authors suggested that the nitro groups of the hapten are coplanar with the Dnp ring and form hydrogen bonds to Asn 36_L and Asn 36_H. The evidence for coplanarity was provided by crystal structures of dinitrobenzene (Trotter, 1961; Trotter and Williston, 1966), but no evidence was cited to support the assertion that the bound hapten formed hydrogen bonds to groups on the protein. An exception to the generally hydrophobic nature of the site is the presence of an aspartic acid 99_H close to (4.5Å from) the bound Dnp ring.

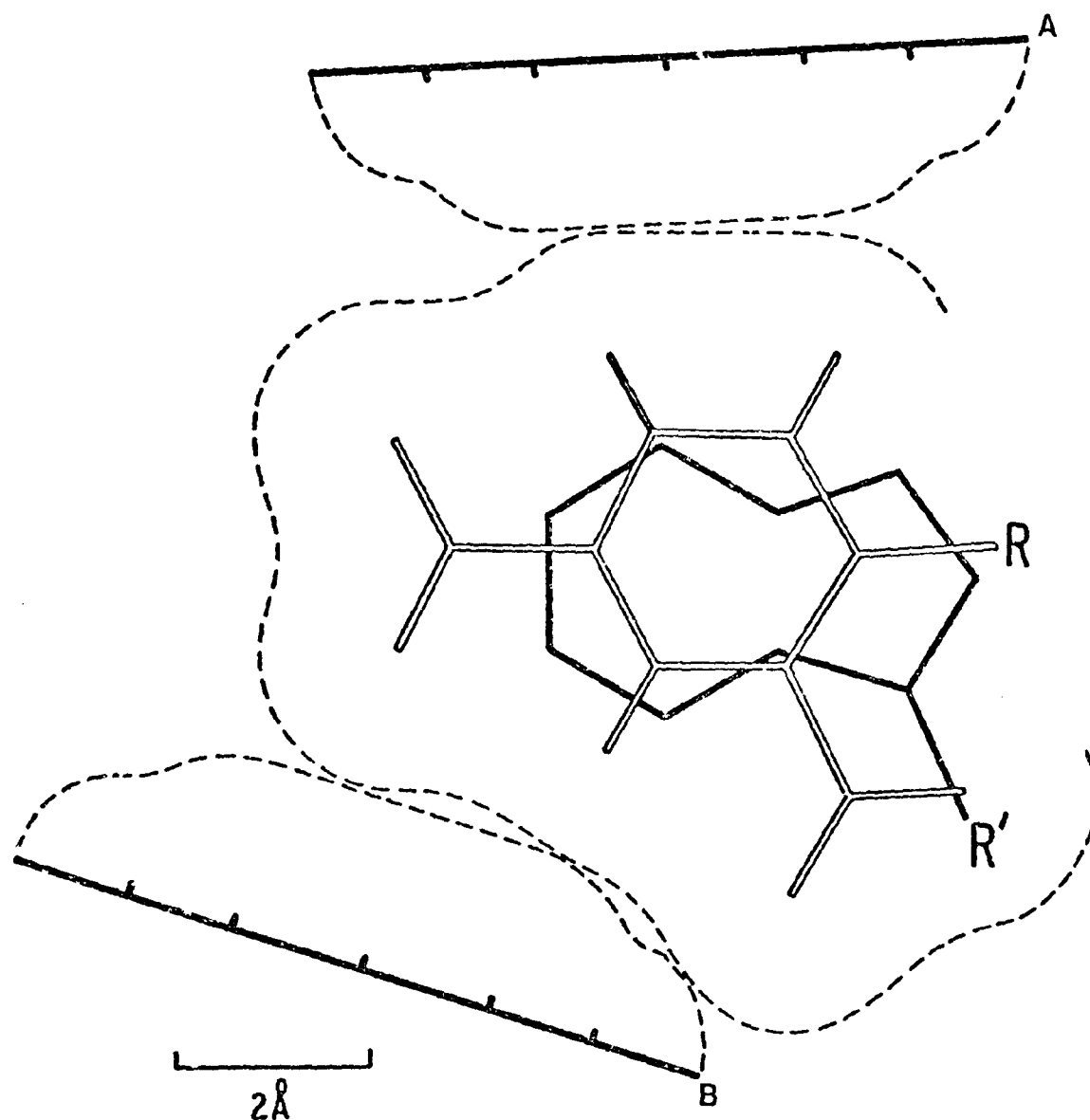
Using this predicted structure the chemical shifts on the Dnp ring ¹H nuclei can be calculated. The two aromatic residues Trp 93_L and Phe 34_H give small upfield shifts (calculated from Johnson-Bovey equation) of 0.37 ppm on H₅, 0.44 ppm on H₆ and 0.025 ppm on H₃. The charge effect of asp 99_H is estimated from the first term of equation 15) to give rise to upfield shifts of c 0.2 ppm on the bound hapten ¹H nuclei. No carbonyl groups lie within 4Å of the Dnp hapten protons, three lie just outside this distance (from Tyr 33_H, asp 101_H and Leu 103_H); the contribution to the shift from these would be small (< 0.1 ppm) (Pople, 1962).

A further possible source of shift changes that must be considered for dinitrophenyl ligands is the distortion of the nitro groups on binding to the protein. It has been shown that these groups are coplanar in solution for 1,3,5 triamino 2,4,6 trinitrobenzene (Cady and Larsen, 1965)

and 2,3,4,6 tetranitroaniline (Dickerson et al, 1966). Comparison of the ^1H n.m.r. spectra of dinitrophenyl, trinitrophenyl and chloronitro anilines and derivatives of these compounds (Gettins, Givol and Dwek, 1978) suggests that nitro groups contribute a shift of 0.6 ppm downfield to the protons ortho to them, when these groups are coplanar with the ring. Thus distortion of these groups on binding could produce upfield shifts of this order. Comparison of the shift changes produced when chloronitro anilines and the N-glycyl derivatives bound to protein 315 did not show any evidence for consistently larger chemical shift changes when the nitro derivatives bound. This shows that nitro group distortion is not a major source of upfield chemical shift change when these haptens bind to protein 315.

In order to account for the observed shift changes on hapten binding, some modification to the predicted structure, is therefore necessary. In doing this the backbone conformations of the hypervariable segments are altered as little as possible from those previously determined in the crystallographic studies, as this structural homology is the fundamental assumption used in the model building procedure. Initially use was made of an uncertainty in the original model building prediction, as to whether the H_3 hypervariable segment was best predicted by the structure of Fab' NEW or that of McPC 603. Padlan et al chose McPC 603, arguing that though the H_3 segment in this latter protein was two residues longer than that in protein 315, the sequences of the C terminal region (positions 103-105) were more similar (protein 315: Leu,-Tyr-Phe McPC 603: Trp-Tyr-Phe) than that of the homologous segment in NEW (Gly-Cys-Ile), in which the H_3 segment is the same length. The model was rebuilt on the basis of the NEW H_3 backbone conformation. This removed Leu 103_H and Asp 99_H from the site and allowed Tyr-34_L to be repositioned under the bound Dnp ring. This would account for one feature of the n.m.r. data, that no consistent

FIG. 16 : Relative geometry of aromatic rings around the dinitrophenyl ring in the combining site of protein MOPC 315



The geometry is calculated on the basis of ring-current effects on the hapten protons, assuming that the dinitrophenyl and tryptophan interaction is as shown in Fig. 10, but with one of the rings rotated through 180°. The van der Waals surfaces of the dinitrophenyl ring and of the two perpendicular aromatic rings are indicated. R and R' represent the side chains of the dinitrophenyl and tryptophan rings. The short vertical lines on the rings A and B represent carbon atom positions for these rings.

TABLE 8 : Comparison of the predicted and observed ring-current shifts (in p.p.m.) in the combining site of protein MU₂PC 315 on addition of Dnp-aspartate based on the structure in Fig. 13

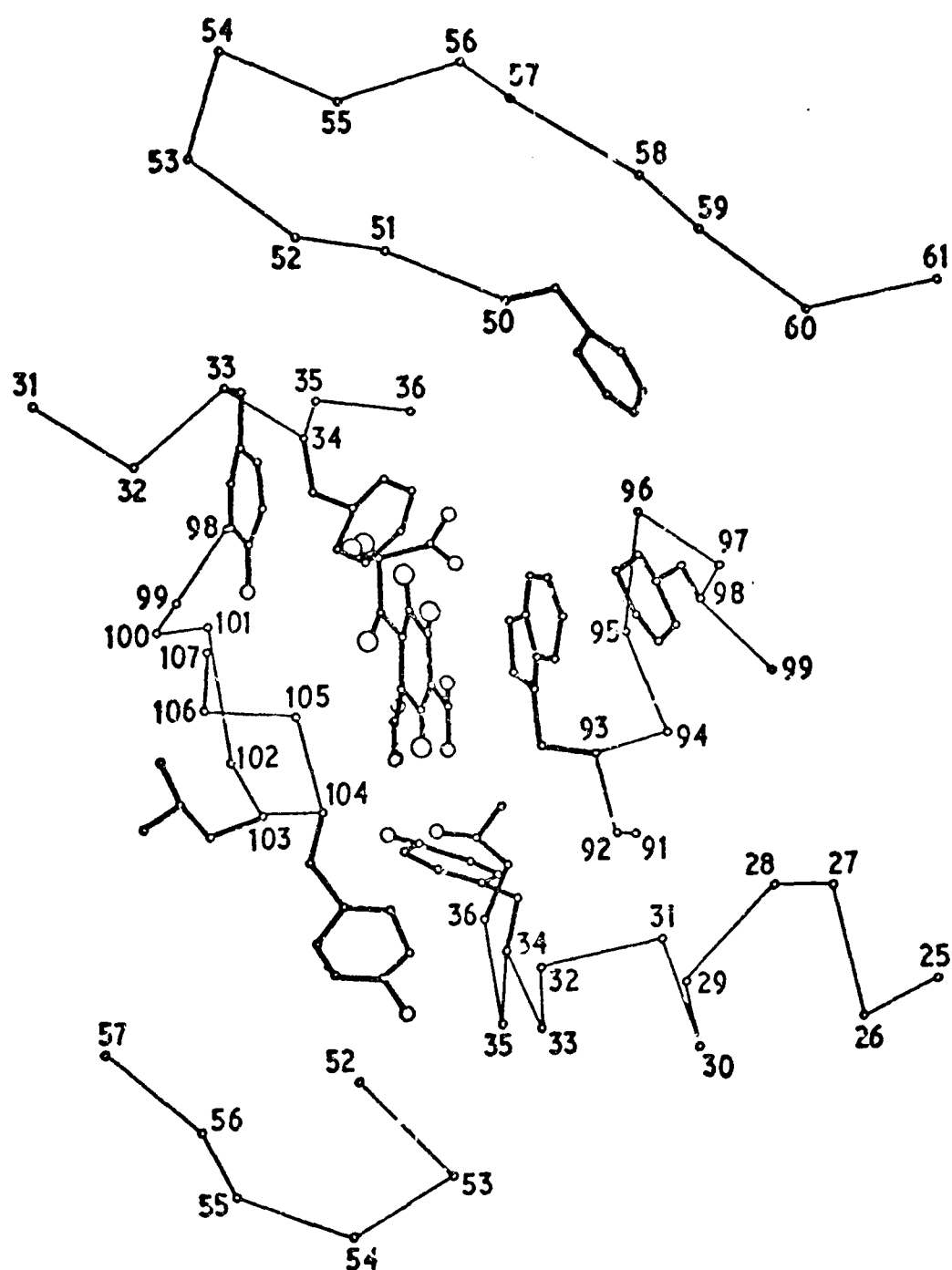
Ring giving rise to shifts ...		Predicted shifts (p.p.m.)					Obs. †
		Dnp	Trp	A	B	Total	
Dnp	H ₍₃₎		0.79	0.80	0.12	1.7	1.68
	H ₍₅₎		0.45	0.17	1.7	2.3	2.3
	H ₍₆₎		0.38	0.08	0.87	1.35	1.31
Trp	H ₍₂₎	0.1				0.1	
	H ₍₄₎	0.3				0.3	
	H ₍₅₎	0.23				0.23	†
	H ₍₆₎	0.27				0.27	
	H ₍₇₎	0.5				0.5	
A*	H ₍₂₎	-0.06				-0.06	
	H ₍₃₎	-0.05				-0.05	
	H ₍₄₎	-0.06				-0.06	
	H ₍₅₎	-0.1				-0.1	
	H ₍₆₎	-0.2				-0.2	
B*	H ₍₂₎	-0.09				-0.09	†
	H ₍₃₎	-0.13				-0.13	
	H ₍₄₎	-0.09				-0.09	
	H ₍₅₎	-0.1				-0.1	
	H ₍₆₎	-0.15				-0.15	

* These rings are as labelled in Fig. 12.

† The protein shifts have not been assigned to particular protons.

perturbation of any aliphatic residues is seen in the ^1H n.m.r. difference spectra, which is at variance with the presence of Leu 103_H as a contact residue for the bound Dnp ring (Fig. 5). A possible alternative explanation for this observation and a caveat is, however, that the Leu 103_H methyl resonances (which would consist of either one 6 proton resonance or two three proton resonances) may be markedly broadened in the Fv fragment ^1H n.m.r. spectrum, and therefore not observable. The modified structure gives Tyr 34_L, Phe 34_H and Trp 93_L as the contact residues for the Dnp ring. If the modified Johnson-Bovey equation (Perkins et al, 1977; Perkins, 1977) is used as a basis for calculation then a structure based on one tryptophan and two phenyl rings, that will give shifts similar to those observed experimentally, can be found. This structure is shown in Fig. 16. No such structure could be found using the Haigh-Mallion treatment (see Appendix I). This is not surprising since this method appears to consistently underestimate upfield shifts in proteins (Perkins and Dwek, 1979). The shift changes predicted from the structure shown in Fig. 16 are given in Table 8. The n.m.r. data thus indicate that from three aromatic amino acid side chains, it is possible to obtain comparable shifts to those observed experimentally. In order to achieve a structure of this type the only change that is necessary to introduce into the model is to rotate the Trp 93_L 180° about the $\text{C}_\beta\text{--C}_\gamma$ bond; this allows the Dnp ring to move under Phe 34_H and minimizes any distortion of the backbone conformation of the protein (see Chapter 6). This structure would predict upfield secondary shifts on the resonances arising from the indole ring of Trp 93_L (Dower et al, 1977). The selective deuteration experiments discussed in section (a) do not support this view. Since a number of complexities (sections(a) and (b)) exist in the interpretation of these data, the orientation of the side chain cannot be established unequivocally. The modified structure is shown in Fig. 17.

FIG. 17 : The combining site of protein MOPC 315



This is based on the n.m.r. data presented in this Chapter.
 The positions of phenylalanine-34_H, tyrosine 34_L, tryptophan-93_L,
 and tyrosine 33_H are obtained from the ring-current calculations.

An alternative structure to that presented in Figure 17 could have been found by a similar movement of the tryptophan but replacing Tyr 34_L by one of the backbone carbonyls in Van der Waals contact with the H₃ nucle. From the values for the diamagnetic susceptibilities of the carbonyl group (Fig. 14) such a conformation would be able to produce a shift similar to that produced by a phenyl or phenoxide ring. If this were attempted then the conformation of the H₃ segment would have to be altered, so that it would be unlike any of the known crystal structures. Further Tyr 34_L would be unable to participate in the site since the L₁ segment would be shielded from the site by the H₃ backbone. This would not be consistent with the affinity labelling studies. The structure shown in Fig. 17, on the other hand, reconciles both the n.m.r. and the chemical modification data.

d) The mode of hapten binding

The three ring structure shown in Fig. 16 is representative of the type of structure that will give rise to the observed shift changes this is a consequence of the qualitative nature of the structural conclusions from the model compound studies (see Fig. 10). Also, it is a consequence of the cylindrical symmetry of the Johnson-Bovey equations that the two six membered rings can be freely rotated about an axis perpendicular to the ring and passing through the centre, and that the tryptophan ring can equally well be placed with the nitrogen atom up or down. It is not theoretically possible to distinguish these conformations. It is a consequence of this that the orientation of the hapten in the combining site shown in Fig. 17 is only one of two theoretically indistinguishable possibilities. In the orientation in which it is shown, it was assumed, following the assertion of Padlan et al that the bound hapten formed hydrogen bonds to Asn 36_L and Tyr 34_L. This

would provide a straightforward explanation for the labelling of this latter residue by MNBDF (Chapter 1, pp.24 and 41). However, work by R. Jackson on the nitro-tyr-34_L-Fv fragment has shown that the affinity of this protein for Dnp haptens is the same (within a factor of 2) as that of the unmodified protein. Further, the pK_a of the nitrotyrosine residue is only slightly affected when Dnp derivatives bind (downwards by 0.3 pH units). No data are as yet available for Tnp derivatives, which, since these have a nitro group in both the 2 and 6 positions might be expected to produce a perturbation. This identifies a particular residue as not forming a hydrogen bond to the hapten. An alternative approach is to determine how many hydrogen bonding interactions (one, two or none) the bound hapten forms, by replacing the nitrogroups with groups known not to form hydrogen bonds. Thus, Gettins, Givol and Dwek (1978) replaced the nitrogroups with chlorine, and showed that the substitution did not affect the binding constant of the haptens for free tryptophan. The affinities of the haptens for the Fv fragment, however, are reduced by a factor of c 10-fold when either nitrogroup was replaced. In a similar study Kooistra and Richards (1978) observed a similar fall in affinity. The latter group specifically interpreted their data in terms of a loss of hydrogen bonding interactions. There is, however, a flaw in this argument. If a group can hydrogen bond in the protein, then it should also do so in water. In consequence, replacing a group that is assumed to hydrogen bond in both environments by one that does so in neither, and which contributes to the total binding energy by this interaction, may well not affect the affinity of the hapten for the protein. Thus, while hydrogen bonding may contribute to the binding energy, there is no direct evidence for this. The observed changes in affinity could equally well arise from changed Van der Waals interactions.

Evidence from comparative binding studies also bears on the question of possible hydrogen bonding interactions, since the structural constraints

imposed by hydrogen bonding are strict (Pauling, 1960 : O-----H bond length 2.45-2.75Å). The n.m.r. data (Table 4) presented in this Chapter show that two Dnp haptens with different side chains will bind differently, but (Appendix II, Fig. 18) these differences in the fully bound chemical shift changes could be accommodated within the tolerances set by the hydrogen bonds, if the shift changes are interpreted in terms of the Johnson Bovey equation. The shift changes for the Tnp derivatives also lie within this range (Chapter 5). Kumar et al (1978) have examined the mode of interaction of the nitro groups of Dnp derivatives with the protein using resonance raman spectroscopy (Chapter 1, sections 6(d) and 7) and conclude that the mode of interaction of the Dnp group with the protein changes as the side chain is changed. No structural information can be obtained from these data, however, since the theoretical basis of changes in resonance raman spectra is not well understood (Carey, 1978).

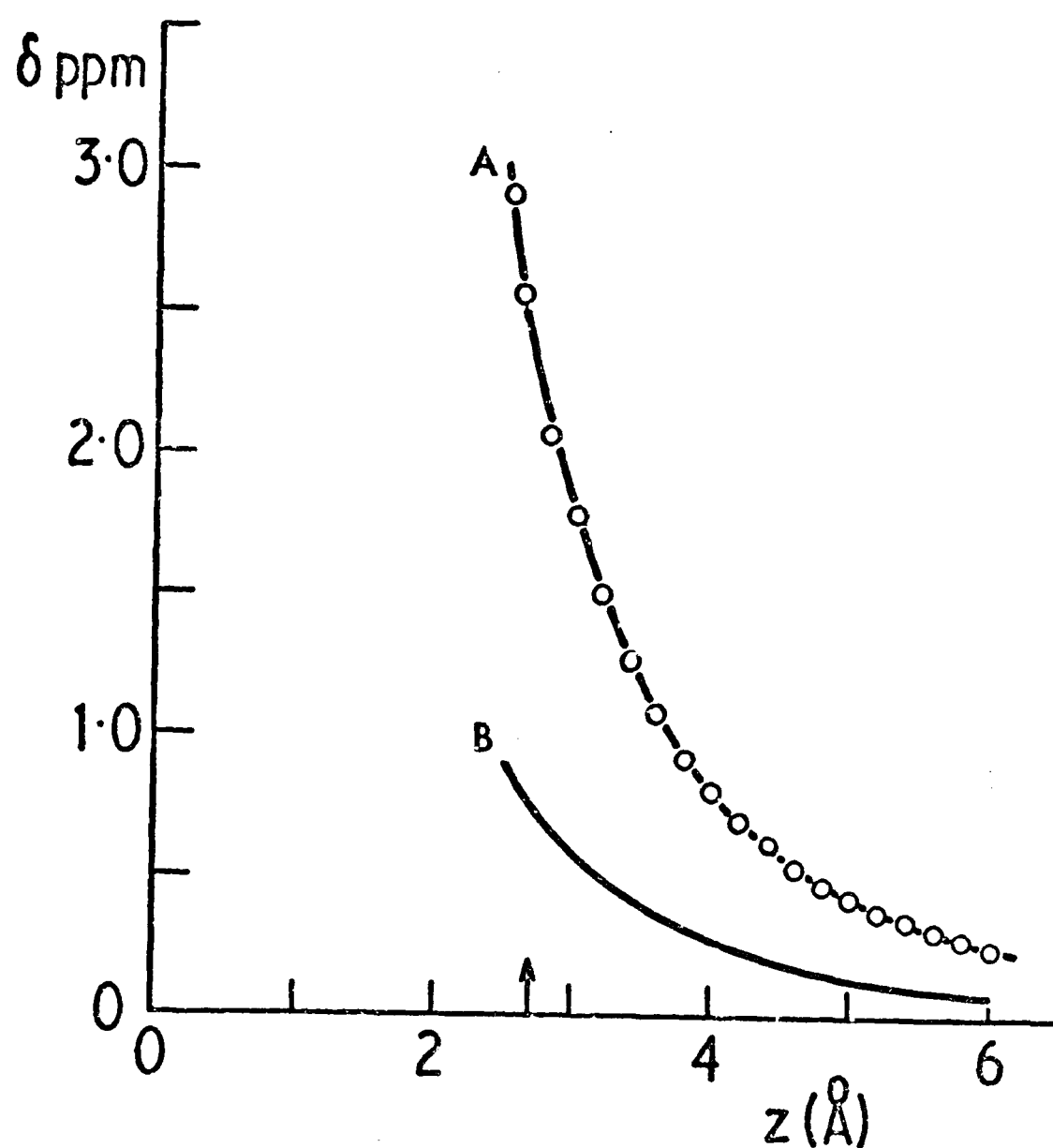
The large shifts upfield changes for the hapten αCH_2 side chain resonances (Table 4; see also Chapter 5) suggest that this part of the hapten molecule also lies close to an aromatic ring or other source of shift perturbation in the protein. If the hapten -NH- group forms an internal hydrogen bond to the 4-nitro group (see section (c) and Padlan et al (1976)) then in the protein-hapten complex the mode of binding shown in Fig. 17 places this group close to Tyr 33_H. The position of this residue is unchanged from that in the original structure, since the conformation of the H₁ hypervariable segment has not been changed. That this part of the hapten is close to the Tyr 33_H is also suggested by the observation that N-Tnp-glycine binding to nitro Tyr-33_H Fv (Jackson, unpublished data) perturbs the pK_a of the nitrotyrosine ionization upwards by 0.6 pH units. Some caution must be exercised in interpreting this result as N-Dnpglycine does not perturb this ionization.

It is concluded that the predicted structure of Padlan et al, is not

consistent with the n.m.r. data in this chapter. However, by initially taking an alternative structure suggested by these authors and minimally modifying this, a structure consistent with the n.m.r. data may be obtained. The final structure is shown in Fig. 17 and the coordinates are given inside the back cover of this thesis. This structure cannot be taken to be definitive in the sense that a protein structure determined by X-ray crystallographic analysis would be. This arises primarily from the assumptions inherent in the model building procedure, and from the reliance that must be placed on this structure in interpreting the n.m.r. data. The mode of hapten binding deduced from the n.m.r. studies places the Dnp group of the hapten in a hydrophobic pocket in contact with the side chains of Tyrosine 34_L, Trp 93_L and Phe 34_H. The -NH-CH- group of the hapten is close to a residue from the H1 hypervariable segment Tyr 33_H. The structure also places the 4-nitro group of the hapten Dnp ring close to Asn 36_L. There is no direct evidence to support the view that the nitro groups form a hydrogen bond interaction.

Finally comparison of the mode of binding of several different haptens, including menadione and compounds other than derivatives of Dnp and Tnp, suggest that all these interact with the same subsite on the protein (Wain-Hobson, 1977).

FIG. 18 : The long range shielding on the z axis from a phenyl ring



A -O- calculated from the Johnson-Bovey equation;

B — calculated from the Haigh-Mallion treatment.

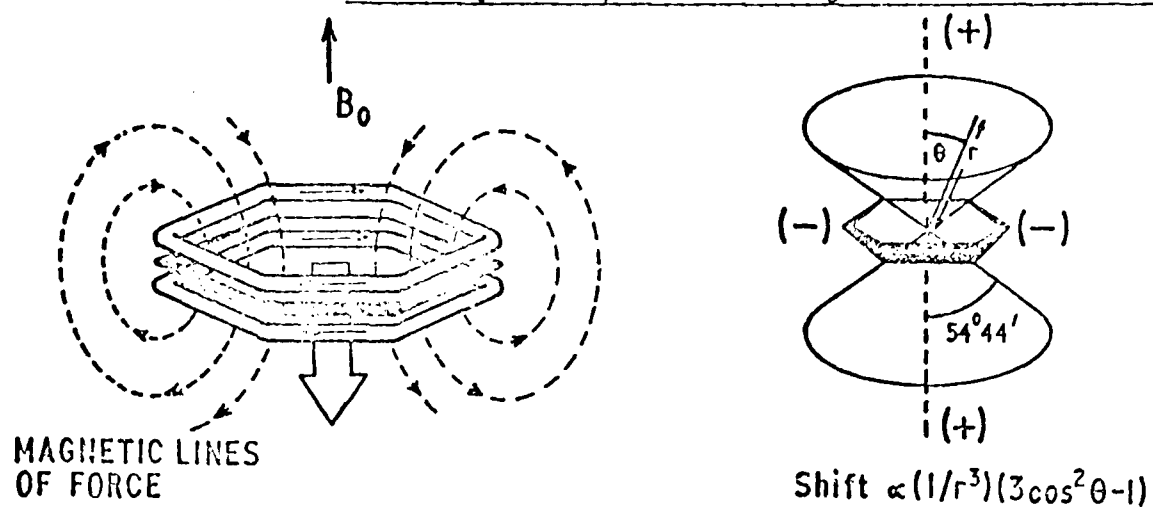
The z-axis is that axis which passes through the centre of the phenyl ring and is perpendicular to the plane of the ring.

Appendix I : Ring Current Calculations

Three theoretical treatments exist for the calculation of long-range shielding effects by aromatic ring systems (Fig. 18(a)). That of Pople (1956) represents the π electron circulation as a dipole located at the ring centre. The treatment of Johnson and Bovey (1958) describes the π electron circulation as two current loops located above and below the ring plane. The treatment of Haigh and Mallion (Haigh, Mallion and Armour, 1970; Haigh and Mallion, 1971, 1972; Mallion, 1971) is based on an extension of McWeeny's (1958) ring current theory. These both use Hückel theory to evaluate the ring current. In the first and last cases a scaling factor is introduced by determining the shift changes due to the ring current in the chemical shifts of the ring-linked ^1H nuclei. That these scaling factors may be inadequate has been suggested by Perkins and Dwek (1979), who have shown that all three calculations underestimate the observed secondary shifts in lysozyme. The interpretation of the model compound and protein data in this chapter was based on a modified form of the Johnson Bovey equation, since of the two more sophisticated treatments this gives better estimates for the upfield shifts due to aromatic ring systems (Fig. 18). The original treatment was modified using scaling factors for the ring current strength calculated from self consistent field theory (Giessner-Prettre and Pullman, 1969) as discussed in Perkins et al (1977). The long range shielding of the dinitrophenyl and trinitrophenyl groups was approximated as that due to benzene. Thus no attempt was made to interpret the shielding effects of these groups quantitatively, as the nitro substituent can be expected to change the ring current of the benzenoid ring, and also contribute further long range shielding.

It is a consequence of the form of the Johnson Bovey equation that

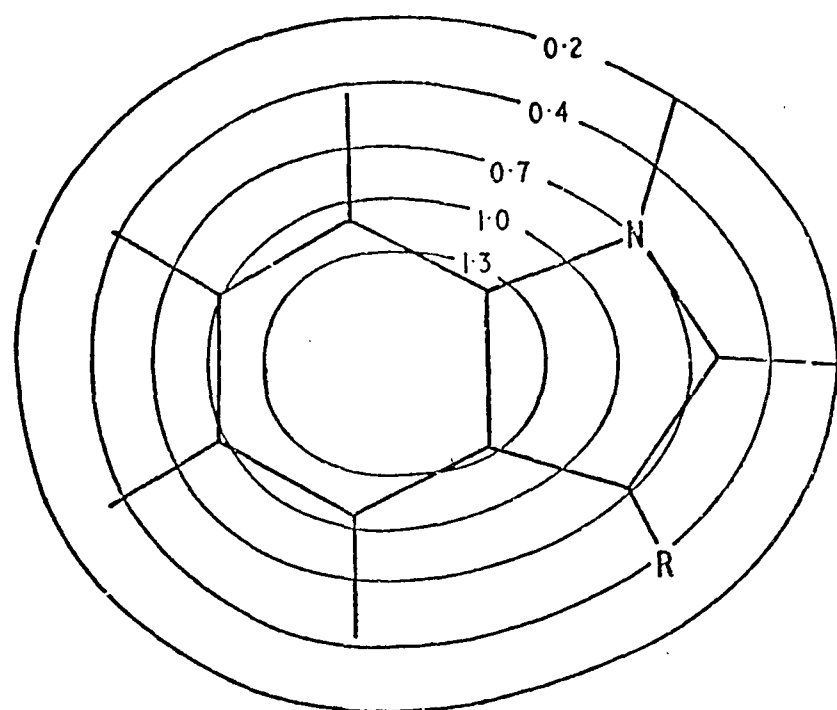
FIG. 19(a) : Shielding envelope for the ring-current shifts from a benzene ring



The cone separates the shielding (+, upfield-shifted) and deshielding (-, downfield-shifted) regions. The z direction is along the hexagonal axis of the benzene ring, and ρ is the direction in the plane of the carbon atoms.

:

FIG. 19(b) : Long range shielding from an indole ring



The diagram shows the chemical shift change contours (in ppm) in a plane 3.3 Å above the ring and parallel to it calculated from the modified Johnson-Bovey equation as described in the text.

all the ring systems treated, except tryptophan, give rise to cylindrically symmetrical long range shielding. This limits the structural inferences that can be made on the basis of ring current theory. The long range shielding from a tryptophan residue in a plane $3.3\text{\AA}$ above the ring and parallel to it is shown in Fig. 19.

The calculated ring current field was used to determine the structures of complexes of aromatic ring systems either by use of a FORTRAN program RSEARCH written by S. Perkins (Perkins et al, 1977, Perkins, 1977) or by direct calculation using tables compiled by JTAB also written by S. Perkins (Perkins et al, 1977).

Appendix II : Linewidth Calculations

In order to determine at what point a broad resonance undergoing a small shift change might be expected to contribute negligible intensity to a ^1H n.m.r. difference spectrum, the intensity of a difference spectrum obtained from a Lorentzian line undergoing a shift change was calculated. The equation describing a Lorentzian line shape is:-

$$g(\nu)^0 = \frac{2\Delta\nu_{\frac{1}{2}}}{\pi} \frac{1}{\Delta\nu_{\frac{1}{2}}^2 + 4\nu^2}$$

this is subtracted from:-

$$g(\nu)^1 = \frac{2\Delta\nu_{\frac{1}{2}}}{\pi} \frac{1}{\Delta\nu_{\frac{1}{2}}^2 + 4(\nu - p\Delta\nu_{\frac{1}{2}})^2}$$

when ν is frequently in Hz $\Delta\nu_{\frac{1}{2}}$ is the linewidth at half height in Hz and $p\Delta\nu_{\frac{1}{2}}$ is the shift change in Hz.

A computer program INTCAL was used to calculate the difference spectrum intensity of the resonances. Each was truncated so that when

$$\frac{g(\nu)}{g(\nu)_{\max}} < 0.1$$

$$g(\nu) = 0$$

to allow for some signal to noise in the spectrum. The results of this calculation are shown in Table 9. It can be seen from Table 9 that the loss of intensity becomes significant in the range $\Delta\nu_{\frac{1}{2}}$ 12-20 Hz, the expected range of linewidths for resonances in the Fv fragment spectrum, when $\Delta\delta_0$ is less than 0.1 ppm.

APPENDIX III: Computer Programmes

BIND

```

        DIMENSION H(100), HT(100)
        DIMENSION TITLE (5), F(100), G(100), XB(100)
        DIMENSION DOBS(100)
        READ(1,50) NP, DDO, TP, TITLE
50      FORMAT(13, F10. 5, F10. 5, A6)
        READ(1,51) C
51      FORMAT(F10. 5)
        DO 100 I=1, NP
        READ(1, 55) HT(1)
55      FORMAT(F10. 5)
100     CONTINUE
        WRITE(2,70) TITLE
70      FORMAT(1X, 5A6)
        DO 200 I=1, NP
        F(I) = 1. 0+(HT(I)/TP)+(C/TP)
        G(I) = F(I) ** 2-4. 0 * (HT(I)/TP)
        H(I) = SQRT(G(I))
        XB(I) = (F(I)-H(I))/20
        DOBS(I) = DDO * XB(I)
        WRITE(2,60) HT(I), XB(I), DOBS(I)
60      FORMAT(1X, F10. 5, 2X, F10. 5, 2X, F10. 5)
200     CONTINUE
        STOP
        END

```

TWO SITE

```

50      CONTINUE

        READ(1,100) AT, BT, C1, C2

100     FORMAT (1X,4F10.5)

        WRITE(2,100) AT, BT, C1, C2

        IF(AT) 9999, 9999, 120

120     AB2=0

150     ABOLD=AB2

        ALPHA=C1

        BETA=C1 * (3.0 * ABOLD-AT-BT) -1.0

        GAMMA=C1 * (AT*BT+2.0*ABOLD*ABOLD-BT*ABOLD-2.0*AT*ABOLD)

        ETA=BETA ** 2-4.0 * ALPHA * GAMMA

        IF(ETA) 900, 199, 199

199     CONTINUE

        WRITE(2,85)

        ABP=(-BETA+SQRT(BETA**2-4.0*ALPHA*GAMMA))/(2.0*ALPHA)

85      FORMAT(/)

        ABM=(-BETA-SQRT(BETA**2-4.0*ALPHA*GAMMA))/(2.0*ALPHA)

        IF(AT-ABP) 200, 300, 300

300     IF(ABP) 200, 200, 250

200     AB=ABM

        GO TO 400

250     AB=ABP

400     AB2=(C2*AB*AT-C2*AB*AB)/(1.0+2.0*C2*AB)

        DIFF=ABS((AB2-ABOLD)/AB2)

        IF(DIFF-0.001) 500, 500, 150

500     WRITE(2,600) AB, AB2

600     FORMAT(1X,3HAB=,1X,E10.5,4X,4HAB2=,1X,E10.5)

900     CONTINUE

        GO TO 50

9999    STOP

```

INTCAL

```
DIMENSION DD(100)
DIMENSION A(100)
DIMENSION DV(100)
DO 100 I=1, 7
  XI=I
  DV(I)=8. 0+XI * 4
100  CONTINUE
  WRITE(2,101), (DV(I), I=1, 12)
101  FORMAT(8X,12F7. 3)
  DO 400 J=1, 10
    XJ=J
    DD(J) = XJ/20
    DO 200 I=1, 7
      A(I)=(ATAN(3.)-ATAN(3.-540.*DD(J)/DV(I)))/ATAN(3.)
      IF(A(I). GT. 2) A(I) = 2
200  CONTINUE
    WRITE(2,300) DD(J), (A(I), I=1, 12)
300  FORMAT(1X, F7. 3, 12F7.3)
400  CONTINUE
  STOP
  END
```

Chapter 3

HIGH RESOLUTION ^1H N.M.R. STUDIES ON THE FV FRAGMENT OF PROTEIN 315: THE USE OF A PARAMAGNETIC CENTRE TO OBTAIN DISTANCE INFORMATION

1. Introduction

The considerable difficulties encountered in obtaining structural information from the ^1H n.m.r. studies described in Chapter 2, arise partly from the poorly resolved spectrum of this protein and partly from the complexity of the origins of secondary shifts in proteins. In the light of this latter consideration a search has been made for suitable paramagnetic probes with which to obtain structural information from the Fv fragment n.m.r. spectrum. If a single extrinsic paramagnetic centre can be introduced into a protein molecule, then it will produce paramagnetic effects that dominate the relaxation behaviour of the system. The analysis of these effects can be treated theoretically, to determine distances of perturbed nuclei from that centre (Dwek, 1973). In the Fv fragment system use was made initially of spin labelled haptens, since the location of the paramagnetic centre could be approximately determined as being in the region of the combining site (Dwek et al, 1975a,b). These haptens were used to obtain the ^1H n.m.r. sub-spectrum of the combining site region of the Fv fragment. An example of these results is shown in Chapter 4, Fig.3. All these spin label derivatives, however, bound tightly to the Fv fragment and could thus not be used for determining distances, since the system exhibited slow exchange conditions on the n.m.r. time scale.

Paramagnetic metal ions, however, can also provide useful probes for obtaining structural information by n.m.r. methods (Dwek, 1973, Campbell and Dobson, 1978). Two series of metal ions have proved useful for this

purpose, transition metal ions (e.g. Mn(II) and Co(II)), and the lanthanide ions. The lanthanides are particularly useful for physical studies as these substitute isomorphously for one another and yet show a wide range of spectroscopic properties. Thus Gd(III) is a n.m.r. broadening probe, Pr(III) is mainly a n.m.r. shift probe whilst Tb(III) is a fluorescence probe. The binding of lanthanides to non immune rabbit IgG and its fragments (Dower et al, 1975) and to the Fv fragment of protein 315 (Dwek et al, 1975a,b) has been studied. It was found that there was only one lanthanide binding site on the Fv fragment of protein 315. The binding of La(III) (Dwek, et al, 1976) and Gd (III) (Dwek et al, 1976; Sutton et al, 1977) to the Fv fragment was investigated further in order to determine whether this paramagnetic centre could prove useful for structural studies. These investigations, however, revealed that the metal ion bound tightly to the protein (10 μ M at pH 7.0) and induced a conformational change in the hapten binding site, possibly altering the structure of this and causing a marked increase in the hapten dissociation constants (20 fold) (Dwek et al, 1976). The use of lanthanides as paramagnetic probes in this system is therefore clearly limited. Thus both in the case of the spin label haptens and the lanthanide ions, the slow exchange conditions preclude the estimation of the paramagnetic broadening of individual ^1H resonances. Therefore, the difference spectra obtained (Dwek et al, 1975; Dwek et al, 1976) only allow an upper limit to be set for the distance of many protons from the paramagnetic centre.

In order to obtain quantitative data from this system the use of divalent metal cations was investigated. It was found that Mn(II) did not bind to non-immune rabbit IgG and its fragments (Dower, 1975). Nor could any Mn(II) binding to the Fv fragment of protein 315 be detected (see below). It was therefore decided to create a specific metal ion binding site on a Fv fragment-hapten complex by the use of suitable Dnp derivatives. In

the experiments reported in this chapter a series of oligophosphate derivatives of N-Dnp-2-aminoethanol have been synthesized. These bind tightly to the Fv fragment and thus provide a specific manganese binding site on the Fv fragment-hapten complex. Additionally, these Dnp derivatives also have the advantage that they can be used as ^{31}P n.m.r. probes, for detecting the presence of charged residues in the protein combining site (Chapter 4).

2. Materials and methods

a) High resolution ^1H n.m.r. studies

These were carried out as described in Chapter 2.

b) Fluorescence measurements

These were carried out as described in Chapter 2, except that in all cases the titrations were carried out at pH 7, the pH as that used for the high resolution ^1H n.m.r. studies. Protein concentrations were $0.5\ \mu\text{M}$.

c) Preparation of samples

Samples for fluorescence and ^1H n.m.r. were prepared as described in Chapter 2. Samples for manganese binding were prepared as described in section (c).

Preparation of N-Dnp-2-aminoethyl Phosphates

i) Preparation of N-Dnp-2-aminoethyl phosphate:

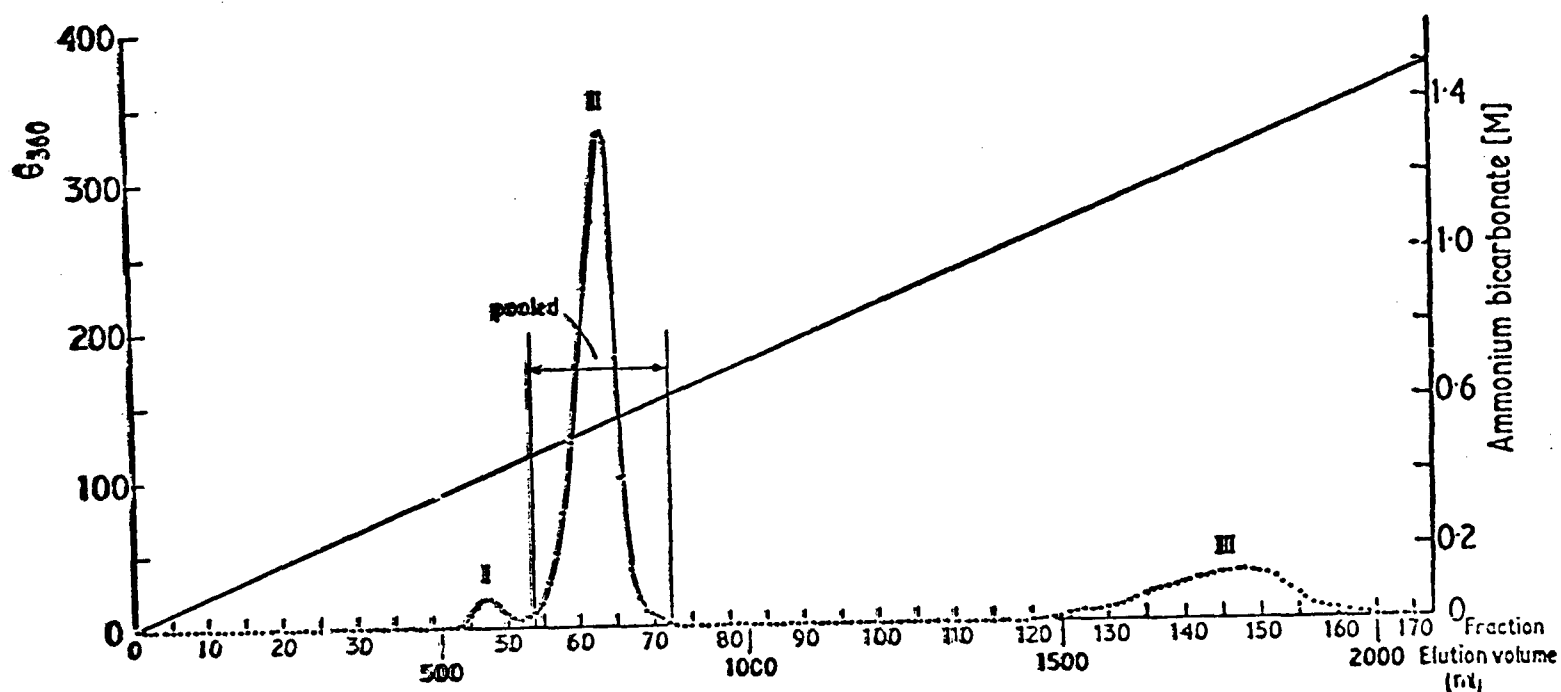
0-phosphorylethanol-2-amine (3.5 mmoles, 0.5g) (Calbiochem, Hereford, U.K.) was reacted with 2,4-dinitrofluorobenzene (3.25 mmoles, 0.6g) (Aldrich Chemicals, Gillingham, Dorset) in 16ml of 0.2M NaHCO_3 . Dinitrofluorobenzene dissolved in 12ml of dioxan was added dropwise over a period of 1 hour to a solution of 0-phosphorylethanol-2-amine at 37°C. The reaction was continued until the solution became clear.

The pH of the solution was adjusted to 1 with concentrated hydrochloric acid, the acid solution was then extracted rapidly with 5 x 1 volumes of ethyl acetate. The ethyl acetate solution was dried over sodium sulphate, and the ethyl acetate was removed by rotary evaporation. The yellow solid was redissolved in a minimum volume of water and lyophilized overnight. Yield 0.89g, 90% initial Dnp. Purity was checked by ^1H n.m.r, ^{31}P n.m.r. (Table 1) and by thin layer chromatography on polyamide sheets (B.D.H. chemicals, Poole, Dorset) in 1:2:17, 0.880 ammonia, water, propan-2-ol, in which the R_f value was 0.74. Analysis: calculated for, $\text{C}_8\text{H}_{10}\text{N}_3\text{O}_8\text{P}$; C, 31.3; H, 3.25; N, 13.7; P 10.1; found; C, 31.2; H, 3.27; N 13.5; P, 9.9.

ii) Preparation of N-Dnp-2-aminoethyldiphosphate and N-Dnp-2-aminoethyltriphosphate.

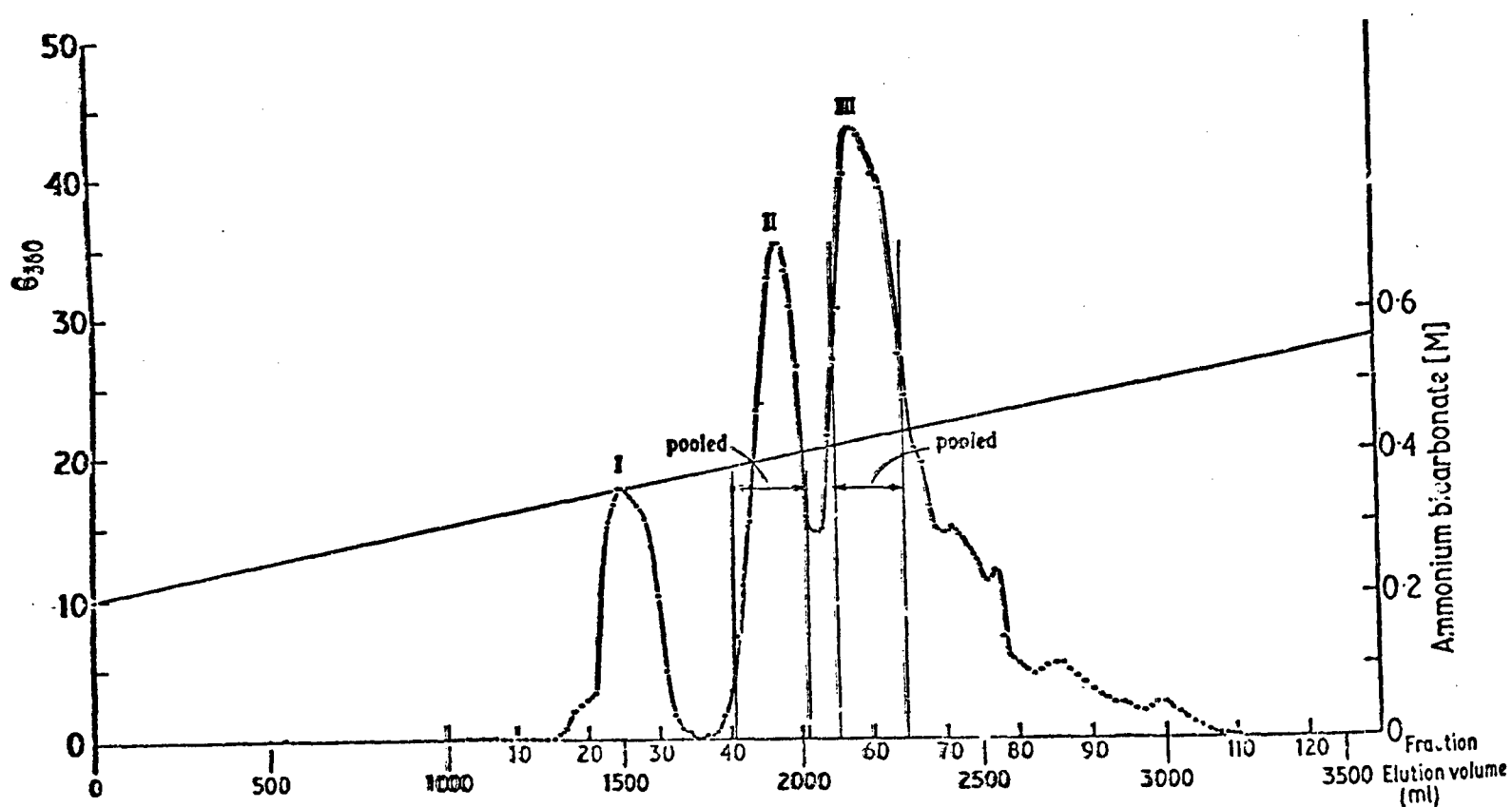
The method is based on a preparation of adenosine-5'-triphosphate by Smith and Khorana (1957). N-Dnp-2-aminoethylphosphate (2 mmoles, 0.614g) (prepared as above) was condensed with orthophosphoric acid (20 mmoles, 2.32g) using dicyclohexylcarbodiimide (100 mmoles, 20.6g) (Aldrich Chemicals, Gillingham, Dorset), for 40-50 hours at 20°C. After reaction, the pyridine solution was filtered to remove dicyclohexylurea, which was washed with water. The aqueous filtrate and washings were extracted

FIG.1: First Ion Exchange Separation Step



I - Dnp ethyl(P), II - Dnp ethyl(P)_n, III - (Dnp ethyl(P))₂. Conditions as in text

FIG.2: Rechromatography of Fraction II: Second Step



I - Dnp ethyl (P), II - Dnp ethyl(P)₂, III - Dnp ethyl(P)₃. Conditions as in text

with 3x70ml of diethyl ether to remove pyridine, and the aqueous phase made up to 100ml with water. The ether washings were discarded and the aqueous solution lyophilised overnight. The yellow solid was redissolved in water (100ml) and allowed to stand overnight to hydrolyse any residual dicyclohexylcarbodiimide. (Recovery 1.97 mmoles Dnp, 99% initial Dnp.) The solution was filtered and applied to a 2.8x17cm Poropak Q column, prepared for use by washing with acetone, ethanol and water (Niederwieser, 1971). Inorganic phosphates were eluted with water and the effluent tested for phosphate by ammonium molybdate precipitation. When no phosphate was present in the effluent the organic phosphates were eluted with 4:1 acetone: water. Acetone was removed from the eluant by rotary evaporation and the aqueous residue made up to 50ml with water. (Total fraction of initial Dnp recovered - 1.64 mmoles, 82%). The solution was applied to a 3.3x35cm DEAE sephadex A25 column. The Dnp phosphates were separated with a 2 litre 0.0-1.5M ammonium bicarbonate (pH 7.8) gradient at a flow rate of 100 mls/hr. (Fig.1). The fractions indicated were pooled, desalted using the Poropak Q column, and, after removing acetone, lyophilised overnight to remove any residual ammonium bicarbonate. The yellow solid (constituting 1.04 mmoles Dnp, 52% initial) was redissolved in 50ml of 0.1M ammonium bicarbonate, applied to the DEAE sephadex column, and eluted with a 4 litre 0.1-0.6M ammonium bicarbonate gradient (Fig.2), at a flow rate of 100 ml/hr. Fractions were pooled as indicated, desalted using the Poropak Q column procedure, and lyophilised. Yield: N Dnp-2-aminoethyl diphosphate 0.14g, 16% initial ; N-Dnp-2-aminoethyltriphosphate 0.26g, 24% initial. Analysis: triammonium N-Dnp-2-aminoethyldiphosphate monohydrate; calculated for $C_8H_{22}N_6O_{12}P_2$; C, 21.04 ; H, 4.82 ; N, 18.4 ; P, 13.6 ; found C, 21.04 ; H, 4.79 ; N, 17.7 ; P 13.5. Tetraammonium N-Dnp-2-aminoethyl triphosphate dihydrate ; calculated for $C_8H_{26}N_7O_{15}P_3$; C, 16.8 ; H, 4.6 ; N, 17.1 ; P, 16.3 ; found ; C, 16.8 ; H, 4.7 ; N, 16.8 ; P 16.5. The purity

ABLE 1 : ^1H and ^{31}P n.m.r. data for 2,4 Dinitrophenyl-2-aminoethyl phosphate derivatives

a) ^1H chemical shifts (ppm)

Compound	H_3	H_5	H_6	1CH	2CH
I	9.17	8.36	7.28	3.85	4.1
II	9.16	8.36	7.31	3.87	4.24
III	9.17	8.37	7.31	3.89	4.30

b) ^1H coupling constants (Hz)

	$J_{\text{H}_3\text{H}_5}$	$J_{\text{H}_5\text{H}_6}$	$J_{\text{H}_1\text{P}}$	$J_{\text{H}_1\text{H}_2}$
I	NS	9.8	5.4	5.4
II	NS	9.8	NS	NS
III	NS	9.8	7.8	4.3

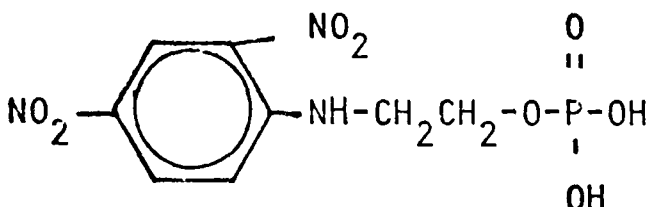
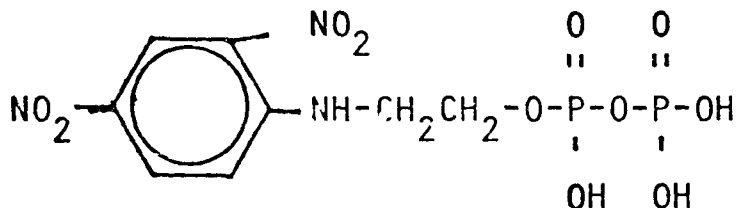
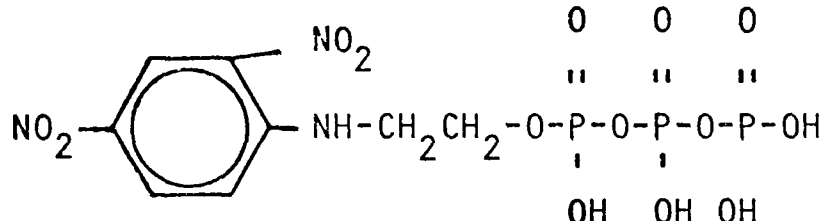
c) ^{31}P chemical shifts (ppm)

	α	β	γ
I	0.75	-	-
II	10.69	9.95	-
III	10.59	22.33	10.15

- ^1H chemical shifts were measured at 270MHz from DSS as an external standard. All samples 2mM in $^2\text{H}_2\text{O}$, pH* 7.1, 0.15M NaCl, 303°K.
- ^{31}P chemical shifts were measured at 36.43MHz from 85% phosphoric acid as an external standard. All samples 5mM, in $^2\text{H}_2\text{O}$: $^1\text{H}_2\text{O}$; 1:4, pH 5.0, NaCl 0.15M, EDTA 80mM, at 303°K.
- For compounds refer to Table 2.
- NS: no coupling observed.

was checked by thin layer chromatography as described above; R_f values; diphosphate derivative 0.56, and triphosphate derivative, 0.22; ^1H n.m.r. and ^{31}P n.m.r. data were also obtained (Table 1). The absorbances of these compounds have been determined at 360 nm. (Table 2.)

TABLE 2. Molar Absorbances of N-Dnp-2-aminoethylphosphates

	Hapten	ϵ_{360}
I	 N-Dnp-2-aminoethylphosphate	17.2×10^3
II	 N-Dnp-2-aminoethyldiphosphate	16.7×10^3
III	 N-Dnp-2-aminoethyltriphosphate	17×10^3

1)

I Absorbances given are for a 1M solution, pH7.0, 1.5M NaCl, in 50mM pipes buffer at 297°K, and 360nm.

2) In Figure 1. Dnp Et(P) is compound I
 (Dnp Et(P))₂ is bis (N-Dnp-2-aminoethyl) pyrophosphate.

Dnp Et(P)_n is a mixture of N-Dnp-2-aminoethyloligophosphates

Dnp Et(P)₂ and Dnp Et(P)₃ are compounds II and III respectively

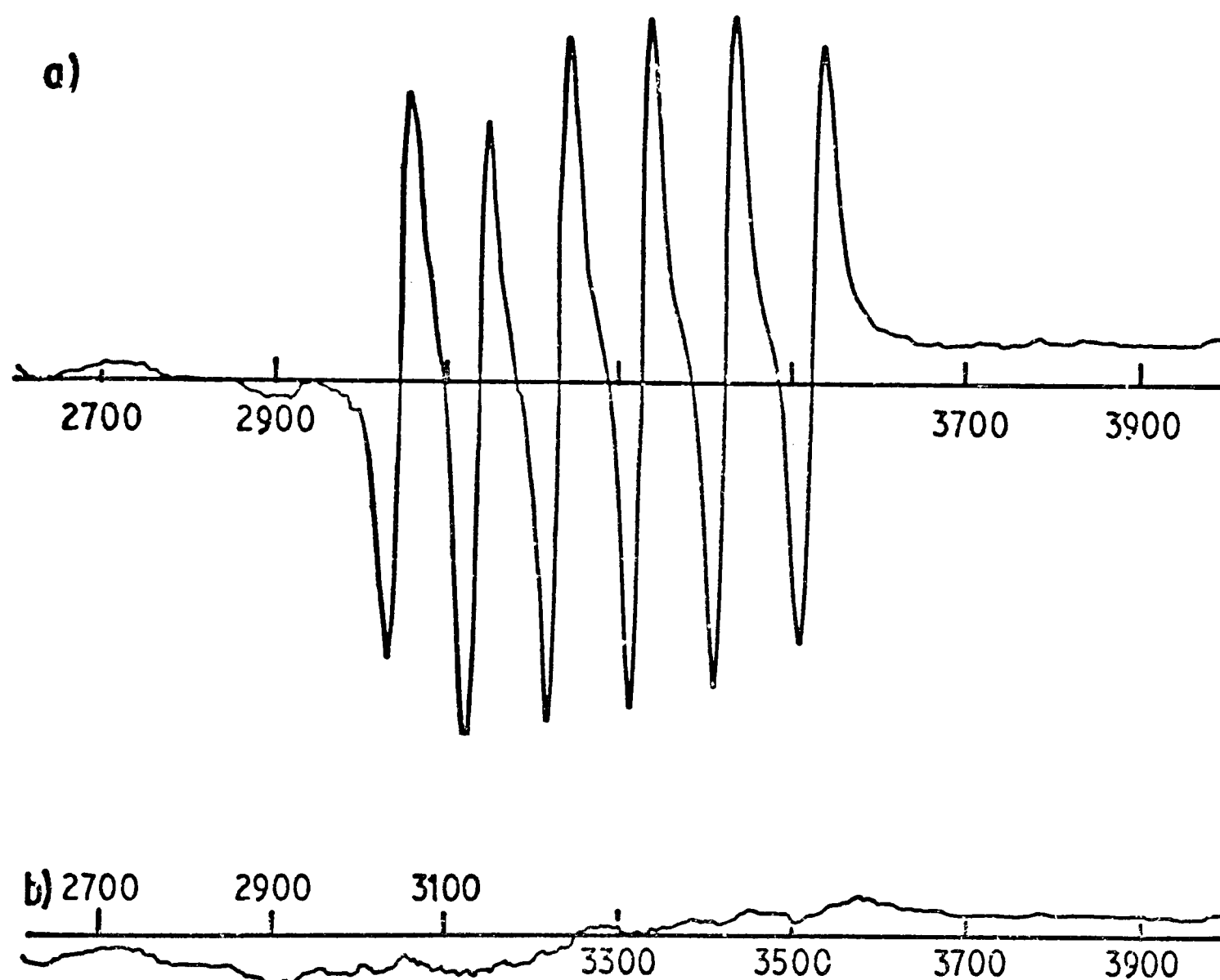
e) Manganese Binding Studies

The binding of manganese to the derivatives of N-Dnp-2-aminoethanol and complexes of these with the Fv fragment of protein 315 was studied by monitoring the manganous ion (Mn(II)) EPR (Electron Paramagnetic resonance) signal. EPR spectra were recorded on a Varian E-line (Century series) EPR spectrometer. The probe temperature was controlled with an Air Products temperature controller monitoring a nitrogen gas stream. Spectra were recorded in the derivative mode with a modulation amplitude of 2 and a frequency of 10 Hz, at a microwave frequency of 9.165 GHz (X-Band) and a field of 3300 Gauss. The scan width was 1×10^3 Gauss. Typical settings for a 200 μ M Mn(II) sample were: sweep rate 125 Gauss/min., time constant 0.5 sec., receiver gain 2×10^3 , power 110-100 mwatts.

All samples were unbuffered, pH 7, 0.15 M NaCl, in $^1\text{H}_2\text{O}$. Measurements were carried out at a temperature of 303° K. The sample cell was a 0.22 cm diameter quartz tube, titrations were performed using a 1 ml plastic disposable syringe attached to the top of the sample cell. Manganese samples were made up from an 18.2 mM stock solution (Spectroscopic standard, manganese nitrate in 1 M nitric acid, B.D.H. Chemicals). The pH was adjusted with NaOH and HCl. Hapten solutions were made up by dissolving the lyophilized solids in $^1\text{H}_2\text{O}$, pH was adjusted as described above. The concentrations were checked using the absorbances given in Table 2. Protein samples were made up by dissolving the lyophilized solid in $^1\text{H}_2\text{O}$. The concentration was checked by measuring the absorbance of the solution at 280 nm and taking the absorbance of a 1 mg/ml solution of the Fv fragment to be 1.5. All solutions were adjusted to an ionic strength of 0.15 with 3 M NaCl, pH 7.

The data were analyzed by equating the intensity of the Mn(II) EPR signal with the concentration of free Mn(II). In order for this to be valid, it must be shown that the intensity of the Mn(II) signal for the bound ion is negligible compared with that of the free ion. This is indeed

FIG. 3: The effect on the EPR spectrum of Mn(II) of the addition of N-Dnp-2-aminoethyl triphosphate



a) 200 μ M Mn(II) pH 7.0, 0.15 M NaCl;

b) 200 μ M Mn(II), 34 mM N-Dnp-2-aminoethyl triphosphate pH 7.0.

so as illustrated for the Mn(II)/N-Dnp-2-aminoethyltriphosphate complex, in Fig. 3. It follows that the fraction of Mn(II) free at any hapten concentration can be calculated as:-

$$\bar{X}_f = \frac{I}{I_0}$$

when I is the intensity of the Mn(II) EPR signal in the presence of hapten, and I_0 is the intensity for the same concentration of free Mn(II). The data were plotted in Scatchard form to obtain n and K_D .

f) Analysis of manganese resonance broadening data

The paramagnetic contribution to the spin-spin relaxation rate of a ^1H nucleus distance r from the bound (Mn(II)) ion is given by the equation:-

$$\frac{1}{T_{2p}} = \frac{\bar{X}_B}{\tau_m + T_{2M}} \quad (2)$$

when τ_m is the chemical exchange time, $\bar{X}_B$ is the fractional saturation of ligand by Mn(II) and T_{2M} is defined by the equation:-

$$\frac{1}{T_{2M}} = \frac{1}{15} \frac{\gamma^2 g^2 S(S+1) \beta^2}{r^6} \left[4\tau_c + \frac{3\tau_c}{1 + \omega_I^2 \tau_c^2} \right] \quad (3)$$

(Dwek, 1973), if the vector connecting the manganese and the ^1H is tumbling isotropically, and the contact term is negligible. Substituting:-

$$\gamma_I = 26.735 \text{ rad. G}^{-1} \text{ sec}^{-1}$$

$$\omega_I = 0.27 \times 10^9 \text{ sec.}^{-1}$$

$$g = 2$$

$$\beta = 0.93 \times 10^{-20} \text{ erg G}^{-1}$$

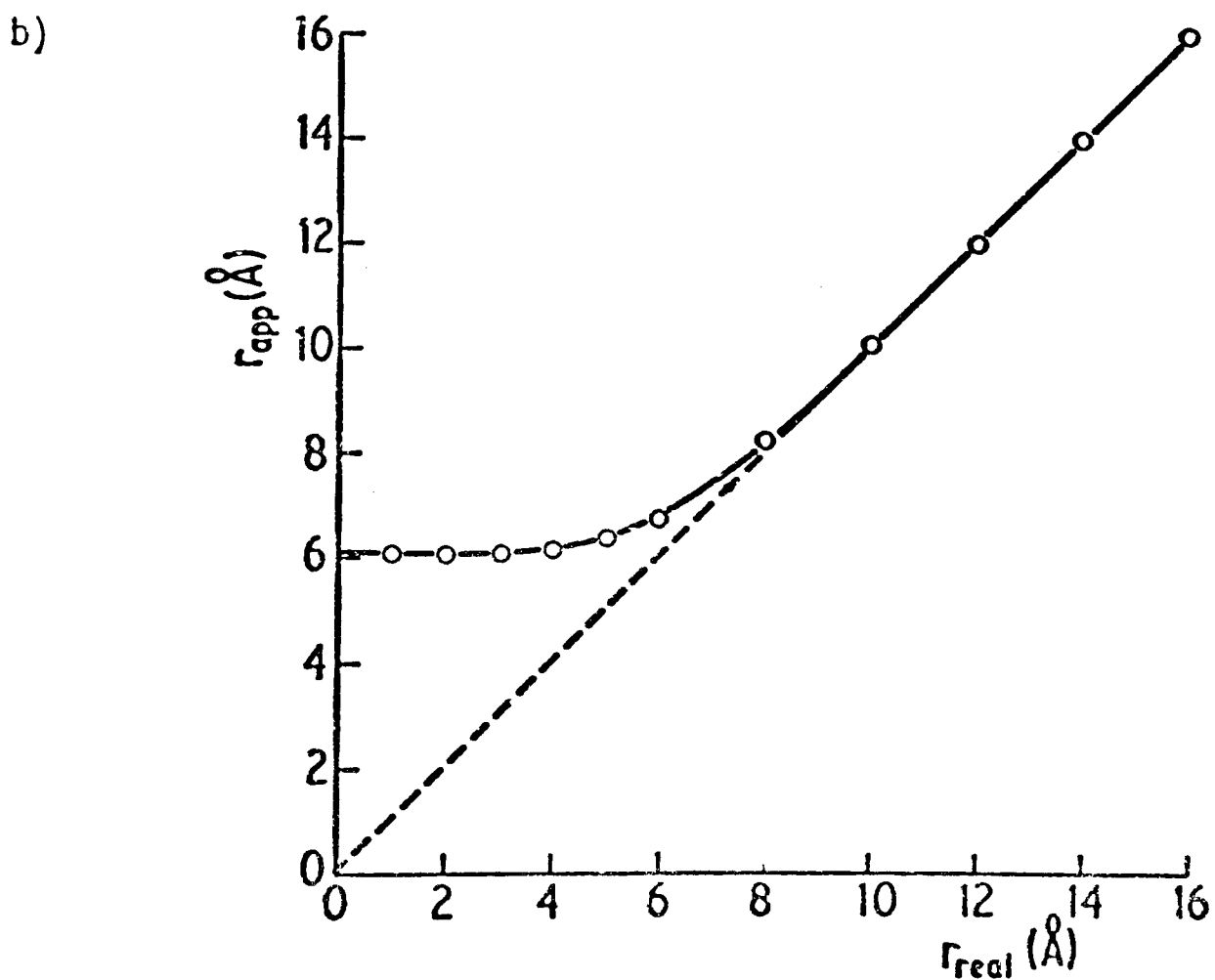
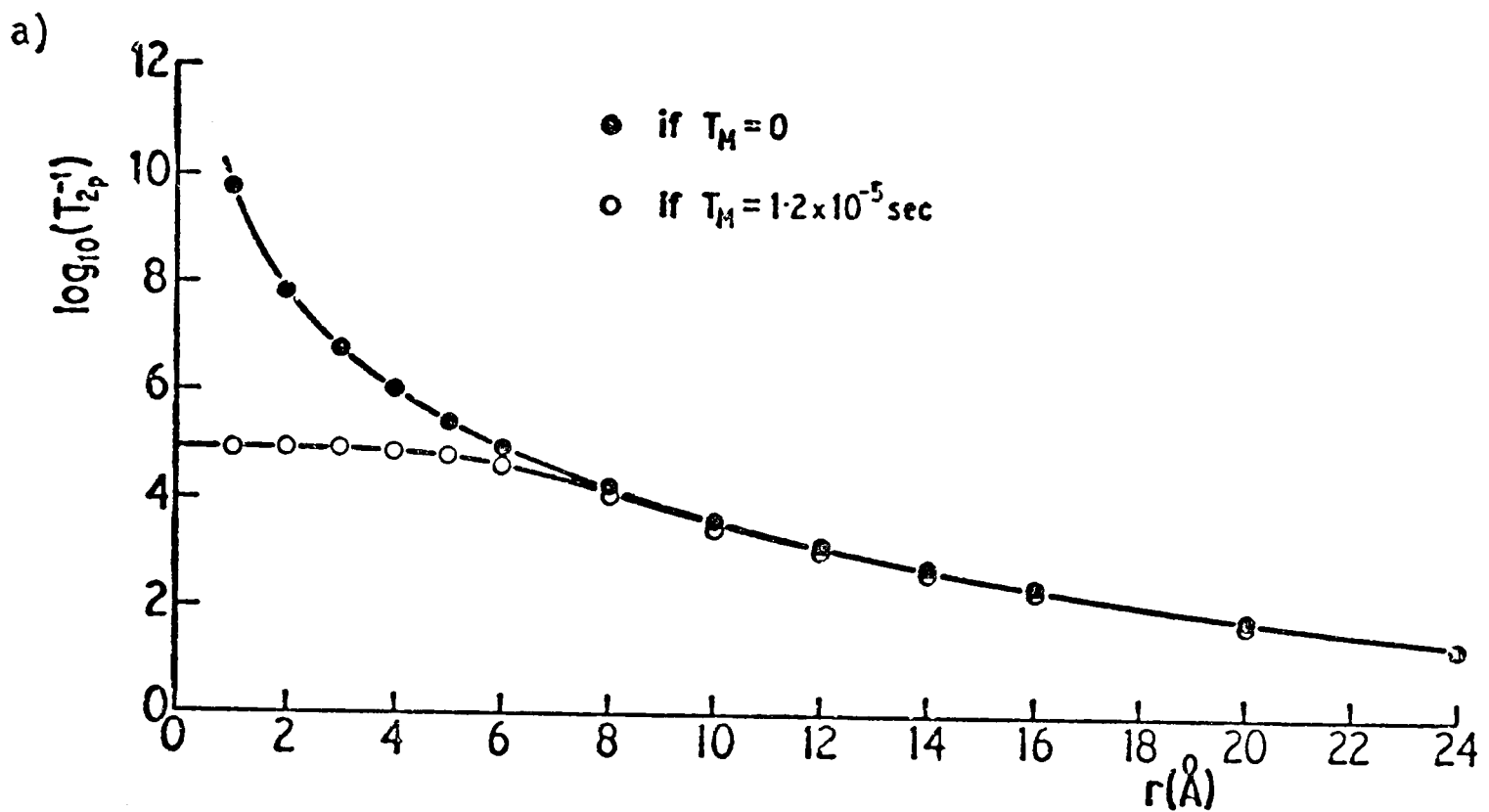
$$S = 5/2$$

$$r = 10^{-8} \text{ cm}$$

and using a value for the correlation time of the Fv fragment of $7 \times 10^{-9} \text{ sec.}$

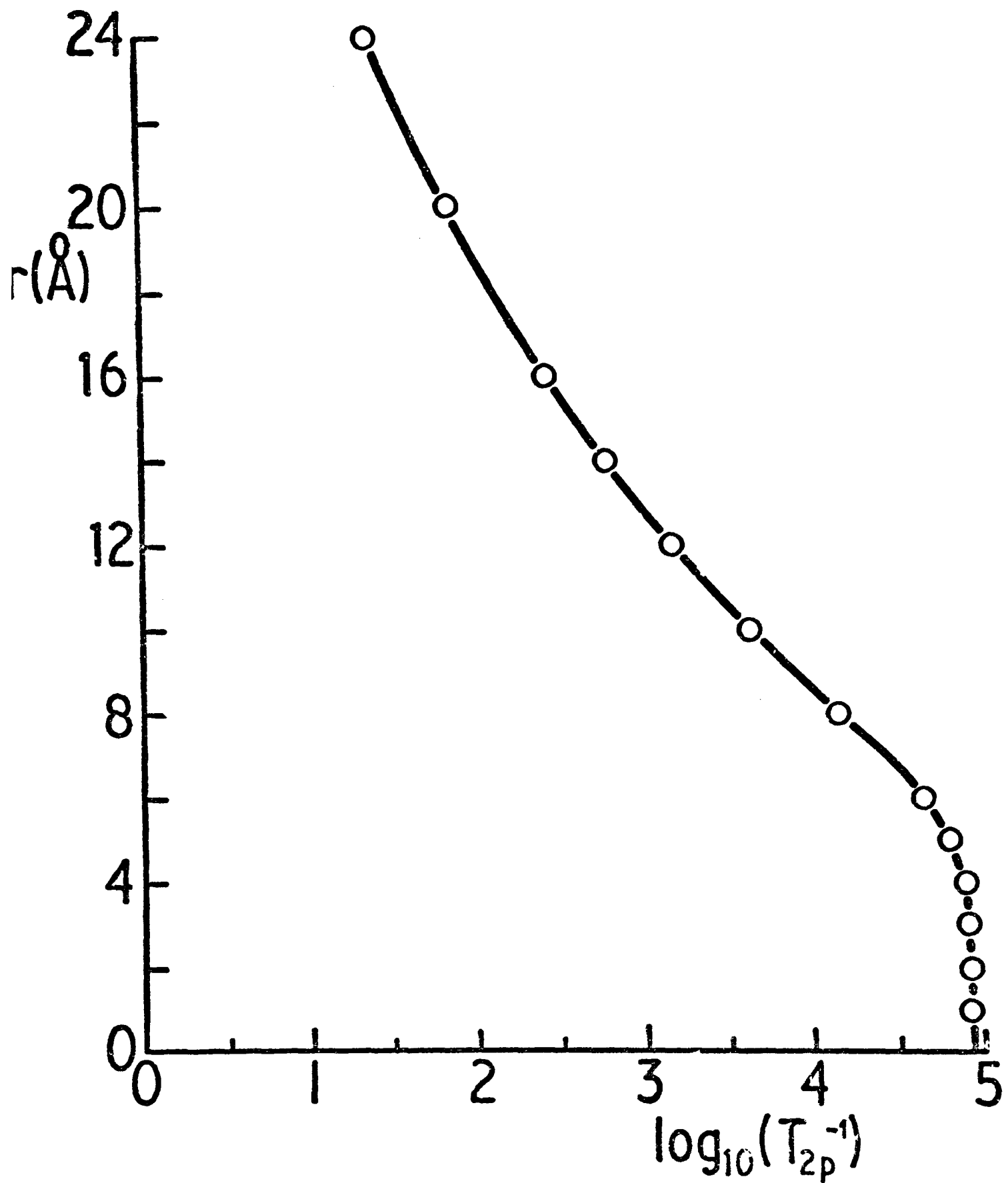
(Sutton et al, 1977; Dwek et al, 1976), we obtain:

Fig. 4:: The effect of chemical exchange on the paramagnetic linebroadening from bound manganoous ion



- a) Deviation between theoretical and observed linebroadening calculated by equations 1 and 2 for a proton at a fixed distance from the metal ion.
- b) Deviation of the distance measured by T_{2p} from the real distance.

FIG. 5 : The dependence of the paramagnetic contribution to the linewidth on the distance of a proton from Mn(II) bound to the Fv fragment/N-Dnp-2-aminoethyldiphosphate complex.



$$\frac{1}{T_{2M}} = (4.23 \times 10^9)/r^6 \quad (4)$$

Whence if $\tau_m < T_{2M}$:-

$$r = (T_{2M} \times 4.23 \times 10^9)^{1/6} \text{ \AA} \quad (5)$$

This assumes fast exchange conditions. In the system used for the paramagnetic distance measurements in this chapter, the Fv N-Dnp-2-aminoethylphosphate/ Mn(II) system, the Mn(II) dissociation constant is 280 μ M.

Comparison with data from studies on adenosine nucleotides (Sternlicht, Jones and Kustin, 1968; Kotowycz and Lemieux, 1973) indicates that τ_m will be c 1×10^{-5} sec. Using this value in equation, the deviations of T_{2p} from T_{2M} can be calculated. This enables the deviation of the measured distance (assuming fast exchange conditions) from the true distance to be assessed, this is shown in Fig. 4. Finally a curve can be constructed, which relates the observed T_{2p} to the distance of the proton from the Mn(II) ion (Fig. 5).

The data for the histidine H2 resonances were obtained by direct measurement, since these are well resolved under the conditions of the n.m.r. experiments (Fig. 8; and see Chapter 4). For other resonances the linebroadening was calculated from the equation:-

$$\frac{I}{I_0} = \frac{\frac{T^{-1}}{2p}}{\frac{T^{-1}}{2p} + \frac{T_0^{-1}}{2}} \quad (\text{Dwek et al, 1975}) \quad (6)$$

when T_2^0 is the spin-spin relaxation time in the absence of the paramagnetic ion. This was estimated to be 1.06×10^{-2} sec. from the linewidth of the histidine 97_L H2 resonance. This has a pK_a of 5.9 (see Chapter 4), and thus will be 5.9% in the protonated form at pH 7.1, the pH of the n.m.r. experiments. Since the resonance is observable at all pH's its maximum linewidth will be 60-100 Hz. Thus the exchange contribution to the linewidth will be negligible, under the conditions of the distance measurement experiments.

3. Results

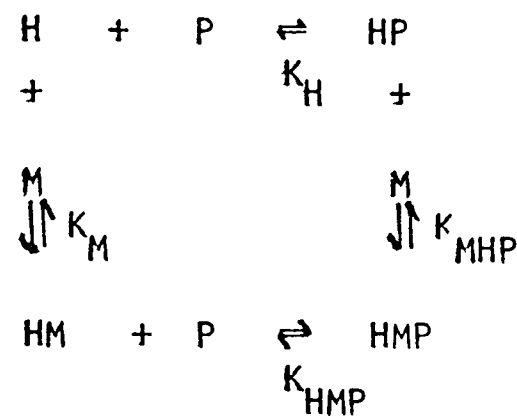
a) Binding Studies

In order to determine the concentrations of the complexes shown in the scheme in Table 3, under the conditions used in the ^1H n.m.r. experiments (described below), the dissociation constants shown in that scheme must be determined. These were determined by fluorescence measurements, for the haptens, and EPR measurements for the Mn(II) ion, as described in the methods section. The dissociation constants of the three haptens from the Fv fragment are very similar (Table 3). This suggests that β and γ phosphate groups of the haptens do not interact with the protein. This view is supported by ^1H (see below) and ^{31}P n.m.r. (see Chapter 4) data. In order to determine the dissociation constants of the hapten/metal ion complexes from the Fv fragment, fluorescence measurements were made of hapten-Fv fragment binding in the presence of 10mM Mg(II) . This metal ion was used in preference to Mn(II) , since the paramagnetism of the latter may introduce spurious quenching effects into the system when it is present in a 10^4 -fold concentration excess over the Fv fragment. The dissociation constants of the haptens all increase by a small amount in the presence of Mg(II) (up to c 30%). (eg, see fig 6)

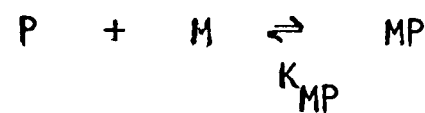
The dissociation constant of the Mn(II) ion from the Fv fragment alone is too weak to be measured (Table 3). The estimated minimum dissociation of the Mn(II) ion from the protein, as measured by EPR studies, is sufficiently large for the concentrations of any Mn(II) /Fv fragment complexes to be negligible under the conditions of the ^1H n.m.r. experiments. This behaviour, with a mouse IgA variable region, resembles that found with non-immune rabbit IgG fragments, where Mn(II) binding could not be detected by EPR measurements or by water proton spin-lattice relaxation rate measurements (Dower, 1975). Thus the only metal ion binding site on the protein/hapten complex will be that introduced by the bound hapten. This behaviour

TABLE 3. Dissociation constants in the Mn(II)-Hapten-Fv fragment system

The dissociation constants are defined in the scheme:



and :-



Hapten	K_{MP}	K_H	K_M	K_{HMP}	K_{MHP}
none	> 24,000	-	-	-	-
N-Dnp-2-aminoethylphosphate	-	0.88	5,000-10,000	1.08	6,000
N-Dnp-2-aminoethyldiphosphate	-	0.62	90	0.63	280
N-Dnp-2-aminoethyltriphosphate	-	0.47	35	0.60	140

1. All dissociation constants are given in μM concentration units;
2. The metal ion binding constants were determined by EPR, and the hapten binding constants were determined by fluorescence; as described in the methods section.
3. The large error in the K_M value for the N-Dnp-2-aminoethylphosphate in the absence of the Fv fragment is due to the insolubility of the manganous salt of this compound, which has a solubility product of $10^{-5} - 10^{-6} \text{ M}^2\text{l}^{-2}$.
4. The minimum dissociation constant of Mn(II) from the Fv fragment of protein 315 was estimated from the scatter of the intensities in the EPR signals from a 1mM Mn(II) solution, as the concentration of the Fv fragment was increased to 2.16 mM. No decrease in the Mn(II) signal was seen that exceeded the experimental error of $\pm 4\%$.

contrasts with that of the lanthanides, which bind tightly to both non-immune rabbit IgG and to the Fv fragment of protein 315 (Dower et al, 1975; Dwek et al, 1975a,b,1976).

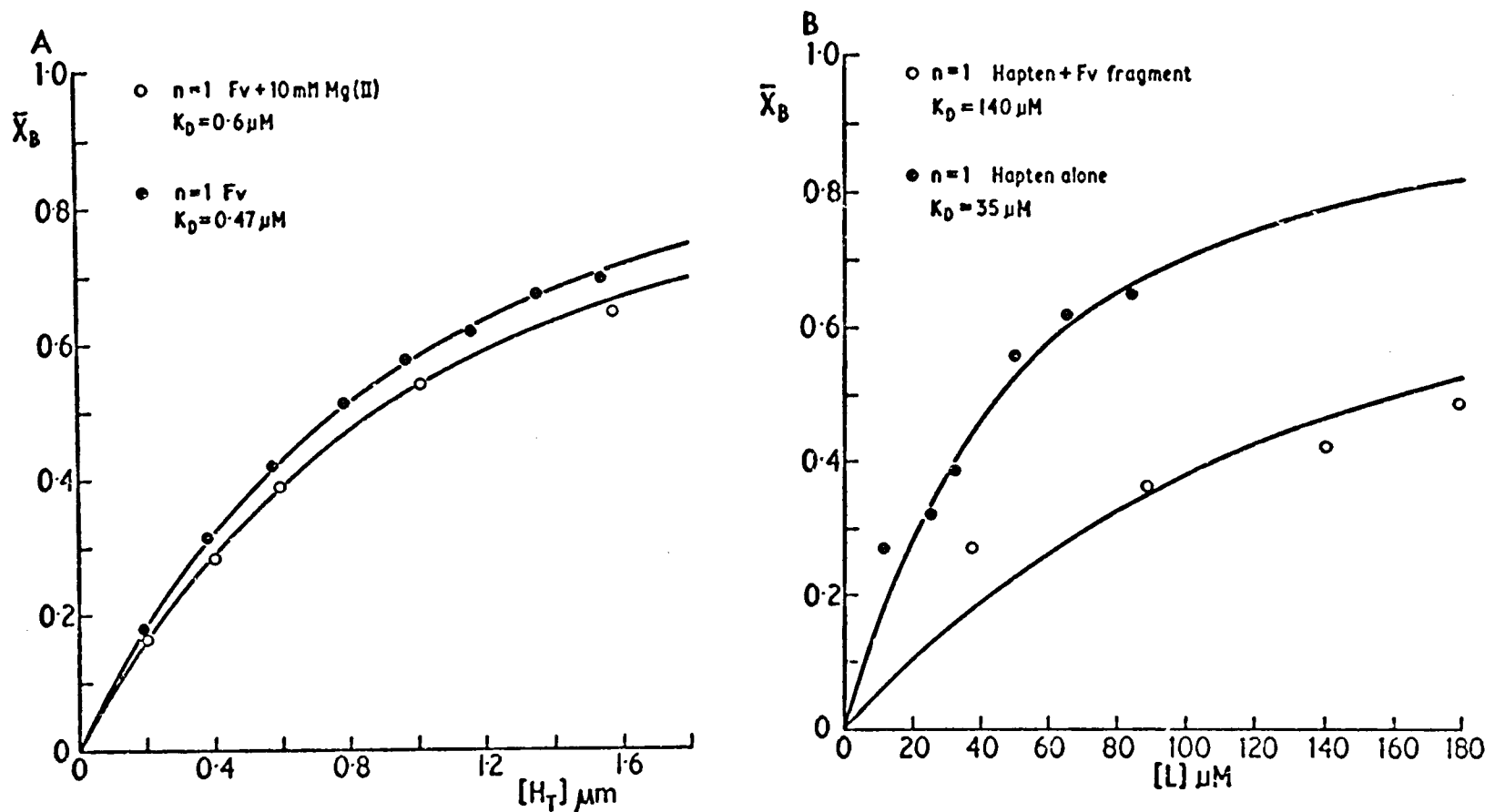
The dissociation constants of the Mn(II) ion from the haptens, both free and bound to the Fv fragment, are given in Table 3. The binding of the metal ion to the phosphate groups on the hapten is weakened by a factor of approximately twofold in the presence of protein. Since:-

$$K_H K_{MHP} = K_M K_{HMP} \quad (7)$$

it follows that this weakening in the Mn(II) ion binding is somewhat larger than is consistent with the weakening in the hapten binding. This discrepancy probably results from small differences in the properties of the Mn(II) and Mg(II) ions. Thus, for example it is shown that the latter binds c five-fold more weakly to the ATP than the former (Gupta and Benovic 1978). The dissociation constants of the Mn(II) ion from the three haptens are in the expected order ; phosphate > diphosphate > triphosphate.

In order for a metal ion binding site to be useful for paramagnetic distance measurements, two conditions must hold. Firstly, it must be a specific site; this is the case for this system. Secondly the metal ion must be in fast exchange with the protein, or protein/ligand, binding site (see Section 2(f)). In this system, the N-Dnp-2-aminoethylphosphate/Fv fragment complex binds the Mn(II) ion too weakly at the major site to be certain, from the minimum estimated dissociation constant for the Fv fragment alone, that some non-specific Mn(II) binding to the protein may not occur. Conversely, the N-Dnp-2-aminoethyltriphosphate/Fv complex binds the Mn(II) ion too tightly to be in fast exchange. Thus the ^1H n.m.r. experiments were carried out with the N-Dnp-2-aminoethyldiphosphate/Fv complex. This system would be expected to have a τ_m in the region of 10^{-5} , based on values for Mn(II) and adenosine nucleotides (Sternlicht, Jones and Kustin, 1968; Kotowycz and Lemieux, 1973).

Fig. 6: The binding of N-Dnp-2-aminoethyltriphosphate and its metal ion complexes to the Fv fragment



- (a) Titrations were performed with $0.53 \mu M$ Fv fragment at 298 K, pH 7, in the presence of 50 mM pipes buffer and 0.15M NaCl. In the titration with 10 mM $MgCl_2$ present the NaCl concentration was reduced to 0.12M to maintain the ionic strength constant.
- (b) Titrations were performed with $20 \mu M$ Mn(II), pH 7, 0.15M NaCl, no buffer, at 298 K. The concentrations on the x axis are either ligand alone, N-Dnp-2-aminoethyltriphosphate; or ligand + 1.2 x that concentration of Fv fragment.

In both cases the continuous curves are calculated using the parameters given.

b) High resolution ^1H n.m.r. studies

In order to examine the mode of binding of the three N-Dnp-2-amino-ethanol derivatives to the Fv fragment, the effect of hapten binding on the 270 MHz ^1H n.m.r. spectrum of the Fv fragment was studied by ^1H n.m.r. difference spectroscopy. The results of these experiments are shown in Fig. 7. The difference spectrum produced by each compound is very similar, as is illustrated in the final (B-D) spectrum which shows the difference-difference spectrum obtained with N-Dnp-2-aminoethylphosphate/Fv fragment and N-Dnp-2-aminoethyltriphosphate/Fv fragment. There is insignificant intensity in the difference-difference spectrum indicating similar modes of binding for each of the haptens, and further suggesting that the β and γ phosphate groups do not interact with the protein. This is supported by the binding data reported in section 3(a) and is consistent with the ^{31}P n.m.r. studies reported in Chapter 4.

Further, the integration shows that in the region 6.9-7.5 ppm c 5-10 protons intensity is present in the Fv fragment spectrum but not in the spectrum of the Fv fragment/hapten complex: indicating that some resonances in the protein spectrum are broadened on hapten binding.

The binding of manganese to the Fv fragment - N-Dnp-2-aminoethyldi-phosphate complex was studied by 270 MHz ^1H n.m.r. The concentration of hapten and protein were such that all the ligand was bound to the protein. Thus only the formation of ternary complex need be considered. The fraction of the hapten protein complex bound by Mn(II) was calculated from the dissociation constants given in Table 3. The experiment was repeated three times. The results of one such experiment are illustrated in Figs. 8 and 9, which show the effect of Mn(II) binding on the ^1H n.m.r. spectrum of the protein-hapten complex. The specific resonance broadening indicated in Fig. 8 is more clearly revealed by the difference spectra

FIG. 7 : 270 MHz ^1H n.m.r. difference spectra obtained on adding N-Dnp-2-aminoethanol phosphate derivatives to the Fv frag

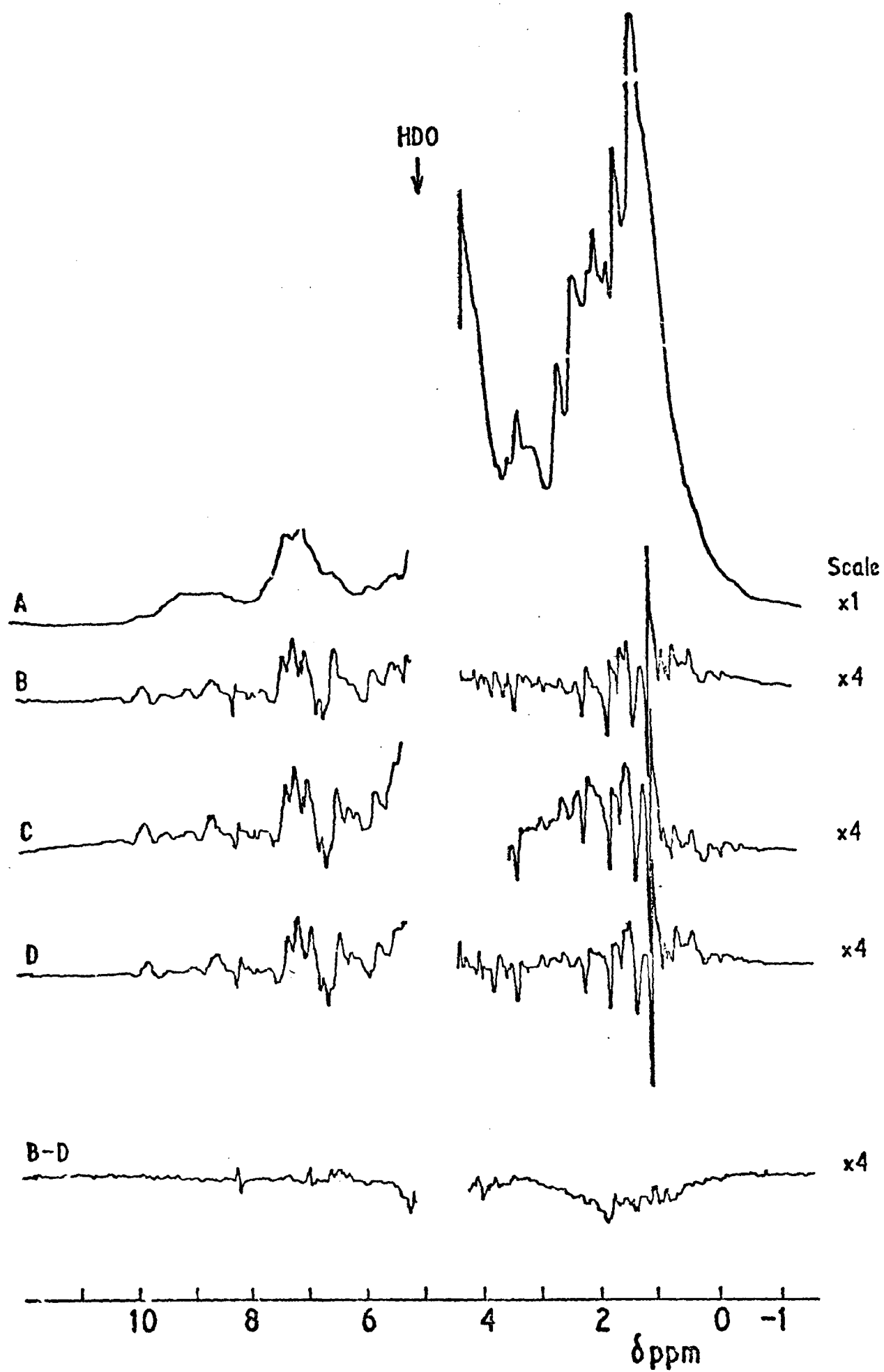


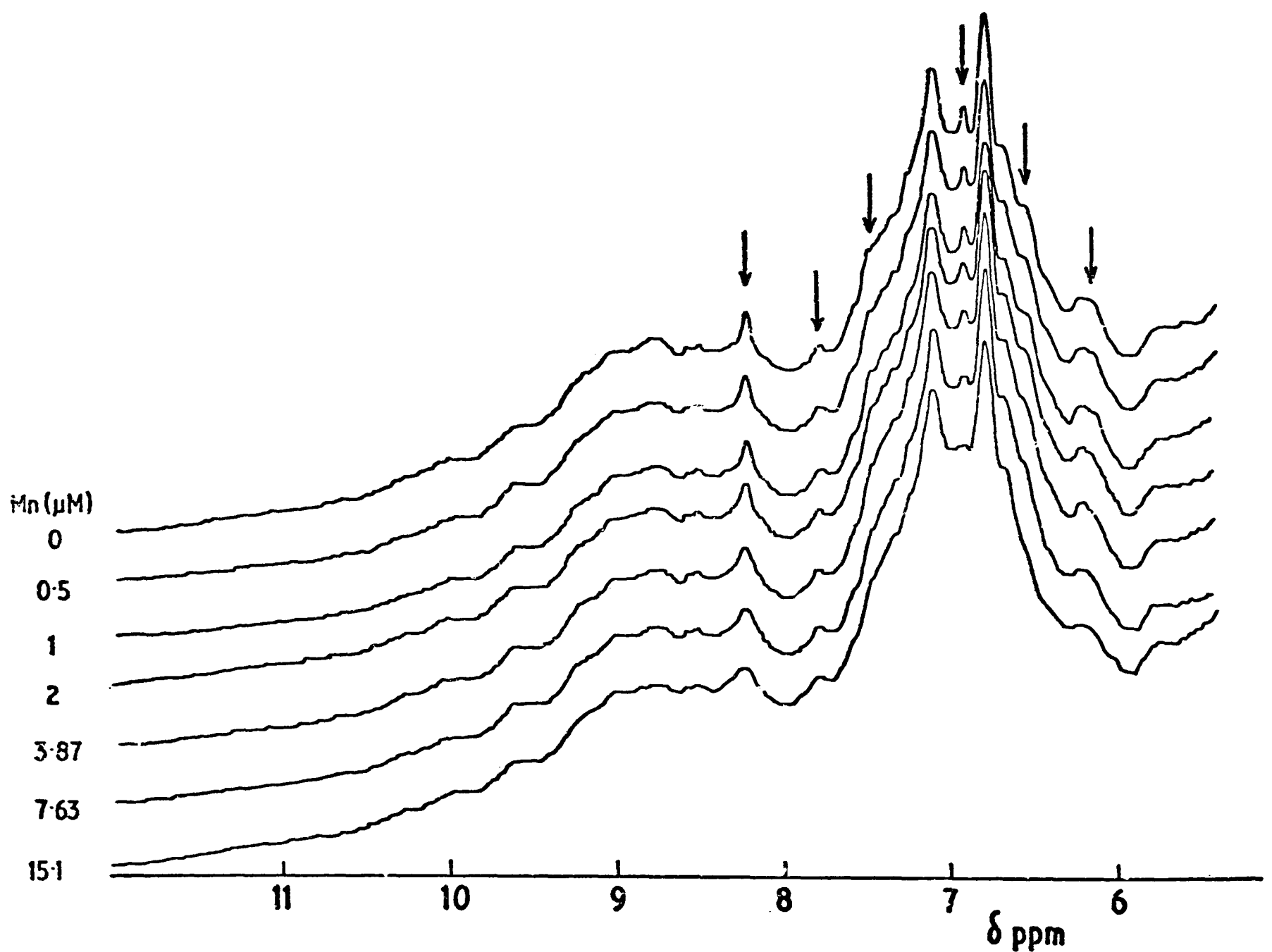
Fig. 7:

All spectra were recorded for 4000 scans; the Fv fragment concentration was 2 mM, pH* 7. The spectra were obtained with:-

- a) B 2 mM Fv fragment alone - 2 mM Fv fragment containing 2 mM N-Dnp-2-aminoethylphosphate
- b) C 2 mM Fv fragment alone - 2 mM Fv fragment containing 2 mM N-Dnp-2-aminoethyldiphosphate
- c) D 2 mM Fv fragment alone - 2 mM Fv fragment containing 2 mM N-Dnp-2-aminoethyltriphosphate.

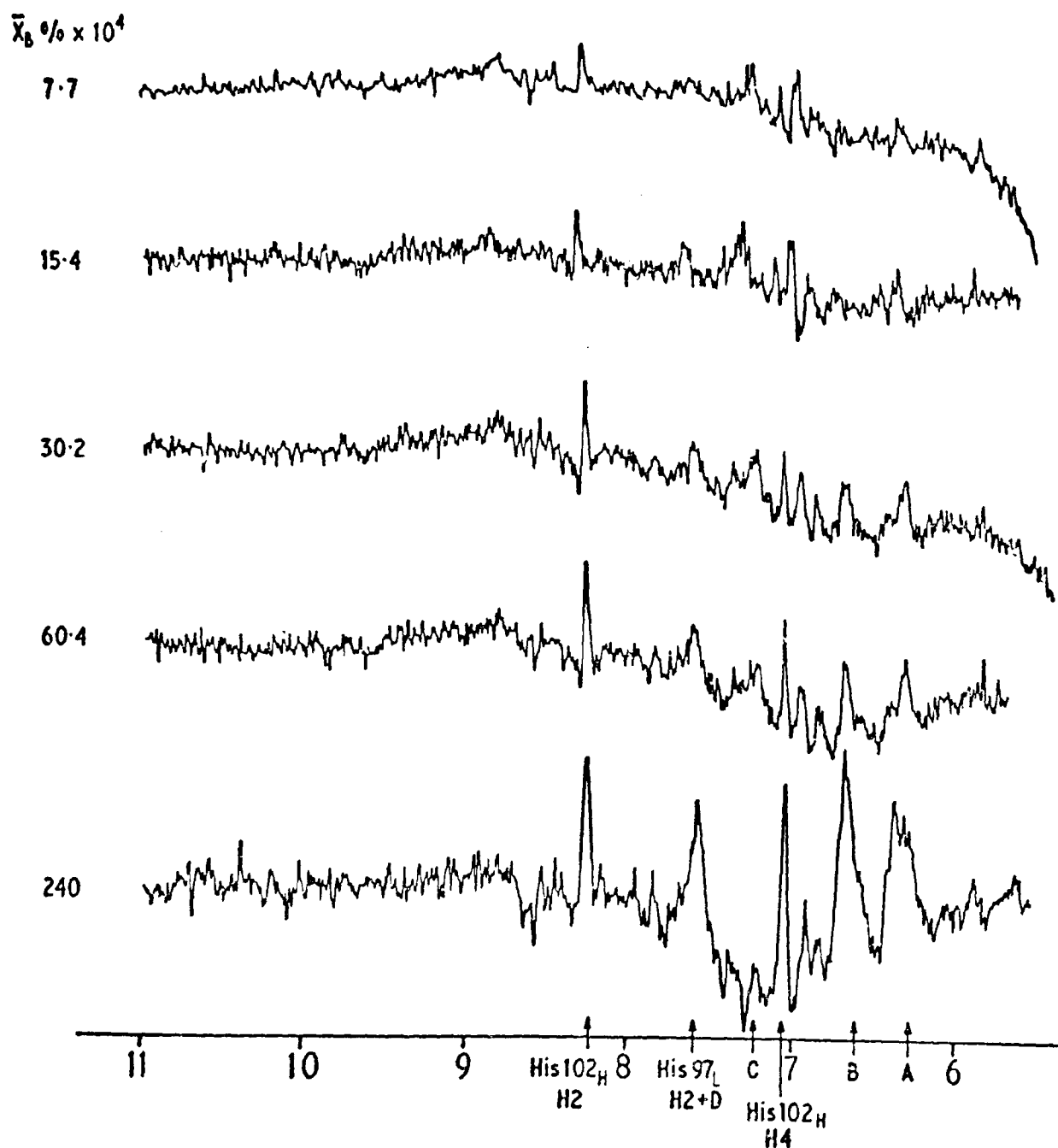
HDO indicates the position of the solvent resonance.

FIG. 8 : The effect of manganese binding on the 270 MHz ^1H n.m.r. spectrum of the Fv fragment/N-Dnp-2-aminoethyl diphosphate complex



Concentration of Fv fragment - 112 mM, concentration of N-Dnp-2-aminoethylphosphate - 1.08 mM, therefore the concentration of free hapten was 13 μM as calculated from the parameters given in Table 3. pH^* 7.05; metal concentration as indicated. The arrows indicate the specific broadening by the bound $\text{Mn}(\text{II})$.

FIG. 9 : The effect of Mn(II) binding on the 270 MHz ^1H n.m.r. spectrum of the Fv fragment N-Dnp-2-aminoethylphosphate complex



Conditions as for figure 8, vertical scale x8, the values of $\bar{X}_B$, the fractional saturation of protein with metal ion, were calculated from the parameters given in Table 3. Difference spectra were generated by subtracting the spectrum recorded with the $\bar{X}_B$ value indicated from that of the Fv fragment-hapten complex alone.

TABLE 4(a) Paramagnetic contribution to the linewidth of resonances in the aromatic region of the 270 MHz¹H n.m.r. spectrum of the N-Dnp-2-aminoethyl diphosphate-Fv fragment complex in the presence of Mn(II)

$\overline{X}_B$ (%)	Resonance ($\Delta\nu_p$ is in Hz)							
	102_H H2	102_H H4	97_L H2	97_L H4	A	B	C	D
0.036	6.1	2.8	-	-	-	-	-	-
0.077	11.5	5.6	-	-	4.	-	-	-
0.154	11.0	9.1	-	-	11.7	4.1	8.	3.0
0.302	17.0	7.8	7.1	7.1	15.	10.5	12.3	8.0
1.2	26.9	7.8	17.0	17.0	20.8	26.6	12.3	21.0
2.4	180.4	26.2	37. ⁰	47. ⁰	55.0	170	12.3	73.0
4.76	-	-	35.6	78.0	47.0	64.0	220	73.0
8.99	-	-	35.6	-	85.0	73.0	850	112.0
18.44	-	-	114.0	-	95.0	112.0	--	95.0

(b) Chemical shifts and distances calculated for resonances tabulated in Table (a)

Resonance	ppm	$\Delta\nu$ $\frac{O}{P}$	T^{-1} $_{2M}$	r (Å)
H1s 102_H H2	8.23	$(6.71 \pm 2.7) \times 10^3$	$(2.1 \pm 0.85) \times 10^4$	7.6 ± 0.7
H1s 102_H H4	6.96	$(0.80 \pm 0.21) \times 10^3$	$(2.51 \pm 0.66) \times 10^3$	11.0 ± 0.5
H1s 97_L H2	7.79	$(5.38 \pm 1.51) \times 10^2$	$(1.69 \pm 0.47) \times 10^3$	11.8 ± 0.5
H1s 97_L H4	6.72	$(1.64 \pm 0.15) \times 10^3$	$(5.15 \pm 0.46) \times 10^3$	9.6 ± 0.2
A	6.21	$(4.87 \pm 1.44) \times 10^2$	$(1.53 \pm 0.45) \times 10^3$	12.1 ± 0.6
B	6.59	$(5.62 \pm 0.20) \times 10^2$	$(1.77 \pm 0.06) \times 10^3$	11.6 ± 0.1
C	7.54	$(1.24 \pm 0.24) \times 10^3$	$(3.90 \pm 0.75) \times 10^3$	10.1 ± 0.4
D	7.10	$(1.13 \pm 0.84) \times 10^3$	$(3.55 \pm 1.70) \times 10^3$	10.5 ± 1.1

Notes for Table :-

- 1) The conditions are given in the legend to figure 8.
- 2) The paramagnetic contribution to the linewidth was measured directly from the spectra for the H₂ resonances of histidines 102_H and 97_L. In these two cases the paramagnetic contribution was also calculated from the intensity in the difference spectra, as described in the methods section. In all other cases this latter method was the only one used.
- 3) $\bar{X}_B$ is the fractional saturation of the N-Dnp-2-aminoethyl-diphosphate-Fv fragment with Mn(II). This was calculated from the concentration of the three components of the system using the parameters determined in the binding studies and summarized in Table 3.

- 4) The values for $\Delta\nu_p^0$ given in Table (b) were determined from the data in Table (a) using linear regression analysis on the equation:

$$\Delta\nu_p = \Delta\nu_p^0 \times \bar{X}_B$$

The errors were calculated from the scatter of the points about the best fit straight line.

- 5) The data in Table (b) are based on three experiments.

shown in Fig. 9. The aromatic region of the spectrum only was investigated, since it was only for this region that shift data could be obtained from the studies reported in Chapter 2. The line broadening data from one experiment are given in Table 4(a). The values for the paramagnetic contribution to the relaxation rates and the calculated distances are given in Table 4(b). The distances are calculated with the assumptions stated in section 2(f).

4. Discussion

a) Reliability of the distance measurements

The assumptions used in calculating distances from paramagnetic broadening data obtained from Mn(II) are discussed in Dwek (1973). Three assumptions are important in this study. First, that the appropriate correlation time is the isotropic rotational correlation time for the Fv-fragment-hapten complex. Second that no through bond effects are involved in the paramagnetic relaxation, i.e. that it is dipolar. Third, that for resonances that can only be resolved by difference spectroscopy, that the intensity in the difference spectra arises from effects on two proton intensity resonances. If this estimate is in error by a factor of twofold it will introduce an error of 12% into the measured distance. Some confidence in the measured distances can be obtained from the data for the four resonances of the two histidines 102_H and 97_L. The sum of the distances between the histidine imidazole ring centres, measured by the manganese experiments is $20 \pm 1 \text{ \AA}$. The distance between the centres of the imidazole rings calculated from the coordinates of the structure given in

Chapter 2, Fig. 17 is 19.4 Å. While this comparison does not act as independent confirmation of the predicted structure the important conclusion is that the metal ion is located approximately on the line of centres for the two histidine imidazole rings.

A further uncertainty could be introduced into these measurements if the metal ion or any of the ^1H nuclei are free to move, in the protein-hapten-metal ion ternary complex. This would invalidate the assumption of slow isotropic tumbling as discussed above, but also, because of the r^{-6} dependence of the dipolar broadening, the average distance calculated from the paramagnetic effects would be an underestimate of the true distance. Thus, for example, the histidine residues may rotate about the $\text{C}_\beta\text{-C}_\gamma$ bonds leading to errors in the measured distances.

A further correlation can be made with the modified model presented in Chapter 2, Fig. 17. The distance from the metal ion (position determined as above) to the guanido group of arginine 95_L was calculated as 7.4 ± 1 Å. The distance that might be expected if the metal ion coordinates between the α and β phosphate groups of Arg 95_L is 7.4 ± 2 Å. It is known from ^{31}P n.m.r. studies that the α -phosphate group of these haptens is in the vicinity of this group on the protein (Chapter 4). Thus, if the metal ion coordinates between the α and β phosphate groups of N-Dnp-2-aminoethyldiphosphate and if the Arg 95_L guanido group is close to the αP of the bound hapten, the expected distance between this group and the metal ion would be 5-7 Å. Thus the calculated distance and the distance expected from the ^{31}P n.m.r. studies are consistent.

b) Conclusions

The paramagnetic distance measurements reported in this Chapter only give quantitative information about the two histidine residues in the Fv fragment, and thus indirectly about the relative position of the H3 and L3 hypervariable segments. Since resonances A-D have not been assigned to particular protons in the Fv fragment, no quantitative conclusions can be drawn from the measured distances for these resonances. The chemical shifts of these resonances correspond to those expected for resonances perturbed by hapten binding. This cannot, however, be taken as proof of identity. In order to determine that resonances perturbed on hapten binding are also affected by the paramagnetic probe, it will be necessary to utilize haptens which contain a metal ion binding site and are in fast-exchange for chemical shift with the Fv fragment. Such haptens should contain less hydrophobic side chains than those used in these studies. A possible series could contain phosphate groups substituted directly onto the Dnp or Tnp ring. In this case, however, the metal ion binding site would be expected to lie within the protein combining site (~12 Å deep). Thus the structure of the ternary complex might be expected to be very different from the binary protein-hapten complex. It may thus be necessary to synthesize a number of these phosphate compounds to search for a suitable compromise.

Finally the series of compounds used in this Chapter are being considered as heavy atom chelating groups for the X-ray crystallographic studies of the Fv fragment. Correlation of such crystallographic data with a number of paramagnetic distance determinations may offer the possibility of obtaining second stage assignments in the Fv fragment ^1H n.m.r. spectrum.

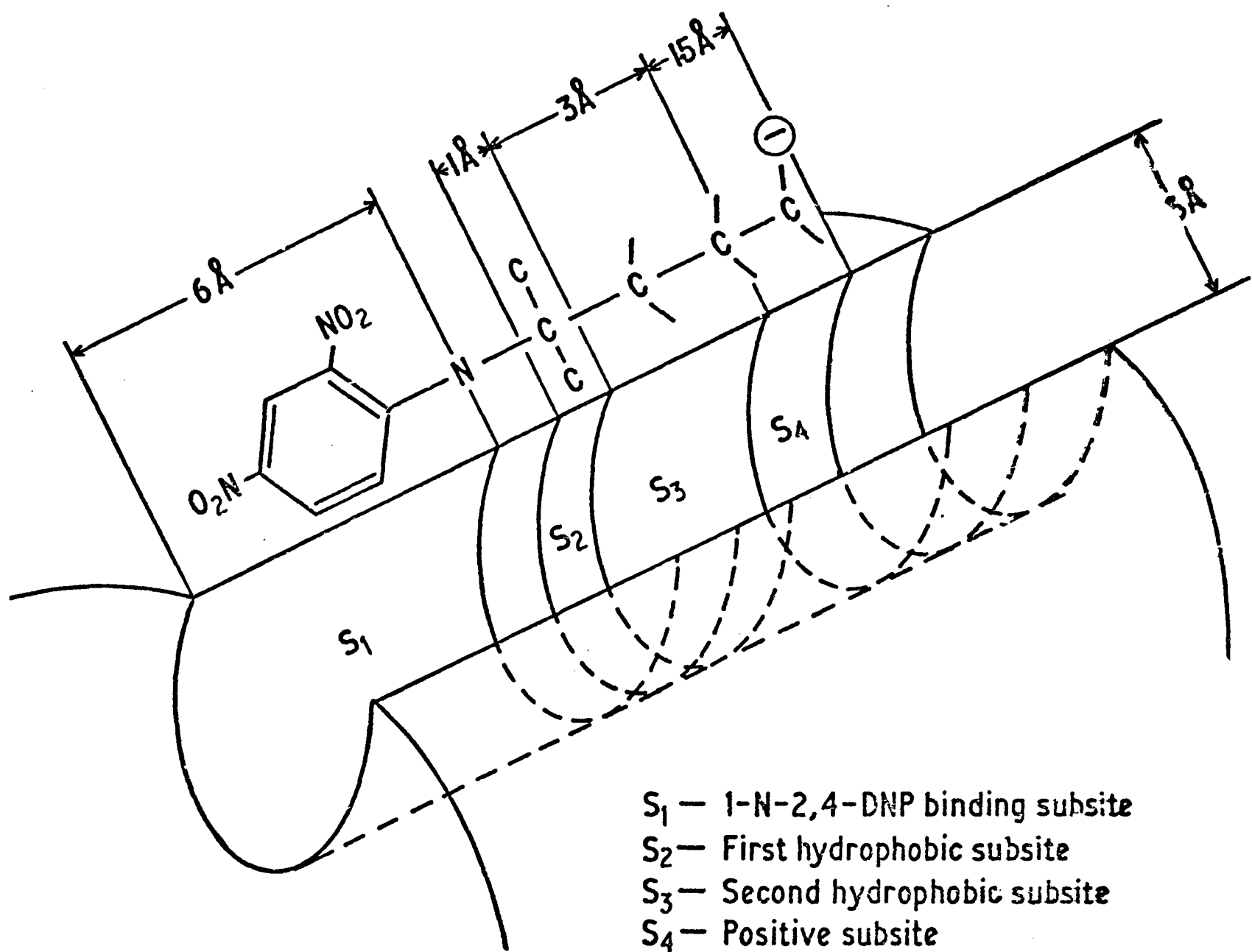
Chapter 4CHARGED RESIDUES IN THE COMBINING SITE OF
THE FV FRAGMENT OF PROTEIN 3151. Introduction

^1H resonances that arise from the H2 and H4 protons of the imidazole ring of histidine residues in proteins are the most straightforward on which to perform first stage assignment (assigned to a type of amino acid) (Campbell, 1977; Campbell, Lindskog and White, 1974, 1975). This is particularly true for globular proteins of high molecular weight because the resulting broad linewidths of the resonance of other amino acid residues obscure the coupling patterns (Kilmarten et al, 1975; Brown and Campbell, 1976; Dwek, 1973). The relative ease of assignment of histidine residues arises for three main reasons; these are normally the only amino acid residues with pK_a 's in the region of pH 5-8.5; the change in ionization state of the imidazole ring markedly affects the chemical shift of the H2 and H4 resonances; and the H2 resonances occur to down-field of the main aromatic amino acid spectrum.

Since both the pK_a 's and the chemical shifts of these residues may be sensitive to changes in local environment, second stage assigned histidine resonances (assigned to a particular residue) can be used to monitor changes in these environments on the protein. There are three histidine residues in the Fv fragment of protein 315, at positions 97_L, 102_H and 44_L respectively. Of these three, 44_L is not in a hypervariable segment and should thus not be close to the combining site. The confirmation of this and the assessment of the involvement of the other two residues in the Fv fragment combining site can be carried out by ^1H n.m.r. studies, using the criteria outlined above. The first and second stage assignment to these three histidine residues of resonances in the 270MHz ^1H n.m.r. spectrum is discussed below.

Since the six histidine resonances are the only clearly resolved and assignable resonances in the Fv fragment spectrum, alternative methods must be used to search for other charged residues in the combining site of this protein. One approach is to investigate the effect that protein-hapten interactions have on the pK_a 's of ionizable groups covalently bound to hapten molecules. If these ionizable groups contain phosphorus in the form of phosphate and phosphonate groups, then ^{31}P n.m.r. studies provide a simple and direct way of measuring the pK_a of the hapten group, both free and bound to the protein. (Gorenstein, Myrwick, and Bode, 1976; Feeney et al, 1975; Coleman et al, 1976; Rao and Cohn, 1977; Huli et al, 1976.) Binding to the protein may affect both the ^{31}P chemical shift and the pK_a of the group. The interpretation of the chemical shift of ^{31}P resonances is complex, three major factors are thought to contribute to this; X-P-Y bond angle (Purdela, 1971; Gorenstein, 1975; Blackburn, Cohen and Weatherall, 1971; Patel, 1974), σ bond electron density (Riess, Van Wazer and Letcher, 1967; Letcher and Van Wazer, 1966), or π orbital overlap (Letcher and Van Wazer, 1966). Since any one of these factors may be affected on binding, and all can produce very large upfield or downfield shifts (Gorenstein, 1975), the attribution of a particular observed shift change to any particular cause is not possible without extra information, such as a crystal structure of the protein-ligand complex. The pK_a of a bound phosphorus-containing group may also be affected by several features of the bound environment; primarily, hydrogen bonding, charged groups and hydrophobicity. The former two effects are believed to cause marked changes in the pK_a of a phosphate group in, for example, tetrahydrofolate reductase (Feeney et al, 1975) and a phosphoryl choline binding antibody, McPC603 (Gettins et al, 1978). The effects of hydrophobicity cannot be satisfactorily accounted for since this cannot be independently measured or calculated; however, comparative data on several ligands can mainly eliminate the uncertainty due to this factor. Thus the effect of

FIG. 1 : The pattern of subsites in the combining site of protein 315



This structure was proposed on the basis of temperature jump data, by Haselkorn et al. (1974)

binding to a protein on the pK_a of a phosphonate or phosphate group covalently bound to a hapten, is a more useful parameter than chemical shift for obtaining information about the bound environment. These effects have been studied using a series of alkylphosphate and alkylphosphonate haptens which bind to the Fv fragment (see also Wain-Hobson, 1977; and Wain-Hobson, et al, 1977).

The major aim of this work is to investigate the 'positive subsite' described by Haselkorn et al (1974), and shown in Fig. 1. This feature of the Fv fragment combining site was detected by measuring the dissociation rates of a homologous series of Dnp haptens bearing carboxyl groups in the side chain, using the temperature jump method. The authors found that if the distance from the Dnp ring to the carboxyl group was 0.8-0.95 nm then the dissociation rate was slower than a simple theoretical treatment would predict. Correlating this data with the affinity labelling studies of Haimovitch et al (1972), who showed that α N-bromoacetyl- ϵ N-Dnp-L-lysine could be used to label Lys 52_H, Haselkorn et al, suggested that this residue was responsible for the positive subsite. Sutton et al (1977) concluded from a later study using spin labelled haptens that a positively charged residue was close to the nitroxide of a hapten which was just contained by the combining site, they placed this residue at the entrance to the site and concluded that this positively charged residue could be identified with Arg 95_L. Since inspection of the predicted structure of Padlan et al (1976) indicates three candidates for the positively charged residue; Arg 95_L, Lys 52_H, and His 102_H, all of which lie in the entrance to the combining site, further data are needed to resolve this problem. The studies reported in this chapter are directed towards this aim.

2. Materials and Methods

a) Haptens

N-Dnp-2-aminoethylphosphate, diphosphate and triphosphate were prepared as described in Chapter 3.

ε-N-Dnp-L-lysine and N-Dnp-L-aspartic acid were purchased from Sigma Chemical Company Ltd. (Poole, Dorset), Dnp-glycine was purchased from BDH Biochemicals (Poole, Dorset), and N-Dnp-2-aminoethylphosphonate N-Dnpaminomethylphosphonate were a gift from Dr. I. Pecht.

N-Tnpaminomethylphosphonate was synthesized from 2,4,6 trinitrobenzene sulphonic acid (TNBS) (4.1 mmoles, 1.52g, purchased from Aldrich Chemical Company, Gillingham, Dorset, as the sodium salt containing 15% water) and aminomethyl phosphonic acid (4.5 mmoles, 500 mg; purchased from Sigma Chemical Company). To 20 mls of aqueous aminomethyl phosphonic acid solution containing 0.15g of sodium bicarbonate, solid TNBS was added with vigorous stirring, reaction was continued for one hour in a silvered flask. The pH of the solution was then adjusted to 1 with concentrated hydrochloric acid and the solution was rapidly extracted with 4x1 volumes of ethyl acetate. The ethyl acetate solution was dried over magnesium sulphate and the solvent removed by rotary evaporation. The yellow solid was dissolved in the minimum volume of water and lyophilized overnight. Theoretical yield of product, calculated for $C_7H_6N_4O_9P$, 1.39g; experimental yield, 1.24g (89%). Atomic analysis, calculated for $C_7H_6N_4O_9P$; C 26.1 ; H 2.17 ; O 44.7 ; N 17.4 ; P 9.77 ; found C 25.8 ; H 2.27 ; N 17.2 ; P 9.63. The purity was checked by ^1H n.m.r.

N-Dnp-3-aminopropylphosphonate was prepared from DNFB (2,4-dinitrofluorobenzene) (1.6 mmoles, 300 mg; purchased from Aldrich Chemical Company,) and 3-aminopropylphosphonic acid (1.8 mmoles, 250 mg ; Sigma Chemical Company) by a similar procedure, except that the insolubility of DNFB in water necessitated its addition in 2 mls of dioxan, dropwise with strong

stirring to 10mls of aqueous solution containing the 3-aminopropylphosphonic acid and 0.65g of sodium bicarbonate.

N-Tnp-3-aminopropylphosphonate was prepared in a similar way to N-Tnp-aminomethylphosphonate. N-Tnp-2-aminoethylphosphate was prepared in the same way as N-Dnp-2-aminoethylphosphate. N-Tnp-L-aspartic acid was prepared in a similar way to N-Tnpaminomethylphosphonate. L-aspartic acid was purchased from B.D.H. Biochemicals.

b) Preparation of the Fv fragment from protein 315

The IgA myeloma protein 315 was isolated, and the Fv fragment prepared as described in Chapter 2.

c) Protein and Hapten Solutions

The lyophilized solids were stored in the deep freeze and solutions made up from the materials after warming. All solutions were discarded after use. Samples for the ^1H n.m.r. studies were dissolved in $^2\text{H}_2\text{O}$ (Ryvan Chemicals, Southampton) containing 0.15M NaCl. Samples for the ^{31}P n.m.r. studies were prepared by dissolving the solid in a solution made from 1 volume $^2\text{H}_2\text{O}$ and 4 volumes 0.1M ethylenediaminetetraacetic acid, sodium salt, in distilled water, and containing 0.15M NaCl. pH was adjusted with dilute sodium deuterioxide and deuterium chloride for the ^1H n.m.r. studies and with 1:4, $\text{NaO}^2\text{H} : \text{NaO}^1\text{H}$ and $^2\text{HCl} : ^1\text{HCl}$ for the ^{31}P n.m.r. studies.

d) High Resolution ^1H n.m.r. studies

^1H n.m.r. spectra were recorded at 270 MHz as described in Chapter 2.

e) Fluorescence measurements

Protein Fluorescence quenching was used to determine hapten binding as described in Chapter 2.

f) ^{31}P n.m.r. studies

^{31}P n.m.r. spectra were recorded at 36.43 MHz on a Bruker WH-90 spectrometer, operating in the fourier transform mode. The field frequency lock was obtained using the sample solvent ^2H resonance. Samples were 1.5ml, in a 5mM tube fitted with a teflon vortex plug. All spectra were collected with a spinning sample. In all cases data were collected with proton decoupling.

The pH was adjusted as described above, measurements were uncorrected meter readings in 1:4 $^2\text{H}_2\text{O}$, : $^1\text{H}_2\text{O}$ from a radiometer pH meter with a glass electrode. pH was measured before and after a spectrum was collected. The two measured values invariably differed by 0.5-0.1 pH unit, and invariably towards the pH previously adjusted from. This effect was more marked with solutions containing protein, and was due to spreading of sample up the inner walls of the tube during removal of the vortex plug. Thus the reading taken after data collection was taken to be the correct value.

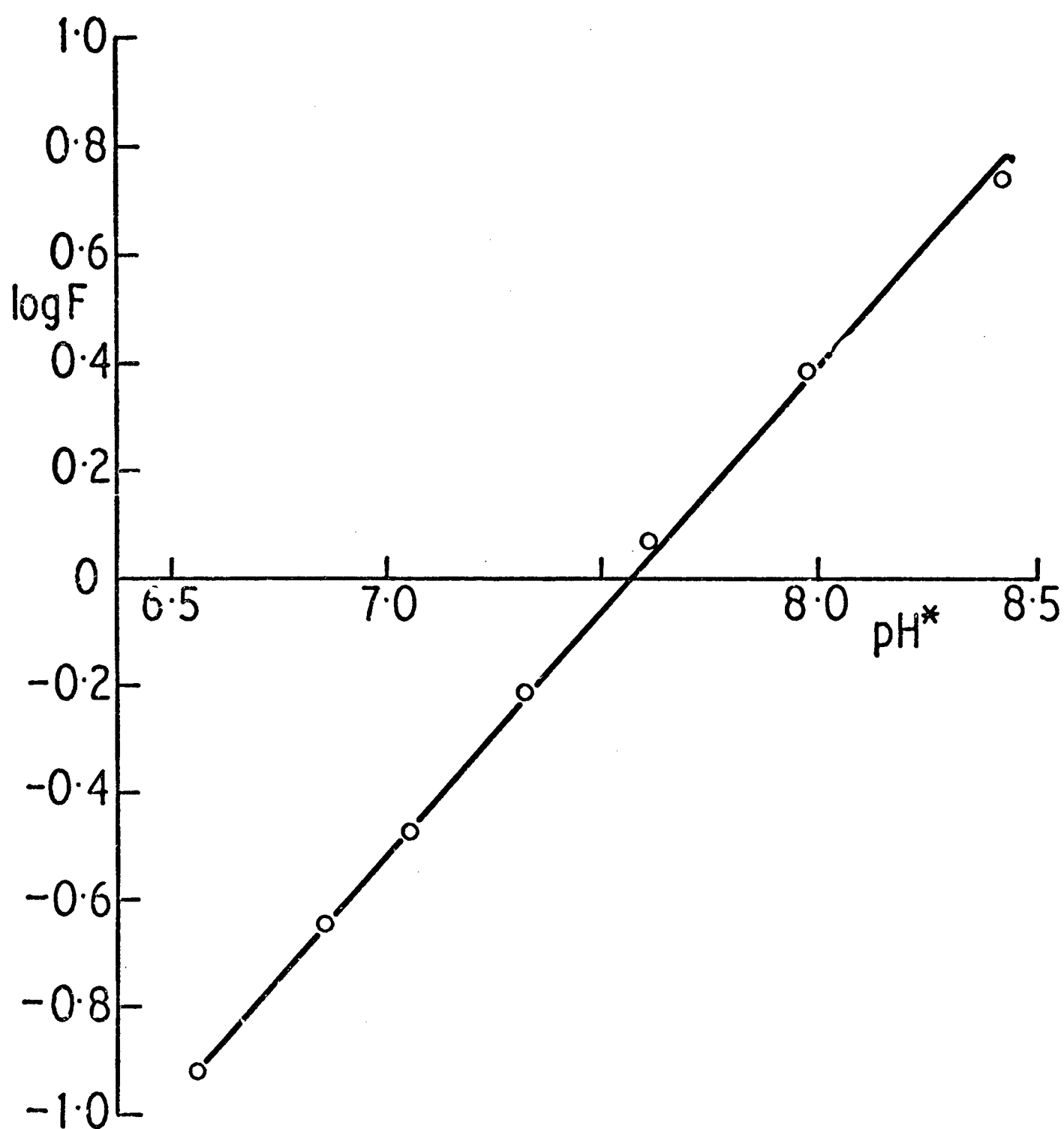
In all spectra a 60° pulse angle was used. For free hapten spectra pulse repetition rates in the range 2-4 seconds were used, for protein-bound spectra a pulse repetition rate of 1 second was used.

Under the conditions used in the protein and hapten experiments (see legends to Tables 3 and 4, and Figures 7, 8, 9) the dissociation constants given in Table 1 show that all the hapten will be bound to the protein.

g) Analysis of pH titration data

pH titration data from either ^1H n.m.r. or ^{31}P n.m.r. studies is in the form of chemical shift of one or more resonances as a function of pH. If a single resonance is seen to change shift continuously in this fashion, then the pKa that the resonance shift change is reflecting can be obtained. The shift (δ_{obs}) at any given pH will be:-

FIG. 2 : Effect of pH on the ^{31}P chemical shift of N-Dnp-3-aminopropylphosphonate: represented as a linear plot



Spectra were recorded using a 10 mM solution of N-Dnp-3-amino-propylphosphonate in 1:4 $^2\text{H}_2\text{O} : ^1\text{H}_2\text{O}$, 80 mM EDTA, 0.15 M $[\text{NaCl}]$, $T = 303^\circ\text{K}$. Chemical shifts were calculated from 85% phosphoric acid as an external standard. Spectra were recorded for 500 scans.

$$\delta_{\text{obs}} = \delta_{\text{HA}} \bar{x}_{\text{HA}} + \delta_{\text{A}^-} \bar{x}_{\text{A}^-} \quad \text{--- (1)}$$

when δ_{HA} , and $\bar{x}_{\text{HA}}$ are the chemical shift of, and fraction of, the protonated form of the system and δ_{A^-} and $\bar{x}_{\text{A}^-}$ are similarly defined for the unprotonated form. (1) can be rewritten as:

$$\frac{\Delta\delta_{\text{obs}}}{\Delta\delta_{\text{O}}} = \frac{[\text{HA}]}{[\text{HA}] + [\text{A}^-]} \quad \text{--- (2)}$$

when

$$\frac{\Delta\delta_{\text{obs}}}{\Delta\delta_{\text{O}}} = \frac{\delta_{\text{obs}} - \delta_{\text{A}}}{\delta_{\text{HA}} - \delta_{\text{A}}} \quad \text{--- (3)}$$

Equation (2) can be rewritten as:-

$$\frac{\Delta\delta_{\text{obs}}}{\Delta\delta_{\text{O}} - \Delta\delta_{\text{obs}}} = \frac{[\text{HA}]}{[\text{A}^-]} \quad \text{--- (4)}$$

this can then be substituted into the Henderson-Hasselbalch equation, to give:-

$$\text{pH} = \text{pKa} + \log_{10} \left(\frac{\Delta\delta_{\text{obs}}}{\Delta\delta_{\text{O}} - \Delta\delta_{\text{obs}}} \right) \quad \text{--- (5)}$$

If $\log_{10} F$ when F , is the term in brackets in (5), is plotted as ordinate, against pH, (5) predicts a straight line of slope 1 which intercepts the x axis at $\text{pH} = \text{pKa}$. This is illustrated for data on N-Dnp-3-propylphosphonate in Figure 2.

To confirm the values for pKa and $\Delta\delta_{\text{O}}$ measured from the experimental curves, theoretical curves were constructed using HICAL, a simple Fortran programme written in this laboratory (see Appendix III).

TABLE 1 : Dissociation Constants of Haptens from the Fv Fragment of Protein 315

Hapten	K_D (μM)
N-Dnp - glycine*	7.8
N-Dnp-L-aspartic acid*	24.5
N-Tnp-L-aspartic acid	19
ϵ -N-Dnp-L-lysine*	0.7
N-Dnpaminomethylphosphonate*	5.0
N-Tnpaminomethylphosphonate	11.0
N-Tnp-2-aminoethylphosphonate*	0.5
N-Tnp-3-aminopropylphosphonate	0.4
N-Dnpbenzylamine [†]	0.26
N-Dnp-2-aminoethylphosphate [†]	0.9
N-Dnp-2-aminoethyldiphosphate [†]	0.62
N-Dnp-2-aminoethyltriphosphate [†]	0.5
N-Tnp-2-aminoethylphosphate	0.8

+ From Haselkorn et al, 1974

* Data of C.A. Sunderland or S. Wain-Hobson

Fluorescence quenching titrations were carried out as described in Chapter 2, at pH 5.5 in the presence of 0.15M NaCl, in 50mM pipes buffer, and at 303° K.

† All conditions as above for these experiments except that pH was 7. The K_D determined for N-Dnp-2-aminoethylphosphate at pH 5.5 was 0.6 μM .

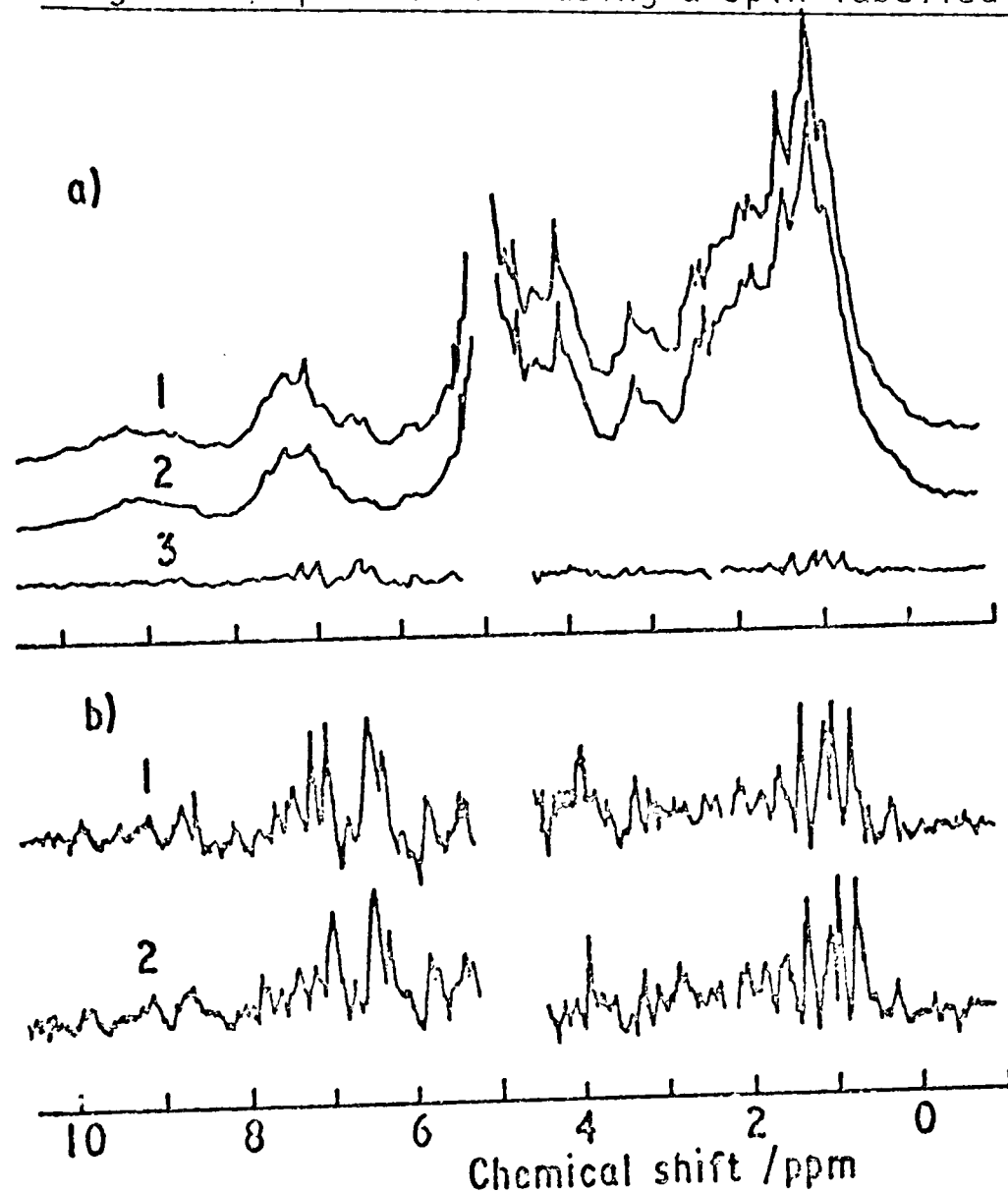
3. Results

a) Binding Studies

The dissociation constants of haptens used for further studies, were determined by the quenching of protein fluorescence (Table 1). It was necessary to ensure for the ^{31}P studies, that no hapten would be free under the conditions of the experiments. This is the case for this system, because all the haptens used in Tables 3, 4 and 5 have $K_D \leq 10\mu\text{M}$ or less. If K_D is $10\mu\text{M}$ and the concentration of the Fv fragment is 1mM and the hapten is 0.9mM then 96% of the hapten will be bound to the protein. This applies to the most weakly binding hapten N-Tnpaminomethylphosphonate.

The data in Table 1 also indicate that there is no striking dependence of binding on the structure of the hapten side chain. The data show that there is a general trend to decreasing dissociation constant as the length of the hydrophobic region of the hapten side chain increases. The dissociation constants of the N-Dnp-2-aminoethyloligophosphates are very similar. This, considered in conjunction with the identical difference spectra that these haptens produce on binding to the Fv fragment (Chapter 3), suggests that the β and γ phosphate groups do not interact with the protein.

FIG.3 : Paramagnetic difference spectra obtained with the F_V fragment of protein.315 using a spin labelled hapten.

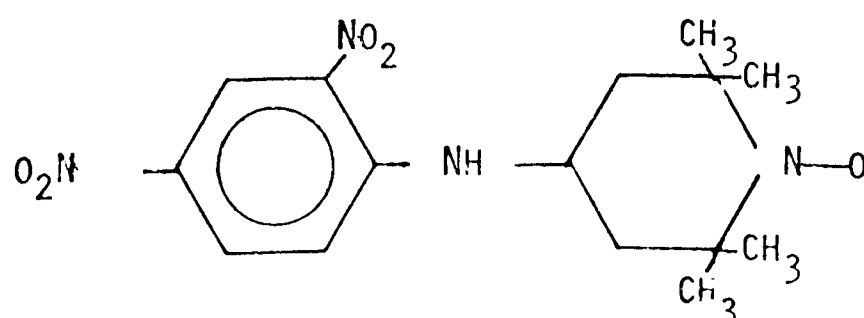


These data are taken from Dwek et al (1975).

- a) F_V fragment of protein 315 - 1.2mM, $T=303^\circ\text{K}$
 $\text{pH}^* = 6.29$, $[\text{NaCl}] = 0.15\text{M}$, 2000 scans

1. F_V fragment
2. F_V fragment + spin labelled hapten
3. difference spectrum

- b) Difference spectra enlarged (x6), at
1. pH 6.29
 2. pH 8.47



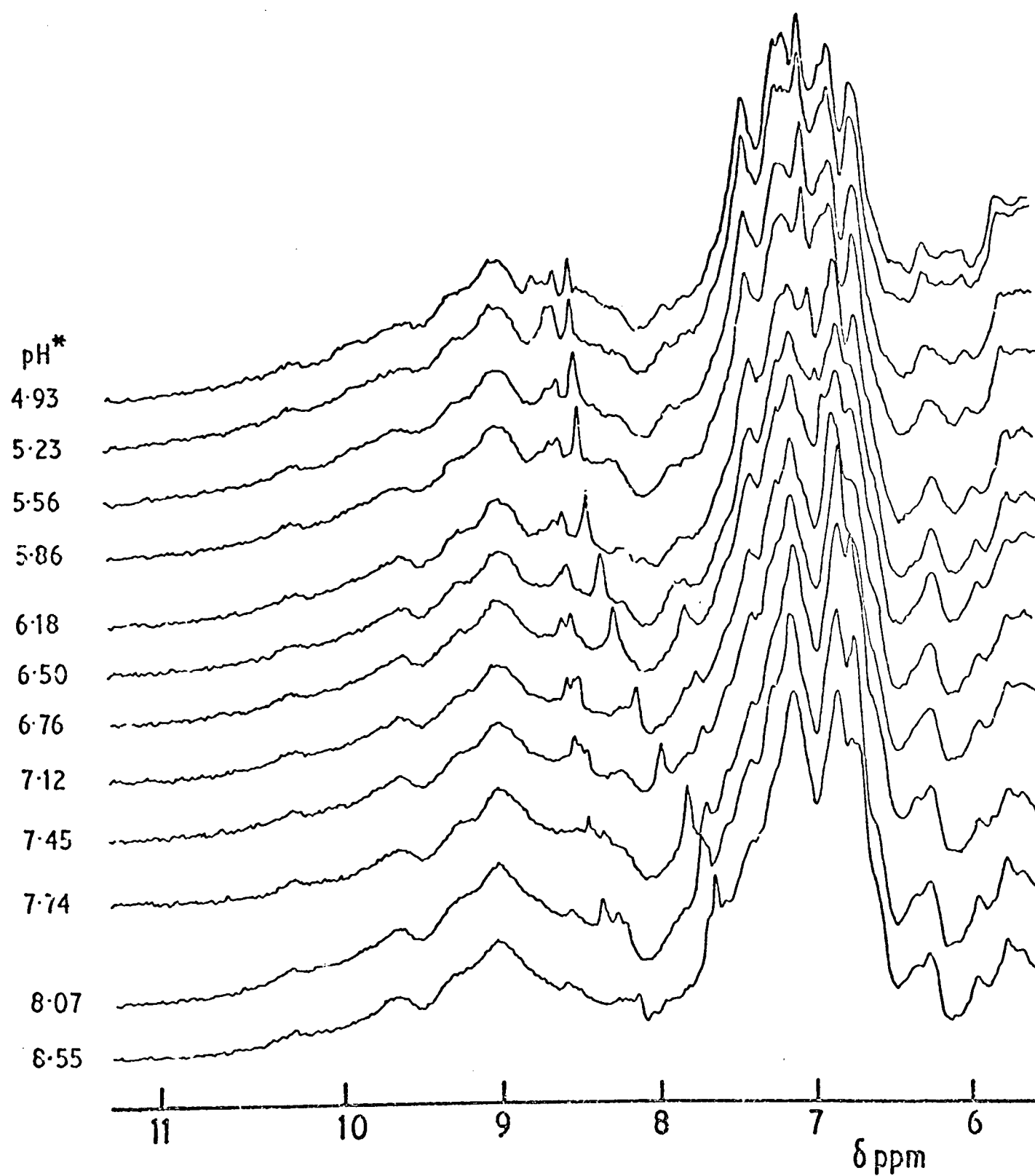
Structure of the spin-labelled hapten.

b) The histidine residues of the F_V fragment of protein 315.

The F_V fragment of protein 315 contains three histidine residues. These are at positions 102 in the heavy (V_H) chain (Francis et al, 1974) and at positions 97 and 44 in the light (V_L) chain. The model of Padlan et al (1976) locates His 44_L at the back of the F_V fragment, His 97_L outside the combining site, on the opposite side of the L3 loop from Trp 93_L , and His 102_H on the edge of the combining site projecting into the combining pocket. The pH dependence of the aromatic region of the ^1H n.m.r. spectrum of the F_V fragment of protein 315 in the presence of N-Dnpaminomethylphosphonate is shown in Fig. 4. Three histidine H2 resonances can be observed changing chemical shift with pH*. The H4 resonances can be observed by difference spectroscopy as shown in Fig. 5. The pH dependence of the chemical shifts of the three H2 resonances are shown in Fig. 6, for the F_V alone, and in the presence of two different haptens. The results of a number of such experiments are summarized in Table 2.

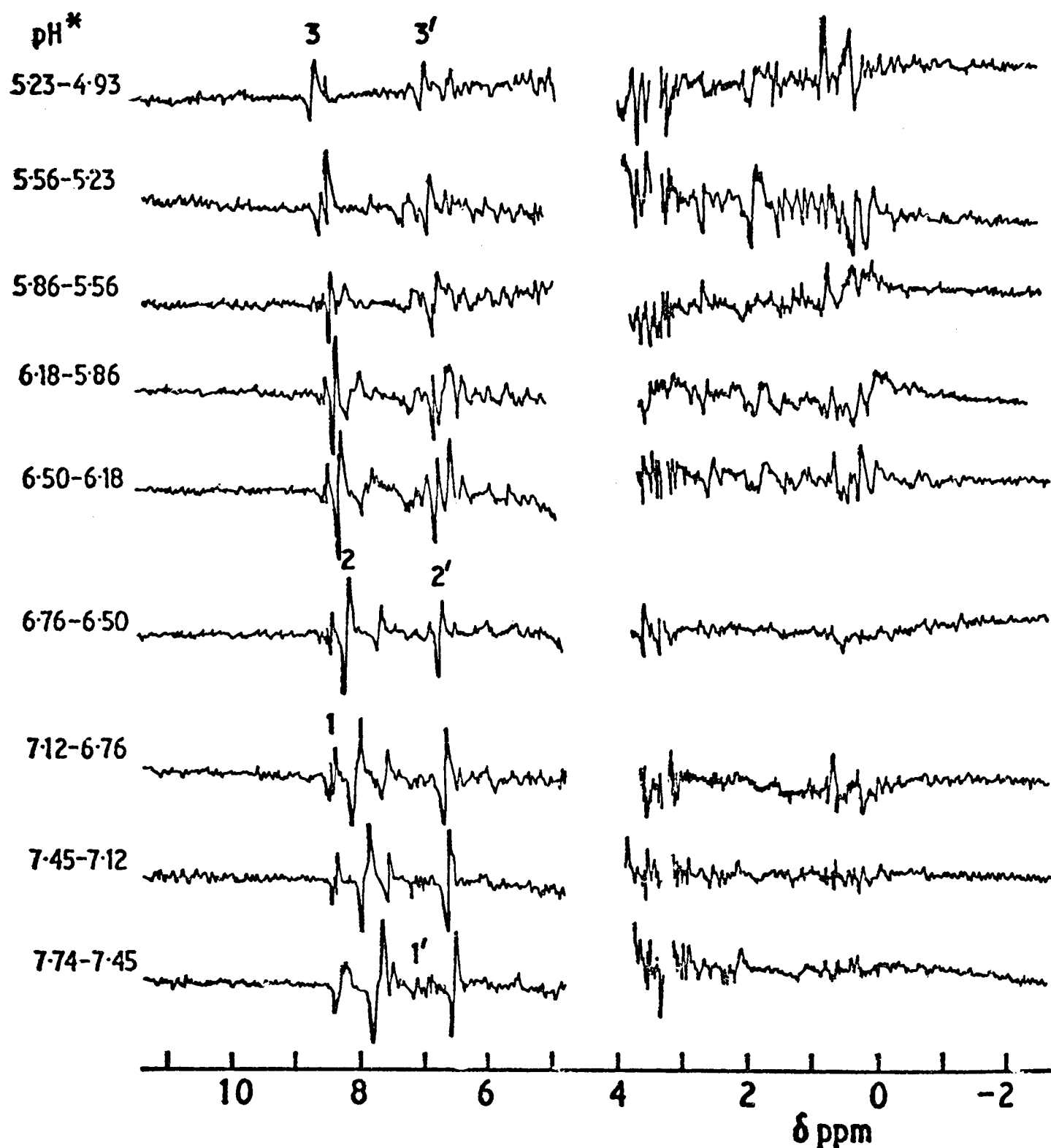
The three histidines of the F_V fragment have different pKa's; that of histidine 1 is 8.2, that of histidine 2 is 6.9 and that of histidine 3 is 5.9. Hence, in the absence of any other evidence, it is clear from the ^1H n.m.r. data, that the F_V fragment contains three different histidine residues. This is consistent with the known sequences of V_L and V_H 315. Fig. 3 (taken from Dwek et al, 1975, a, b) shows that addition of a spin label hapten causes the linewidth of the resonances of histidines 2 and 3 to be broadened. Further, the manganese binding experiments in Chapter 3 indicate that the order of distance of the three histidines from the bound metal ion, known to be located at the hapten side chain, is, 2 closer than 3, much closer than 1. Thus since histidine 97_L and 102_H are in hypervariable segments whilst 44_L is not, histidine 44_L is histidine 1 with a pKa of 8.2.

FIG.4 : pH* dependence of the aromatic region of the 270MHz ^1H n.m.r. spectrum of the F_V fragment of protein 315 in the presence of N-Dnpaminomethylphosphonate



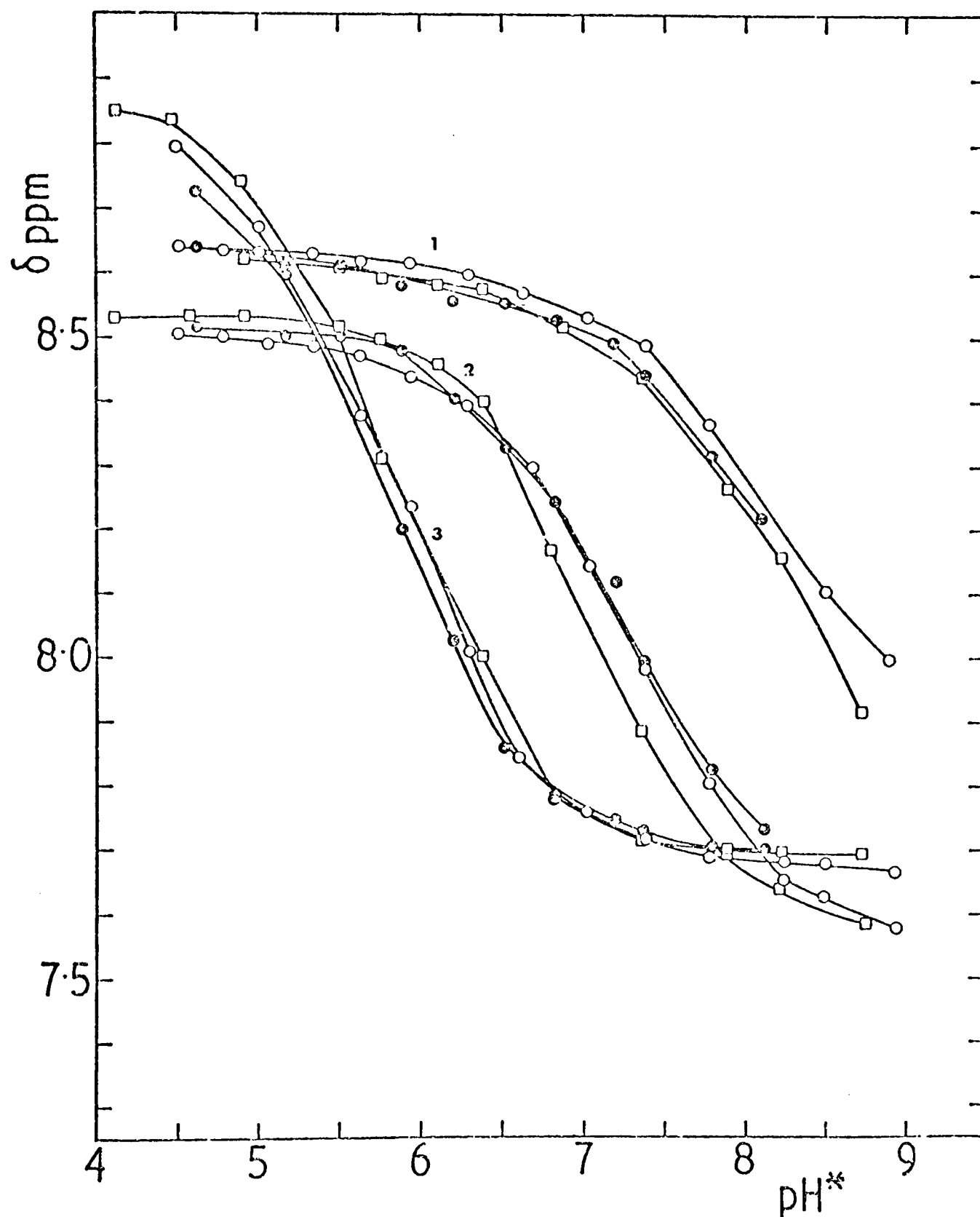
Spectra were recorded using a 1.67mM F_V fragment solution containing 1.67 mM N-Dnpaminomethylphosphonate, 0.15M NaCl in $^2\text{H}_2\text{O}$ $T = 303^\circ\text{K}$.

Fig. 5 : Difference spectra illustrating the pH* dependence of the 270MHz ^1H n.m.r. spectrum of the F_γ fragment of protein 315 in the presence of N-Dnpaminomethylphosphor



Sample as described in Fig. 4, spectra were generated by subtracting as indicated in the figure.

FIG. 6 : pH* dependence of the ^1H chemical shifts of the H2 resonances of the histidine residues of the F_v fragment of protein 315.



- □ - 1.7mM F_v fragment⁺
- ● - 1.7mM F_v fragment + 1.7mM N-Tnpamino-ethylphosphonate[‡]
- ○ - 1.4mM F_v fragment + 1.4mM N-Dnp-2-aminoethylphosphate.

All samples in $^2\text{H}_2\text{O}$, 0.15M NaCl, T=303°K

+ Solvent suppression 35 dB on $^1\text{H}^2\text{H}_0$

‡ Solvent suppression 40 dB on $^1\text{H}^2\text{H}_0$

TABLE 2 : pKa values of the three histidine residues in the
F_v fragment of protein 315

Hapten	(pKa values)		
	1	2	3
None	8.2	6.9	5.9
N-Dnp-glycine	8.2	7.5	5.9
N-Tnp-L-aspartic acid	8.2	7.3	5.9
N-Dnp-L-aspartic acid	8.2	7.3	5.9
ε-N-Dnp-L-lysine	8.2	7.1	5.9
N-Dnpaminomethylphosphonate	8.2	7.3	5.9
N-Tnpaminomethylphosphonate	8.2	7.3	5.8
N-Tnp-2-aminoethylphosphonate	8.2	7.3	5.7
N-Tnp-3-aminopropylphosphonate	8.2	7.2	5.9
N-Dnp-2-aminoethylphosphate ⁺	8.2	7.4	5.9
N-Dnpbenzylamine ⁺	8.2	7.1	5.9

All values were determined from the pH dependence of the aromatic region of the 270 MHz ¹H n.m.r. spectrum of the F_v fragment of protein 315. Spectra were obtained and analyzed as described in Chapter 2. An example is shown in Figures 3 and 4. Protein concentrations were in the range 1-2mM, all solutions were in ²H₂O and contained 0.15M NaCl; T = 303°K. In those cases where haptens were added, the hapten and protein concentrations were the same. These data were obtained from analysis of the chemical shift changes of the H2 resonances only.

+ Data from Dwek et al, 1975.

TABLE 3 : pKa values of a series of phosphonate haptens free and bound to the F_v fragment of protein 315

Hapten	pK_a^f	pK_a^b	$\Delta\delta_A$	$\Delta\delta_B$
N-Tnpaminomethylphosphonate ⁺	5.7	5.9±0.2	0	0
N-Tnp-2-aminoethylphosphonate ⁺	6.9	7.1±0.2	±0.2	±0.5
N-Tnp-3-aminopropylphosphonate [*]	7.45	6.85	0.7	0.7

+ Data of Simon Wain-Hobson, measured at 129MHz and room temperature in the absence of EDTA, in 1:4 ²H₂O:¹H₂O, 0.15M NaCl.

* Experiments performed both at 129MHz, no EDTA (S. Wain-Hobson) and at 36.43 MHz, 80 mM EDTA, 0.15M NaCl, T = 303°K, Hapten concentration for the 'free' (pK_a^f) measurement 10 mM. For bound (pK_a^b) measurement, sample was 0.54 mM F_v, 0.5 mM N-Tnp-3-aminopropylphosphonate. 61° pulse angle, 2000 scans accumulated.

$\Delta\delta_B$ is the shift change of the -2 anion in ppm on binding to the protein:-

$$\Delta\delta_B = \delta_B \text{ (bound)} - \delta_B \text{ (free)}$$

$\Delta\delta_A$ is similarly defined for the -1 anion.

pK_a^f is the pKa of the hapten alone.

pK_a^b is the pKa of the hapten when bound to the F_v fragment.

The data in Table 2 show that the pKa of one of the three histidine residues is consistently perturbed on hapten binding. This is histidine 2, with a pKa of 6.9 in the F_V fragment alone. This histidine is also close to the metal ion bound to the side chain of N-Dnp-2-aminoethylphosphate (see Chapter 3). As shown in Chapter 3 the structure of Padlan et al is correct to the extent that it has predicted the overall relative orientations of the hypervariable loop backbones (see Chapter 1, section (c)) thus it follows that histidine 2 is histidine 102_H. Therefore, by elimination, histidine 3 is histidine 97_L.

Irrespective of the correctness of these last assignments, the data in Tables 1 and 2 indicate that the perturbation of the pKa of histidine 2, which projects into the combining site, shows no marked dependence on the structure of the hapten side chain. This suggests that histidine 2 does not make any strong interactions with the hapten side chain. It is therefore unlikely to be the residue responsible for the positive subsite detected by Haselkorn et al (1974) and Sutton et al (1977).

c) P³¹ n.m.r. studies of hapten binding to the F_V fragment

Data from the P³¹ n.m.r. investigations of Wain-Hobson et al (1977), into the binding of haptens containing phosphate and phosphonate groups, to the F_V fragments are summarized in Table 3. These data indicate that if all three haptens bind to the F_V fragment in a fully extended conformation then there is a positively charged residue 8.0-10.0Å from the Dnp ring binding site. The marked downward perturbations in the pKa of the second ionization of the phosphonate group of N-Tnp-3-amino-propylphosphonate, on which the above conclusion is based, was not observed with N-Dnp-2-aminoethylphosphate. These two haptens are of identical length, according to crystallographic results. (Kraut, 1961; Okaya, 1966.) Wain-Hobson et al, argued that different conformations exist about the Tnp-NHR and Dnp-NHR bonds. In order to test this hypothesis, the data shown in Table 4 were collected. These data

TABLE 4 : pKa values for a set of phosphate and phosphonate haptens free and bound to the Fv fragment of protein 315.

	pK_a^f	pK_a^b	$\Delta\delta_A$	$\Delta\delta_B$
N-Dnp-2-aminoethylphosphate	6.3	6.6 ⁺	+0.2	+1.3
N-Tnp-2-aminoethylphosphate [*]	6.2	6.2±0.2	—	—
N-Dnp-3-aminopropylphosphonate [‡]	7.55	6.85	-8.5	-9.58
N-Tnp-3-aminopropylphosphonate [‡]	7.45	6.85	+0.7	+0.7

+ Data of Simon Wain-Hobson; measured at 129MHz. pK_a^f for this hapten was determined by a ^1H n.m.r. titration at 270MHz, monitoring the chemical shift of the ^1H 's.

* For this hapten, experiments were carried out at 129MHz, in 80mM EDTA, 0.15M NaCl at 303°K. In the absence of protein 10mM N-Tnp-3-aminopropylphosphonate was used. In the presence of protein, 1.5mM F_v fragment and 1mM hapten. The error is calculated simply from ΔV_{Y_2} .

‡ All experiments were carried out at 36.43MHz in 80mM EDTA, 0.15M NaCl; T=303°K. The free hapten measurements were carried out on a 10mM solution. The conditions for N-Tnp-3-aminopropylphosphonate and F_v fragment are described in Table 2. For N-Dnp aminopropylphosphonate the sample was 0.93mM, hapten 0.8mM.

All samples were 1:4 ^2H_2O : ^1H_2O . For the free haptens 200 scans with a 60° pulse angle and a pulse repetition rate of 1.024 seconds was used. For the bound haptens 1000 scans were used with a 60° pulse angle and a pulse repetition rate of 0.512 seconds.

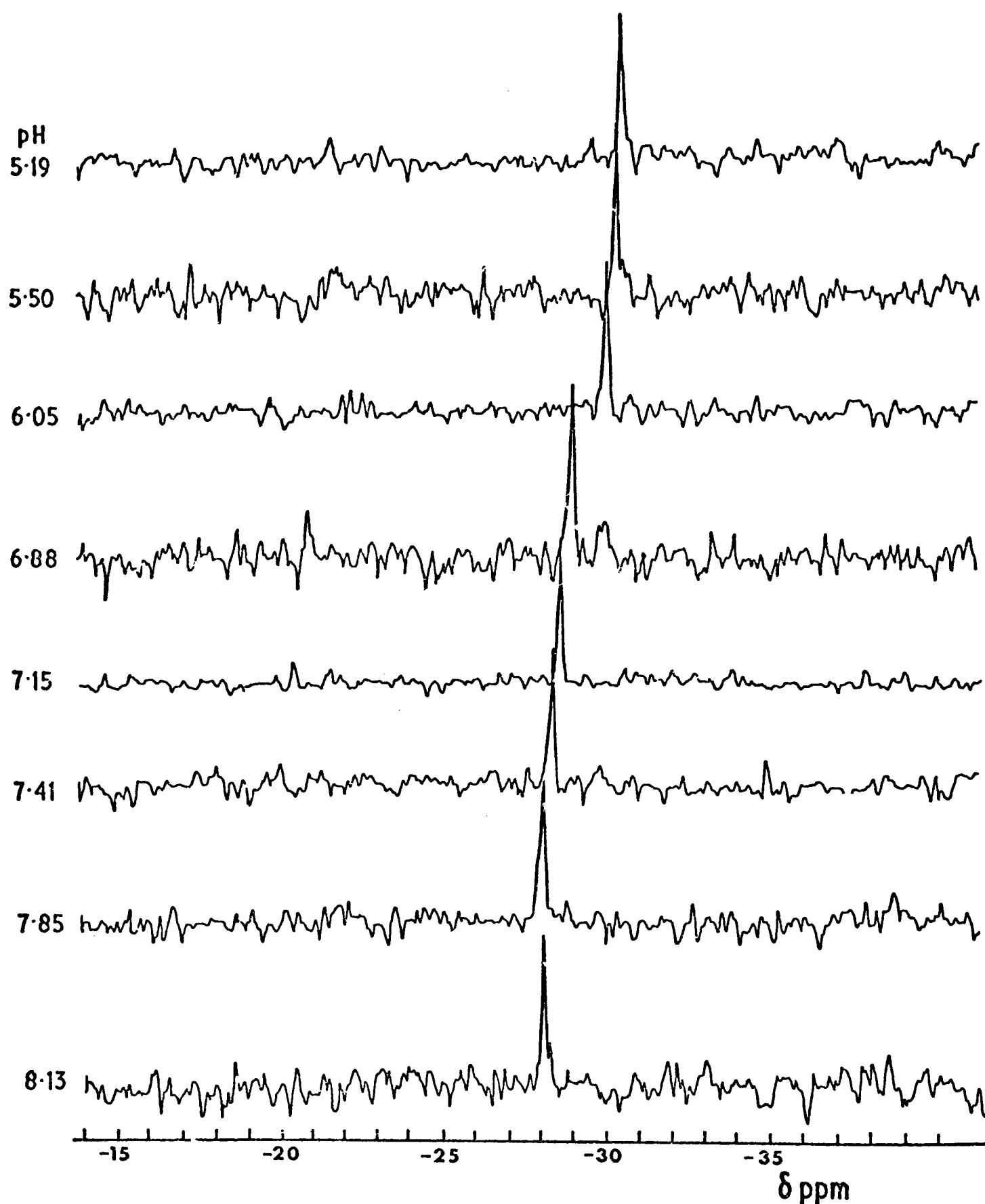
$\Delta\delta_A$, $\Delta\delta_B$, pK_a^f , pK_a^b as for Table 2.

FIG. 7(a) : pH dependence of the ^{31}P n.m.r. spectrum of N-Dnp-3-aminopropylphosphonate



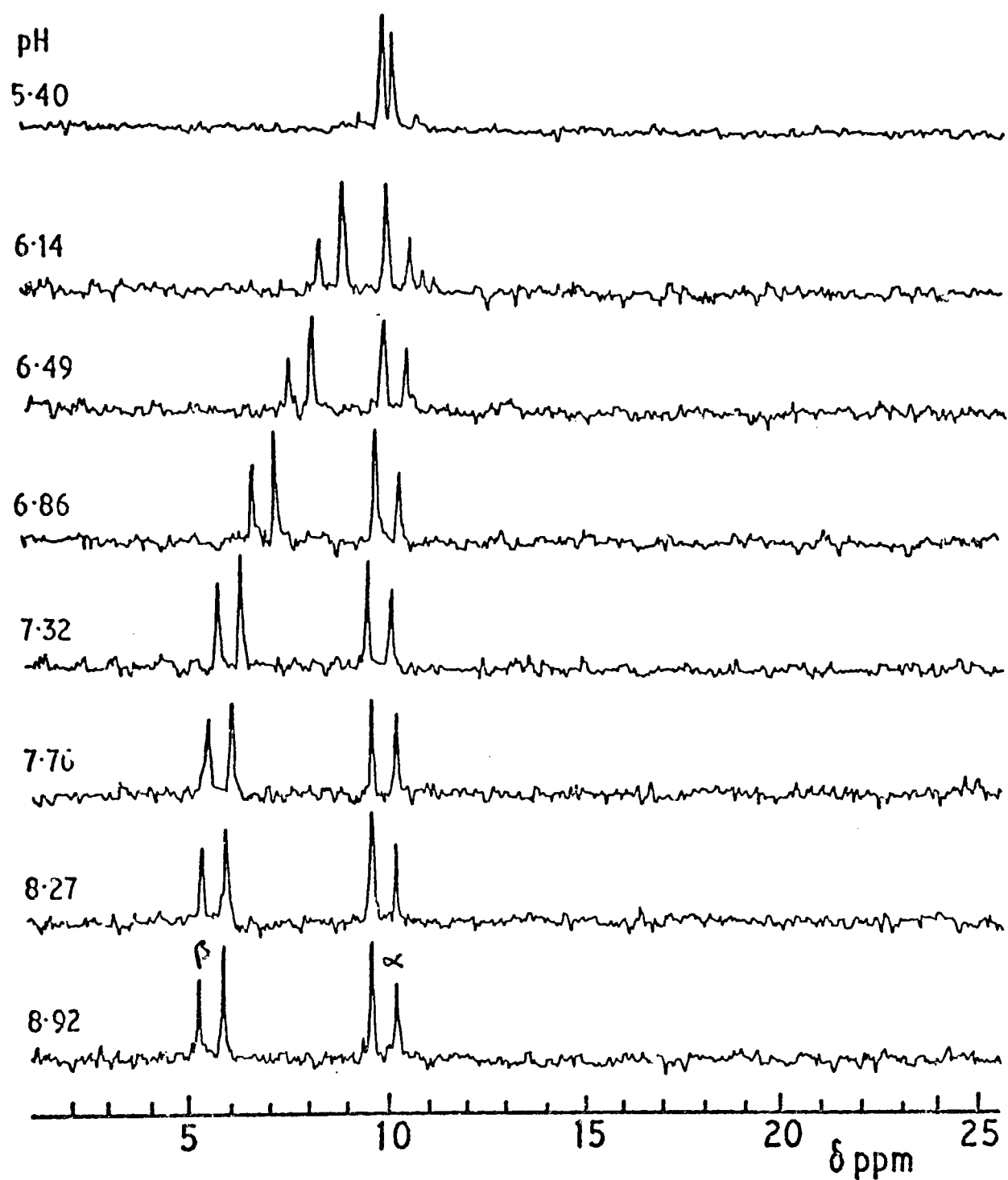
The sample was 10mM K-Dnp-3-aminopropylphosphonate, 100mM EDTA 0.15M NaCl in 1:4, ^2H O ; ^1H O ; $T=303^\circ\text{K}$. Linebroadening 1Hz, 100 accumulations per spectrum.

FIG. 7(b) : pH dependence of the ^{31}P n.m.r. spectrum of
N-Dnp-3-aminopropylphosphonate in the presence
of the F_V fragment of protein 315.



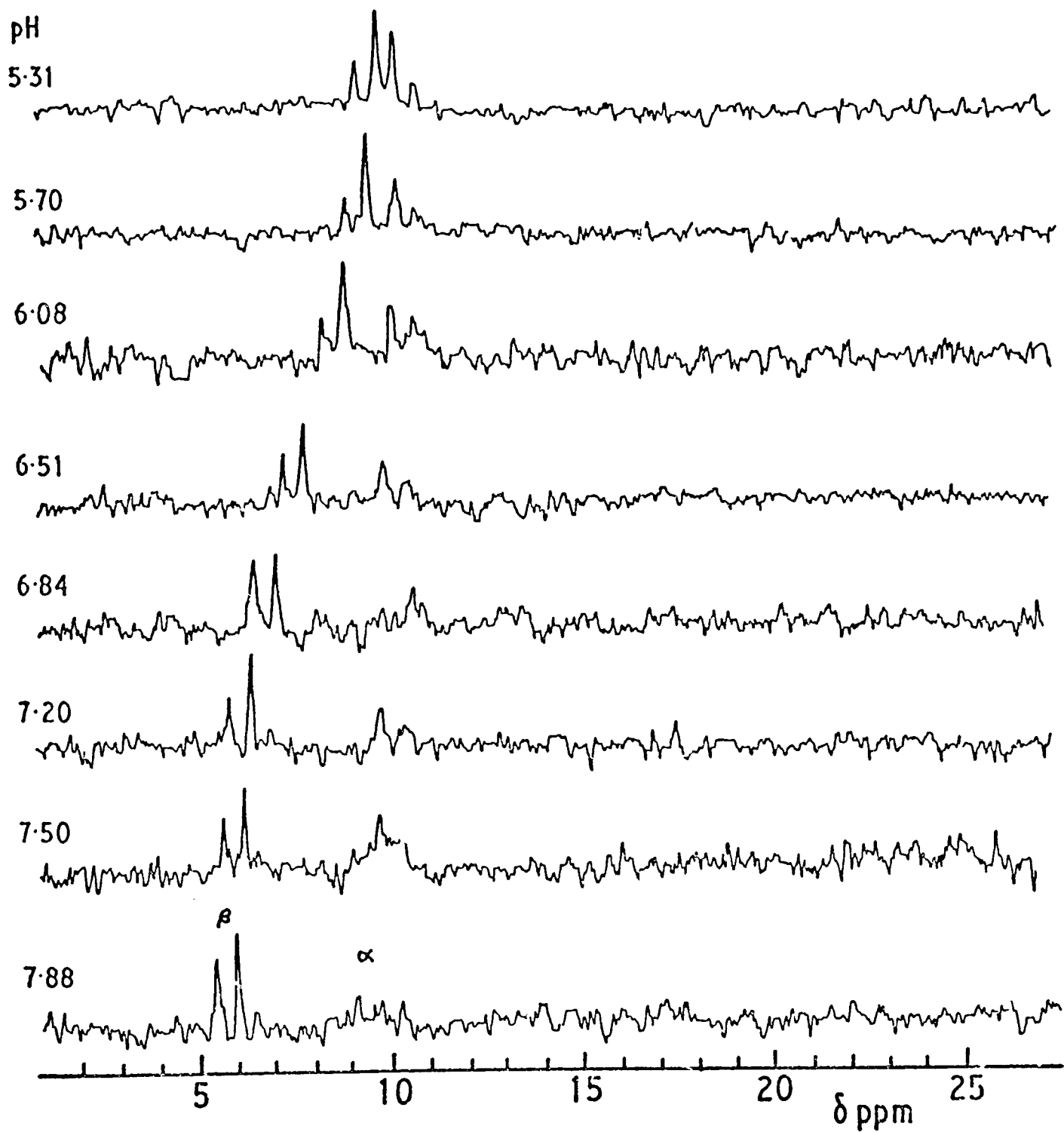
The sample was 0.8mM N-Dnp-3-aminopropylphosphonate, 0.93 mM F_V fragment, 0.15M NaCl, 100mM EDTA in 1:4, $^2\text{H}_2\text{O} : ^1\text{H}_2\text{O}$ $T=303^\circ\text{K}$. Linebroadening 2Hz. 1000 scans accumulated per spectrum.

FIG. 8(a) : pH dependence of the ^{31}P n.m.r. spectrum of N-Dnp-2-aminoethyldiphosphate



The sample was 5mM N-Dnp-2-aminoethyldiphosphate, 80mM EDTA, 0.15M NaCl; in 1:4, $^2\text{H}_2\text{O} : ^1\text{H}_2\text{O}$; $T=303^\circ\text{C}$ linebroadening 1Hz, 200 scans accumulated per spectrum.

FIG. 8(b) : pH dependence of the ^{31}P n.m.r. spectrum of N-Dnp-2-aminoethyldiphosphate in the presence of the F_V fragment of protein 315.



The sample was; N-Dnp-2-aminoethyldiphosphate 0.95mM,
 F_V fragment 1.03mM, 80mM EDTA, 0.15M NaCl. 1:4 $^2\text{H}_2\text{O}$:
 $^1\text{H}_2\text{O}$. $T=303^\circ\text{K}$. Linebroadening 2Hz, 1000 scans
 accumulated per spectrum.

FIG. 9(a) : pH dependence of the ^{31}P n.m.r. spectrum of N-Dnp-2-aminoethyltriphosphate.

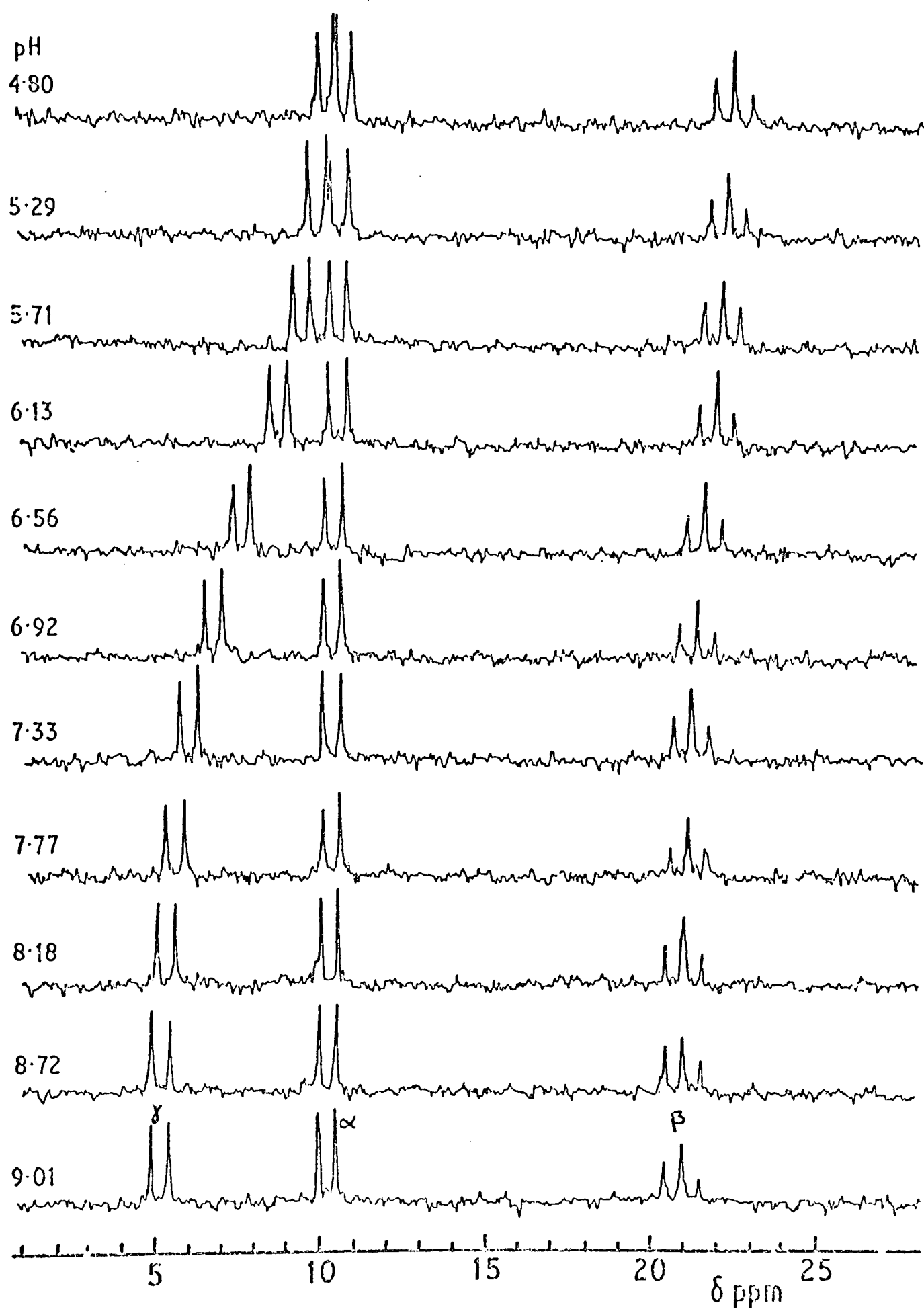
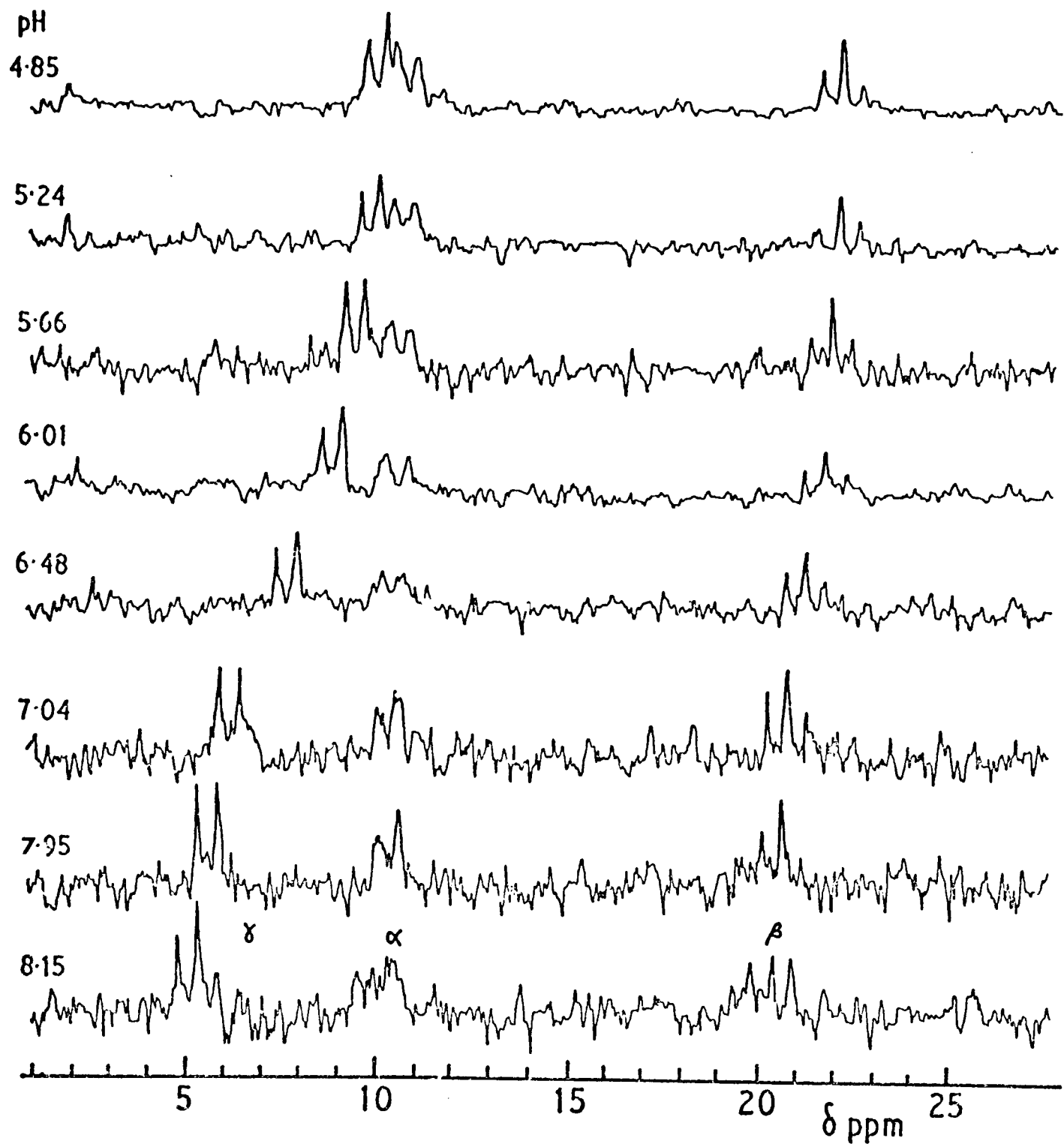


Fig. 9(b) : pH dependence of the ^{31}P n.m.r. spectrum of N-Dnp-2-aminoethyltriphosphate in the presence of the F_V fragment of protein 315.



The sample was, 0.95mM N-Dnp-2-aminoethyltriphosphate, 1.03mM F_V fragment, 80mM EDTA, 0.15M NaCl, 1:4 $^2\text{H}_2\text{O}$: $^1\text{H}_2\text{O}$, $T=303^\circ\text{K}$. 2Hz linebroadening, 1000 scans accumulated² per spectrum.

Indicate that the presence or absence of a pKa perturbation, is a function of the chemical nature of the phosphorus derivative and not of the possible internal conformation of the hapten. It is not clear why this marked difference between phosphates and phosphonates should occur in this system. This is discussed further below.

Shift changes of the ^{31}P resonance are also seen for many of these haptens (Tables 3 and 4). The largest of these were observed on N-Dnp-3-aminopropylphosphonate, this is shown in Figs. 7(a)(b). The theory of phosphorous chemical shifts is not well defined, however an explanation for this effect, in terms of possible interactions with the protein, can be made (see discussion).

The series of N-Dnp-2-aminoethanol derivatives, N-Dnp-2-aminoethyl-phosphate, diphosphate and triphosphate were studied by ^{31}P n.m.r. (Figs. 8(a),(b) and 9(a),(b), to search for polar groups further away from the Dnp binding site than 8.0 Å. Only in the case of the N-Dnp-2-aminoethyl-phosphate was any perturbation of the pKa of the last ionization of the phosphate group seen. Indeed, none of the pKa's, none of the chemical shifts and none of the ^{31}P coupling constants of the β and γ groups of the diphosphate and triphosphate haptens are significantly perturbed when these bind to the Fv fragment (Tables 5 and 6). These data indicate that the β and γ phosphate groups do not interact with the protein. This conclusion is supported by the 270MHz ^1H n.m.r. difference spectra presented in Chapter 3 (see page 100).

Finally, the data shown in Figs. 8(a),(b) and 9(a),(b) indicate that the α phosphate group resonances are broadened when the N-Dnp-2-aminoethyl-diphosphate and N-Dnp-2-aminoethyltriphosphate bind to the protein, whilst the resonances of the β and γ phosphates are not. This is shown more clearly in Figure 10. The probable causes of this differential broadening, are discussed below.

It is clear from the small perturbations in pKa and shift, or line-width of the α phosphate group of these three haptens, that the α

TABLE 5 : pKa's free and bound to the F_V fragment for
N Dnp-2-aminoethyloligophosphates

Hapten	pK _a ^f	pK _a ^b	Δδ _A	Δδ _B
N-Dnp-2-aminoethylphosphate	6.3	6.6*	+0.2	+1.3
N-Dnp-2-aminoethyldiphosphate	6.53	6.53	+0.3	0
N-Dnp-2-aminoethyltriphosphate	6.53	6.53	0	+0.1

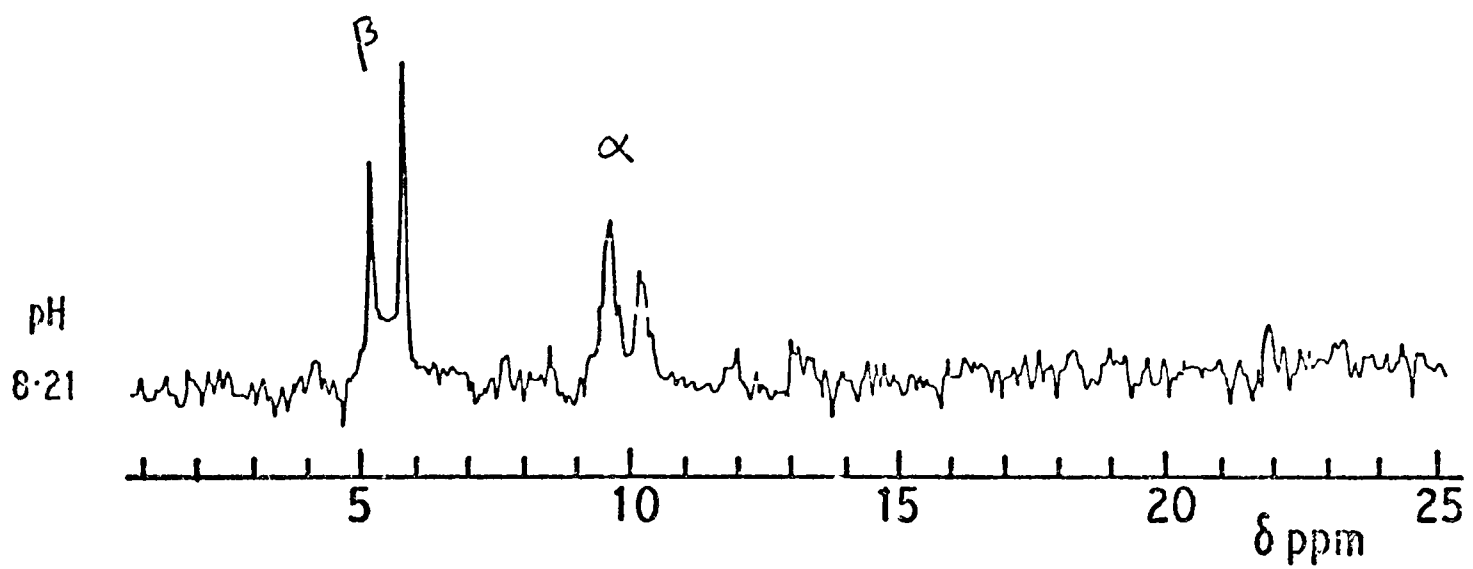
* Data of Simon Wain-Hobson, see Table 3.

TABLE 6 : ³¹P coupling constants in Hz, for phosphate haptens
free and bound to the F_V fragment of protein 315

	free		bound	
	pH	pH	pH	pH
N-Dnp-2-aminoethyldiphosphate	5.40	8.27	5.31	8.21
J α β	20	22	20	21
N-Dnp-2-aminoethyltriphosphate	5.29	8.18	5.24	8.15
J α β	19	20	20	20
J β γ	19	20	19	20

Conditions as given in Figs 8 & 9

FIG. 10 : The effect on the ^{31}P n.m.r. spectrum of N-Dnp-2-amino-ethylidiphosphate of the binding of this hapten to the F_V fragment of protein 315.



Conditions as for Figure 8(b), except for 3000 scans accumulated.

phosphate environment is changed on hapten binding. Thus this group is within the site when the hapten is bound, the β and γ groups of these haptens, however, are not. Thus the site is between 8.0 Å and 12.0 Å in depth, from the centre of the Dnp ring to the protein surface. This value is consistent with those obtained by other methods (Sutton et al, 1977; Wilder, Green and Schumaker, 1975; Pecht, 1974).

4. Discussion

a) The histidine residues of the Fv fragment

Six resonances in the 270 MHz ^1H nmr spectrum of the Fv fragment of protein 315 have been assigned to the three histidine residues located at positions 44_{L} , 97_{L} and 102_{H} in the sequences (Francis et al, 1974; Schulenberg et al, 1971). In order to assign the resonances of the histidine pK_{a} 's 6.9 and 5.9 to histidines 102_{H} and 97_{L} respectively, it must be assumed that the model of Padlan et al correctly predicts the conformation (of the polypeptide chain backbones) of the L_3 and H_3 hypervariable loops.

The data presented in this chapter indicate that none of the three histidine residues contributes significantly to the free energy of binding of the hapten.

b) ^{31}P n.m.r. studies

There are four measurable parameters obtainable from the ^{31}P nmr spectra that may change when phosphonate or phosphate ligands bind to a protein; chemical shift, linewidths, coupling constants, and the pK_{a} of the ^{31}P -containing group.

i) Chemical shifts. It has been proposed that the ^{31}P chemical shift changes of alkyl phosphonates can be described by the equation:-

$$\Delta\delta = 100\Delta X_{\text{R}} - 4\Delta\theta_{\text{O-P-O}} - 147\Delta\pi_{\text{P-O}} \quad \sim (6)$$

When ΔX_{R} is the change in the effective electronegativity of the alkyl substituent, $\Delta\theta_{\text{O-P-O}}$ is the change in the O-P-O bond angle and $\Delta\pi_{\text{P-O}}$ is the change in the oxygen/phosphorous π -orbital overlap (Riess, Letcher and Van Wazer, 1967; Letcher and Van Wazer, 1966 a, b). Gorenstein (1975) has suggested that, in the case of phosphates, large shift changes

(± 10 -20 ppm) can be produced by small bond angle distortions (1 - 5°) alone. This is supported by studies on cyclic phosphates and phosphonates (Blackburn, Cohen and Weatherall, 1971), which show that 5-membered ring compounds and 6-membered ring compounds show shifts of -15 and +8 ppm respectively compared with the acyclic derivative.

Most of the haptens show small shifts in the ^{31}P resonances on binding to the Fv fragment (0-2 ppm) (Tables 3 and 4). In one case, that of N-Dnp-3-aminopropylphosphonate (Figs. 7a, b, and Table 4) a downfield shift of c9 ppm occurs on binding to the Fv fragment. The origin of this shift may well be a small bond angle distortion (Purdela, 1971). This effect has been postulated to account for the shift changes in ^{31}P resonances in nucleotide stacking interactions (Patel, 1974). It is not clear what relationship exists between thermodynamic stability, O-P-O bond angle, and ^{31}P chemical shift. However, Blackburn et al, report that downfield shifted 5-membered ring compounds are in a strained conformation. Thus the data suggest that the bound hapten phosphate group, in the case of N-Dnp-3-aminopropylphosphonate in particular, may be in a strained conformation.

ii) Linewidths. Figures (8(b) and 9(b) show the ^{31}P nmr spectra of N-Dnp-2-aminoethyltriphosphate and N-Dnp-2-aminoethyldiphosphate bound to the Fv fragment. In both cases it can be seen that the linewidth of the α -P resonance is increased relative to those of the β or, β and γ resonances. From the data presented in Fig. 10, it can be estimated that the linewidth of the α -P resonance increases to 8 Hz on binding of N-Dnp-2-aminoethyldiphosphate to the Fv fragment at pH 8.21. The (1 Hz) linewidth of the β resonance is not detectably affected by this process.

In the absence of paramagnetic centres, three mechanisms dominate ^{31}P spin relaxation; chemical shift anisotropy, dipolar interactions with nearby protons, and chemical exchange. The correlation time for the first two mechanisms is taken to be the rotational correlation time for

the Fv fragment (7×10^{-9} sec.; Sutton et al, 1977; Dwek et al, 1976), which is the longest correlation time in the system. Using the value of the chemical shift anisotropy of adenosine-5' -diphosphate (150 ppm; Gadian, 1974), the contribution of this mechanism to the linewidth of the α -P resonance of N-Dnp-2-aminoethyldiphosphate is calculated to be 0.35 Hz (see Appendix I). The contribution from dipolar broadening would be expected to be dominated by the two protons on the 1' CH₂ group of the hapten, which are 0.25 nm from the phosphorous nucleus (Kraut, 1961). The dipolar broadening contribution is calculated using this distance, to be less than 1 Hz (Appendix I).

In considering chemical exchange contributions to linewidth, two equilibria must be taken into account, the binding of the hapten to the Fv fragment, and the third ionization of the diphosphate group. Since the dissociation constant of the hapten from the Fv fragment is 0.63 μ M (Table 1), the hapten will be 99.5% bound to the protein under the conditions of the nmr experiments. Further, the shift change of the α -P resonance on binding of hapten to the Fv fragment is small (>0.2 ppm), it can thus be calculated that the contribution to the linewidth from this process is $c 10^{-3}$ Hz (Appendix I). Finally, the proton exchange process cannot account for the α -P resonance linebroadening. Since both the α -P and the β -P resonances chemical shifts reflect the same proton exchange process, and the chemical shift change of the latter on ionization is much greater than that of the former, and yet the latter shows no increase in linewidth (Fig 8[b]), on binding to the Fv fragment.

Thus none of the mechanisms can account for the observed line broadening effect. A possible explanation for this discrepancy is that chemical shift anisotropy of α -P group is markedly increased on binding of the hapten to the protein. It would be necessary to postulate an increase to 500 ppm to account for the observed linebroadening effects (Appendix I). This is rendered more probable by the observed shift in the case of N-Dnp-3-amino-propylphosphonate. In one case distortion of the phosphorus-containing

group produces a shift, and in the other case this produces no net shift but an increase in chemical shift anisotropy. If this is the case one cannot estimate a correlation time for the system, since it is possible that the correlation time is shorter than 7×10^{-9} sec. but that the chemical shift anisotropy is larger than 500 ppm.

iii) Coupling constants. Two types of coupling constant are observable in ^{31}P nmr spectra of alkylphosphates and phosphonates, due to ^1H - ^{31}P and ^{31}P - ^{31}P coupling. All spectra reported in this chapter were recorded with proton decoupling, thus only ^{31}P - ^{31}P coupling constant were obtained from experiments on the di- and triphosphate derivatives of N-Dnp-2-aminoethanol. These are c 20 Hz for both haptens, at all pH's, whether free, or bound to the Fv fragment (Table 6). Since the value of coupling constants depends on the conformation of the oligophosphate group, these data show that the average conformation of these groups does not change on binding of the haptens to the Fv fragment. This is again consistent with the hypothesis that the β and γ phosphate groups do not interact with the Fv fragment.

iv) pK_a values. The pK_a values of alkylphosphates and phosphonates have been related to the structure of these compounds by Kumler and Eiler (1943) and Crofts and Kosolapoff (1953) respectively. In the former report the authors argue that the difference in pK_a 's between phosphoric acid (1.97 and 6.82) and methyl phosphoric acid (1.54 and 6.31) are explicable on the basis of the differences in solvation for the different charge states of the two compounds. This is necessary since, on the basis of inductive effects alone, the pK_a value of a phosphoric acid derivative containing an electron donating alkyl group would be expected to be higher than that of phosphoric acid. Indeed, replacing a methyl group by an ethyl group causes an increase in pK_a of 0.4 pH units. The authors conclude that the solvation effect causes a fall in pK_a of

c 1 pH unit when an alkyl group is substituted for an ionizable hydrogen atom in alkyl phosphates. Since alkyl phosphonates are derivatives of phosphonic acid, in which the alkyl group is substituted for a non-ionizable hydrogen atom, this effect would be expected to be less important for these compounds (Crofts and Kosolapoff, 1953).

The data presented in Tables 3 and 4 are consistent with this difference in the chemistry of the two types of phosphorus derivative, if there is a significant change in the solvation of the phosphate or phosphonate group on binding to the protein. In particular, the data in Table 4 are consistent with this explanation for the observed pK_a perturbations, if it is also postulated that all four phosphorus-containing groups are close to (c 4.0 Å) a positively charged residue on the protein (Appendix II). The Dnp and Tnp aminopropylphosphonic acid derivatives show a marked downward perturbation in pK_a (-0.8 pH units) due to the presence of a positively charged residue, because the pK_a 's of alkylphosphonates are relatively insensitive to changes in solvation. Whereas, in the case of the aminoethylphosphoric acid derivatives, the electrostatic effect is cancelled out by an effect due to solvation. The hypothesis that phosphonic acid derivatives are insensitive to solvation changes, is supported by the observation that the pK 's of N-Tnp-2-aminoethylphosphonate (c 0.6 nm long) and N-Tnpaminomethylphosphonate (c 0.5 nm long) are not perturbed on hapten binding to the Fv fragment. despite the fact that the region of the site in which the phosphonate group would be located is hydrophobic, according to the structure proposed by Haselkorn et al (1974) (see Figure 1). If this explanation is correct, then it follows that it will be a general property of phosphonate analogues of phosphate ligands, that the pK_a perturbation will be up to one pH unit more in the downward direction than that of the corresponding phosphate.

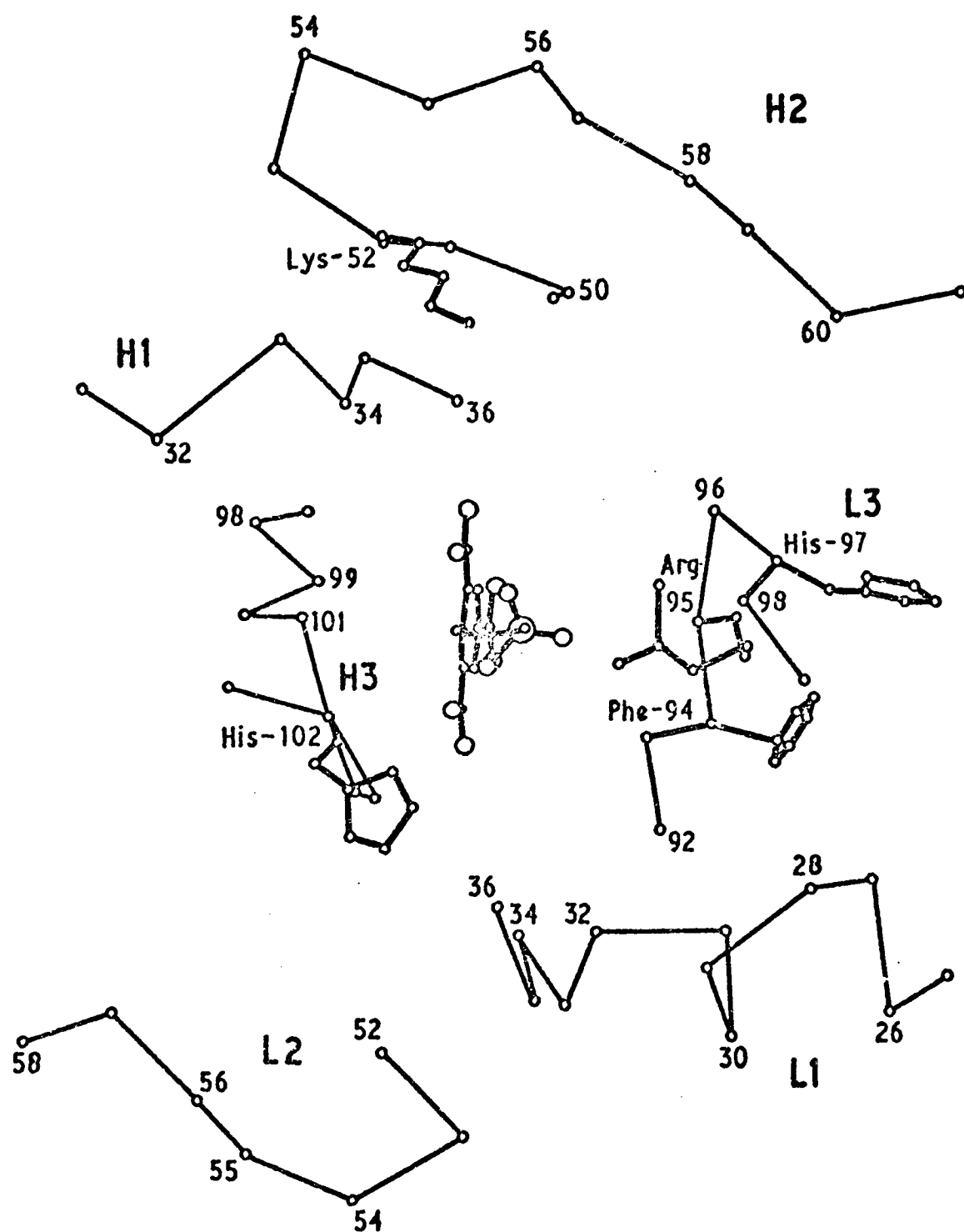
These considerations show that caution must be exercised in interpreting changes in the pK_a values of phosphorus derivatives on binding to

proteins (see Appendix II). In this system, however, where a large amount of comparative data have been obtained, one can conclude that a positively charged amino acid side chain lies close to the phosphorus-containing group of the compounds in Table 4.

c) Conclusions

The data presented in this chapter show that at least one positively charged amino acid residue is located in the entrance of the combining site of the Fv fragment of protein 315. If it is assumed that the side chains are in an extended conformation when the haptens are bound to the Fv fragment, then, using the results of X-ray studies on aminoethylphosphate (Kraut, 1961) and aminoethyl phosphonate (Okaya, 1966), the distance from the Dnp/Tnp binding site to this amino acid side chain will be 8.0-10.0 Å. The lack of any observable perturbation of the environment of the β and γ phosphate groups of the di and triphosphate derivatives of N-Dnp-2-aminoethanol, indicates that the protein surface is c 11.0 Å from the Dnp ring binding site. These conclusions are in agreement with those of Haselkorn et al (1974) and Sutton et al (1977). There are two amino acid residues that could potentially be the positively charged residue that is detected by ^{31}P nmr. These are Lys 52_H and Arg 95_L. The former has been shown to be present in the combining site region by affinity labelling studies (Weinstein, Wilchek and Givol, 1969; Haimovitch, Givol and Eisen, 1970; Haimovitch, Eisen and Givol, 1971; Givol et al, 1971). In these studies a reagent, ϵ -N-Dnp- α -N-bromoacetyl-L-lysine, which has an overall length of 16.0 Å was shown to be much more efficient at labelling this Lys (52_H) as opposed to Tyr 34_L which was labelled by shorter reagents. It is difficult to draw unequivocal structural conclusions from these kind of studies, although the authors point out that the lysine residue must be closer than 16.0 Å from the Dnp ring binding site.

FIG. 11: Charged residues in the combining site of the Fv fragment of protein 315



The haptent is N-Tnp-3-aminopropylphosphonate, the structure is that discussed in Chapters 2 and 3.

Chemical modification studies (Klostergaard, 1977a, b) have shown that one arginine residue in protein 315 can be protected against glyoxalation by hapten, and that protein 315 modified with glyoxal shows complete loss of binding activity towards N-Dnpaminocaproate. No such effects are seen when lysine residues are maleylated. These studies suggest that the positively charged residue detected by the nmr studies is Arg 95_L. The depth of the site estimated from the studies reported in this chapter is in agreement with that measured by a variety of other methods (Pecht, Givol and Sela, 1972; Pecht, Haselkorn and Friedman, 1972; Haselkorn et al, 1974; Pecht, 1974; Wilder, Green and Schumaker, 1975; Rosenstein and Richards, 1976). All these studies are consistent with the conformation of the combining site residues shown in Fig. 11. In this conformation the distance of the guanido group of Arg 95_L from the centre of the bound Dnp ring is 10.5 Å, whilst the distance of the amino group of Lys 52_H from the same point is 15.5 Å. These distances are subject to considerable (c 5.0 Å) uncertainty since both Arg and Lys side chains are c 5.0 Å long and are flexible.

Appendix I : ^{31}P relaxation mechanisms

The contribution of chemical shift anisotropy to the spin-spin relaxation rate of a ^{31}P nucleus is described by the equation:

$$\frac{1}{T_2} = \frac{2}{15} \gamma_I^2 B_0^2 \sigma_0^2 \left[\tau_c + \frac{\tau_c}{1 + \omega_I^2 \tau_c^2} \right] \quad 7$$

Using the following values to describe the Fv fragment hapten system:-

$$\gamma_I = 1.084 \times 10^4 \text{ rad G}^{-1} \text{ sec.}^{-1}$$

$$B_0 = 2.11 \times 10^4 \text{ G}$$

$$\sigma_0 = 150 \text{ ppm}$$

$$\tau_c = 7 \times 10^{-9} \text{ sec.}$$

$$\omega_I = 3.643 \times 10^7 \text{ Hz}$$

the contribution to T_2^{-1} is 1.09 sec.^{-1} . This will contribute 0.35 Hz to the linewidth, since:-

$$\frac{1}{T_2} = \pi \Delta\nu \quad 8$$

when $\Delta\nu$ is the linewidth at half height.

The contribution from phosphorus-proton dipolar interaction is calculated from:-

$$\frac{1}{T_2} = \frac{g_1^2 g_2^2 \beta_N^4 S(S+1)}{15 \hbar^2 r^6} \left[4\tau_c + \frac{\tau_c}{1 + (\omega_I - \omega_S)^2 \tau_c^2} + \frac{6\tau_c}{1 + \omega_I^2 \tau_c^2} + \frac{3\tau_c}{1 + \omega_S^2 \tau_c^2} + \frac{6\tau_c}{1 + (\omega_I + \omega_S)^2 \tau_c^2} \right] \quad 9$$

If the x-ray structure of Kraut (1961) is used then there are two protons at a distance of 0.25 nm from the ^{31}P nucleus, which will dominate the dipolar relaxation. Thus substituting the values:-

$$g_1(^1\text{H}) = 5.585$$

$$g_2(^{31}\text{P}) = 2.263$$

$$\beta_N = 0.50504 \times 10^{-23} \text{ erg G}^{-1}$$

$$S = 0.5$$

$$\hbar = 1.0544 \times 10^{-27} \text{ erg sec.}$$

$$r = 2.5 \times 10^{-8} \text{ cm}$$

$$\tau_c = 7 \times 10^{-9} \text{ sec}$$

$$\omega_I(^{31}\text{P}) = 3.643 \times 10^7 \text{ Hz}$$

$$\omega_S(^1\text{H}) = 9.000 \times 10^7 \text{ Hz}$$

the dipolar contribution is only 10^{-2} Hz.

The exchange contribution to the spin-spin relaxation rate is calculated according to:-

$$\frac{1}{T_2} = \frac{X}{T_{2B}} + \frac{X_F}{T_{2F}} + 4\pi^2 (1 - X_B)^2 X_B \frac{\Delta_o^2}{k_{-1}} \quad 10$$

(Campbell and Dobson, 1977);

Thus substituting values:-

$$T_{2B}^{-1} = 3 \text{ sec.}^{-1}$$

$$T_{2F}^{-1} = 3 \text{ sec.}^{-1}$$

$$\Delta_o = 0.2 \text{ ppm} = 7 \text{ Hz}$$

$$X_B = 0.995$$

$$X_F = 0.005$$

$$k_{-1} = 10^2 \text{ sec.}^{-1} \quad (K_D = 0.42 \mu\text{M}, k_1 = 2.2 \times 10^8)$$

When the on and off rates are defined for the equilibrium:-

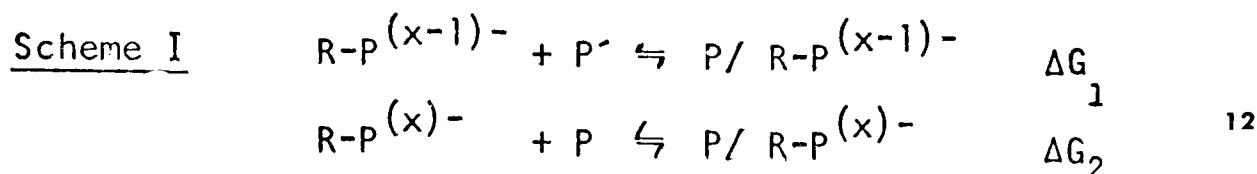


the exchange contribution to the linewidth is calculated to be

$$1.5 \times 10^{-3} \text{ Hz.}$$

Appendix II : Changes in the pK_a 's of phosphate and phosphonate groups

The change in the pK_a of a ligand phosphate or phosphonate group on binding of the ligand to a protein, arises from the difference in affinity of the protein for the protonated and non-protonated forms of the ligand. This is formally stated as:



When R-P is the phosphate or phosphonate ligand, P is the protein, ΔG_1 is the free energy of binding of the non-protonated form of the ligand and ΔG_2 is the free energy of binding of the protonated form of the ligand. Since:-

$$pK_a = -\log_{10} K_a \quad 13$$

when:-

$$K_a = \frac{[H^+][R-P^{(x)-}]}{R-P^{(x-1)-}} \quad 14$$

and since:-

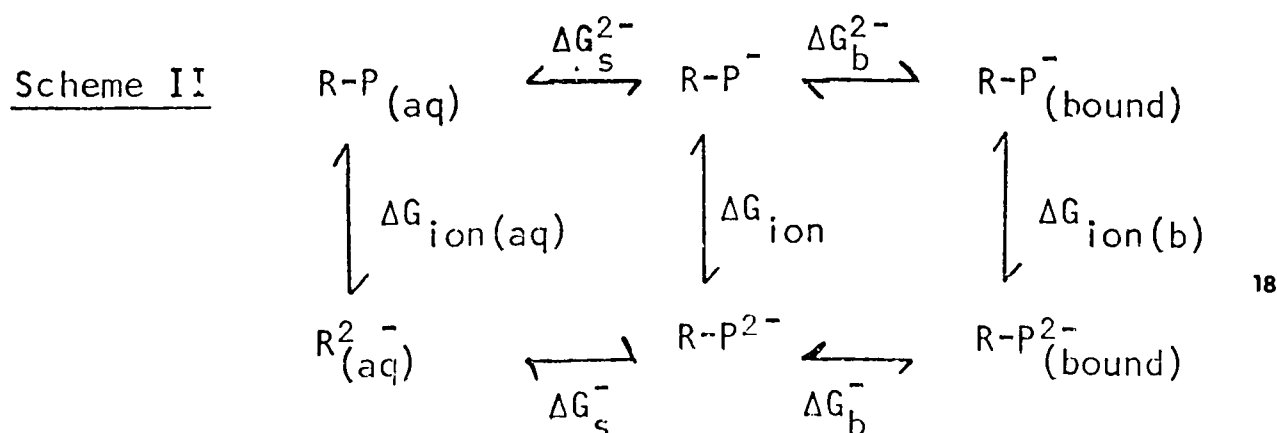
$$\Delta G = -RT \ln K \quad 15$$

It follows that:-

$$\Delta pK_a = pK_a(\text{free}) - pK_a(\text{bound}) \quad 16$$

$$= \frac{\Delta G_1 - \Delta G_2}{2.303 RT} \quad 17$$

If the second ionization of a phosphonate or phosphate group is considered, then the binding can be broken down into two steps as follows:-



when the ΔG_s 's are the free energy changes for the transfer of ligand from vapour to water, and the ΔG_b 's are the free energy changes for transfer from vapour to protein.

Thus from Schemes I and II it follows that:-

$$\Delta G_1 = \Delta G_b^- - \Delta G_s^- \quad 19$$

$$\Delta G_2 = \Delta G_b^{2-} - \Delta G_s^{2-}$$

Whence from equation 17 it follows that:-

$$pK_a = \frac{\Delta G_b^- - \Delta G_b^{2-} - (\Delta G_s^- - \Delta G_s^{2-})}{2.303 RT} \quad 20$$

In this equation the differential solvation terms appear. In the F_V /hapten system the approximate value of ΔG_1 can be estimated since the hapten binding studies are performed at pH 5.5.. (see legend to Table 1) Thus ΔG_1 for N-Dnp-3-aminopropylphosphonate can be calculated from the value of K_D (Table 1) to be $-8.93 \text{ Kcal M}^{-1}$, thus from the data in Table 4 ΔG_2 is $-9.92 \text{ Kcal M}^{-1}$. Here it can be seen that the binding free energy difference between the two ionization states is small (10%) compared with the overall free energy of binding. However, the free energy of binding is itself a composite term, as shown in Scheme II. Estimated values for the solvation term ΔG_s and the total binding free energy terms ΔG_b , can be made from the free energies of vapourization and aqueous solubility products of organic phosphate esters. These estimates lie in the range -15 to -30 Kcal M^{-1} . Thus the total free energy of binding may be as large as -40 Kcal M^{-1} . Clearly this refers to the whole molecule not to phosphate group alone, but indicates that the free energy contribution from the pK_a perturbation is very small (c 1- 5%) compared to the other free energy changes in the system.

Appendix III

TITLE - HHCAL.

```

MASTER NMR
DIMENSION DOBS(100)
DIMENSION TITLE (5), XL(100), XM(100), PH(100)
READ (1,10) NP, PKA, DA, DB, TITLE
10  FØRMAT (1X, 3F 10.5, 5A6)
    WRITE (2,50) TITLE
50  FØRMAT (1X, 5A6)
    WRITE (2,51)
52  FØRMAT (1X, 4H PH, 6X, 6HD(OBS))
    DO 100 I=1, NP
    READ (1,20) pH (I)
20  FØRMAT (F 10.5)
    XL(I) = EXP10 (- pH(I))
    XM(I) = EXP10 (- pKA)
    DOBS = DA*(XL(I)/(XL(I) + XM(I))) + DB* (XM(I)/(XL(I) + XM(I)))
    WRITE (2,30) pH(I), DOBS(I)
30  FØRMAT (1X, F 10.5, 2X, F 10.5)
100 CØNTINUE
    STOP
    END

```

Chapter 5THE BINDING OF 2,4,6-TRINITROPHENYL DERIVATIVES
TO THE FV FRAGMENT OF PROTEIN 3151. Introduction

Comparison of immunoglobulin-sequence data (Wu and Kabat, 1970) with the results of X-ray diffraction studies (Davies, Padlan and Segal, 1975) suggests that the variable domains of these proteins consist of regions of conserved three-dimensional structure (the immunoglobulin fold) to which are attached short sections of polypeptide chain (the hypervariable loops) (Chapter 1). It is these loops that form the antibody-combining site. By using such sequence homologies and structural similarities, it has been possible to build a model of the combining site of the mouse myeloma protein MOPC 315 (Padlan et al, 1976), by fitting the sequence of this protein to the α -carbon co-ordinates of other myeloma proteins, mainly McPC603. This model has been refined (see Chapter 2) to give a solution structure of the combining site (Dwek et al, 1977). The major feature of the combining site, a subsite formed by tryptophan-93_L, phenylalanine-34_H and tyrosine-34_L, was investigated by using the binding of Dnp derivatives.

Tnp derivatives have been shown to bind to protein MOPC315 (Eisen et al, 1968). In this chapter the binding of four Tnp derivatives to the Fv fragment of protein MOPC315 is investigated, to compare the mode of binding of these haptens with that of the Dnp derivatives.

2. Materials and Methods

a) Preparation of solutions of Fv fragment from protein 315

The Fv fragment of the mouse myeloma IgA protein MOPC315 was prepared as described in Chapter 2. Samples for the n.m.r., fluorescence and absorption-difference-spectroscopy studies were prepared as described in Chapter 2.

b) Synthesis of haptens

N-Tnp-L-aspartate. Aspartic acid (1.6g; BDH Biochemicals, Poole, Dorset, U.K.) was allowed to react with 4.2g of 2,4,6-trinitrobenzenesulphonic acid (Sigma [London] Chemical Company, Norbiton Station Yard, Kingston-upon-Thames, Surrey), in 10 ml. of water at room temperature (25°C) for 1 h, in a flask silvered to exclude light. NaHCO_3 (2g) was added to maintain the pH at 7.

After 1 h the reaction mixture was acidified with 1M-HCl, and extracted with ethyl acetate. The ethyl acetate was removed by evaporation under vacuum, and the yellow solid thus obtained was dissolved in the minimum volume of acetone. Water was added to the acetone solution until a slight turbidity appeared; the solution was then left to crystallize overnight. The crystals were separated by filtration and dried under vacuum.

The purity of the product was checked by t.l.c. in butan-1-ol/acetic acid/water (5:3:1, by vol.) on polyamide plates (BDH Biochemicals) in which it ran as one spot, with no ninhydrin-staining impurity co-chromatographing with aspartic acid (Found: C, 34.6; H, 2.4; N, 15.6 Calc. for $\text{C}_{10}\text{H}_8\text{N}_4\text{O}_{10}$; C, 35; H, 2.5; N, 16.5%).

N-Tnp-glycine. This was prepared similarly, from 1.2g of glycine (BDH Biochemicals) and 5.6g of 2,4,6-trinitrobenzenesulphonic acid.

N-Tnp-aminomethylphosphonate. This was prepared as described in Chapter 4.

c) Preparation of hapten and tryptophan solutions

The solutions were prepared as described in Chapter 2.

d) Fluorescence measurements

Fluorescence spectra were recorded on a Perkin-Elmer/Hitachi MPF-2A recording spectrofluorimeter, as described in Chapter 2.

e) Optical-difference spectroscopy

Optical-difference spectra were recorded on a Cary 17 recording spectrophotometer, using Hellma QS tandem cells, as described in Chapter 2.

f) High-resolution ^1H n.m.r. studies

270 MHz ^1H n.m.r. spectra were recorded on a Bruker spectrometer as described in Chapter 2.

g) Analysis of n.m.r. data

Data from the model-compound studies were analysed as described in Chapter 2.

Data for the hapten in the Fv-fragment-binding studies were analysed graphically according to:

$$\frac{\delta_{\text{obs.}}}{\Delta\delta_0 [\text{protein}]_{\text{total}}} = \frac{1}{[\text{hapten}]_{\text{total}}} \quad (1)$$

(where $\Delta\delta_0$ is the maximum shift change and $\delta_{\text{obs.}}$ is the observed shift), which applies when $[\text{hapten}], [\text{protein}] \gg K_D$, such that when $[\text{hapten}]_{\text{total}} > [\text{protein}]_{\text{total}}$, $[\text{hapten}]_{\text{bound}} = [\text{protein}]_{\text{total}}$. This is illustrated in Fig. 5(a), which shows that when $[\text{hapten}]_{\text{total}}$ is approximately equal to $[\text{protein}]_{\text{total}}$ the assumption breaks down and the plot of $\delta_{\text{obs.}}$ versus $[\text{hapten}]_{\text{total}}^{-1}$ becomes non-linear. However, by using either data in this region of the hapten-shift plot, and applying:-

$$\bar{X}_B = \frac{\Delta\delta_{\text{obs.}}}{\Delta\delta_0} \quad (2)$$

the Scatchard equation:

$$\frac{\bar{X}_B}{[A_F]} = \frac{1}{K_D} (n - \bar{X}_B) \quad (3)$$

(where A is either hapten or protein, $\bar{X}_B$ is the fraction of A bound and n is number of sites) can be applied (Fig. 5b) to determine n and K_D (dissociation constant).

3. Results

a) Model-compound studies

The binding of Tnp-L-aspartate and Tnp-glycine to tryptophan was studied by 270MHz proton n.m.r., at pH 6.8 and 303°K, in the presence of 0.15 M-NaCl. When hapten (40mM) was added to tryptophan (2mM), in both cases the resonances of the tryptophan were shifted upfield.

Shift titrations were performed by varying the Tnp-glycine concentration (0-46.5mM) against a fixed concentration of tryptophan (2mM), and varying the tryptophan concentration (0-20mM) against a fixed concentration of Tnp-glycine (2 mM). It was not possible to fit the data of both titrations to binding in a simple binary complex. The simplest model consistent with the data is formation of a ternary complex $(\text{Trp})_2\text{-Tnp}$. Analysis of the data (Chapter 2; Dower et al, 1977) gave a dissociation constant (K_{D1}) of 57mM, and the shift ratios shown in Table 1.

From these data and the geometry of the ring-current field as predicted by the Johnson-Bovey equation (Johnson & Bovey, 1958), it follows that the Tnp-glycine and the tryptophan stack with the trinitrophenyl ring located over the centre of the tryptophan ring. This is consistent with the results of crystallographic analyses (Hansen, 1964).

The data in Table 1(b) show that the chemical shift of the Tnp ring proton resonances in particular, are independent of the charge state of the carboxyl groups of the molecule. All the shifts are < 0.4 ppm and

TABLE 1. a) Shift ratios in the complex of Tnp-glycine and L-tryptophan

Measurements were made at 270 MHz and 303 °K, in the presence of 0.15 M-NaCl.

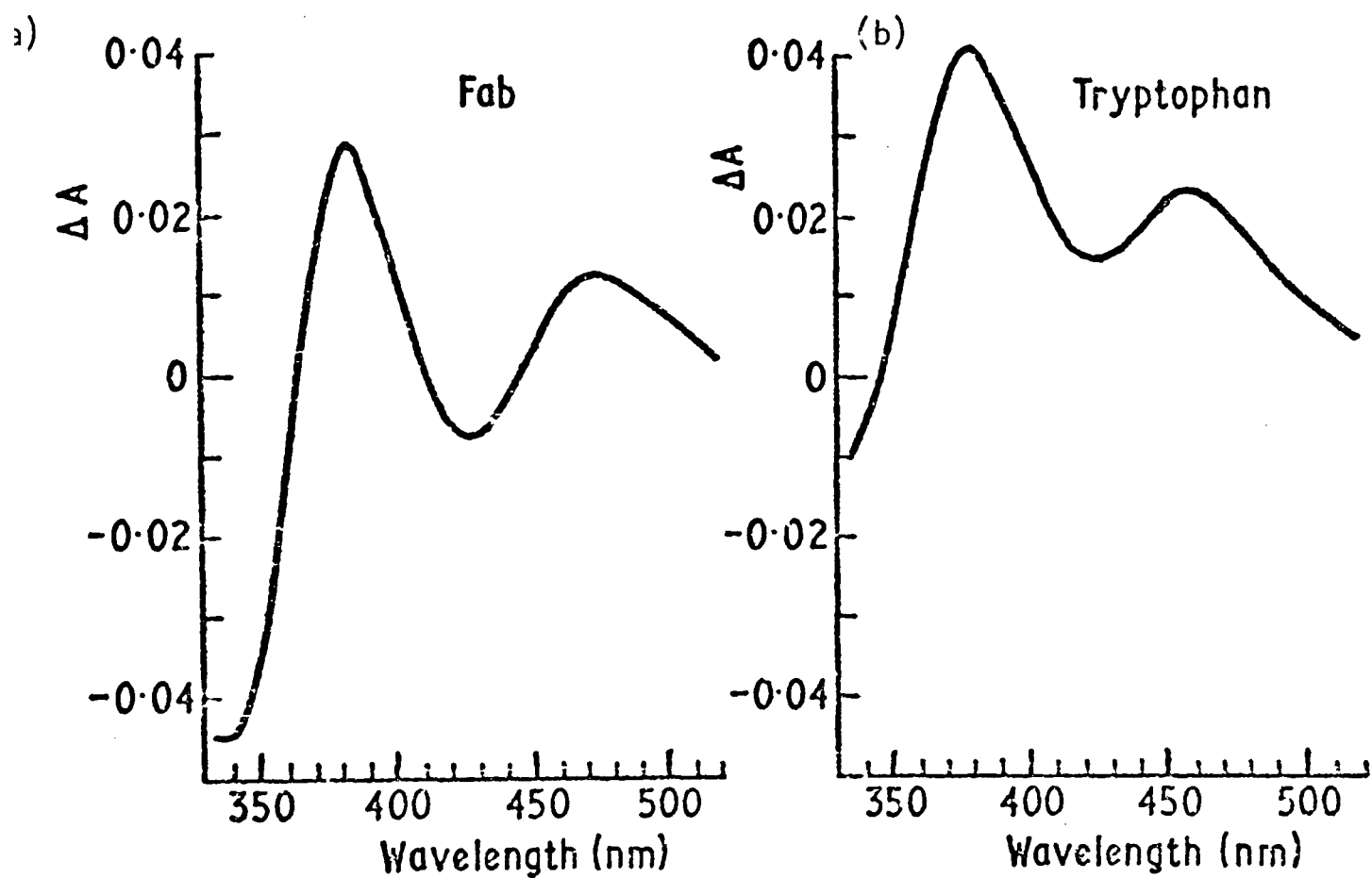
Proton	Shift ratio
Tnp-glycine H ₍₃₎ +H ₍₅₎	1
α-CH ₂	0.44
L-Tryptophan H ₍₂₎	0.45
H ₍₇₎	0.64
H ₍₄₎	0.59
H ₍₅₎	0.54
H ₍₆₎	0.54

TABLE 1. b) pH*dependence of ¹H chemical shift of Tnp haptens

N-Tnp-L-aspartate	pH* 2.2	pH* 12.5
H ₃ +H ₅	9.22	9.15
αCH	4.28	4.09
βCH ₂	3.07	2.68
N-Tnpglycine	pH* 4	pH* 12.5
H ₃ +H ₅	9.14	9.11
αCH ₂	3.74	3.71

All samples were 20mM, in ²H₂O, spectra recorded for 100 scans, at 303°K. The chemical shifts are relative to DSS as an external standard.

Fig. 1. Optical-difference spectra of Tnp-aminomethylphosphonate with (b) L-tryptophan and (a) protein MOPC315 Fab^r fragment



Conditions were as follows.

- (a) Spectrum obtained by subtracting the spectrum of 95 μ M-Tnp-aminomethylphosphonate from that of the same concentration of Tnp-aminomethylphosphonate in the presence of 65 μ M-Protein MOPC315 Fab^r fragment. All solutions were pH7.4 in phosphate-buffered saline (0.15 M-NaCl/0.02M-phosphate)
- (b) Spectrum obtained by subtracting the spectrum of 0.19mM-Tnp-aminomethylphosphonate from that of the same concentration of Tnp-aminomethylphosphonate in the presence of 33mM-L-tryptophan. All solutions were pH6.0 and were unbuffered
(Data of R. Jackson)

decrease as the concentration of the charged form of the carboxyl group increases. It is difficult to attribute the observed shift changes to a particular cause since at least three causes can be envisaged. These are chemical shift anisotropy, through bond induction, and through space charge effects as discussed in Chapter 2.

These data show that the shift observed on binding to the Fv fragment can be interpreted as largely arising from ring current effects as discussed in Chapter 2.

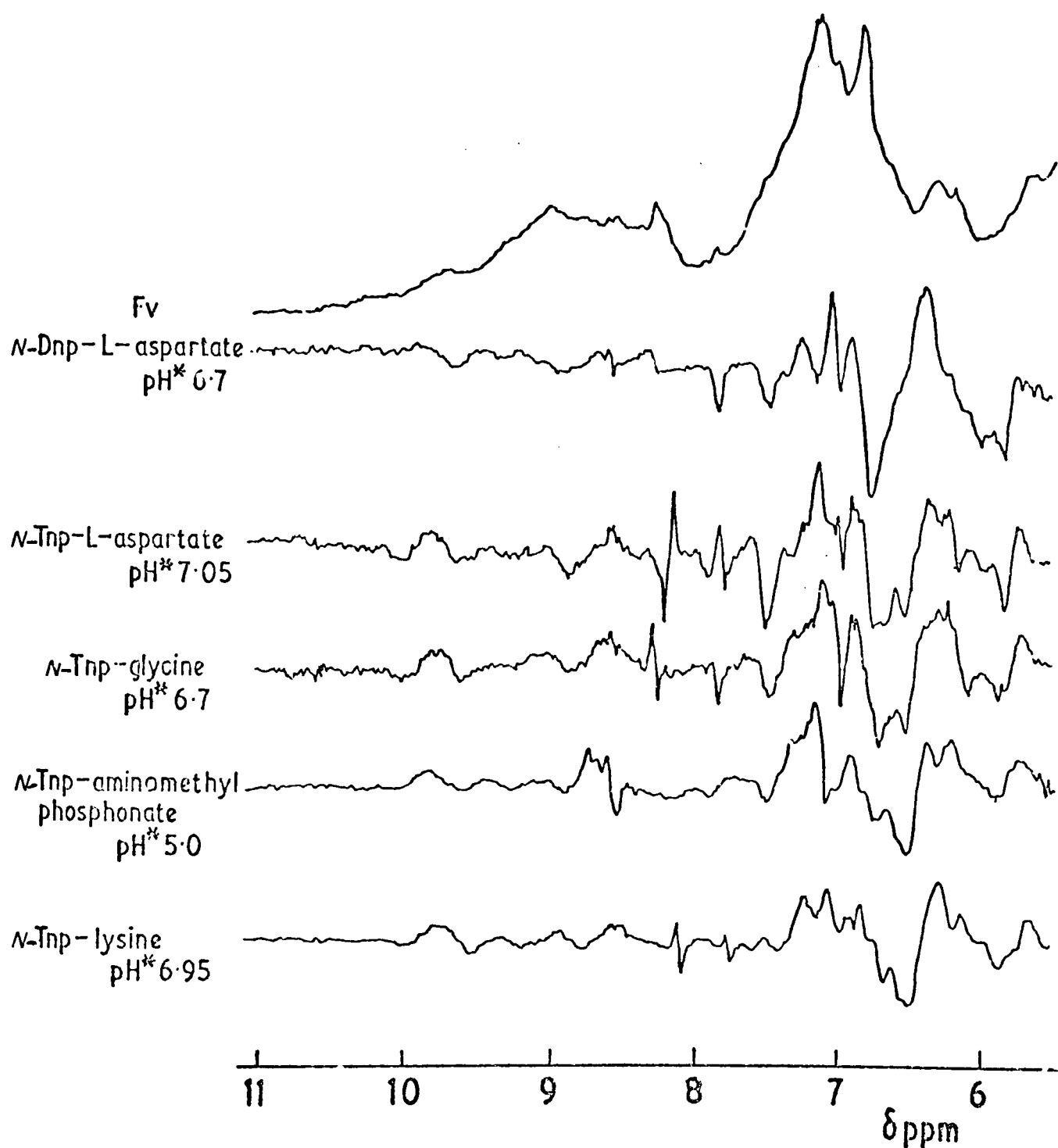
b) Optical-difference spectroscopy

The Optical -difference spectra for Tnp-aminomethylphosphonate with the Fab' fragment of protein MOPC 315 and tryptophan are shown in Fig. 1. The similarity of these difference spectra indicates that the Tnp-aminomethylphosphonate interacts with a tryptophan residue in the combining site of the Fab' fragment of protein MOPC315, in the same way as it interacts with tryptophan in solution (Little & Eisen, 1967). In conjunction with the results of the preceding section, this suggests that Tnp-aminomethylphosphonate stacks with a tryptophan residue in the combining site of the Fab' fragment of protein MOPC315.

c) Binding Studies

The binding of four different haptens to the Fv fragment from protein MOPC315 was studied by titrations using quenching of protein fluorescence. The dissociation constants thus determined are listed in Table 2. Also shown in Table 2 is the expected exchange-rate range (see below) for each hapten under the conditions of the n.m.r. studies of the hapten-Fv-fragment interactions (see below). These are based on the on-rate of $2.2 \times 10^8 \text{ s}^{-1} \text{ M}^{-1}$ determined for trinitrotoluene binding to the Fv fragment (Haselkorn et al 1974).

FIG. 2. Downfield region of the 270 MHz proton n.m.r. difference spectra of protein MOPC315 Fv fragment with several haptens



All spectra were recorded in the presence of 0.15 M-NaCl, in $^2\text{H}_2\text{O}$. All samples were c 1.3 mM Fv fragment, or 1.3 mM Fv fragment + equimolar hapten, 0.15 M NaCl, $T=303$ K, pH* as indicated. The data for α -N-Tnp-L-lysine were collected by Mr. P. Gettins.

TABLE 2 : Binding of 2,4,6-trinitrophenyl derivatives to
protein MOPC 315

All experiments were performed in the presence of 0.15 M-NaCl and 20 mM-Pipes at $25 \pm 2^\circ\text{C}$.

Hapten	K_D (μM)	pH	N.m.r. exchange properties
Tnp-glycine	12	6.8	Fast
Tnp-L-aspartate	19	6.8	Fast
Tnp-aminomethylphosphonate	20	6.6	Fast

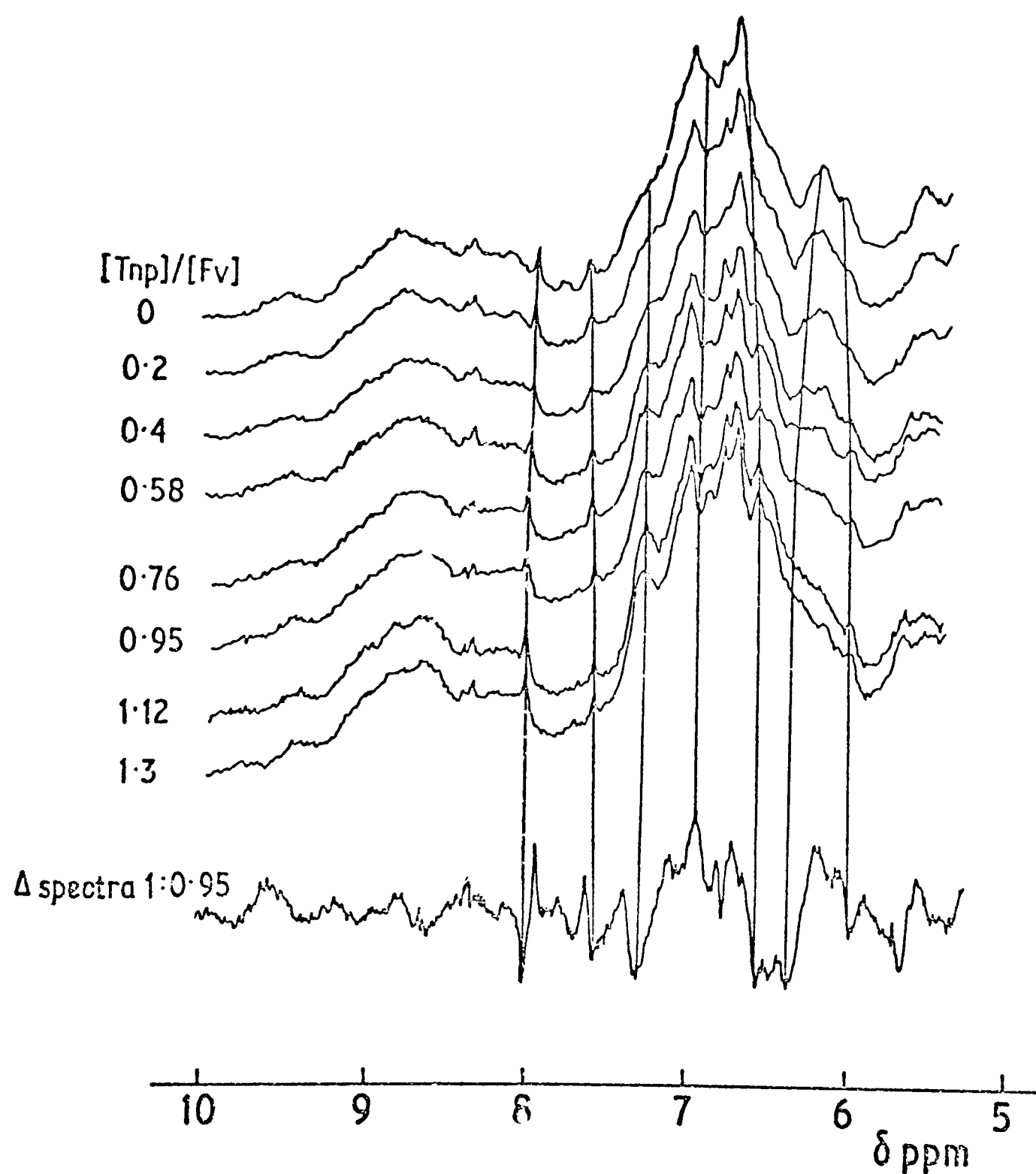
d) ^1H n.m.r. studies

(i) Difference spectroscopy. The region 5.5-11 p.p.m. of the 270 MHz proton n.m.r. difference spectra of the four Tnp-haptens with that of Fv fragment from protein MOPC315 is shown in Fig. 2, together with that for Dnp-L-aspartate. These spectra show that in the chemical shift range 5.5-7.5 p.p.m. the same resonances of the Fv fragment are perturbed by all the haptens. The differences between the spectra in the region 7.5-9.0 p.p.m. are due to the variation in the shifts of the three histidine H2 resonances with pH* (meter reading in $^2\text{H}_2\text{O}$ obtained with a pH-meter standardized with $^1\text{H}_2\text{O}$ standard buffers) (Wain-Hobson et al., 1977). It is concluded that all these haptens interact similarly with the Fv fragment.

(ii) Shift titrations. The difference spectra in Fig. 2 show the initial and final positions of resonances from the Fv fragment perturbed on hapten binding, and also the resonances of the bound hapten molecule. However, it is not possible, from such spectra, to assign a resonance in the spectrum of the Fv fragment-hapten complex to a resonance in the

FIG. 3 Titration of the Fv fragment of protein MOPC315
with Tnp-L-aspartate

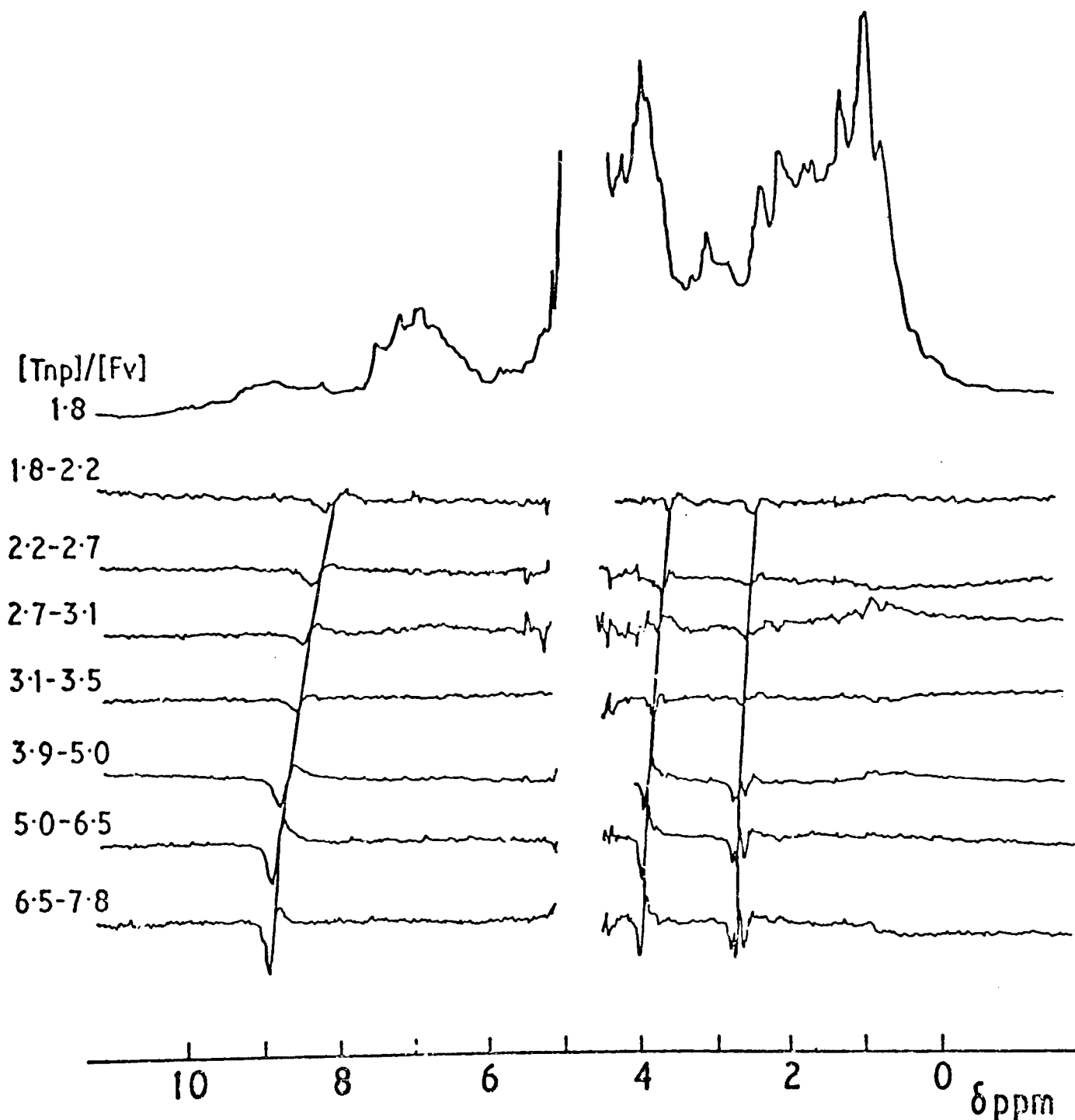
(a)



Shift changes of the protein resonances on hapten binding illustrated by the Fv 270 MHz n.m.r. spectra and a difference (Δ) spectrum, showing the final positions of resonances perturbed.

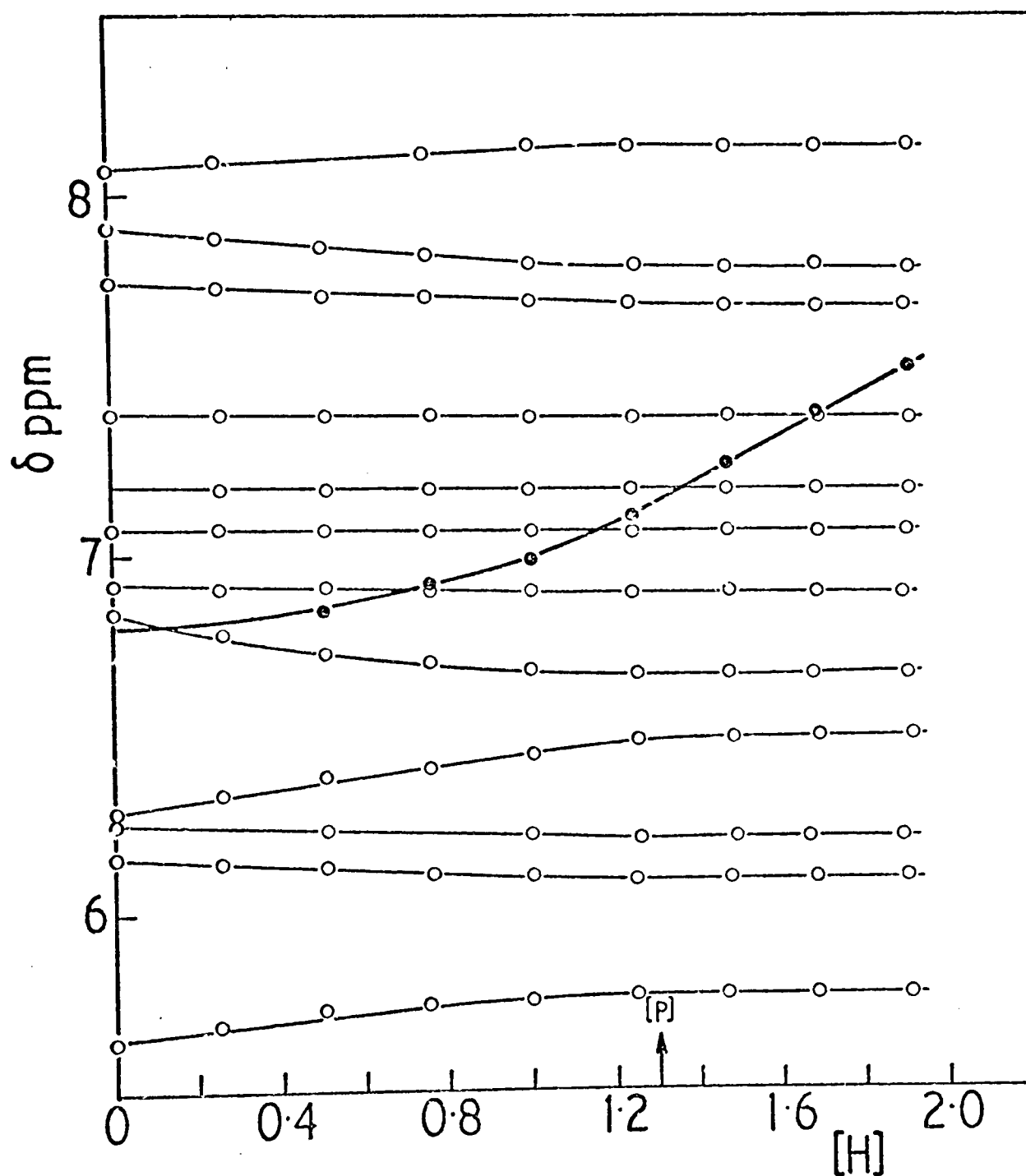
FIG. 3. Titration of the Fv fragment of protein MOPC315 with Tnp-L-aspartate

(b)



Shift changes of hapten resonances, illustrated by serial differences taken from spectra of samples in which the concentration of hapten exceeded the concentration of the Fv fragment. The concentration of Fv was 1.3 mM, the pH* was 7.05. All other conditions were as in Fig. 2. Chemical shift was measured from that of the sodium salt of 3-(trimethylsilyl)propanesulphonic acid as an external standard.

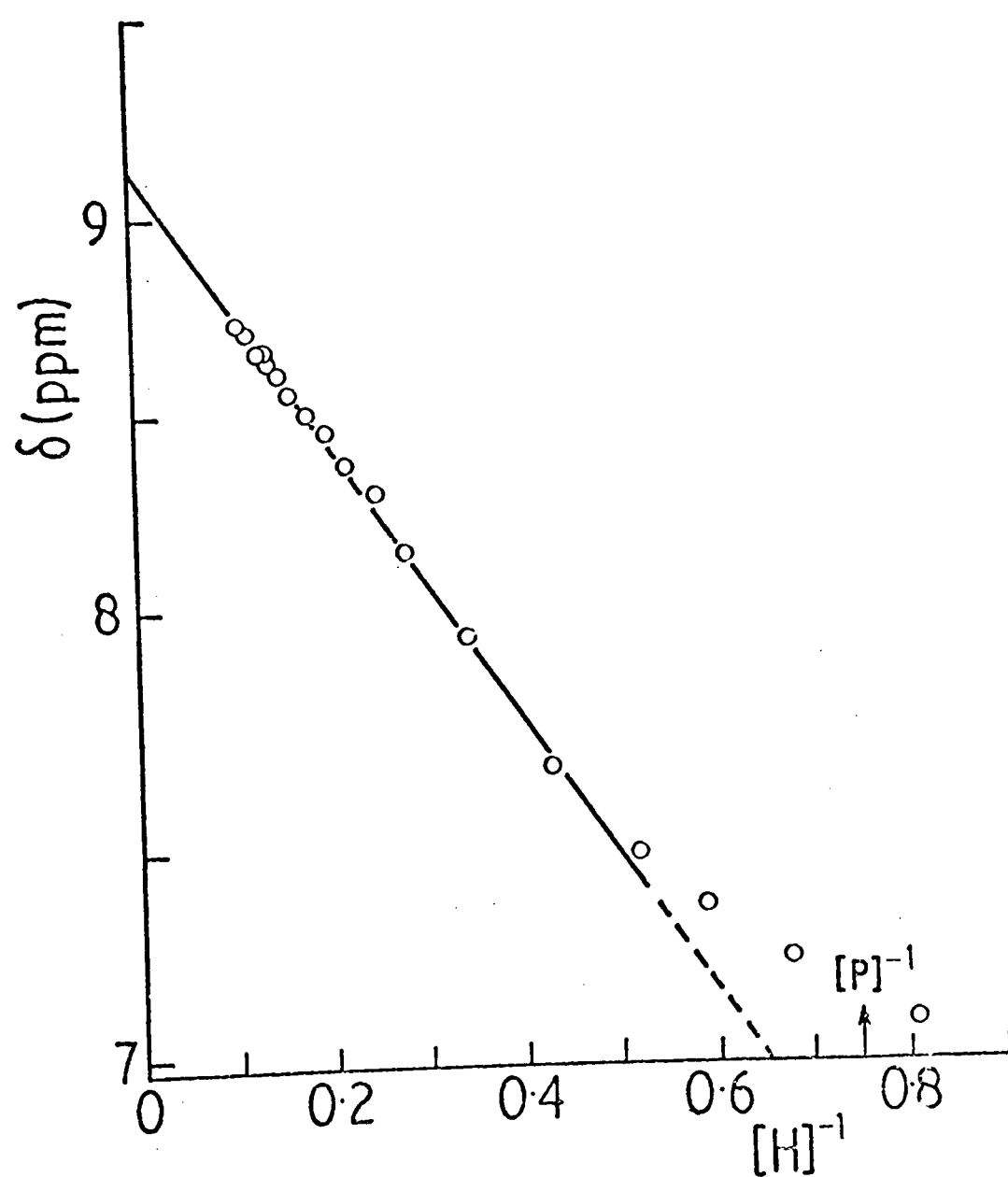
FIG. 4. Changes in chemical shift in the aromatic region (5.5-8.5p.p.m.) of protein and hapten resonances perturbed on addition of Inp-L-aspartate to the Fv fragment of protein MOPC315



Protein concentration is indicated by $[P]$. The shift-change behaviour of the hapten $H_{(3)} + H_{(5)}$ resonance is indicated by $-\bullet-$. The curve fitted to these points is a theoretical line calculated with $n=1$, $K_D=27\mu M$, $\delta=2.38$ p.p.m. Conditions are as given in Table 3.

FIG. 5. Analysis of 270 MHz proton n.m.r. data from the titration of Tnp-L-aspartate with the Fv fragment from protein MOPC315

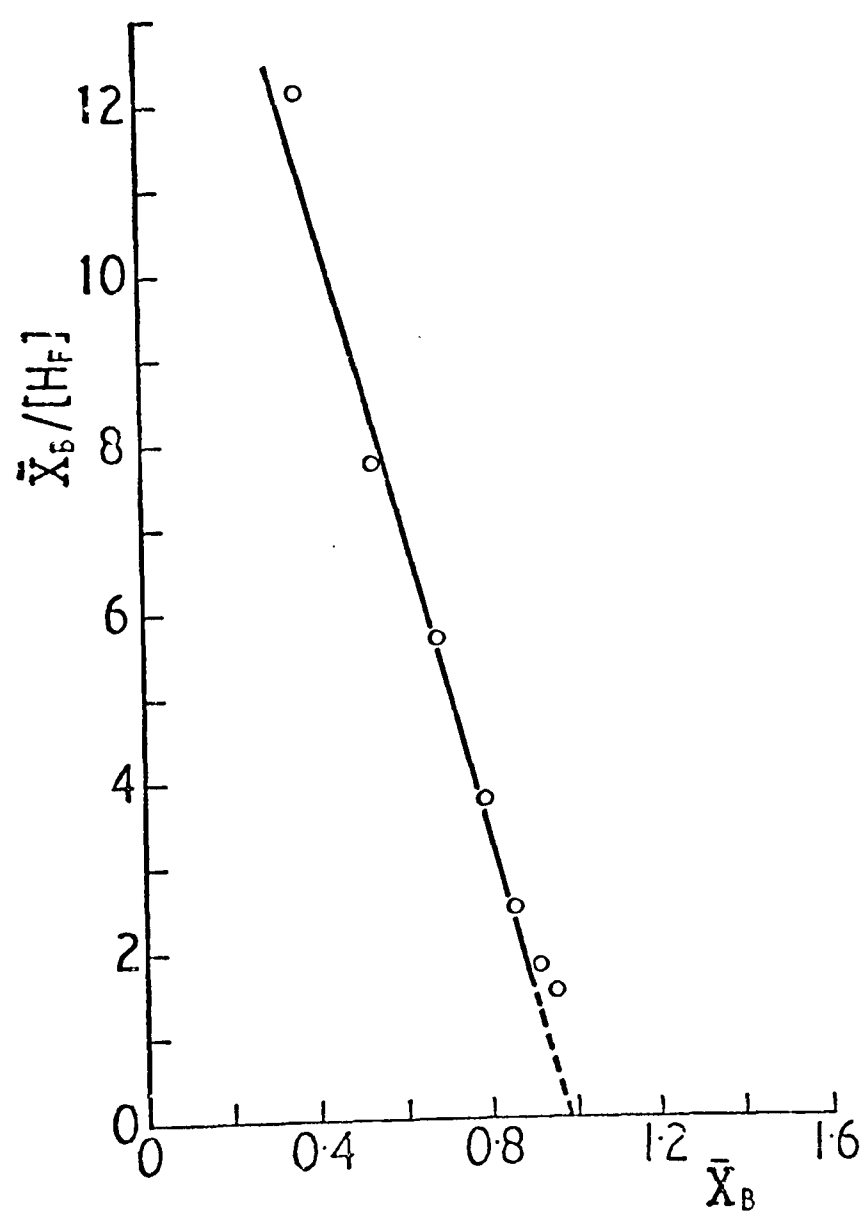
(a)



Single-reciprocal plot, as described in the Materials and Methods section, of the shift-change behaviour of the hapten $H_{(3)}+H_{(5)}$ resonance.

FIG. 5. Analysis of 270 MHz proton n.m.r. data from the titration of Tnp-L-aspartate with the Fv fragment from protein MOPC315

(b)



Scatchard plot obtained from the shift-change behaviour of the non-linear region of (a). Conditions were as in Table 3.

unperturbed hapten or protein spectra. Hence it is not possible to determine the shift change of such a resonance on hapten binding. To do this, titrations must be performed in which the chemical shift of a perturbed resonance changes continuously as a function of fraction of hapten (or protein) complexed. Such conditions are termed fast exchange and are given when $k_{\text{off}} > \Delta\omega_0$, where k_{off} is the rate of dissociation of hapten from protein and $\Delta\omega_0$ is the change in chemical shift (in Hz) of a resonance between bound and unbound environments.

The haptens listed in Table 2, Tnp-glycine, Tnp-aminomethylphosphonate and Tnp-L-aspartate are suitable for such titrations. Titrations were performed with Tnp-glycine and Tnp-L-aspartate.

The results for Tnp-L-aspartate are illustrated in Fig. 3, and the shift behaviour of resonances is shown in Fig. 4. Fig. 5(a) shows the use of a single-reciprocal plot of the hapten $H_{(3)} + H_{(5)}$ resonance chemical-shift behaviour (see Fig. 3b) to determine the shift change for this resonance (see above). Fig. 5(b) shows the shift data of the resonance plotted according to the Scatchard equation; this shows that $n=1$ and $K_D = 27\mu\text{M}$, in good agreement with the values determined by fluorescence studies. The results for both haptens are summarized in Table 3.

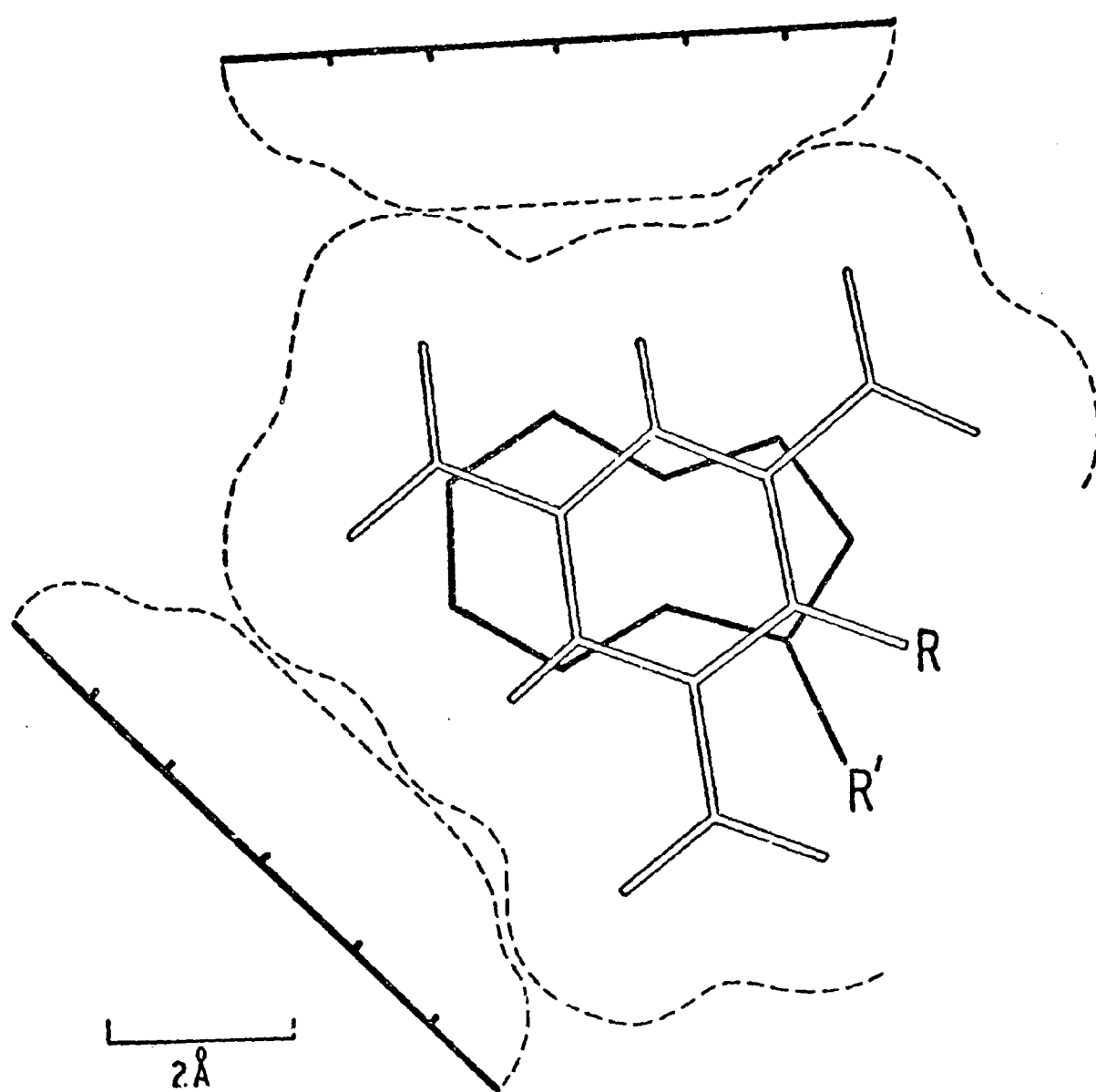
Comparison of the data in Table 3 with those for Dnp-L-aspartate and Dnp-glycine shows that the same resonances in the protein spectrum are perturbed by Tnp-L-aspartate, Tnp-glycine and Dnp-haptens, and that the hapten aromatic proton resonances experience similar large shifts in all cases. It is concluded that the Tnp-haptens stack with tryptophan-93_L, in the hydrophobic subsite of the Fv-fragment-combining site (tryptophan-93_L, phenylalanine-34_H, tyrosine-34_L).

TABLE 3 : Chemical shifts and changes in chemical shifts of resonances perturbed on binding of Tnp-glycine and Tnp-L-aspartate to the Fv fragment of protein MOPC315

Measurements were made at 270 MHz and 303K, in the presence of 0.15 M-NaCl. Shifts were measured from that of the sodium salt of 3-(trimethylsilyl)propanesulphonic acid as an external standard. In the Tnp-glycine experiment the pH* was 6.7 and the concentration of the Fv fragment was 1.4mM; in the Tnp-L-aspartate experiment the pH* was 7.05 and the concentration of Fv fragment was 1.3mM. A negative sign indicates a downfield shift.

$\Delta\delta_0$ (p.p.m.)				
Hapten resonances	δ_0 (p.p.m.)	Tnp-Gly	Tnp-L-Asp	Nb. of protons
Tnp-L-Asp	9.07	—	2.3	2
$H_{(3)}^*H_{(5)}$				
α -H	3.83	—	1.24	1
β -H	2.50	—	0.88	2
Tnp-Gly				
$H_{(3)}^+H_{(5)}$	9.13	Approx. 2.6	—	2
α -H	3.72	Approx. 1.4	—	2
Protein resonances				
His-102 _H H2	8.07	No shift	-0.07	1
	7.91	No shift	0.10	1
His-97 _L H2	7.76(7.87)	< 0.05	0.06	1
	7.04	Broadens out in both		2
His-97 _L H4	6.93(7.01)	< 0.05	< 0.05	1
	6.93	-0.05	—	1-2
	6.88	0.15	0.2	1-2
	6.35	0.17	0.22	2
	6.31	0.15	0.05	2
	6.20	0.14	0.07	2
	5.65	—	-0.13	2
	7.23	Decreases in intensity in both		2
	7.43	Increases in intensity in both		2

FIG. 6 : Mode of binding of Tnp derivatives to the Fv fragment of protein MOPC 315.



The Figure shows the orientation of the trinitrophenyl ring in the aromatic box of the combining site, determined by ring-current calculations, as described in the text. R is the hapten side chain; R' is the link of tryptophan-93_L to the polypeptide chain. Phenylalanine-34_H and tyrosine-34_L are shown in cross-section (the section passing through C-1 and C-4). Carbon atom positions and the ring centre are indicated.

TABLE 4 : Comparison of calculated and experimental ring-current shifts in the combining site of protein M0PC315 on the addition of Tnp-haptens, based on the structure of Fig.6 .

All values are expressed as p.p.m.

Shifts on hapten resonances Contribution from residues					
Hapten resonance	Trp-93 _L	Tyr-34 _L	Phe-34 _H	Total	Observed
H ₍₃₎	0.94	1.52	—	2.4	2.4
H ₍₅₎	0.6	1.6	—	2.2	

Shift on protein resonances caused by the trinitrophenyl ring

	Calculated	Observed
--	------------	----------

Trp-93_L

H ₍₂₎	
H ₍₄₎	0.33
H ₍₅₎	0.11
H ₍₆₎	0.13
H ₍₇₎	0.44

Phe-34_H

H ₍₂₎	-0.08	}	approx. -0.2 approx. -0.2 -0.1
H ₍₃₎	-0.12		
H ₍₄₎	-0.08		
H ₍₅₎	-0.10		
H ₍₆₎	-0.12		

Tyr-34_L

H ₍₂₎	-0.12	}	-0.1
H ₍₃₎	-0.08		
H ₍₅₎	-0.16		
H ₍₆₎	-0.10		

e) Ring-current calculations

The data presented in the previous section were used to determine the mode of binding of the Tnp-haptens to the Fv fragment. This was accomplished by using the ring-current contour-map approach (Chapter 2) based on the Johnson-Bovey equation and assuming a plane-to-plane separation between tryptophan-93_L and the trinitrophenyl ring of 3.3 Å (Hansen, 1964). A complete redetermination of the geometry of the Tnp contact residues was not attempted. Rather, the previously determined solution structure of the Fv-fragment-combining site (Chapter 2) was used as the basis for the calculations. The resulting structure is shown in Fig. 6 and the comparison of the shifts predicted by this structure with the experimental shifts is shown in Table 4.

The large shifts on the hapten are well predicted (Table 4) for both Tnp-glycine and Tnp-L-aspartate. The shifts on the protein are only poorly predicted, though of the right order of magnitude. This arises from two causes; firstly, because the shifts are so small (all ≤ 0.2 p.p.m.) and, secondly, because the ring-current properties of the trinitrophenyl ring system are poorly predicted by the Johnson-Bovey treatment, and no allowance can be made for the chemical-shift anisotropy of the nitro groups.

4. Discussion

In Chapter 2, it was shown that the aromatic ring of Dnp-haptens bound to the Fv fragment of protein MOPC315 is located in a subsite of aromatic amino acid residues, consisting of tryptophan-93_L, tyrosine-34_L and phenylalanine-34_H. It was also shown that the most important interaction between the hapten and the protein is the stacking of the hapten aromatic ring with the indole ring of tryptophan-93_L. The data described in this chapter show that this is also the major interaction between Tnp-haptens and the Fv fragment. Comparison of the mode of binding of the Tnp-haptens

(Fig. 6) with that of the Dnp-haptens (Chapter 2) shows that they are similar but not identical, since the trinitrophenyl ring is located approximately 0.5 Å further out of the site. However, the trinitrophenyl ring is located centrally over the indole ring of tryptophan-93_L, as indicated by the model-compound n.m.r. data and the red shift of the absorption spectrum. The shift data for the trinitrophenyl ring protons of the haptens Tnp-glycine, Tnp-L-aspartate show that the trinitrophenyl ring of these haptens is bound very similarly in each case, as suggested by the proton n.m.r. difference spectra. Further, the similarity of the difference spectra in Fig. 2 shows that all the haptens bind in the same way. This demonstrates that, although interactions between the hapten side chain and the Fv fragment make a significant contribution to the binding energy, the mode of binding of the whole hapten is determined by the mode of binding of the trinitrophenyl ring.

The similarity in the modes of binding of Tnp- and Dnp-haptens to protein 315 arises primarily because the hydrophobic subsite of the combining site is sufficiently large to allow the trinitrophenyl ring to stack effectively with tryptophan-93_L, despite the extra nitro group at position 6. Further, the solution structure shows that only tyrosine-34_L, and asparagine-36_L are available for hydrogen-bonding interactions; hence there is no hydrogen bond formed between the 6-nitro group of the trinitrophenyl ring and the protein. Thus 6-nitro group of 2,4,6-trinitrophenol has no strong interactions with the protein (either favourable or unfavourable). Therefore protein 315 discriminates poorly between Dnp and Tnp derivatives, and cannot be said to be more like an anti-Dnp antibody than an anti-Tnp antibody, or vice versa (Johnston et al., 1974; Elsen et al., 1970). This is supported by the dissociation constants given in Table 5, which are very similar for corresponding Dnp and Tnp

derivatives. Finally, studies of the cross-reactivity of natural anti-Dnp and anti-Tnp antisera for Tnp and Dnp respectively (Little and Eisen, 1969) show that the properties of protein MOPC315 in this respect lie well within the range found for such antisera, and also that for most of the antibodies in the antisera the stacking with a tryptophan residue demonstrated for protein 315 is a major interaction (Little and Eisen, 1967).

TABLE 5 : Comparison of dissociation constants from the Fv fragment of protein MOPC 315 of Dnp-and Tnp-haptens with identical side chains

All compounds shown are of the form Tnp(Dnp)-R; in the lysine derivatives the di- or tri-nitrophenyl group is linked to the ϵ -amino group. The binding was measured by fluorescence quenching, and the conditions as described in Table 2.

Side chain	Dnp derivative	Tnp derivative
Aminomethylphosphonate	5.0	20
L-Aspartate	24	19
Glycine	12	8

Chapter 6

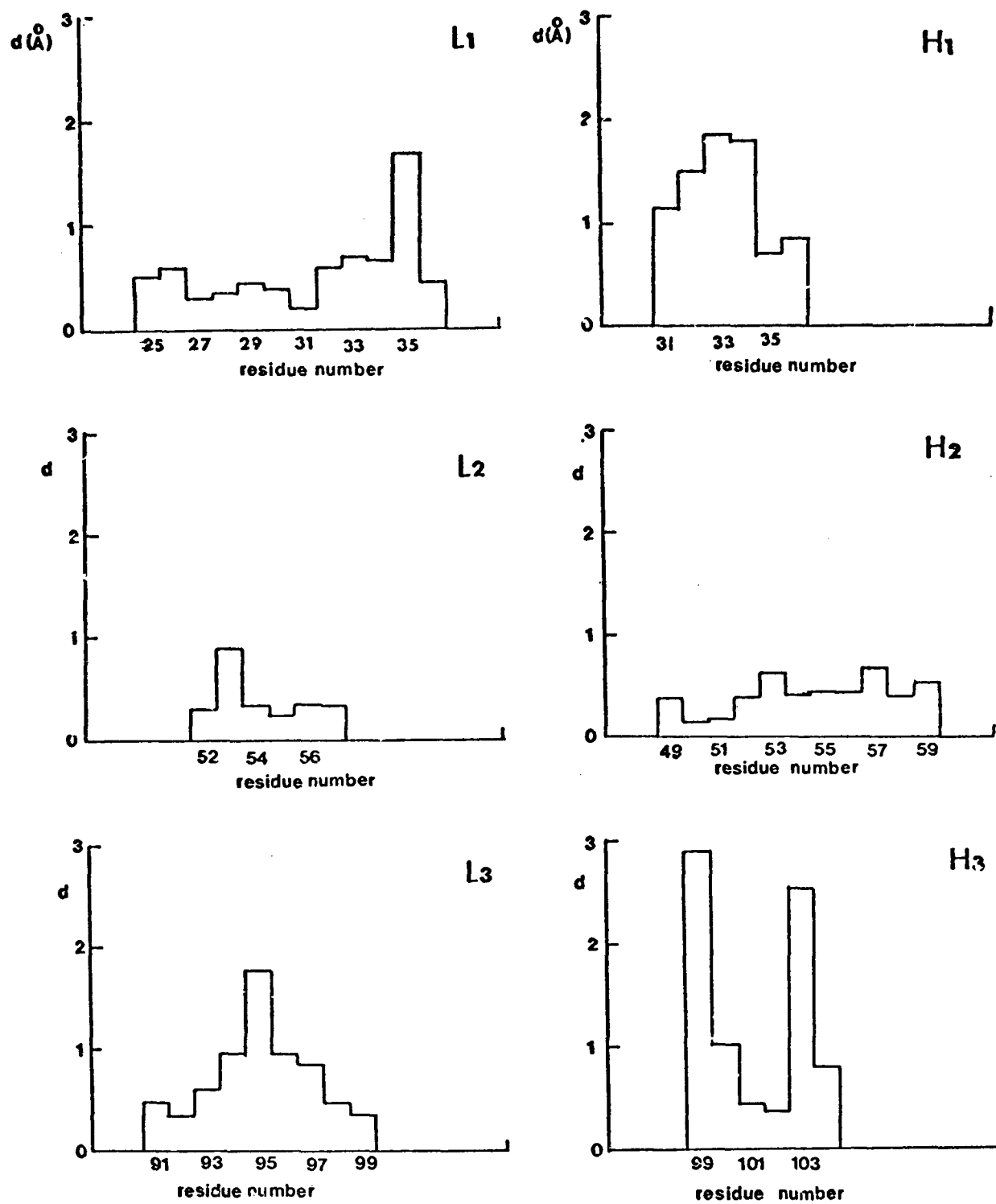
GENERAL CONCLUSIONS

1. N.m.r. and Model Building: an approach to antibody combining site structures: an assessment

The structural homologies between antibody molecules have led to the view that it should be possible to extrapolate from the crystal structures that are known, and thus predict the structures of the combining sites of antibodies for which only sequence data are available. In this thesis, the application of n.m.r. methods to the study of the interaction of the Fv fragment of protein 315 with a variety of Dnp and Tnp derivatives has been described. The results of these studies have been used in conjunction with a predicted structure for the combining site of this protein in an attempt to define the mode of interaction of haptens with the protein, and the conformation of the amino acid residues in the vicinity of the bound hapten.

It is clear from the discussion in Chapters 2, 3 and 4, that the interpretation of n.m.r. data in terms of any particular structure rests heavily on the predicted structure. This is in part due to the problems of resolution and assignment encountered with proteins of molecular weight $\geq 25,000$. If, however, it is assumed that these are technical problems that will be solved with improving equipment and sufficient selective perturbations of the system, then a fundamental problem remains. This is the difficulty in determining the structural features giving rise to observable changes in n.m.r. parameters. Thus, for example, secondary shifts in proteins arise from; ring current effects, diamagnetic anisotropy of groups containing double bonds in the protein, for example, carbonyl groups charge effects, solvation effects, changes in intramolecular structure due to bond angle strain and changes in average conformation. Thus several

Fig. 1 : A comparison of the polypeptide backbone conformations of the predicted and refined structures of the combining site of Protein 315



The histograms represent the root mean square distance between the α carbon coordinates of the residues in the hypervariable region of both structures. These distances were calculated using a FORTRAN program SUPERB, written by J. Thornton and made available by M. Lewis. The program generates a rotation matrix from a non linear least squares fit between the coordinate sets and superimposes the α carbon coordinates.

Independent effects contribute to one measurable change - the change in chemical shift between free in solution and bound to the protein. It may be possible to estimate the magnitude of these effects by suitable model compound studies, but some or all of these remain functions of the unique protein environment. It is thus difficult to see in the absence of other information, how ^1H n.m.r. can be used to determine a unique structure of a protein. These difficulties can be avoided by the use of extrinsic paramagnetic probes (Chapter 3), although here also several assumptions have to be made to extract structural conclusions from the n.m.r. data. Thus the value of n.m.r. as a structural tool in the Fv fragment-hapten system resides in determining the fine detail of the structure of the protein combining site close to the bound hapten. In order for this to be possible the model building must provide a close approximation to the backbone conformations of the hypervariable segments of the polypeptide chains.

The above considerations lead to the view that in the Fv fragment system, one is not justified in extensively modifying the predicted structure on the basis of the n.m.r. data. Thus the predicted and refined structures are similar. This is illustrated in Fig. 1, which shows a non-linear least squares fit superposition of the α -carbon coordinates. The errors inherent in building wire models and measuring coordinates from them can be appreciated from the III segment superposition. In the coordinates of Padlan et al, this was not put through a diamond refinement procedure because the segment was found to have a sequence error and was rebuilt. This shows that a rms error of 1.5 Å is 'experimental' for the model building procedure. The only segment to show changes above this is H3 which was rebuilt to resemble the conformation of NEW as discussed in Chapter 2.

The structural conclusions that these studies thus lead to is that a conformation of the amino acid residues in the combining site of the Fv

TABLE 1 : A summary. The data used to define the positions of particular amino acid residues in the combining site of the Fv fragment of protein 315

<u>Residue</u>	<u>Data used</u>	<u>References</u>
Tyr 34 _L	¹ H n.m.r., affinity labelling, chemical modification	Dower <u>et al</u> , 1977; Haimovitch <u>et al</u> , 1971; Goetzl and Metzger, 1970; Klostergaard <u>et al</u> , 1978; Jackson, unpublished data.
Trp 93 _L	Optical difference spectroscopy, C.D., ¹ H n.m.r., esr	Eisen, Simms and Potter, 1968; Freed, Rocky and Davis, 1976; Dower <u>et al</u> , 1977; Sutton <u>et al</u> , 1977.
Arg 95 _L	³¹ P n.m.r., esr, chemical modification, rapid reaction kinetics	Wain-Hobson <u>et al</u> , 1977; Sutton <u>et al</u> , 1977; Klostergaard <u>et al</u> , 1973; Haselkorn <u>et al</u> , 1974.
His 97 _L	¹ H n.m.r.	Wain-Hobson <u>et al</u> , 1977.
Phe 34 _H	¹ H n.m.r.	Dower <u>et al</u> , 1977.
His 102 _H	¹ H n.m.r.	Wain-Hobson <u>et al</u> , 1977.

Asp 99_H and Leu 103_H repositioned when the H3 segment was rebuilt.

fragment of protein 315 can be found, which is consistent with the n.m.r. data and provides a structural basis for the specificity of protein 315, with only limited modification of one of the two alternative starting structures. The crucial question that remains to be answered is whether the original structure of Padlan et al is a good approximation to the real structure of this protein. If it is, then the n.m.r. refinement will be a useful method of improving that approximation. Therefore, in order to provide a test of the model building prediction an independent crystallographic structure determination of an antibody for which a structure has been predicted, must be undertaken. Two such crystal structure determinations are at present in progress; In Oxford the structure of the Fv fragment of protein 315 is being determined, and at N.I.H. that of J359, a galactan binding mouse myeloma protein, is being determined. If these studies demonstrate that the model building provides a good prediction of polypeptide backbone conformation of the hypervariable segments, and therefore, of which amino acid side chains participate in the combining site, then n.m.r. methods will be useful in future to provide further information about the mode of interaction of haptens with these proteins.

2. Summary of structural data available for protein 315

Table 1 summarizes the data currently available on the role of amino acid residues in the combining site of protein 315. The data are all consistent with the structure illustrated in Fig. 17, Chapter 2, except for those regarding Tyr 34_L, which will therefore be discussed in detail. It has been shown that two affinity labels react with this residue (Chapter 1, section 7). The labelling of this residue with BADE abolishes the activity of protein 315, whereas labelling with MNBDF leads to only 50-fold reduction in affinity. Reduction of the MNBDF labelled protein to give amino Tyr 34_L protein 315 leads to complete restoration of activity. Nitration of this residue in (V_L)₂ also leaves the activity of the protein

unaffected, and reduction of the nitrotyrosine 34_L protein 315 or Fv fragment thus giving the aminotyrosine 34_L protein by a different route also leads to no loss of activity. Iodination of this residue, however, leads to 20-fold fall in affinity and hapten protects against this modification. It is not known whether haptens will protect against modification of $(V_L)_2$ by tetranitromethane. At present this body of data appears inconsistent, and at least suggests that caution should be exercised in the interpretation of chemical modification data.

The modified structure derived from the incorporation of all the available data into the original structure of Padlan et al, is given in microfiche form in the back of this thesis. The coordinates were measured from the wire model by Mr. P. Gettins.

3. The mode of interaction of haptens with protein 315

The binding site of the Fv fragment of protein 315 is a hydrophobic pocket formed by side chains from the L3, H1 and L1 hypervariable segments. The H2 and L2 hypervariable segments do not contribute. One side of the pocket is probably formed by part of the polypeptide backbone of the H3 loop, although this does not form any direct contacts with the bound hapten. The polynitrophenyl binding subsite is formed by the side chains of Trp 93_L with which the hapten forms a stacking interaction, and Phe 34_H and Tyr 34_L . The hapten side chain is in contact with Tyr 33_H and Arg 95_L . The side chain of Asn 36_L is oriented so that it may be able to form a hydrogen bond to a nitro group in the 4 position on the Dnp ring. The contributions of hydrogen bonding to the affinity of haptens for protein 315 has, however, not been proven. This hydrophobic pocket contains a high concentration of aromatic amino acid side chains, and appears to be able to accommodate a wide range of aromatic ligands in approximately the same orientation. Thus data presented in Chapter 5 indicate that Tnp haptens bind in a very

similar way to Dnp haptens. Also data presented by Wain-Hobson (1977) indicate that menadione binds to the Fv fragment of protein 315 in such a way that it interacts with the same amino acid residues as polynitro-phenyl derivatives. These data thus indicate that the multispecificity of protein 315 arises from the relative lack of selectivity of the binding site for aromatic ligands rather than the existence of spatially distinct sites on the protein for different ligands. The presence of the positively charged Arg 95_L near the entrance to the combining site may suggest that a negatively charged group c 10 Å from the Dnp ring will contribute to the total binding energy, but comparison of the binding constant of a large number of haptens does not support this view. (Chapter 4)

The interaction of the Dnp or Tnp ring with the protein is dominated by the light chain. This probably explains why the light chain dimer retains significant affinity for Dnp derivatives (Chapter 1, section 7). The contribution of the light chain to the side chain binding appears to be less however; residues from the H1 and H3 hypervariable segments also contributing, in particular Tyr 33_H. This further explains why affinity of the light chain dimer for Dnp derivatives is less dependent on the structure of the side chain.

The Fv fragment is a part of the protein 315 7s monomer produced by the action of the protein pepsin and thus the binding site conformation might be different in this fragment, from that in the whole IgA 7s monomer. Two n.m.r. studies suggest that the conformation of the site is the same in the Fv fragment, the Fab fragment and the IgA 7s monomer. Morris Givol and Dwek (1978) have shown that the chemical shift changes in the hapten ¹H resonances of N-Dnp-L-aspartate, which reflect mainly through space effects, are the same on binding to the three proteins. Koolstra and Richards (1978) have shown that the shift changes produced in the ¹⁹F resonances, which reflect mainly Van der Waals contacts, of Dnp and Tnp analogues in which nitro groups are replaced by trifluoromethyl

groups, are the same when these ligands bind to the three proteins. The biologically important form of an IgA is the secretory dimer $(7s\ IgA)_2$ -J-SC (Chapter 1, section 5(e)). This form of protein 315 has not been purified and no detailed studies have been carried out on it. Thus no data are available on the structure of the combining site in the biologically important form of the IgA. The data for the 7s monomer, the Fab' fragment and the Fv fragment show, by two techniques which reflect quite different aspects of the bound environment, that the bound environment of the hapten is conserved. This is also supported by the complete retention of activity by the Fab' and Fv fragments. The significance of these results must, however, be regarded with some caution since the unique ease of cleavage of protein 315 at the V/C junction which allows production of an Fv fragment suggests that there may be some unusual features about the longitudinal interactions between the variable domains and the C_L-C_H pair in this protein. It may thus not be the case in all antibodies that the combining site conformation is unmodulated by the presence of the constant domains.

This caveat also applies to any generalization that might be made concerning the failure to detect any conformational changes in the Fv fragment when Dnp and Tnp derivatives bind to this protein (Chapter 2). Two further caveats should be stressed in connection with this point. First, that protein 315 is an IgA, which does not fix complement by the classical pathway (Chapter 1, sections 4 and 5(d)). Second, that the studies in this thesis have been applied to the binding of small molecules to the Fv fragment of protein 315 and not to the binding of large antigens. The possibility that larger molecules might induce some conformation change cannot be ruled out. The Fv fragment-hapten system is thus too far removed from real immune complexes for the data obtained from it to be brought to bear with any confidence on the question of effector activation (Metzger, 1978) (Chapter 1, section 6(d)).

Finally, in relation to the question of hapten-induced conformational changes it has been shown that the on-rate for haptens to the Fv fragment, whilst slower than that calculated for a diffusion controlled reaction, reflects only a single binding step. N.m.r. data discussed in Chapter 2 are consistent with this view.

4. The relationship of the protein 315 - hapten system to other Dnp binding myeloma proteins and antibodies

Other than protein 315, two BALB/c Dnp-binding mouse myeloma proteins have been isolated. These are MOPC 460 and XRPC 25; both are chain bearing IgA proteins. The affinity of both of these proteins for Dnp derivatives is lower than that of protein 315 (Potter, 1977) by approximately two orders of magnitude. The overall dimensions of the binding sites of these proteins have been compared with those for 315 by Willan et al (1977) using a spin label method. It was shown that all three proteins had sites of the same depth (11-12 Å) but that the width of the sites at the entrance was different in each case being MOPC 460 > MOPC 315 > XRPC 25. This is consistent with the absence of affinity of XRPC 25 for bulky haptens such as menadione and 2,4, dinitronaphthol.

Few detailed structural data are available for XRPC 25 but Richards and coworkers have performed some structural studies on MOPC 460 using affinity labelling (Lifter et al, 1974; Richards et al, 1974; Haimovich et al, 1972) and fluorescence energy transfer (Rosenstein and Richards 1976). Labelling has been obtained for Lys 52_L and Tyr 33_H. It is interesting that a residue in the L2 hypervariable segment can be affinity labelled in this protein as this segment does not participate in the combining site of protein 315. Fluorescence energy transfer measurements also showed that ε-N-Dnp-L-lysine and menadione bind in distinct sites on MOPC 460. It is difficult to reconcile this with the observation that these haptens bind competitively to the protein. As yet only limited

sequence data are available for this protein and an understanding of its specificity must await more detailed structural data.

The relationship of protein 315 to induced anti-Dnp antibodies has been clarified by recent gene sequencing studies (Tonegawa et al, 1978) and the demonstration that the Dnp-binding antibodies produced in a primary response to Dnp-Keyhole limpet hemocyanin are predominantly λ -2 In type (Cotner and Eisen, 1978) (see Chapter 1, section 7). If this is correlated with the finding that the Dnp specificity of protein 315 appears to reside with the V_L domain rather than the V_H then it would appear that a $V_{\lambda 2}$ germ line gene encodes for a closely related group of antibodies produced by a limited somatic diversification, which carry specificity for Dnp and related aromatic groups. The role of the heavy chain of protein 315 in determining its specificity is less clear at present; it is however important to note that the heavy chain variable region subgroups of MOPC 315 (VH-8), XRPC 25 (VH-9) and MOPC 460 (VH-9) are similar. It will be interesting to see how similar the sequences of the V_H regions of these three proteins are. Finally, the affinities of induced anti-Dnp antibodies for Dnp derivatives can be 2-3 orders of magnitude greater than that of protein 315 (Pink and Askonas, 1974). It will be interesting to see what changes in combining site structure give rise to these higher affinities, when such proteins, purified using hybridoma cell lines, are characterized in detail.

BIBLIOGRAPHY

- Amzel, L.M., Poljak, R.J., Saul, F., Varga, J., and Richards, F.F., (1974),
Proc. Natl. Acad. Sci. U.S.A. 74, 1427.
- Arnon, R., (1973), The Antigens, Vol.1, 88, Ed. Sela, M., Academic Press,
New York and London.
- Arnon, R. and Sela, M., (1969), Proc. Natl. Acad. Sci., U.S.A. 62, 163.
- Athey, T.W. and Cathou, R.E., (1977), Immunochemistry, 14, 397.
- Barisas, B.G., Singer, S.J. and Sturtevant, J.M., (1972), Biochemistry 11, 2741
- Barisas, B.G., Singer, S.J., and Sturtevant, J.M., (1975), Immunochemistry,
12, 411.
- Barisas, B.G., Sturtevant, J.M. and Singer, S.J., (1971), Biochemistry 10, 2816.
- Barstad, P., Hubert, J., Hunkapiller, M., Goetze, A., Schilling, J., Black, B.,
Eaton, B., Richards, J., Weigert, M. and Hood, L., (1978),
Eur. J. Immunol. 8, 497.
- Beale, D., and Feinstein, A., (1976), Quart. Rev. Biophys. 9, 135.
- Benedict, A.A., Lawson, C., and Nik-Khan, H., (1963), Science 139, 1303.
- Bennich, H. and Johansson, S.G.O., (1971), Adv. Immunol. 13, 1.
- Bennich, H., Milstein, C., and Secher, D.C., (1973), FEBS Letts. 33, 49.
- Berggard, I. and Bearn, A.G., (1968), J. Biol. Chem. 243, 4095.
- Bjork, I. and Tanford, C., (1971), Biochemistry, 10, 1271.
- Blackburn, G.M., Cohen, J.S. and Weatherall, I., (1971), Tetrahedron 27, 2903.
- Blow, D.M., (1971), The Enzymes, Vol.III, 185, Ed. Boyer, P., Academic Press,
New York and London.
- Blundell, T.L. and Johnson, L.N. (1976), Protein Crystallography, Academic
Press, New York and London.
- Bourgois, A., Fougereau, M. and Rocca-Sera, J., (1974), Eur. J. Biochem.
43, 423.
- Brandtzaeg, P., (1977), Immunochemistry 14, 179.

- Brient, B.W., Haimovich, J. and Nisonoff, A., (1971), Proc. Natl. Acad. Sci., U.S.A., 68, 3136.
- Brient, B.W., and Nisonoff, A., (1970), J. Exp. Med., 132, 951.
- Brown, F.F. and Campbell, I.D., (1976), FEBS Letts, 65, 322.
- Buckingham, A.D., (1960), Canad. J. Chem. 38, 300.
- Bundi, A. and Wüthrich, K., (1978), Biopolymers, in the press.
- Bunting, J.R., and Cathou, R.E., (1973), J. Mol. Biol. 77, 223.
- Burgen, A.S.V., Jardetsky, O., Metcalfe, J.C. and Wade-Jardetsky, N., (1967), Proc. Natl. Acad. Sci. U.S.A. 58, 447.
- Burnet, F.M., (1959), The Clonal Selection Theory of Acquired Immunity, Cambridge University Press, Cambridge.
- Burton, D.R., Forsén, S., Karlström, G., Dwek, R.A., McLaughlin, A.C. and Wain-Hobson, S., (1977), Eur. J. Biochemistry, 75, 445.
- Cady, H. and Larsen, A.L., (1965), Acta Cryst. 18, 485.
- Campbell, I.D., (1977), NMR in Biology, 13, Eds. Dwek, R.A., Campbell, I.D., Richards R.E. and Williams, R.J.P., Academic Press, London, New York and San Francisco.
- Campbell, I.D., and Dobson, C.M. (1978), Methods in Biochemical Analysis, 26, in the press.
- Campbell, I.D., Dobson, C.M., Moore, G.R., Perkins, S.J., and Williams, R.J.P., (1976), FEBS Letts. 70, 96.
- Campbell, I.D., Lindskog, S. and White, A.I., (1974), J. Mol. Biol. 90, 469.
- Campbell, I.D., Lindskog, S. and White, A.I., (1975), J. Mol. Biol. 98, 597.
- Capra, J.D. and Kehoe, J.M., (1975), Adv. Immunol. 20, 1.
- Carey, P.R., (1978), Quart. Rev. Biophys. 11, 309.
- Carey, P.R., Froese, A. and Schneider, H., (1973), Biochemistry 12, 2198.
- Cathou, R.E. and Dorrington K. (1975), Biological Macromolecules - Subunits in Biological Systems, 7, 91, Eds. Fasman G.D. and Timasheff, S.N., Marcel Dekker, New York.

- Cesari, I.M. and Weigert, M.G., (1973), Proc. Natl. Acad. Sci. U.S.A. 70, 2112.
- Chess, L. and Schlossman, S.F., (1977), Adv. Immunol. 25, 213.
- Chipman, D.M. and Sharon, N., (1969), Science 165, 454.
- Coates, H.B., McLaughlin, K.A., Campbell, I.D. and McColl, C.E., (1973), Biochem. Biophys. A 310, 1.
- Coleman, J.E., Anderson, R.A., Ratcliffe, R.G. and Armitage, I.M., (1976), Biochemistry 15, 5419.
- Coleman, J.E., Anderson, R.A., Ratcliffe, R.G. and Armitage, I.M., (1976), Biochemistry 15, 5419.
- Colman, P.M., Deisenhofer, J., Huber, R. and Palm, W., (1976), J. Mol. Biol., 100, 257.
- Connell, G.E. and Porter, R.R., (1971), Biochem. J. 124, 53P.
- Cookson, D.R., Moore, G.R., Pitt, R.C., Williams, R., J.P., Campbell, I..D., Ambler, R.P., Bruschi, M. and Le Gall, J., (1978), Eur. J. Biochem. 83, 261.
- Cooper, M.D., Lawton, A.R. and Kincade, P.W., (1972), Clin. Exp. Immunol. 11, 143.
- Cotner, T. and Eisen, H.N., (1978), J. Exp. Med. 148, 1388.
- Crofts, P.C. and Kosolapoff, G.M., (1953), J. Amer. Chem. Soc. 75, 3379.
- Cunningham, A.J., (1974), Contemp. Top. Mol. Immunol. 3, 1.
- Davies, D.R., Padlan, E.A. and Segal, D.M., (1975), Ann. Rev. Biochem. 44, 639.
- Dawson, G. and Clamp, J.R., (1968), Biochem. J., 107, 341.
- Day, L.A., Sturtevant, J.M. and Singer, S.J., (1963), Ann. N. Y. Acad. Sci, 103, 611.
- Deisenhofer, J., Colman, P.M., Epp, O. and Huber, R., (1976), Hoppe-Seylers Z. Physiol. Chem., 357, 1421.
- Deisenhofer, J., Colman, P.M., Huber, R., Haupt, and Schwick, G., (1976), Hoppe-Seylers, Z. Physiol. Chem. 357, 435.
- Deisenhofer, J. and Steigman, W., (1975), Acta Cryst. Sect. B, 31, 238.

- Della-Corte, E., and Parkhouse, R.M.E., (1973), *Biochem. J.* 136, 589.
- Dickinson, C., Stewart, J.M. and Holden, J.R., (1966), *Acta. Cryst.* 21, 663.
- Dorrington, K.J., and Tanford, C., (1970), *Adv. Immunol.* 12, 333.
- Dourmaskin, R.R., Virella, G., and Parkhouse, R.M.E., (1971),
J. Mol. Biol. 56, 207.
- Dower, S.K., (1975), Part II Thesis, Oxford University.
- Dower, S.K., Dwek, R.A., McLaughlin, A.C., Mole, L.E., Press, E.M. and
Sunderland, C.A., (1975), *Biochem. J.* 149, 73.
- Dower, S.K., Wain-Hobson, S. Gettins, P., Givol, D., Jackson, W.R.C.,
Perkins, S.J., Sunderland, C.A., Sutton, B.J., Wright, C.E.
and Dwek, R.A., (1977), *Biochem. J.* 165, 207.
- Dreyer, W.J. and Bennet, J.C., (1965), *Proc. Natl. Acad. Sci. U.S.A.*
54, 864.
- Dwek, R.A., (1973), *Nuclear Magnetic Resonance in Biochemistry:
Applications to Enzymes*, Clarendon Press, Oxford.
- Dwek, R.A., Givol, D., Jones, R., McLaughlin, A. C., Wain-Hobson, S.,
White, A.I. and Wright, C.E., (1976), *Biochem. J.* 155, 37.
- Dwek, R.A., Knott, J.C.A., Marsh, D., McLaughlin, A.C., Press, E.M.,
Price, N.C., and White, A.I., (1975a), *Phil. Trans. Roy. Soc.*
London, B 272, 53.
- Dwek, R.A., Knott, J.C.A., Marsh, D., McLaughlin, A.C., Press, E.M.,
Price, N.C. and White, A.I., (1975b), *Eur. J. Biochem.* 53, 25.
- Dwek, R.A., Wain-Hobson, S., Dower, S., Gettins, P., Perkins, S.J. and
Givol, D., (1977), *Nature (London)*, 266, 31.
- Edelman, G.M., (1959), *J. Amer. Chem. Soc.* 81, 3155.
- Edelman, G.M., (1970), *Biochemistry* 9, 3197.
- Edelman, G.M., Cunningham, B.A., Gall, W.E., Gottlieb, P.D., Rutishauser, U.
and Waxdal, M.J. (1969), *Proc. Natl. Sci. U.S.A.* 63, 78.
- Edelman, G.M. and Gally, J.A., (1962), *J. Exp. Med.* 116, 207.
- Edelman, G.M. and Galley, J.A., (1964) *Proc. Natl. Acad. Sci. U.S.A.* 51, 846.
- Edelman, G.M., Heremans, J.F., Heremans, M. and Kunkel, H.G., (1960),
J. Exp. Med. 112, 203.

- Edelman, G.M. and Poulik, M.D., (1961), J. Exp. Med. 113, 861.
- Edmundson, A.B., Wood, W.K., Schiffer, M., Hardman, K.D., Ainsworth, C.F. and Ely, K.R., (1970), J. Biol. Chem. 245, 2763.
- Edmundson, A.B., Ely, K.R., Abola, E.E., Schiffer, M.M. and Panagiotopoulos, N.C., (1975), Biochemistry 14, 3953.
- Edmundson, A.B., Ely, K.R., Girling, R.L., Abola, E.E., Schiffer, M., Westholm, F.A., Faush, M.D. and Deutsch, H.F., (1974), Biochemistry 13, 3816.
- Eichman, K., Braun, D.G. and Krause, R.M., (1971) J. Exp. Med 134, 18.
- Eisen, H.N., Michaelides, M.C., Underdown, B.J., Schulenberg, E.P. and Simms, E.S., (1970) Fed. Proc. Fed. Amer. Soc. Exp. Biol. 29, 78.
- Eisen, H.N., Simms, E.S. and Potter, M., (1968) Biochemistry 7, 4126.
- Eisen, H.N. and Siskind, G.W., (1964) Biochemistry 3, 996.
- Ellerson, J.R., Yasmeen, P., Painter, R.H. and Dorrington, K.J., (1976), J.Immunol. 116, 510.
- Ely, K.R., Colman, P.M., Abola, E.E., Hess, H.C., Peabody, D.S., Parr, D.M., Connell, G.E., Laschinger, C.A. and Edmundson, A.B., (1978), Biochemistry 17, 820.
- Ely, K.R., Firca, J.R., Williams, K.J., Abola, E.E., Fenton, J.M., Schiffer, M., Panagiotopoulos, N.C. and Edmundson, A.B., (1978) Biochemistry 17, 159.
- Ely, K.R., Girling, R.L., Schiffer, M., Cunningham, D.E., and Edmundson, A.B., (1973), Biochemistry 12, 4233.
- Emsley, J.W., Feeney, J. and Sutcliffe, L.H., (1966), High Resolution Nuclear Magnetic Resonance Spectroscopy (2 vols.), Pergamon Press ; Oxford, London, Edinburgh, New York, Paris, Frankfurt.
- Epp, O., Colman, P., Fehlhammer, H., Bode, W., Schiffer, M., Huber, R. and Palm, W., (1974), Eur. J. Biochem. 45, 513.
- Epp, O., Lattman, E.E., Schiffer, M., Huber, R. and Palm, W., (1975), Biochemistry 14, 4943.
- Erwin, P.M. and Aladjem, F., (1976) Immunochemistry 13, 873.
- Farah, F.S., Kern, M. and Eisen, H.N., (1960), J. Exp. Med. 112, 1195.

- Feeney, J., Birdsall, B., Roberts, G.C.K. and Burgen, A.S. (1975)
Nature (London), 257, 565.
- Feeney, J., Birdsall, B., Roberts, G.C.K. and Burgen, A.S.V., (1977),
N.M.R. in Biology, III, Eds. Dwek, R.A., Campbell, I.D., Richards,
R.E., and Williams, R.J.P., Academic Press, London, New York and
San Francisco.
- Fehlhammer, H., Schiffer, M., Epp, O., Colman, P.H., Lattman, E.E.,
Schwager, P. and Steigman, W., (1975), Biophys. Struct. Mech. 1, 139.
- Feinstein, A., (1974) J. Physiol. 242, 32P.
- Feinstein, A. and Rowe, A.J., (1965) Nature (London) 205, 147.
- Fett, J.W. and Deutsch, H.F., (1974), Biochemistry, 12, 4102.
- Firca, J.R., Ely, K.R., Kremer, P., Westholm, F.A., Dorrington, K.J., and
Edmundson, A.B., (1978), Biochemistry 17, 148.
- Fleet, G.W.J., Knowles, J.R. and Porter, R.R., (1972) Biochem. J. 128, 499.
- Fleischman, J.B., Pain, R.H. and Porter, R.R., (1962), Arch. Biochem.
Biophys. Suppl. 1, 174.
- Foster, R. and Fyfe, C.A., (1969) Prog. NMR. Spectroscopy 4, 1, Eds.
Emsley, J.W., Feeney, J. and Sutcliffe, L.H., Pergamon Press.
- Fothergill, J.E. and Anderson, W.H.K., (1978) Curr. Top. in Cell Reg.
13, 259.
- Francis, S.H., Leslie, R.G.Q., Hood, L.E. and Eisen, H.N., (1974), Proc.
Natl. Acad. Sci. U.S.A. 71, 1123.
- Frangione, B., Milstein, C. and Franklin, E.C., (1969), Nature (London)
221, 149.
- Freed, R., Rockey, J. and Davis, R., (1976) Immunochemistry 13, 509.
- Freedman, M.H., Grossberg, A.L. and Pressman, D., (1968), Biochemistry, 7, 1941.
- Freeman, R., Hill, H.D.W. and Kaptein, R., (1972), J. Mag. Res. 7, 327.
- Froese, A., (1968), Immunochemistry 5, 253.
- Froese, A. and Schon, A.H., (1975), Cont. Top. Mol. Immunol. 4, 23.
- Froese, A., Schon, A.H. and Eigen, M., (1962) Can. J. Chem. 40, 1786.
- Gadian, D.G., (1974), D. Phil Thesis, Oxford University.

- Gall, W.E., and Edelman, G.M., (1970) *Biochemistry* 9, 3188.
- Gally, J.A., (1973), *The Antigens*, Vol. I, 162, Ed. Sela, M., Academic Press, New York and London.
- Gartland, G.L., Freeman, G.R. and Bugg, C.E., (1974), *Acta. Cryst. B*, 30, 841.
- Georgey, J., Fudenberg, H.H. and Van Loghem, E., (1970) *Immunochemistry*, 7, 1.
- Gergely, J., Medgyesi, G.A. and Stanworth, D.R., (1967), *Immunochemistry* 4, 369.
- Geschichter, R. and Copeland, M., (1928), *Arch. Surg.* 16, 807.
- Gettins, P., (1979), D. Phil Thesis, Oxford University.
- Gettins, P., Givol, D. and Dwek, R.A., (1978) *Biochem. J.* 173, 713.
- Gettins, P., Potter, M., Rudikoff, S. and Dwek, R.A., (1977) *FEBS Letts.* 84, 87.
- Giessner-Prettre, C., and Pullman, B., (1969) *C.R. Hebd. Séances Acad. Sci. Ser D* 268, 1115.
- Givol, D., (1967), *Biochem. J.* 104, 39c.
- Givol, D., (1974) *Essays in Biochem.* 10, 73, Eds.; Campbell, P.N. and Dickens, F., Academic Press, London, New York and San Francisco.
- Givol, D., private communication.
- Givol, D., Strausbach, P.H., Hurwitz, E., Wilchek, M., Haimovich, J. and Eisen, H.N., (1971) *Biochemistry* 10, 3461.
- Glaser, M. and Singer, S.J., (1971), *Proc. Natl. Acad. Sci. U.S.A.* 68, 2477.
- Goetze, A.M. and Richards, J.H., (1977a), *Proc. Natl. Acad. Sci. U.S.A.* 74, 2109.
- Goetze, A.M. and Richards, J.H. (1977b) *Biochemistry* 16, 228.
- Goetze, A.M. and Richards, J.H. (1978) *Biochemistry* 17, 1733.
- Goetzl, E.J. and Metzger, H. (1970a) *Biochemistry* 9, 1267.
- Goetzl, E.J., and Metzger, H. (1970b) *Biochemistry* 9, 3862.
- Goldstein, D.J., Humphrey, R.L. and Poljak, R.J., (1968), *J. Mol. Biol.* 35, 247.

- Gorenstein, D.G., (1975), J. Amer. Chem. Soc. 97, 898.
- Gorenstein, D.G., Myrwicz, A.M. and Bode, J., (1976) J. Amer. Chem. Soc. 98, 2308
- Götze, O. and Muller-Eberhard, H.J., (1976), Adv. Immunol. 24, 1.
- Gowans, J.L., (1969), Harvey Lectures 64 87.
- Grabar, P. and Williams, C.A. Jr., (1953), Biochim. Biophys. Acta 10, 193.
- Granato, D., Braun, D.G. and Vassalli, P., (1974) J. Immunol. 113, 417.
- Green, N.M., Dourmashkin, R.R. and Parkhouse, R. M.E., (1971), J. Mol. Biol. 56, 203.
- Grey, H.M., Abel, C.A., and Zimmerman, B., (1971) Ann. N.Y. Acad. Sci. 190, 37.
- Grey, H.M., Sher, A. and Shalatin, No., (1970), J. Immunol. 105, 75.
- Gupta, R.K. and Benovic, J.L., (1978), Biochem. Biophys. Res. Comm. 84, 130.
- Hadler, N.M. and Metzger, H.H., (1971) Proc. Natl. Acad. Sci. U.S.A. 68, 1421.
- Haigh, G.W. and Mallion, R.B., (1971a), Mol. Phys. 22, 945.
- Haigh, C.W. and Mallion, R.B., (1971b), Mol. Phys. 22, 955.
- Haigh, C.W. and Mallion, R.B., (1972), Org. Mag. Reson. 4, 203.
- Haigh, C.W., Mallion, R.B. and Armour, E.A.G., (1970) Mol. Phys. 18, 751.
- Haimovich, J., Eisen, H.N. and Givol, D., (1971) Ann. N.Y. Acad. Sci. 190, 352
- Haimovich, J., Eisen, H.N., Hurwitz, E. and Givol, D., (1972), Biochemistry 11, 2389.
- Haimovich, J., Givol, D. and Eisen, H.N., (1970) Proc. Natl. Acad. Sci. U.S.A. 67, 1656.
- Halpern, M.S. and Koshland, M.E., (1970), Nature (London) 228, 1276.
- Halpern, M.S. and Koshland, M.E., (1971) Biochemistry 10, 3843.
- Hammes, G.G., (1974) Investigations of Rates and Mechanisms II : Investigations of Elementary Steps in Solution and very Fast Reactions, 3rd edn., John Wiley, New York.

- Hammes, G.G., and Wu, C.W., (1974) *Ann. Rev. Biophys. Bioeng.* 3, 1.
- Hanson, A.W., (1964), *Acta Cryst.* 17, 559.
- Hardy, R.R. and Richards J.H., (1978), *Biochemistry* 17, 3867.
- Harris, H. and Watkins, J.F., (1965), *Nature (London)* 205, 640.
- Haselkorn, D., Friedman, S., Givol, D. and Pecht, I., (1974),
Biochemistry 13, 2210.
- Heidelberger, M. and Pederson, K.O. (1937), *J. Exp. Med.* 65, 393.
- Helman, M., Shreier, I. and Givol, D., (1976), *J. Immunol* 117, 1933.
- Heremans, J.F., (1974), *The Antigens*, Vol.III, 368, Ed. Sela, M.,
Academic Press, New York and London.
- Hill, R.L., Delany, R., Fellows, R.E. and Lebovitz, H.E., (1966),
Proc. Natl. Acad. Sci. U.S.A., 56, 1762.
- Hilschman, N. and Craig, L.C., (1965), *Proc. Natl. Acad. Sci.*
U.S.A. 53, 1403.
- Hochman, J., Inbar, D. and Givol, D., (1973) *Biochemistry*, 12, 1130.
- Hornick, C.L. and Karush, F., (1972) *Immunochemistry* 9, 325.
- Hsia, J.C. and Little, J.R., (1973), *FEBS Letts.* 31, 80.
- Hsia, J.C.C. and Piette, L.H., (1969), *Arch. Biochem. Biophys.* 129, 296.
- Hsia, J.C., Wong, L.T.L. and Kaiow, W., (1973) *J. Immun. Meth.*, 3, 17.
- Hsia, J.C., Wong, L.T.L., Pryse, K. and Little, J.R., (1973)
Immunochemistry 10, 517.
- Huber, R., Deisenhofer, J., Colman, P.M., Matsushima, M. and Palm, W.,
(1976) *Nature (London)* 264, 415.
- Hull, W.E., Halford, S.E., Gutfreund, H. and Sykes, B.D., (1976),
Biochemistry 15, 1547.
- Humphrey, P.L., (1976) *J. Mol. Biol.* 29, 525.
- Inbar, D., Givol, D. and Hochman, J., (1973), *Biochemistry* 12, 4541.
- Inbar, D., Hochman, J. and Givol, D., (1972), *Proc. Natl. Acad. Sci.*
U.S.A., 69, 2659.

- Inbar, D., Rotman, M. and Givol, D., (1971), J. Biol. Chem. 246, 6272.
- Ishizaka, K., Ishizaka, T. and Hornbrook, M.M., (1966), J. Immunol. 97, 75.
- Jackman, L.M. and Sternhell, S., (1969), International Series of Monographs in Organic Chemistry, Vol.5: Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry, Pergamon Press.
- Jackson, R., (1979), D. Phil. Thesis, Oxford University.
- Jefferis, R., Weston, P.D., Stanworth, D.R. and Clamp, J.R., (1968), Nature (London), 219, 646.
- Johnston, M.F.M., Barisas, B.G. and Sturtevant, J.M., (1974), Biochemistry 13, 390.
- Johnston, M.F.M. and Eisen, H.N., (1976), J. Immunol. 117, 1189.
- Johnson, C.E. and Bovey, F.A., (1958) J. Chem. Phys. 24, 1012.
- Kabat, E.A., (1978), Adv. Prot. Chem. 32, 1.
- Kabat, E.A. and Wu, T.T., (1971) Ann. N.Y. Acad. Sci. 190, 382.
- Kabat, E.A., Wu, T.T. and Bilofsky, H., (1976) Variable Regions of Immunoglobulin Chains, Medical Computer Systems Inc.
- Kakimoto, K. and Onoue, K., (1974) J. Immunol 112, 1373.
- Kalk, A. and Berendsen, H.J.C., (1976) J. Mag. Res. 24, 343.
- Karush, F., (1962) Adv. Immunol 2, 1.
- Kelly, K.A. Sehon, A.H. and Froese, A., (1971) Immunochemistry 8, 613.
- Kilmarten, J.V., Breen, J.J., Roberts, G.C.K. and Ho, C., (1975), Proc. Natl. Acad. Sci. U.S.A. 70, 1246.
- Kindt, T.J., (1975), Adv. Immunol. 21, 35.
- Klinman, N.R., (1971), J. Immunol. 106, 1338.
- Klinman, N.R., (1972) J. Exp. Med. 136, 241.
- Klinman, N.R. and Sigal, N.H., (1978), Adv. Immunol. 28, 255.
- Klostergaard, J., Grossberg, A.L., Krausz, L.H. and Pressman, D., (1977a), Immunochemistry 14, 37.

- Klostergaard, J., Krausz, L.H., Grossberg, A.L. and Pressman, D., (1977b), *Immunochemistry*
- Klostergaard, J., Mayers, G.L., Grossberg, A. L. and Pressman, D., (1978), *Immunochemistry* 15, 225.
- Köhler, G. and Milstein, C., (1975) *Nature (London)*, 256, 495.
- Köhler, G. and Milstein, C., (1976), *Eur. J. Immunol* 6, 511
- Koo, P.H. and Cebra, J.J., (1974), *Biochemistry* 13, 184.
- Kooistra, D.A. and Richards, J.H. (1978) *Biochemistry* 17, 345.
- Kooistra, D.A., Hardy, R.R. and Richards, J.H., to be published.
- Kornfeld, R. and Kornfeld, S., (1976) *Ann. Rev., Biochem.* 45, 217.
- Korngold, L. and Lipari, R., (1956), *Cancer* 9, 262.
- Koshland, M.E., (1975), *Adv. Immunol* 20, 41.
- Kotowycz, G. and Lemieux, P., (1973), *Chem. Revs.* 73, 669.
- Kratzin, H., Altevogt, P., Ruban, P., Kortt, A., Staroscik, K. and Hilschmann, N., (1975), *Hoppe-Seylers, Z. Physiol. Chem.* 356, 1337.
- Krause, R.M., (1970) *Adv. Immunol.* 12, 1.
- Kraut, J., (1961), *Acta Cryst.* 14, 1146.
- Kreth, H.W. and Williamson, A.R., (1973) *Eur. J. Immunol.* 3, 141.
- Kubo, R.T., Zimmerman, B. and Grey, H.M., (1973), *The Antigens*, Vol. I, 417, Ed. Sela, M., Academic Press, New York and London
- Kumar, K., Phelps, D.J., Carey, P.R. and Young, N.M., (1978), *Biochem. J.* 175, 727.
- Kumler, W.D. and Eiler, J.J., (1943), *J. Amer. Chem. Soc.* 65, 2355.
- Lafferty, K.J. and Oertlis, S., (1963), *Virology* 21, 91.
- Lancet, D. and Pecht, I., (1976), *Proc. Natl. Acad. Sci. U.S.A.* 73, 3549.
- Landsteiner, K., (1944), *The Specificity of Serological Reactions*, Harvard University Press, Cambridge, Mass.
- Laschinger, C.A. and Connell, G.E., (1978), *Immunochemistry* 15, 119.

- Letcher, J.H. and Van Wazer, J.R., (1966), J. Chem. Phys. 45, 2916.
- Letcher, J.H. and Van Wazer, J.R., (1966), J. Chem. Phys. 44, 815.
- Licht, A., Lancet, D., Schechter, J. and Pecht, I., (1977), FEBS Letts. 78, 211.
- Lifter, J., Hew, C-L., Yoshioka, M., Richards, F.F. and Konigsberg, W.H., (1974), Biochemistry 13, 3567.
- Lin, L-C. and Putnam, F.W., (1978), Proc. Natl. Acad. Sci. U.S.A. 75, 2649.
- Little, J.R. and Eisen, H.N., (1967), Biochemistry 6, 3119.
- Little, J.R. and Eisen, H.N., (1969), J. Exp. Med. 129, 247.
- Low, T.L.K., Liu, V.Y. and Putnam, F.W., (1976), Science 191, 390.
- Lumbroso, N., Wu, T.K. and Daily, B.P., (1963), J. Phys. Chem. 67, 2469.
- Lynch, R.G., Graff, P.J., Sirisinha, S., Simms, E.S. and Eisen, H.N., (1972), Proc. Natl. Acad. Sci. U.S.A. 69, 1540.
- Mallion, R.B., (1971), J.Chem. Soc. B, 687.
- Marchalonis, J.J., (1977), Immunity in Evolution, Edward Arnold, London.
- McDonald, C.C. and Phillips, W.D. (1969), J. Amer. Chem. Soc. 91, 1515.
- McCammon, B., Gelin, B.R. and Karplus, M., (1977), Nature (London) 267, 585.
- McKean, D.J., Bell, M. and Potter, M., (1978), Proc. Natl. Acad. Sci., U.S.A. 75, 3913.
- McWeeny, R., R., Hoffman, T. and Kunkel, H., (1977), J. Exper. Med. 145, 760.
- Metzger, H., (1974), Adv. Immunol. 18, 169.
- Metzger, H., (1978), in the press.
- Michaelides, M.C. and Eisen, H.N. (1974) J. Exp. Med. 140, 687.
- Miller, J.F.A.P. and Mitchell, G.F., (1969). Transplantation Revs. 1, 3.
- Milstein, C., (1967), Nature (London) 216, 330.
- Milstein, C. and Pink, J.R.L., (1970), Prog. Biophys. Mol. Biol. 21, 209.
- Milstein, C. and Svasti, I., (1971), Progress in Immunol. Vol. I, 35, Ed. Amos, B., Academic Press, New York and London.

- Mitchison, N.A., (1971) *Eur. J. Immunol.* 1, 10.
- Morris, A.T., Givol, D. and Dwek, R.A., (1978) *Immunochemistry* 15, 519.
- Nakamura, H., Yoo, T.J., Grossberg, A.L. and Pressman, D., (1970),
Immunochemistry 7, 637.
- Natvig, J.B. and Kunkel, H.G., (1973), *Adv. Immunol.* 16, 1.
- Niederwieser, A., (1971), *J. Chromatog.* 54, 215.
- Nisonoff, A., Hopper, J.E. and Spring, S.B., (1975) *The Antibody Molecule*,
Academic Press, New York, San Francisco and London.
- Nisonoff, A. and Pressman, D., (1975), *J. Amer. Chem. Soc.* 79, 1616.
- Nisonoff, A., Winkler, E.C., Lipmann, L.N. and Woernly, D.L., (1960),
Arch. Biochem. Biophys. 89, 230.
- Nossal, G.J.V., Szenberg, A., Ada, G.L. and Austin, C.H., (1964),
J. Exp. Med. 119, 485.
- Novotny, J. and Franek, F., (1975) *Nature (London)*, 258, 641.
- O'Donnell, I.J., Frangione, B., and Porter, R.R., (1970) *Biochem. J.* 116, 261.
- Okaya, J., (1966), *Acta Cryst.* 20, 712.
- Onoue, K., Kishimoto, T. and Yamamura, Y., (1968) *J. Immunol.* 100, 238.
- Orin, G., Davis, R., Freed, R. and Rockey, J., (1976), *Immunochemistry*, 13, 517.
- Oudin, J., (1974), *The Antigens*, Vol. II, 278, Ed. Sela, M., Academic Press,
New York, San Francisco and London.
- Padlan, E.A., (1977), *Quart. Rev. Biophys.* 10, 35.
- Padlan, E.A., Segal, D.M., Spande, T.F., Davies, D.R., Rudikoff, S. and
Potter, M. (1973), *Nature New Biol.* 245, 165.
- Padlan, E.A. and Vrana, M., unpublished data.
- Padlan, E.A., Davies, D.R., Pecht, I., Givol, D. and Wright, C., (1976),
Cold Spring Harbour, Symp. Quant. Biol. 41, 627.
- Parkhouse, R.M.E. (1972), *Nature New Biol.* 236, 9.
- Patel, D.J., (1974) *Biochemistry* 13, 2388.

- Pauling, L., (1960) The Nature of the Chemical Bond, 3rd Edn. Oxford University Press, Oxford.
- Pecht, I., (1974) The Immune System, Genes, Receptors and Signals, 15: Academic Press, New York, San Francisco and London.
- Pecht, I., Givol, D. and Sela, M., (1972) J. Mol. Biol. 68, 241.
- Pecht, I., Haselkorn, D. and Friedman, S., (1972) FEBS Letts. 24, 331.
- Perkins, S.J., (1977), D. Phil. Thesis, Oxford University.
- Perkins, S.J., Dower, S.K., Gettins, P., Wain-Hobson, S. and Dwek, R.A., (1977) Biochem. J. 165, 223.
- Perkins, S.J. and Dwek, R.A., (1979), to be published.
- Peterson, P.A., Cunningham, B.A., Berggard, I. and Edelman, G.M., (1972), Proc. Natl. Acad. Sci. U.S.A. 69, 1697.
- Piette, L.H., Hsia, J.C.C., Kosman, D.J. and Spallholz, J.E., (1971), Abstract: First European Biophysics Congress 113, Eds. Broada, E., Locher, A. and Spinter-Lederer, H.
- Pilz, I., Kratky, O. , and Karush, F., (1974) Eur. J. Biochem. 41, 91.
- Pilz, I., Schwarz, E. and Palm, W., (1977) Eur. J. Biochem 75, 195.
- Pink, J.R.L. and Askonas, B.A., (1974) Eur. J. Immunol. 4, 426.
- Poljak, R., (1975) Adv. Immunol. 21, 1
- Poljak, R.J., Amzel, L.M., Avey, H.P., Becker, L.N. and Nisonoff, A., (1972) Nature New Biol. 235, 137.
- Poljak, R.J., Amzel, L.H. Avey, H.P., Chen, B.L., Phizackerly, R.P. and Saul, F., (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 3305.
- Poljak, R.J., Amzel, L.H., Chen, B.L., Phizackerly, R.P. and Saul, F., (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 3440.
- Pople, J.A., (1956), J. Chem. Phys. 24, 1111.
- Pople, J.A., (1962) J. Chem. Phys. 37, 60.
- Porter, R.R., (1959), Biochem. J. 73, 119.
- Porter, R.R. and Weir, R.R., (1966) J. Cell Physiol. Suppl. 67 (part II), 51.
- Potter, M., (1972) Physiol. Revs. 52, 631.

- Potter, M., (1977) *Adv. Immunol.* 25, 141.
- Press, E.M. and Hogg, N.M., (1970), *Biochem. J.* 117, 641.
- Purdela, D., (1971) *J. Mag. Res.* 5, 23.
- Putnam, F.W., Florent, G., Paul, C., Shmoda, T. and Shimizu, A., (1973), *Science*, 182, 281.
- Rabbitts, T.H., (1978) *Nature (London)* 275, 291.
- Raff, M.C., Sternberg, M. and Taylor, R.B., (1970) *Nature (London)* 225, 553.
- Rao, B.D.N. and Cohn, M., (1977) *Proc. Natl. Acad. Sci. U.S.A.* 74, 5355.
- Ray, A. and Cebra, J.J., (1972), *Biochemistry* 11, 3647.
- Reid, K.B.M and Porter, R.R., (1975) *Cont. Top. Mol. Immunol.* 4, 1.
- Reth, M., Hämmerling, G.J. and Rajewsky, K., (1978) *Eur. J. Immunol* 8, 393.
- Richards, F.F., Konigsberg, W.H., Rosenstein, R.W. and Varga, J.M., (1975) *Science* 187, 130.
- Richards, F.F., Lifter, J.L., Hew, G.L., Yoshioka, M. and Konigsberg, W.H., (1974) *Biochemistry* 13, 3572.
- Riess, J.G., Van Wazer, J.R. and Letcher, J.H., (1967), *J. Phys. Chem.* 71, 1925.
- Roberts, G.C.K. Feeney, J., Birdsall, B., Kimber, B., Griffiths, D.V., King, R.W. and Burgen, A.S.V., (1977) *NMR in Biology*, 95, Eds. Dwek, R.A., Campbell, I.D., Richards, R.E., and Williams, R.J.P., Academic Press, London, New York and San Francisco.
- Roberts, G.C.K. and Jardetsky, O., (1970) *Adv. Prot. Chem.* 24, 447.
- Rockey, J.H., Dorrington, K.J. and Montgomery, P.C., (1971), *Nature (London)*, 232, 192.
- Roholt, O., Radzimski, G. and Pressman, D., (1965) *Science*, 147, 613.
- Ropartz, C., Lenoir, J. and Rivat, L., (1961) *Nature (London)* 189, 586.
- Rosenstein, R.W. and Richards, F.F., (1976), *Immunochemistry* 13, 939.
- Rossi, G., Choi, T.K. and Nisonoff, A., (1969), *Nature (London)* 223, 837.
- Rossi, G. and Nisonoff, A., (1968) *Biochem. Biophys. Res. Comm.* 31, 914.

- Rowe, D.S. and Fahey, J.L., (1965), J. Exp. Med. 121, 171.
- Rubinstein, W.A. and Little, J.R., (1970) Biochemistry 9, 2106.
- Rudikoff, S. and Claflin, J.L., (1976) J. Exp. Med. 144, 1294.
- Rudikoff, S., Potter, M., Segal, D.M., Padlan, E.A. and Davies, D.R., (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 3689.
- Sarma, V.R., Silverton, E.W., Davies, D.R. and Terry, W.D., (1971), J. Biol. Chem. 246, 3753.
- Schaefer, T. and Schneider, W.E., (1960) Canad. J. Chem. 32, 1244.
- Schechter, I., Ziv, E. and Licht, A., (1976), Biochemistry 15, 2785.
- Schiffer, M., Girling, R.L., Ely, K.R. and Edmundson, A.B., (1973) Biochemistry 12, 4620.
- Schulenberg, E.P., Simms, E.S., Lynch, R.G., Bradshaw, R.A. and Eisen, H.N., (1971), Proc. Natl. Acad. Sci. U.S.A. 68, 2623.
- Schweizer, M.P., Chan, S.I., Helmkamp, G.K. and Ts'o, P.O.P., (1964), J. Amer. Chem. Soc. 86, 696.
- Segal, D.M., Padlan, E.A., Cohen, G.H., Rudikoff, S., Potter, M. and Davies, D.R. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 4298.
- Sehon, A.H., (1963) Ann. N.Y. Acad. Sci. 103, 626.
- Seidman, J.G., Leder, A., Edgell, M.H., Polisky, F., Tilghman, S.M., Tiemeier, D.C. and Leder, P., (1978), Proc. Natl. Acad. Sci. U.S.A. 75, 3881.
- Sharon, J. and Givol, D., (1976) Biochemistry 15, 1591.
- Shimizu, A., Putnam, F.W. and Paul, C., (1971) Nature New Biol. 231, 73.
- Singer, S.J. and Doolittle, R.F., (1966) Science 153, 13.
- Sirisinha, S. and Eisen, H.N., (1971) Proc. Natl. Acad. Sci. U.S.A. 68, 3130.
- Smith, M. and Khorana, H.G., (1958), J. Amer. Chem. Soc. 80, 1141.
- Snyder, G.H., Rowan III, R., Karplus, S. and Sykes, B.D., (1975), Biochemistry 14, 3765.
- Sox, H.C. and Hood, L.E., (1970), Proc. Natl. Acad. Sci. U.S.A. 66, 975.
- Steiner, L.A. and Eisen, H.N., (1967) J. Exp. Med. 126, 1143.

- Sternlicht, H., Jones, D.E. and Kustin, K., (1968), J. Amer. Chem. Soc. 90, 7110.
- Sternlicht, H. and Wilson, D., (1967), Biochemistry 6, 2881
- Strausbach, P.H., Weinstein, Y., Wilchek, M., Shaltiel, S. and Givol, D., (1971) Biochemistry 10, 4342.
- Strosberg, A.D., Fraser, K.J., Margolies, M.N. and Haber, E., (1972) Biochemistry 11, 4978.
- Strosberg, A.D., Margolies, M.N. and Haber, E., (1974) Fed. Amer. Soc. Exp. Biol. Proc. 33, 726.
- Stryer, L. and Griffiths, O.H., (1965) Proc. Natl. Acad. Sci. U.S.A. 54, 1785.
- Sturtevant, J.M., Wofsy, L. and Singer, S.J., (1961), Science 134, 1434
- Sutton, B.J., Gettins, P., Givol, D., Marsh, D.M., Wain-Hobson, S., Willan, K.J. and Dwek, R.A., (1977) Biochem. J. 165, 177.
- TaImage, D.W., (1959), Science 129, 1643.
- Tiselius, A. and Kabat, E.A., (1939) J.Exp. Med. 69, 119.
- Tomasi, T.B. Jr. and Calvinico, N., (1968) Fed. Amer. Soc. Exp. Biol. Proc. 27, 617.
- Tomasi, T.B. and Grey, H.M., (1972), Prog. Allergy 16, 81.
- Tonegawa, S., Brack, C., Hozumi, N., Matthysens, G. and Schuller, R., (1977) Immunol. Revs. 36, 73.
- Tonegawa, S. Maxam, A.M., Tizard, R., Bernard, O. and Gilbert, W., (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 1485.
- Tourville, D.R., Adler, R.H., Bienenstock, J. and Tomasi, T.B., (1969), J. Exp. Med. 129, 411.
- Trotter, J., (1961) Acta Cryst. 14, 244.
- Trotter, J. and Williston, C.S., (1966) Acta Cryst. 21, 285.
- Turner, M.W. and Bennich, H., (1968), Biochem. J. 107, 171.
- Underdown, B.J., Simms, E.S. and Eisen, H.N., (1971) Biochemistry 10, 4359.
- Valentine, R.C. and Green, N.M., (1967), J. Mol. Biol. 27, 615.

- Veillard, A. and Pullman, B., (1961), C.R. Hebd. Séances Acad. Sci. 253, 2277.
- Voss, E.W. and Watt, R.M., (1977) *Immunochemistry* 14, 237.
- Wagner, G., De Marco, A. and Wüthrich, K., (1976) *Biophys. Struct. Mech.* 2, 139.
- Wain-Hobson, S., (1977) D. Phil. Thesis, Oxford University.
- Wain-Hobson, S., Dower, S.K., Gettins, P., Givol, D., McLaughlin, A.C., Pecht, I., Sunderland, C. and Dwek, R.A., (1977), *Biochem. J.* 165, 227.
- Waldenström, J., (1943) *Nord. Med.* 20, 2288.
- Waldenström, J., (1944) *Acta Med. Scand.* 117, 216.
- Wang, A.C., Wang, I.Y.F., McCormick, J.H. and Fudenberg, H.H., (1969) *Immunochemistry* 6, 451.
- Wang, A.C., Wilson, S.K., Hopper, J.E., Fudenberg, H.H. and Nisonoff, A., (1970) *Proc. Natl. Acad. Sci. U.S.A.* 66, 337.
- Watanabe, S., Barnikol, H.N., Horn, J., Bertram, J. and Hilschmann, M., (1973) *Hoppe-Seylers Z. Physiol. Chem.* 354, 1505.
- Weinstein, Y., Wilchek, M. and Givol, D., (1969) *Biochem. Biophys. Res. Comm.* 35, 694.
- Wells, J.V., Fudenberg, H.H. and Givol, D., (1973) *Proc. Natl. Acad. Sci. U.S.A.* 70, 1585.
- Werblin, T.P. and Siskind, G.W. (1972) *Immunochemistry* 9, 987.
- Wilde, C.E.III and Koshland, M.E., (1978) *Biochemistry* 17, 3209.
- Wilder, R.L., Green G. and Schumaker, V.N., (1975) *Immunochemistry* 12, 55.
- Willan, K.J., Marsh, D.M., Sunderland, C.A., Sutton, B.J., Wain-Hobson, S., Dwek, R.A. and Givol, D., (1977) *Biochem. J.* 165, 199.
- Williamson, A.R., (1976) *Ann. Rev. Biochem.* 45, 467.
- Winkelhalke, J.L., (1978) *Immunochemistry* 15, 695.
- Wofsy, L., Metzger, H. and Singer, S.J., (1962) *Biochemistry* 1, 1031.
- Wong, L.T.L., Piette, L.H., Little, J.R. and Hsia, J.C., (1974) *Immunochemistry* 11, 377.

Wright, C., (1977) D. Phil. Thesis, Oxford University.

Wu, T.T. and Kabat, E.A., (1970) J. Exp. Med, 132, 211.

Yguerabide, J., Epstein, H.F. and Stryer, L., (1970), J. Mol. Biol. 51, 573.

Yoo, T.J., Nakamura, H., Grossberg, A.L. and Pressman, D., (1970)
Immunochemistry, 7, 627.